TESIS DOCTORAL

Diseño, desarrollo y aplicación de la tecnología LAMP para el diagnóstico de la esquistosomosis: del laboratorio al campo

FACULTAD DE FARMACIA

Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola

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*Que la Tesis Doctoral titulada "**Diseño, desarrollo y aplicación de la tecnología LAMP para el diagnóstico de la esquistosomosis: del laboratorio al campo",** que se presenta para optar al grado de Doctor por la Universidad de Salamanca en la modalidad de Tesis por compendio de publicaciones, ha sido realizada por **Javier Gandasegui Arahuetes**, Licenciado en Farmacia por la Universidad Complutense de Madrid, bajo nuestra dirección en el Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola de la Universidad de Salamanca y en el Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca y dentro del programa de doctorado de Salud y Desarrollo en los Trópicos. Consideramos que reúne, a nuestro juicio, originalidad y contenidos suficientes,por lo que autorizamos su presentación para ser evaluada.*

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Fdo. Dr. Antonio Muro Álvarez *Fdo. Dr. Pedro Fernández Soto*

Dedicatoria

A Fernando Gandasegui Alonso, el
Abito, por enseñarme a ser coherente
e íntegro, por tu imborrable ejemplo

Nada te turbe,
Nada te espante,
Todo se pasa, [...]

Santa Teresa de Jesús

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Estamos juntos

Índice

Resumen

La esquistosomosis, una enfermedad parasitaria producida por trematodos del género *Schistosoma*, es una de las principales enfermedades tropicales desantendidas. En la actualidad no se dispone de un método de diagnóstico adecuado ya que las técnicas parasitológicas y serológicas presentan problemas de sensibilidad y especificidad. El diagnóstico molecular no se utiliza de forma rutinaria, particularmente en zonas endémicas y de bajos recursos. El LAMP (*loop-mediated isothermal amplification*) es una técnica de amplificación de ADN que se realiza en condiciones isotérmicas. Presenta las ventajas de ser rápida, sensible, específica, permite la discriminación visual de los resultados y puede ser aplicada como diagnóstico *point-of-care*. En este trabajo se diseñaron, desarrollaron y aplicaron métodos moleculares basados en la tecnología LAMP para el diagnóstico de *Schistosoma mansoni* y *Schistosoma haematobium*, así como para la detección de caracoles infectados con *S. mansoni*.

En primer lugar, se desarrolló una técnica de amplificación de ADN de *S. mansoni*, denominada SmMIT-LAMP, que ha mostrado su efectividad para la detección del parásito en la fase aguda de la infección. Su aplicación en muestras humanas y de caracoles en un área endémica de baja transmisión en Brasil ha demostrado una alta sensibilidad en comparación con las técnicas de diagnóstico clásicas, así como su utilidad para la identificación de focos de transmisión de la enfermedad. Estos estudios se detallan en los trabajos 1 y 5.

A continuación se diseñó otro método LAMP (Sh-LAMP) para la detección de *S. haematobium.* Se puso a punto en muestras clínicas de orina en el laboratorio con y sin extracción previa de ADN (Rapid-Heat LAMPellet method) y posteriormente se evaluó con éxito en una zona de alta transmisión en Angola. Además, Sh-LAMP mostró una elevada reproducibilidad en un laboratorio de referencia. Estos estudios se encuentran recogidos en los trabajos 2 y 4.

Finalmente, Biompha-LAMP mostró ser una herramienta útil para la detección de *S. mansoni* en caracoles infectados experimentalmente. Este estudio se encuentra detallado en el trabajo 3.

Resumo

Esquistossomose, uma doença parasitária causada por trematódeos do gênero Schistosoma, é uma das principais doenças tropicais desantendidas. Atualmente não há um método diagnóstico adequado, como técnicas parasitológicas e sorológicas têm problemas de sensibilidade e especificidade. O diagnóstico molecular não é rotineiramente utilizada, particularmente em áreas endémicas e pobre. A técnica LAMP é uma amplificação de ADN é realizada sob condições isotérmicas. Tem as vantagens de ser rápida, sensível, específica, permite que os resultados de discriminação visual e pode ser aplicada como um diagnóstico point-of-care. Neste trabalho foram concebidos, desenvolvidos e aplicados métodos moleculares baseados na tecnologia LAMP para o diagnóstico de Schistosoma mansoni e Schistosoma haematobium, e para detectar caracóis infectados com S. mansoni.

Em primeiro lugar, uma técnica foi desenvolvida para a amplificação de ADN de S. mansoni, chamada SmMIT-LAMP, o qual provou ser eficaz para a detecção do parasita na fase aguda da infecção. A sua aplicação em amostras humanas e de caracois numa área endémica de baixa transmissão no Brasil tem mostrado alta sensibilidade em relação às técnicas convencionais de diagnóstico e sua utilidade para identificar focos de transmissão da doença. Estes estudos são detalhados no trabalho 1 e 5.

Em seguida, outro método LAMP (Sh-LAMP) para a detecção de S. haematobium foi concebido. Ele foi otimizado em amostras clínicas de urina no laboratório com e sem extracção de ADN (Rapid-Heat LAMPellet method) e, subsequentemente, avaliadas com sucesso numa zona de transmissão em Angola. Além disso, Sh-LAMP mostrou elevada reprodutibilidade no laboratório de referência. Estes estudos são recolhidos no trabalho 2 e 4.

Finalmente, Biompha-LAMP mostrou-se útil para a detecção de S. mansoni em caracóis infectados experimentalmente. Este estudo é o trabalho detalhado 3.

C.I

Capítulo I: Introducción

El tema principal de este trabajo es el desarrollo de nuevos métodos de diagnostico molecular aplicables en zona endémica de esquistosomosis, una de las enfermedades tropicales olvidadas más importantes. Por este motivo, el primer apartado de la introducción de esta tesis doctoral comenzará explicando brevemente el concepto y las principales características de las **enfermedades olvidadas**. Se enumerarán los patógenos que producen estas infecciones y daremos una visión global sobre el impacto que provocan en la salud.

Dado que la enfermedad objeto de estudio de este trabajo es la **esquistosomosis**, a continuación se describirá el parásito causante de la enfermedad, prestando atención a sus características morfológicas, su genoma, ciclo de vida y epidemiología, así como la patogenia y las manifestaciones clínicas. Se hará especial hincapié en los métodos de diagnóstico más habituales y los más innovadores, mostrando la necesidad del desarrollo de nuevas técnicas. Finalmente, se comentará brevemente las pautas terapéuticas y las medidas preventivas frente a la esquistosomosis. En este apartado nos centraremos especialmente en *Schistosoma mansoni* y *Schistosoma haematobium*, ya que son las dos especies de estudio en esta tesis doctoral.

Puesto que la técnica de diagnóstico desarrollada y evaluada es la **amplificación de ADN mediante LAMP** (*loop-mediated isothermal amplification*), en el último apartado se explicará con detalle el mecanismo, las principales características de la técnica, su utilización para la detección de diferentes enfermedades producidas por helmintos y su potencial aplicación como test de diagnóstico *point-of-care*

1.1. Enfermedades olvidadas

El grupo de las enfermedades olvidadas, o como indican sus siglas en inglés *Neglected Tropical Diseases* (NTDs), son un grupo heterogéneo de enfermedades transmisibles presentes en 149 países de las regiones tropicales y subtropicales del planeta. Son enfermedades fuertemente relacionadas con la pobreza que afectan mayoritariamente a las poblaciones más pobres y excluidas de la sociedad (WHO, 2017). Tras la Declaración del Milenio en el año 2000 empezó a gestarse el concepto de enfermedades olvidadas, pero no fue hasta 2005 cuando aparece por primera vez el término *"Neglected Tropical Disease*" en un artículo publicado en la revista *PLOS Medicine* (Molyneux et al., 2005). En principio fueron trece las enfermedades consideradas como NTDs (Hotez et al., 2006). Posteriormente, la Organización Mundial de la Salud (OMS) incluye cuatro más. (WHO, 2010). Recientemente se han añadido ectoparásitos, micosis profundas y envenenamiento por mordedura de serpiente, resultando un total de 20 las entidades incluidas como enfermedades olvidadas (Tabla 1).

Las características generales de las NTDs son (Hotez, 2011):

- Enfermedades que afectan especialmente a la población que vive bajo el umbral de la pobreza, particularmente en países del África Subsahariana, Asia, Latinoamérica y el Caribe (aproximadamente 1.400 millones de personas).
- Son de curso crónico, produciendo desajustes que dan como resultado problemas en el crecimiento infantil, en el desarrollo cognitivo, durante el embarazo o disminución de la capacidad de trabajo.
- A pesar de mostrar una elevada morbilidad, la mayoría de las NTDs se caracterizan por presentar una mortalidad relativamente baja.
- La mayoría de las NTDs son enfermedades que se conocen desde hace miles de años y están recogidas en diferentes textos antiguos (por ejemplo, la hematuria producida por la esquistosomosis urogenital se recoge en el papiro de Ebers en el año 1500 a.C. (Kloos y David, 2002).

Tabla 1. Listado de las 20 enfermedades tropicales olvidadas (NTDs) y número de personas que padecen las distintas enfermedades. Tomado de la página web de la OMS (http://www.who.int/neglected_diseases/diseases/en/).

Las 20 NTDs descritas por la OMS	**Prevalencia global aproximada (millones)**
Geohelmintosis (Ascaridiosis, Tricuriosis, Uncinariosis)	807 604 576
Esquistosomosis	207
Filariosis linfática	120
Oncocercosis	37
Dracunculosis	<0,01
Trematodosis de origen alimentario	20-40
Equinococosis	Sin datos
Teniosis y cisticercosis	Sin datos
Leishmaniosis	12
Enfermedad de Chagas	8-9
Tripanosomosis humana africana	<0,01
Tracoma	84
Lepra	0,4
Úlcera de Buruli	<0,01
Trepanomatosis endémica	Sin datos
Dengue y otras arbovirosis	50
Rabia	0,05
Micosis profundas	Sin datos
Ectoparásitos	Sin datos
Mordedura de serpiente	Sin datos

Las NTDs tienen especial impacto en determinados colectivos (Hotez, 2009). Afectan principalmente a la población africana y a los americanos afrodescendientes del comercio trasatlántico de esclavos (Lammie et al., 2007). También son vulnerables las poblaciones indígenas y las personas que viven o han vivido recientemente en situaciones de conflictos armados, o que han sufrido violaciones en sus derechos humanos (Hotez, 2008; Hotez y Thomson, 2009).

Para entender el impacto global de las NTDs, es necesario recurrir al concepto de DALYs (*Disability-Adjusted Life-Years*), el cual hace referencia al número de años de vida saludable perdidos por muerte o enfermedad. Se calcula que las NTDs producen entre 20 y 70 millones de DALYs (Hotez, 2011). Este

amplio rango se atribuye, entre otras razones, a la falta de consenso para estimar la morbilidad asociada a infecciones causadas por helmintos (King y Dangerfield-Cha, 2008; King et al., 2005).

Desde el punto de vista geográfico, las NTDs tienen lugar en regiones tropicales y subtropicales del planeta, especialmente en el África Subsahariana, Sudeste asiático y regiones tropicales de las Américas. En África es donde se encuentra la mayor carga de estas enfermedades y es normal encontrar áreas donde coexisten más de seis NTDs. En este continente tienen lugar una tercera parte de las geohelmintosis del mundo, casi todos los casos de esquistosomosis y oncocercosis, y todos los casos de dracunculosis y tripanosomosis humana africana (Hotez y Kamath, 2009). Latinoamérica y el Caribe también sufren altos índices de estas enfermedades, especialmente Brasil, donde a excepción de la enfermedad de Chagas, todas fueron importadas a lo largo de 500 años a través del tráfico trasatlántico de esclavos (Lammie et al., 2007). El sudeste asiático es responsable de una tercera parte de las geohelmintosis, la mayoría de los casos de trematodosis de origen alimentario y un elevado número de afecciones por Dengue y otras arbovirosis (Hotez y Ehrenberg, 2010).

Recientemente la OMS publicó el último informe sobre las NTDs, siendo las principales medidas para su controlla quimioterapia preventiva o PCT (*Preventive Chemotherapy)*, el control vectorial y veterinario, las mejoras en el suministro de agua potable y en el saneamiento público, la intensificación en la asistencia clínica y el desarrollo de métodos de diagnóstico más eficaces. Se espera que con estas medidas se alcancen los objetivos de control y eliminación de estas enfermedades para el año 2020 (WHO, 2017).

1.2. Esquistosomosis

La esquistosomosis es una enfermedad parasitaria producida por diferentes especies del género *Schistosoma* incluidas dentro del phylum *Platyhelminthes*, clase *Trematoda*, subclase *Digenea*, familia *Schistosomatidae*. Las 21 especies descritas hasta el momento se clasifican en 4 grupos (Tabla 2). *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum*, *S. japonicum* y *S. mekongi* son las que afectan principalmente a las personas (Webster et al., 2006).

Tabla 2. Grupos y especies del género *Schistosoma*. Tomada de Loker y Mkoji, 2005.

GRUPO ***S. haematobium***	**GRUPO** ***S. mansoni***	**GRUPO** ***S. japonicum***	**GRUPO** ***S. indicum***
S. haematobium	*S. mansoni*	*S. japonicum*	*S. indicum*
S. intercalatum	*S. rodhaini*	*S. mekongi*	*S. nasale*
S. bovis	*S. hippopotami*	*S. malayensis*	*S. spindale*
S. matthei	*S. edwardiense*	*S. sinensium*	*S. incognitum*
S. curassoni		*S. ovuncatum*	
S. margrebowiei			
S. leiperi			

1.2.1. Morfología y ciclo biológico

Los esquistosomas son trematodos dioicos sanguíneos que presentan dimorfismo sexual. El gusano macho tiene una hendidura o canal ginecóforo en el que aloja a la hembra, de morfología más estrecha y alargada (Figura 1).

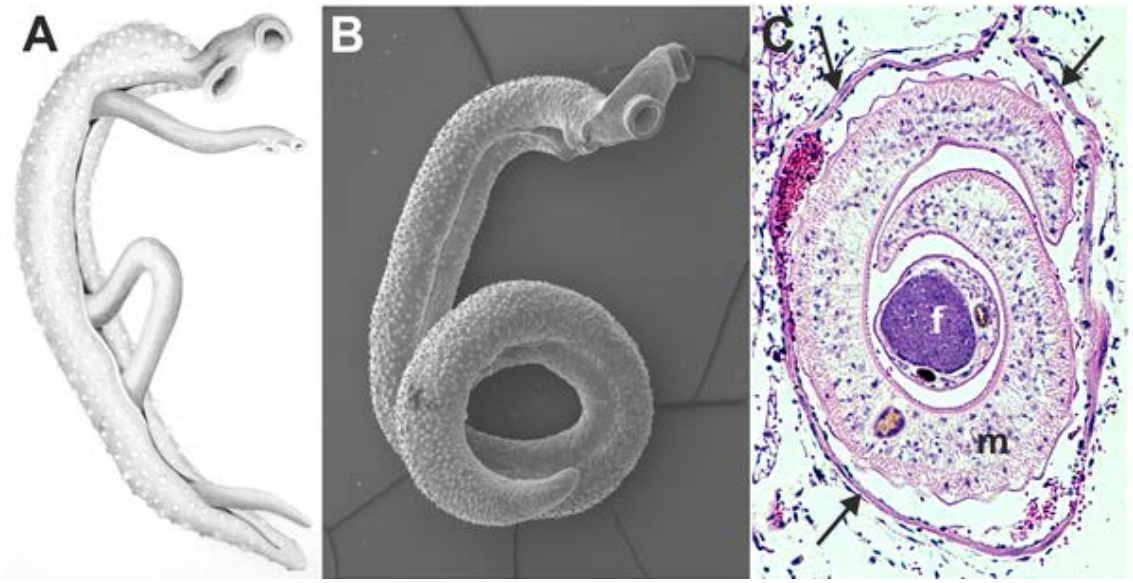

Figura 1. Imágenes de adultos de *Schistosoma* spp.. A) Dibujo de una pareja de adultos, B) fotografía de un macho mediante microscopía electrónica y C) preparación al microscopio de un corte transversal de una pareja de adultos. f, hembra, del inglés *female*; m, macho del inglés, *male*. Tomada de Mebius et al., 2013.

El ciclo de vida de todas las especies de esquistosomas se mantiene en un hospedador definitivo y en un caracol de agua dulce que actúa como hospedador

intermediario (HI) (Figura 2). Los humanos adquieren la infección al entrar en contacto directo con agua contaminada con la fase infectiva del parásito, llamada cercaria. De hecho, la esquistosomosis es considerada una de las principales enfermedades relacionadas con el agua (Steinmann et al., 2006). Esta fase larvaria de vida libre es capaz de atravesar activamente la piel del hospedador definitivo y llegar al torrente sanguíneo. Durante este proceso, la cercaria pierde la cola que le permite moverse en el medio acuático, transformándose en una nueva fase llamada esquistosómula, la cual inicia la migración a través de la sangre hasta alcanzar su localización definitiva. Tras producirse su maduración sexual, los machos y hembras se aparean, desplazándose hasta llegar a diferentes localizaciones: *S. haematobium* a las venas del plexo vesical y *S. mansoni* a las venas mesentéricas superiores. Los parásitos pueden permanecer en estas localizaciones durante 3-10 años, aunque se han descrito casos en los que han sobrevivido hasta 40 años (Chabasse et al., 1985). A continuación tiene lugar la reproducción y la consecuente puesta de huevos, los cuales son capaces de atravesar la pared vascular, entrar en el lumen vesical o intestinal y ser expulsados con la orina (en el caso de *S. haematobium*) o con las heces (en el caso de *S. mansoni*). Desde la penetración de las cercarias hasta la expulsión de los huevos pueden transcurrir cerca de 90 días en el caso de *S. haematobium* y entre 4 y 6 semanas en el de *S. mansoni* (Secor, 2005).

Los huevos son de morfología no operculada y poseen una “espina” que varía en tamaño y posición dependiendo de la especie (*S. haematobium* tiene una espina terminal y *S. mansoni* una espina lateral). Un pequeño porcentaje de los huevos queda atrapado en diferentes tejidos formando granulomas que son la causa de los daños originados en la fase crónica de la enfermedad. Los huevos eclosionan cuando entran en contacto con el agua y liberan una nueva fase larvaria llamada miracidio, capaz de nadar hasta encontrar e invadir al hospedador intermediario. La relación entre la especie de caracol y la especie de esquistosoma es género-especie específica, es decir, *S. mansoni* infecta únicamente a ciertas especies del género *Biomphalaria* y *S. haematobium* a especies del género *Bulinus*. Dentro del caracol tiene lugar una serie de reproducciones asexuales que finalizan con la emisión de cercarias a las colecciones acuáticas. Estos hospedadores

intermediarios pueden expulsar cientos de cercarias de forma continua, actuando como amplificadores de la enfermedad (Stensgaard et al., 2013).

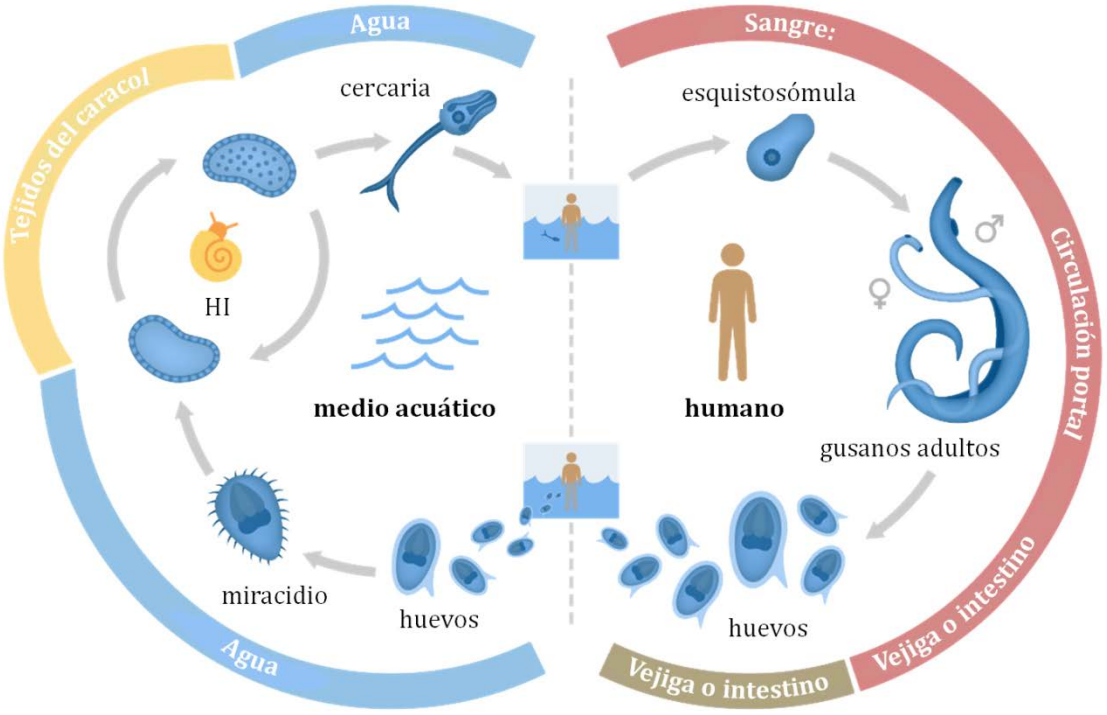

Figura 2. Ciclo biológico de los esquistosomas. Adaptado de Genome Research Limited (http://www.yourgenome.org/facts/what-is-schistosomiasis).

1.2.2. Genoma, transcriptoma y proteoma

En 1994 la OMS en colaboración con *Schistosoma Genome Network* comenzó el proyecto para secuenciar el genoma de *S. mansoni*. Finalmente en 2009 se describe la secuencia completa y se realiza el mapeo de los genes (Berriman et al., 2009). Tres años más tarde se describe el genoma completo de *S. haematobium* (Young et al., 2012). El tamaño del genoma de *Schistosoma* es mayor que el de la mayoría de los patógenos descritos con 385 Mb para *S. haematobium* y 381 Mb para *S. mansoni* (LoVerde et al., 2004). Prácticamente la mitad del genoma ha sido mapeado utilizando la técnica de hibridación "in situ" (FISH) y ambas especies presentan 8 pares de cromosomas, incluyendo 7 autosomas y un cromosoma sexual. La mayoría de los genes descritos se componen de grandes intrones y exones mucho más cortos. Además, los intrones en el extremo 5' son más pequeños que los intrones del extremo 3' (Tabla 3). Una característica a resaltar es el gran

contenido de secuencias repetitivas como hecho importante desde el punto de vista de la relación evolutiva y filogenética con el hospedador (Crellen et al., 2016).

Tabla 3. Características principales del genoma de *S. haematobium* y *S. mansoni*. Adaptada de Young et al., 2011.

Características del genoma	***S. haematobium***	***S. mansoni***
Tamaño aproximado del genoma (Mb)	385	381
Número de cromosomas (2**n**)	8	8
Proporción codificante del genoma (%)	4,43	4,72
Número atribuido de genes	13.073	13.184
Tamaño de los genes (media de pb ± s.d.; rango)	11.952 ± 16.273; 30–20	13.397 ± 18.029; 84–240.193
Longitud de dominios codificantes (media pb ± s.d.; rango)	1.319 ± 1.502; 30–28.212	1.344 ± 1.447; 60–22.983
Número de exones por gen (media de pb ± s.d.; rango)	5,4 ± 5,80; 1–136	6,2 ± 6,24; 1–94
Longitud de los exones (media de pb ± s.d.; rango)	246 ± 287; 1–9.737	218 ± 236; 1–9.291
Longitud de los intrones (media pb ± s.d.; rango)	2.442 ± 2.958; 1–68.754	2,331 ± 3,200; 1–67.221
Contenido en GC (%)	34,3	34,7
Grado de repetición (%)	47,2	45

La descripción del transcriptoma de *S. mansoni* comenzó a realizarse mediante lo que se conoce como *Expressed Sequence Tag* (EST) a partir de librerías de ADNc obtenidas mediante RT-PCR, en las que se amplificó la región codificante de los ARNm. Recientemente, gracias a la tecnología de RNA-seq (Mortazavi et al., 2008) se están estudiando los diferentes perfiles transcripcionales dependiendo de la fase del ciclo en el que se encuentre el parásito. A partir de los resultados obtenidos se hallaron evidencias de cómo los esquistosomas interaccionan con las señales inmunológicas y endocrinas del hospedador. Un hecho que apoya esta teoría es que el 20% de sus genes muestran similitud con secuencias descritas en

mamíferos (Hu et al., 2003). Los resultados de estos estudios están recogidos en diferentes bases de datos como GeneDB (www.genedb.org) (Logan-Klumpler et al., 2012) y SchistoDB (www.schistodb.net) (Zerlotini et al., 2013). Por otro lado, hay múltiples trabajos que han estudiado la composición proteica de las distintas fases del ciclo de *S. mansoni* (Curwen et al., 2004), siendo las de mayor interés las moléculas que interactúan con el sistema inmunitario durante la infección ya que pueden ser dianas para el desarrollo de fármacos y vacunas eficaces contra la esquistosomosis. Recientemente se acaba de publicar el genoma de *Biomphalaria glabrata*, el cual posee un tamaño de 916 Mb distribuidos en 18 cromosomas que contienen 14.423 genes. El estudio de los diferentes perfiles de expresión de los genes ha ayudado a identificar posibles marcadores moleculares que intervienen en la interacción con *S. mansoni* durante la infección, avanzando en el conocimiento de la relación parásito-hospedador (Adema et al., 2017).

1.2.3. Epidemiología

La esquistosomosis es una de las enfermedades más prevalente de las zonas tropicales y subtropicales del planeta, teniendo serias consecuencias en la salud global y produciendo un grave impacto económico y social en los países en desarrollo (King et al., 2005). Se calcula que hay más de 200 millones de personas infectadas en 78 países diferentes (la mayoría en el África subsahariana), 120 millones con sintomatología, 20 millones con enfermedad grave y una mortalidad anual que supera las 200.000 muertes al año (WHO, 2014). La mayor prevalencia en áreas endémicas ocurre entre los adolescentes y disminuye generalmente en la edad adulta. Se calcula un 60-80% de infección activa en niños de edad escolar y un 20-40% en adultos (Colley et al., 2014). Algunos autores señalan el gran impacto de la morbilidad causada por la esquistosomosis, reflejada en una pérdida de 3,3 millones de DALYs (Murray et al., 2012).

S. haematobium y *S. mansoni* son las especies más importantes desde un punto de vista global ya que entre las dos acumulan más del 90% de los casos de esquistosomosis en el mundo (WHO, 2017). La primera es el único agente causal de la esquistosomosis urogenital y supone más de la mitad de los casos de la enfermedad. Por otro lado, *S. mansoni* es la especie más importante en términos de morbilidad y distribución geográfica causante de la esquistosomosis intestinal y

hepática (King, 2010), siendo la única especie presente en el continente americano (Figura 3).

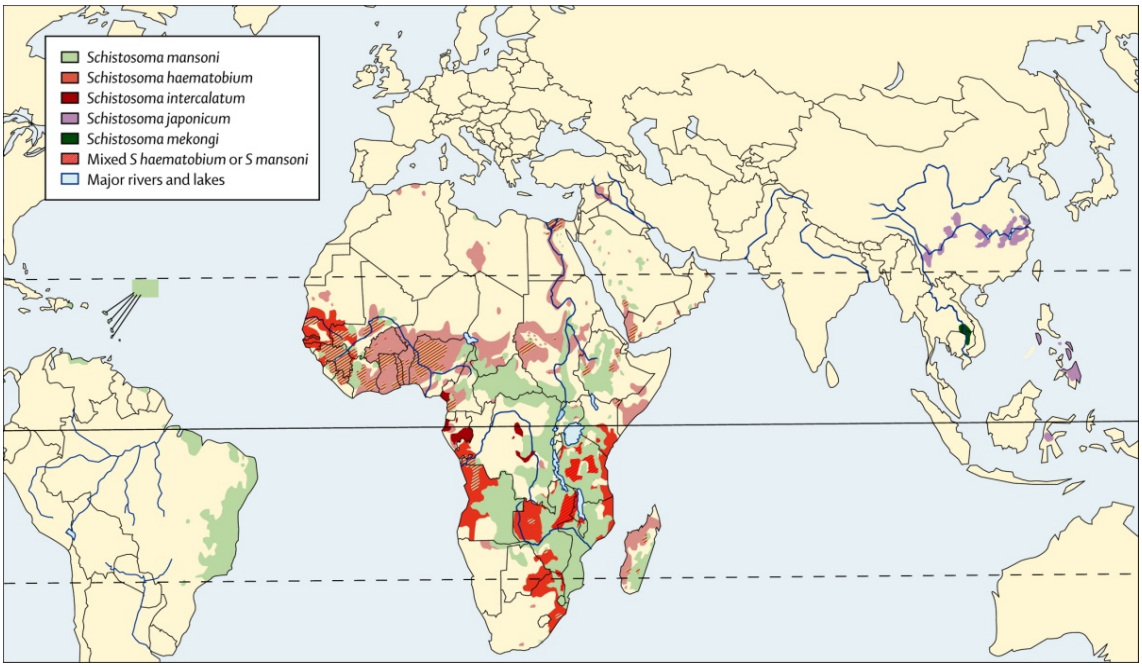

Figura 3. Distribución geográfica de las diferentes especies de esquistosomas. Tomada de Colley et al., 2014.

Para que ocurra una correcta transmisión de la esquistosomosis deben concurrir una serie de condiciones. La primera y más importante es que existan poblaciones de caracoles susceptibles de mantener el ciclo biológico y transmitir la enfermedad. Estos hospedadores son resistentes a la sequía, a la contaminación, y a los cambios climáticos. Si en un área se encuentra una especie de caracol que presenta resistencia natural a la infección la tasa de transmisión será menor (Carvalho et al., 2008). Normalmente las poblaciones de caracoles son estables y la enfermedad presenta un carácter fuertemente endémico y local. Sin embargo, cambios en las condiciones hidrográficas como inundaciones o las construcciones de grandes presas (como la de Aswan en Egipto o diferentes presas en Nigeria) han permitido que los caracoles proliferen y se expandan llevando la enfermedad a zonas no endémicas de esquistosomosis (Malek, 1975; Oladejo y Ofoezie, 2006). Otra característica importante para la transmisión es su asociación a la falta de desarrollo. Para que siga manteniéndose el ciclo de vida del parásito se requiere la

contaminación del agua con heces y orina por falta de un saneamiento adecuado. La exposición al agua contaminada suele asociarse a actividades diarias y rutinarias como el lavado de utensilios y el baño, así como a actividades relacionadas con la agricultura, la pesca o la ganadería (King, 2010). Al contrario de lo que cabría esperar, la transición demográfica rural-urbana no ha eliminado la enfermedad, si no que en las periferias de las grandes ciudades de Sudamérica y África se mantiene la transmisión de la esquistosomosis (Barbosa et al., 2010; Ugbomoiko et al., 2010).

Debido al contexto globalizado en el que vivimos, es importante señalar el incremento de casos de esquistosomosis importada como consecuencia de la movilidad internacional. Ya sea por viajeros, expatriados por motivos laborales o por la emigración sur-norte, cada vez es más frecuente encontrar casos de esquistosomosis en países no endémicos. De hecho, según datos de *GeoSentinel*, la esquistosomosis es la causa de morbilidad en el 4% de los viajeros que regresan del África subsahariana (Freedman et al., 2006). Un estudio realizado en 788 inmigrantes subsaharianos que presentaban eosinofilia absoluta describe que el 17% padecían esquistosomosis (Pardo et al., 2006). Asimismo, esta helmintosis fue la primera causa descrita en otro estudio llevado a cabo en inmigrantes subsaharianos que presentaban eosinofilia relativa (Carranza-Rodríguez et al., 2008).

Este fenómeno tiene gran importancia a nivel epidemiológico. En el año 2014 una niña de 4 años acudió a consulta médica en Toulouse, Francia, por presentar hematuria. A pesar de no haber viajado a zona endémica, tras una serie de exámenes se encontraron huevos de esquistosomas alojados en la pared vesical. Fue el primer caso reportado de transmisión endémica de esquistosomosis en Europa, particularmente en Córcega (Berry et al., 2014). En la isla hay caracoles del género *Bulinus* capaces de servir como hospedador intermediario a las especies del grupo *S. haematobium*. Posteriormente, se registraron más casos en Francia, Alemania e Italia, en los que todos los pacientes habían contraído la enfermedad también en la isla de Córcega. Más de 120 personas, entre residentes en el área y turistas, contrajeron esquistosomosis urinaria. Tras una serie de estudios moleculares estudiando los genes correspondientes a COX-1 e ITS se vio que las

especies causantes eran *S. haematobium*, híbridos *S. haematobium-S. bovis* y *S. bovis*. Un análisis más exhaustivo de las secuencias de ADN mostró la estrecha relación entre los parásitos encontrados en la isla y los procedentes de África occidental. De hecho, se asocia la introducción de la esquistosomosis en Europa con la llegada de inmigrantes infectados procedentes de Senegal, los cuales activaban el ciclo biológico del parásito al orinar en el río (Boissier et al., 2016).

1.2.4. Patogenia y manifestaciones clínicas

Los **mecanismos de agresión** se pueden dividir en varias fases: *(i) fase de penetración cercariana* (Mountford y Trottein, 2009): este complejo proceso necesita de la presencia de estímulos químicos por parte del hospedador y de la liberación de proteasas secretadas por la cercaria. El componente mayoritario de estas proteasas es una serin-proteasa de 30 kDa cuya actividad elastasa degrada la elastina de la piel facilitando la penetración de la cercaria. Varios estudios realizados en la piel demuestran que los productos de excreción-secreción cercarianos inducen la formación de edema e infiltración de neutrófilos, además de ser capaces de degradar otras macromoléculas presentes en la piel del hospedador como la queratina, fibronectina, laminina o colágeno. Sin embargo, esta reacción inflamatoria no induce protección en el individuo sino que favorece la supervivencia del parásito. *(ii) Fase de migración de la esquistosómula* (Caldas et al., 2008): durante las 4-6 semanas siguientes a la infección, la esquisotosómula migra por la circulación sanguínea hasta llegar a su sitio de maduración, produciéndose altos niveles de citocinas pro-inflamatorias (principalmente TNFα, IL-1, IL-6 e IFNγ) cuya máxima expresión se produce a la decimosexta semana post-infección. Esta situación se asocia a una respuesta Th1 predominante, responsable de la fiebre de Katayama. *(iii) Fase de formación del granuloma alrededor del huevo* (Pearce y Mac Donald, 2002): esta fase tiene lugar a partir de la séptima semana post-infección. Aunque una gran cantidad de huevos son liberados al exterior, parte de ellos quedan atrapados en el hígado, intestino o vejiga urinaria e inducen una respuesta inmunológica responsable de la patogenia de la fase crónica de la esquistosomosis. Alrededor de ellos se produce un infiltrado celular compuesto por macrófagos, eosinófilos, linfocitos CD4+ y colágeno que da lugar a una amplia reacción granulomatosa.

Desde un punto de vista general, **las manifestaciones clínicas** también se puede dividir en tres fases: fase de inicio, fase aguda y fase crónica.

La *fase de inicio* o también denominada dermatitis cercariana, se caracteriza por el desarrollo de prurito en las primeras 24 horas siguientes a la penetración de las cercarias a través de la piel. Se produce una respuesta de hipersensibilidad mediada por IgE que ocurre en el 7-36% de los pacientes infectados (Kolarova et al., 2013). Se observa más en viajeros y pasa desapercibida en personas residentes en áreas endémicas (Freedman et al., 2006).

La *fase aguda* o Síndrome de Katayama se origina entre las 2-8 semanas después de la exposición y se debe a la reacción inmunológica desencadenada frente a la fase de migración de la esquistosómula. Se caracteriza principalmente por fiebre, lesiones cutáneas (exantema, urticaria), afectación pulmonar (tos, disnea) y eosinofilia (Ross et al., 2007). En general es autolimitada, aunque en algunas ocasiones los síntomas pueden persistir más de 10 semanas presentando diarrea, pérdida de peso, dolor abdominal, hepatoesplenomegalia, etc. Radiológicamente aparecen infiltrados pulmonares y engrosamiento bronquial en radiografía de tórax y nódulos hepáticos hipoecogénicos o hipodensos en ecografía y tomografía computerizada. El síndrome de Katayama es más frecuente en viajeros procedentes de áreas endémicas y se ha descrito clásicamente en infecciones con alta carga parasitaria, aunque actualmente se sabe que no es especie-específica ni guarda relación con la intensidad de la infección (Ross et al., 2013).

La *fase crónica* se presenta con más frecuencia en personas residentes en áreas endémicas de esquistosomosis. Aparece meses o años después de la infección y se debe a la reacción granulomatosa formada en torno a los huevos atrapados en hígado, bazo, intestino, vejiga urinaria y otras localizaciones más lejanas como pulmones y sistema nervioso. Las personas sin exposición previa pueden desarrollar esquistosomosis crónica tras una corta exposición, observada hasta en un 20% de los viajeros, aunque las complicaciones graves se producen siempre tras infecciones repetidas y con alta carga parasitaria. Sus principales manifestaciones clínicas se pueden agrupar en (Gryseels et al., 2006): *(i) manifestaciones habituales*: presencia de eosinofilia y de microhematuria (en las

infecciones ocasionadas por *S. haematobium*). *(ii) Manifestaciones clásicas frecuentes*: las más típicas son las urinarias, las hepatoesplénicas y las intestinales. Las manifestaciones urinarias se producen principalmente por la localización de los huevos de *S. haematobium* en plexos venosos del tracto urinario, provocando inflamación granulomatosa, ulceración y poliposis en la mucosa uretral y vesical. Cursan clínicamente con un síndrome miccional irritativo con disuria, polaquiuria, proteinuria y especialmente hematuria terminal. Suele ser más sintomática en niños y adultos jóvenes. El cuadro puede complicarse con litiasis y sobreinfección bacteriana. Si la enfermedad progresa puede provocar fibrosis y calcificación de uréteres y vejiga, produciendo hidrouréter e hidronefrosis (Kahlaf et al., 2012) (Figura 4A). Si se afecta el parénquima se puede producir fracaso renal. Una complicación grave de la esquistosomosis urinaria es la aparición de carcinomas malignos de las células escamosas vesicales (Parkin, 2006). Las manifestaciones hepatoesplénicas se producen cuando los huevos de *S. mansoni* localizados en las venas mesentéricas acceden a la circulación venosa portal, ocasionando una oclusión gradual de las venas intrahepáticas que puede causar fibrosis de Symmers. Esto da lugar a una hipertensión portal con ascitis, esplenomegalia, desarrollo de varices esofágicas con sangrado digestivo alto y encefalopatía (Figura 4B). Teniendo en cuenta la localización de las lesiones, no aparecen datos de insuficiencia hepatocelular. Todo ello se manifiesta sin signos o estigmas de hepatopatía como ictericia, arañas vasculares, eritema palmar, atrofia testicular o ginecomastia. Las manifestaciones intestinales se producen cuando los huevos atraviesan la pared intestinal produciendo hiperplasia, ulceración, formación de microabscesos y poliposis. Las lesiones se localizan frecuentemente en el intestino delgado y en el recto. Clínicamente presentan dolor abdominal y diarrea de tipo inflamatorio con o sin sangre (Gray et al., 2011). En ocasiones puede complicarse produciendo una enteropatía, cuadro oclusivo o suboclusivo, prolapso anal o incluso fístulas anorectales. Todo esto plantea un diagnóstico diferencial con la enfermedad inflamatoria intestinal.

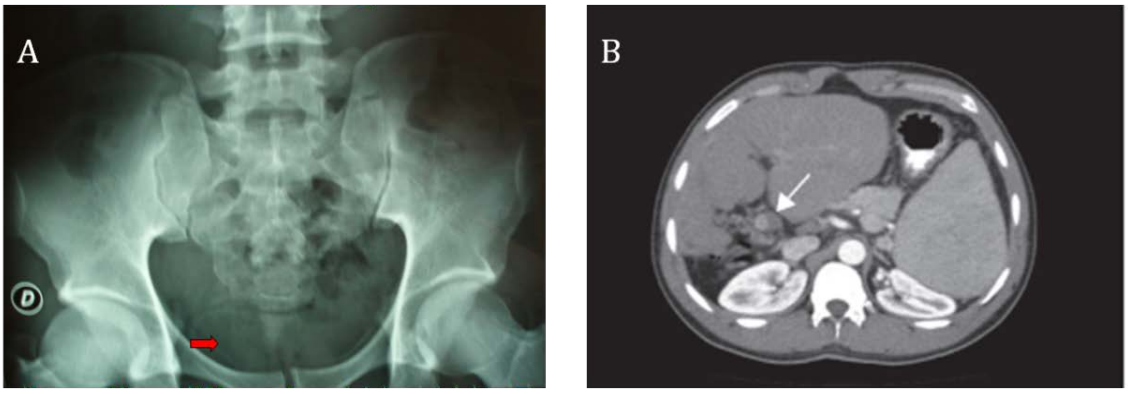

Figura 4. (A) Calcificación de la vejiga (flecha roja) en la esquistosomosis urinaria en radiografía de abdomen; tomada de Muro et al., 2010. (B) TAC de un paciente con hepatoesplenomegalia debido a una esquistosomosis intestinal crónica y trombosis portal secundaria (flecha blanca); tomada de Pérez del Villar Moro et al., 2012b.

(iii) Manifestaciones clásicas poco frecuentes: se denominan también esquistosomosis ectópicas. Las más frecuentes son las cardiopulmonares, renales, genitales y las de afectación del sistema nervioso central. Las manifestaciones cardiopulmonares se producen como consecuencia de la salida de huevos desde las venas vesicales (en el caso de *S. haematobium*) o desde la circulación portal a través del *shunt* porto-cava (en el caso de *S. mansoni*). Como consecuencia de la localización de los huevos en la circulación pulmonar se produce fibrosis de las arterias pulmonares presentándose hipertesión pulmonar con insuficiencia cardíaca derecha (Bethem et al., 1997). Ocasionalmente la esquistosomosis provoca lesiones a nivel parenquimatoso renal afectando específicamente el glomérulo, originando glomerulonefritis (Barsoum, 2004). En general la glomerulonefritis se asocia a la existencia de una esquistosomosis hepatoesplénica con *shunt* porto-sistémico. El tipo más frecuente es la glomerulonefritis mesangial que suele presentarse con una discreta proteinuria y microhematuria. Las lesiones a nivel genital son de dos tipos: inflamatorias en relación a huevos viables y fibrosas e hipertróficas en relación a huevos no viables o calcificados. En mujeres afecta a vulva, vagina y cérvix. Son indoloras pero en el caso de ulcerarse, fistulizarse o sobreinfectarse pueden producir dispareunia y leucorrea (Poggensee et al., 2000) que facilitan la transmisión de otras enfermedades infecciosas, como el VIH. En los hombres las más frecuentes son la prostatitis crónica y la infección de las vesículas seminales. La clínica más habitual es la alteración en la eyaculación

y la hematoespermia (Corachán et al., 1994). Las dos manifestaciones más típicas de la neuroesquistosomosis son el síndrome cerebral y el medular. La localización de los huevos de *Schistosoma* spp. en territorio cerebral puede debutar como una crisis comicial y suele progresar hasta una encefalitis focal cerebral. Se describe hasta en un 6% y se relaciona en menor medida con *S. haematobium* (Ferrari, 2008). La localización medular de los huevos de esquistosoma es más frecuente que la encefálica y puede producir varias formas de mielitis siendo las más características la mielitis granulomatosa, la mieloradiculitis, la mielitis isquémica o vascular, siendo la más frecuente la mielitis transversa. Los pacientes presentan de forma prácticamente constante una vejiga neurógena asociada en ocasiones a un nivel sensitivo-motor con debilidad en piernas, incapacidad para la marcha, dolor lumbar y parestesias (Ross et al., 2012).

Además, todas las especies de esquistosomas provocan afectaciones sistémicas debilitantes e inespecíficas como consecuencia de la inflamación continuada y alteraciones en el metabolismo (Bustinduy et al., 2011), provocando malnutrición, anemia, retraso en el crecimiento y trastornos en el desarrollo intelectual (King, 2008).

1.2.5. Diagnóstico de la esquistosomosis

Actualmente disponemos de un amplio arsenal de técnicas diagnósticas, desde técnicas parasitológicas básicas hasta métodos moleculares altamente sofisticados. Se pueden agrupar en tres categorías principales: (*i*) diagnóstico parasitológico, (*ii*) diagnóstico inmunológico y (*iii*) diagnóstico basado en la detección de ácidos nucleicos o molecular.

1.2.5.1. Diagnóstico parasitológico

Los métodos parasitológicos fueron los primeros empleados para el diagnóstico de la esquistosomosis. Están basados en la detección de huevos en heces (en el caso de la esquistosomosis intestinal y hepática) o en orina (en el caso de la esquistosomosis urogenital). No son apropiados para el diagnóstico temprano de la enfermedad, ya que la puesta de huevos se inicia al menos a la cuarta semana post-infección (Wang et al., 2011).

Para la detección microscópica de huevos en heces se utiliza la técnica de Kato-Katz (KK). Fue desarrollada en 1972 (Katz et al., 1972) y actualmente está recomendada por la OMS como *gold standard* (WHO, 2013). Es sencilla, de bajo coste, muy específica, permite estimar la intensidad de la infección y puede ser realizada por personal con un entrenamiento relativamente básico. Sin embargo, cuando la carga parasitaria es baja la sensibilidad del test disminuye notablemente (Enk et al., 2008; Kongs et al., 2001). No obstante, la sensibilidad puede mejorarse si se aumenta el número de láminas examinadas en muestras recogidas en días consecutivos (Sayasone et al., 2015). Se han desarrollado otros métodos como la concentración con formol-éter (Polderman et al., 1994), la flotación con una solución saturada de cloruro sódico (Glinz et al., 2010) o la técnica del FLOTAC (Truant et al., 1981; Coulibaly et al., 2016), la cual permite analizar una cantidad de muestra mayor, pero necesita cierto nivel de infraestructura en el laboratorio, lo que dificulta su aplicación en campo (Cringoli et al., 2010). También hay una técnica que tras provocar la enclosión de los huevos permite observar el miracidio al atraerlos a una fuente de luz (Zhu et al., 2014).

Para la detección de huevos de *S. haematobium* se utiliza la filtración o la sedimentación/centrifugación de la orina y posterior observación bajo el microscopio. Al igual que en la detección de huevos en heces, el principal problema es la baja sensibilidad, especialmente en infecciones leves (Stete et al., 2012). Por otro lado, la presencia de proteinuria y principalmente hematuria son indicadores que se han usado en el diagnóstico de la esquistosomosis urogenital (Morenikeji et al., 2014). Las tiras reactivas de análisis de orina en combinación con cuestionarios de información demográfica se han utilizado ampliamente como tests de diagnóstico rápido, sencillo y de bajo coste (Bogoch et al., 2012). Sin embargo, la hematuria no es un signo específico de la esquistosomosis ya que se puede presentar en otras patologías. Por eso, la efectividad de las tiras reactivas está directamente relacionada con la prevalencia de la enfermedad (Krauth et al., 2015).

1.2.5.2. Diagnóstico inmunológico

El diagnóstico inmunológico consiste en la detección de anticuerpos anti-esquistosomas o la detección de antígenos de esquistosomas en diferentes fluidos

(orina, suero, plasma o esputo). Hay una gran variedad de tests diagnósticos con diferente nivel de sofisticación. Estos métodos son particularmente útiles cuando los tests parasitológicos no son efectivos, especialmente en situaciones donde la carga parasitaria es muy baja (Alarcón de Noya et al., 2007). Muchas de las técnicas inmunológicas se han desarrollado con la finalidad de su aplicación para que sirvan como *point-of-care tests* (POCT).

Se han desarrollado técnicas para la **detección de anticuerpos** contra las diferentes fases del parásito. Estas técnicas son capaces de diagnosticar infecciones por *S. mansoni* y *S. haematobium* y han resultado útiles en zonas de baja endemicidad (Gonçalves et al., 2006).

El *test de precipitación circumoval* o COPT detecta anticuerpos en suero de pacientes infectados empleando huevos del parásito liofilizados (Rodríguez-Molina et al., 1962). Ha resultado ser sensible y específico y empleándolo junto con la microscopía ha mejorado notablemente la eficacia del diagnóstico (Carvalho et al., 2014a). Las principales desventajas del COPT son la complejidad de la técnica y que el test continúa siendo positivo una vez que se ha eliminado el parásito del organismo (Doenhoff et al., 2004).

El *test de inmunofluorescencia indirecta* o IFI detecta anticuerpos en suero empleando secciones de gusanos adultos, huevos o cercarias mediante microscopía de fluorescencia (Azab et al., 1984). Se ha utilizado solo o en combinación con otros métodos de diagnóstico en zonas de baja prevalencia. Este test es de difícil aplicación ya que este tipo de microscopía y los reactivos, así como el entrenamiento de los técnicos de laboratorio, suponen una barrera para su uso en zonas endémicas (Burlandy-Soares et al., 2003).

La *técnica de hemaglutinación indirecta* o IHA es capaz de detectar anticuerpos de pacientes infectados empleando eritrocitos recubiertos de antígenos de esquistosomas. Gracias a su simplicidad, este test se ha utilizado para estudiar la prevalencia y la vigilancia en áreas endémicas. Sin embargo, a parte del elevado título de anticuerpos tras el tratamiento, ha mostrado mucha reactividad cruzada con otras especies de helmintos, lo que implica una baja especificidad (Shorgo et al., 2005).

La *técnica de ELISA* utilizaba en un primer momento extractos crudos de adultos o huevos como antígenos, dando reactividad cruzada con otros helmintos (Sarhan et al., 2014). Posteriormente se desarrollaron ELISAs con proteínas o fracciones de proteínas purificadas para mejorar la especificidad de la técnica (Dunne et al., 1984; Carvalho et al., 2014b; Grenfell et al., 2013; Tanigawa et al., 2015). Existe una proteína de *S. mansoni* denominada SmCFT (*Cercarial Transformation Fluid*), utilizada mediante ELISA para el diagnóstico de *S. mansoni* y *S. haematobium* (Smith et al., 2012) y que posteriormente se adaptó como test de diagnóstico rápido o RDT (*Rapid Diagnostic Test*). La técnica mostró la misma sensibilidad que dos láminas de KK para el diagnóstico de *S. mansoni* y una filtración de orina para *S. haematobium*. Sin embargo, es necesario evaluar esta técnica en otro tipo de situaciones, como en zonas de baja endemicidad o tras una campaña post-tratamiento (Coulibaly et al., 2013a; Nausch et al., 2014).

Actualmente existen diferentes *kits* comerciales basados en la detección de anticuerpos (ELISA e IHA), utilizándose en la práctica clínica particularmente en países no endémicos, para diagnosticar casos de esquistosomosis importada. No obstante, la especificidad de estos *kits* continúa siendo baja (Kinkel et al., 2012).

La **detección de antígenos** de esquistosomas es un método de diagnóstico directo utilizado en diferentes fluidos biológicos (suero, orina, etc). Los dos antígenos más empleados son el antígeno circulante catódico (CCA) y el antígeno circulante anódico (CAA), llamados así por su comportamiento cuando son sometidos a inmunoelectroforesis (Gonçalves et al., 2006). Son antígenos género-específicos, es decir, idénticos para todas las especies de esquistosomas y se excretan durante la regurgitación de la sangre tras ser ingerida por los gusanos adultos. Son útiles para evaluar la eficacia post-tratamiento ya que dejan de detectarse (Agnew et al., 1995; Stohard, 2009). Además, se identifican a la 3ª semana post-infección, permitiendo realizar el diagnóstico en fase aguda (Van Dam et al., 1996).

En los últimos años se utiliza una tira reactiva comercial como test de diagnóstico rápido que detecta CCA en orina para el diagnóstico de *S. mansoni* (Colley et al., 2013). Desafortunadamente, este test no es efectivo para la detección de *S. haematobium* (Ochodo et al., 2015) y por tanto no es útil en áreas donde

cohabitan ambas especies (Ashton et al., 2011). Se cuestiona su aplicación en áreas de baja prevalencia, ya que su positividad está directamente relacionada con la intensidad de la infección (Tchuem et al., 2012). Sin embargo, ha demostrado su utilidad en zonas de media y alta endemicidad y se puede emplear para estimar la eficacia al tratamiento (Colley et al., 2013; Coulibaly et al., 2013b).

También se ha empleando la tecnología UCP-LF (*Up-Converting Phosphor-Lateral Flow*) para detectar CAA en suero y orina y poder diagnosticar la esquistosomosis urogenital de forma rápida y sencilla (Corstjens et al., 2008). Un estudio reciente demuestra que es más sensible que la microscopía (Knopp et al., 2015). Sin embargo, requiere un mayor desarrollo técnico para su aplicación en condiciones de campo (Corstjens et al., 2014).

1.2.5.3. Diagnóstico molecular

El diagnóstico molecular consiste en la detección de ácidos nucleicos (ADN o ARN) mediante técnicas de amplificación, principalmente la reacción en cadena de la polimerasa o PCR y todas sus variantes.

La detección de ADN de esquistosomas en muestras de heces, orina o biopsias de tejidos se ha empleado como diagnóstico altamente sensible y específico. La PCR convencional diseñada sobre segmentos de genes nucleares es más sensible que la microscopía, tanto en heces para el diagnóstico de *S. mansoni* (Pontes et al., 2002; Pontes et al., 2003), como en orina para el diagnóstico de *S. haematobium* (Ibironke et al., 2011). Se ha aumentando la sensibilidad de la técnica estudiando genes mitocondriales, debido al elevado número de copias que existen en cada célula. Además, al ser secuencias únicas de cada especie, presentan un alto grado de especificidad (Gobert et al., 2005). También se han desarrollado y aplicado con excelentes resultados diferentes variantes de PCR, como Touchdown-PCR (Helmy, 2007) o ELISA-PCR (Gomes et al., 2010). La técnica de PCR a tiempo real (qPCR) presenta una serie de ventajas en comparación con la PCR convencional: detecta concentraciones más bajas de ADN, permite realizar un análisis cuantitativo, y es más rápida ya que no requiere de electroforesis para visualizar los resultados. La qPCR se ha empleado para diferenciar las distintas especies de esquistosomas (Sady et al., 2015; Vinkeles et al., 2014) y para estimar la intensidad de la infección (Gomes et al., 2006; Pillay et al., 2014). Además, se ha

desarrollado una qPCR-múltiplex que permite la amplificación simultánea de ADN de *S. mansoni* y *S. haematobium* (Ten Hove et al., 2008).

La detección mediante PCR del ADN libre circulante o CFPD (*Cell-Free Parasite DNA*) también se ha sido utilizado en el diagnóstico molecular de la esquistosomosis. Este ADN proviene de la muerte de las esquistosómulas durante su migración, de la renovación del tegumento de los gusanos adultos o de la desintegración de los huevos inactivos (Xu et al., 2013). Una vez que el CFPD está en circulación puede detectarse en suero y plasma (Pontes et al., 2002). Además, pequeños fragmentos de ADN se pueden filtrar y aparecer en la orina (Sandoval et al., 2006), saliva o líquido cefalorraquídeo (Kato-Hayasi et al., 2013). Esta técnica es más sensible que los métodos parasitológicos y que la detección de antígenos en orina, demostrando su utilidad en la fase prepatente (Lodh et al., 2013; Lodh et al., 2014; Suzuki et al., 2006). Asimismo, se ha usado para el diagnóstico de nueroesquistosomosis (Härter et al., 2014). Es importante señalar que la detección de CFPD en orina o en saliva elimina los inconvenientes de obtener y trabajar con muestras de sangre (Sandoval et al., 2006; Wichmann et al., 2013). Sin embargo, los pequeños fragmentos de ADN en este tipo de muestras son muy susceptibles a la degradación enzimática y tras un periodo largo de almacenaje en congelación las muestras no son aptas para ser utilizadas (Fernandez-Soto et al., 2013).

El uso de la PCR y sus variantes ha sido de gran utilidad y algunos autores las proponen como técnicas *gold standard* para el diagnóstico de la esquistosomosis (Meurs et al., 2015). No obstante, son muy caras y requieren de personal y equipamiento especializados por lo que no son útiles para el diagnóstico bajo condiciones de campo y su uso queda restringido a unos pocos laboratorios de referencia.

Estudios recientes han demostrado que los micro-ARNs (miARNs) pueden emplearse como biomarcadores de la infección. Los miARNs son pequeñas moléculas de ARN no codificantes producidas por diferentes tipos de seres vivos que desarrollan un papel fundamental en procesos fisiológicos y patológicos. Se incorporan en el complejo silenciador inducido por ARN mediante unión específica al ARN mensajero, inhibiendo su traducción y desestabilizando su estructura. Se han caracterizado e identificado miARNs específicos de esquistosomas,

detectándose a partir de la octava semana post-infección (Hoy et al., 2014). También se ha analizado el perfil de los miARNs de los hospedadores definitivos; sin embargo, dado que estas moléculas pueden verse alteradas por otras infecciones es necesario realizar más estudios para evaluar su utilidad en el diagnóstico de la esquistosomosis (Hoy et al., 2014; He et al., 2013).

En la Tabla 4 se resumen las principales características de los métodos empleados en el diagnóstico de la esquistosomosis.

Tabla 4. Métodos de diagnóstico más empleados en la esquistosomosis y sus principales características. Adaptada de Utizinguer et al., 2015.

Diagnóstico	Método	Sensibilidad	Especificidad	Cuantificación	Aplicabilidad
Parasitológico	KK	+	+++	+++	+++
	Filtración de orina	+	+++	+++	+++
Inmunológico	ELISA	+++	+	+	+
	POCT-CCA	++	++	+	+++
Molecular	PCR	+++	+++	+	+
	qPCR	+++	+++	+++	+

1.2.6. Tratamiento y control

El fármaco de elección para el tratamiento de la esquistosomosis es el praziquantel (PZQ). Actúa frente a todas las especies de esquistosomas paralizando y dañando el tegumento de las formas adultas y reduciendo la producción de huevos. Este fármaco no es efectivo frente a las formas inmaduras del parásito, las esquistosómulas, por lo que ni previene la infección ni puede emplearse como profilaxis. En los últimos años se están buscando alternativas al PZQ. Los derivados de la artemisinina empleados para el tratamiento de la malaria, como el artemeter, son una opción prometedora (Pérez del Villar et al., 2012a; Liu et al., 2014).

En 1985 la OMS propuso un plan estratégico para controlar la esquistosomosis a través del tratamiento masivo con PZQ a la población en riesgo

de infección, lo que se conoce como PCT (WHO, 1985). En la Figura 5 se muestra el porcentaje de población que requiere PCT en función de los países endémicos de *S. mansoni* y *S. haematobium*.

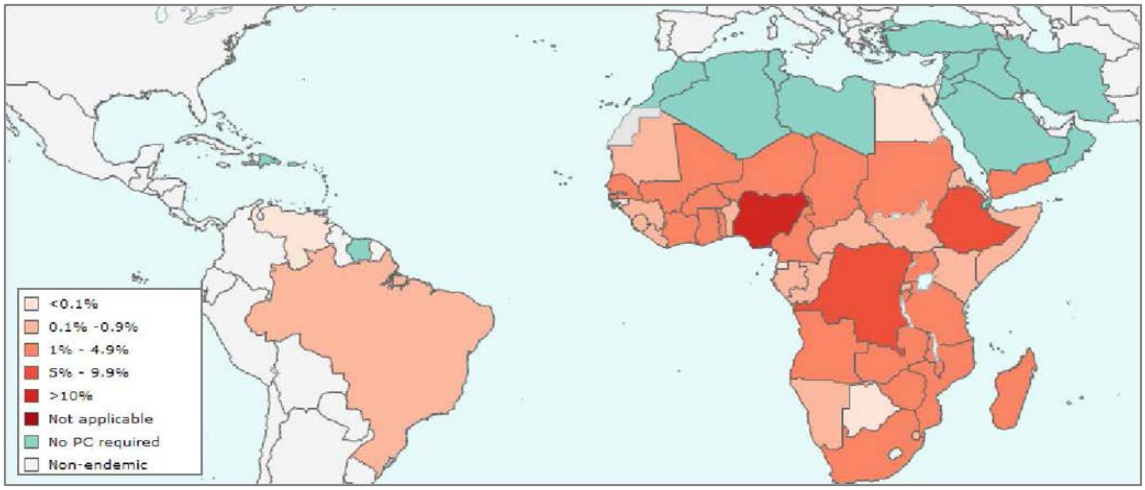

Figura 5. Porcentaje de individuos por país que requieren quimioterapia preventiva (PCT) con PZQ para la esquistosomosis en África y América. Mapa generado desde el mapa interactivo de la OMS (http://apps.who.int/neglected_diseases/ntddata/sch/sch.html).

En la asamblea del 2012 de la OMS se animó a los países que estaban desarrollando programas de control de la morbilidad a que dieran un paso más hacia la eliminación (WHO, 2013). Estas acciones consisten principalmente en poner en marcha programas integrales compuestos por PCT, control de los hospedadores intermediarios y educación de la población (Knopp et al., 2012). Sin embargo, estos programas han resultado ser menos efectivos de lo esperado (WHO, 2014).

El descubrimiento de una vacuna contra la esquistosomosis se plantea como la opción más efectiva para su control y prevención. En la actualidad no hay ninguna vacuna comercial y únicamente tres candidatos vacunales se están evaluando en ensayos clínicos (fase I-III): glutatión S-transferasa de *S. haematobioum* (rSh28GST), proteína unida a ácidos grasos de *S. mansoni* (Sm14) y tetraspanina de *S. mansoni* (Sm-TSP-2) . Otra molécula (Smp-80 de *S. mansoni*) va a comenzar los ensayos clínicos en fase I (Siddiqui y Siddiqui, 2017).

1.3. LAMP: mecanismo, utilidad y aplicabilidad

En el año 2000, Notomi y colaboradores describen la amplificación isotérmica de ácidos nucleicos mediada por bucles o LAMP(*loop-mediated isothermal amplification*) (Notomi et al., 2000). Desde entonces se han descrito multitud de aplicaciones de la técnica (Fu et al., 2011) entre las que se incluyen la detección y diagnóstico de una gran variedad de enfermedades infecciosas (Dhama et al., 2014). El LAMP está basado en el principio de síntesis de ADN por desplazamiento de cadena a temperatura constante (60ºC-65ºC), el cual es llevado a cabo por una polimerasa con alta actividad de desplazamiento, siendo la *Bst* polimerasa (obtenida de la bacteria *Bacillus stearothermophillus*) la más empleada (Mori et al., 2013). La técnica utiliza un sistema de 4 cebadores que reconocen 6 secuencias distintas dentro del ADN diana (Figura 6): 2 cebadores internos, *Forward Inner Primer* (FIP) que contiene las secuencias F1c-F2 y *Backward Inner Primer* (BIP) que contiene las secuencias B1c-B2; y 2 cebadores externos, *Forward Outer Primer* (F3) y *Backward Outer Primer* (B3). También pueden emplearse 2 cebadores adicionales llamados *loop-primers* (*Loop Forward* y *Loop Backward*) que permiten acelerar la reacción (Nagamine et al., 2002). En algunas reacciones tipo LAMP se incluye en la mezcla de reacción la betaína que, entre otras funciones, mejora el rendimiento de la reacción reduciendo la formación de estructuras secundarias en regiones ricas en GC (Reka et al., 2014).

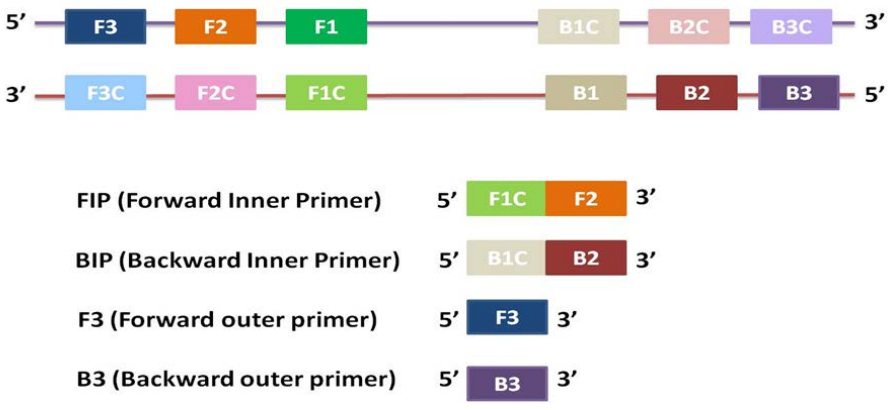

Figura 6. Sistema de *primers* empleados para el LAMP. Tomada de Rekha et al., 2014.

El mecanismo de una reacción tipo LAMP consta de dos fases: una fase no cíclica y una fase cíclica (Figura 7). La **fase no cíclica** comienza con la unión del

cebador FIP mediante la interacción F2-F2c (*primer*-ADN) en el extremo 5' del ADN diana y la posterior elongación gracias a la actividad de desplazamiento de cadena de la *Bst* polimerasa. A continuación, se une el *primer* externo F3 a la secuencia F3c que se sitúa fuera de la unión del FIP desplazando a la previamente formada, liberando una hebra de gran longitud con el primer FIP en un extremo. Después tiene lugar el proceso complementario mediante la unión del primer BIP (B2-B2c), posterior elongación e interacción del *primer* B3 con la secuencia B3c. Al replicar de nuevo el ADN libera una hebra de pequeña longitud que presenta los *primers* FIP y BIP en cada extremo lo que genera una estructura de bucle o lazo. La **fase cíclica** comienza a partir de la hebra formada durante la fase previa y que presenta un bucle en cada extremo. Esta fase presenta un mecanismo bastante complejo en el que sólo intervienen los *primers* internos. Utilizando la hebra de ADN se forma la cadena complementaria también con un bucle en cada extremo, y viceversa. Durante este proceso se forman multitud de productos amplificados intermedios de ADN de diferente tamaño a los que también se pueden unir los *primers* internos y comenzar nuevas emplificaciones. Este ciclo de repite una y otra vez de forma que, partiendo de una cantidad muy baja de ADN, se produce una gran cantidad de material genético amplificado (Ushikubo, 2004).

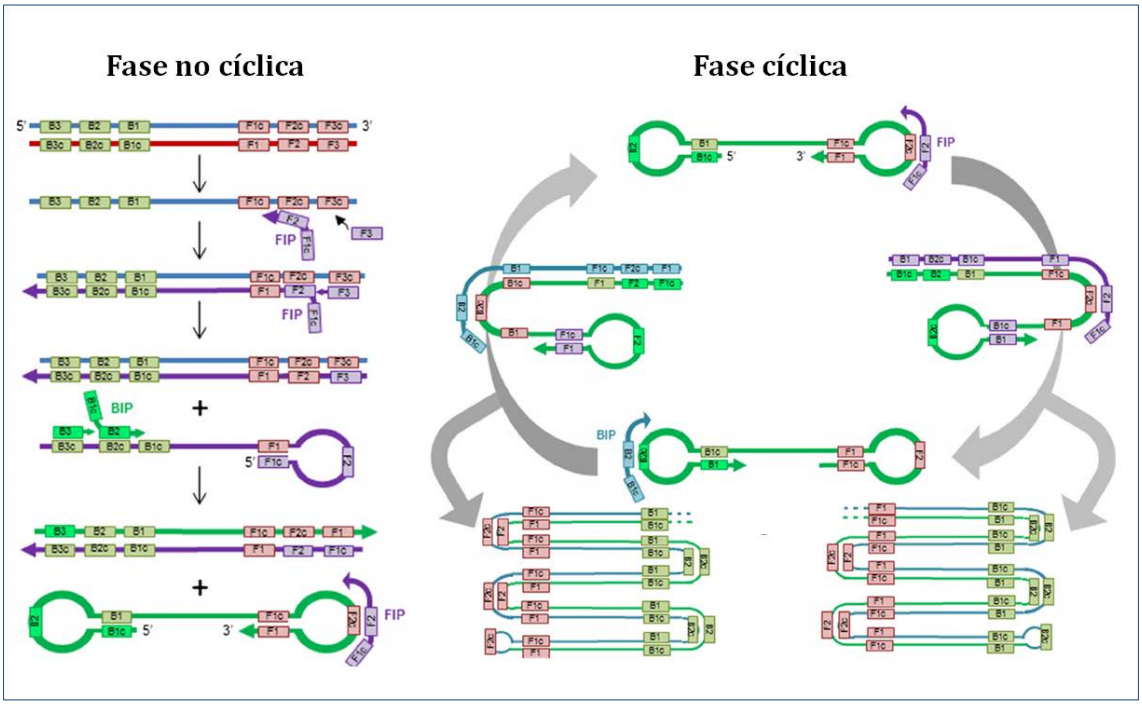

Figura 7. Mecanismo de la reacción de LAMP mostrando las fases de amplificación Tomado de Bruce et al., 2015.

Una de las grandes ventajas de este mecanismo de amplificación es su alto rendimiento. La gran cantidad de material genético generado partiendo de unas pocas copias de ADN y el anillamiento de los múltiples cebadores hacen que la sensibilidad y la especificidad sean muy elevadas (Notomi et al., 2000). Además, el hecho de que la amplificación tenga lugar a temperatura constante permite que la reacción pueda realizarse en un simple termobloque o baño de agua, haciendo innecesario el uso de aparataje más sofisticado como los termocicladores (Reka et al., 2015).

Otra de las grandes ventajas del LAMP es su potencial aplicación como método molecular en zonas endémicas de bajos recursos, ya que los reactivos pueden almacenarse a temperatura ambiente durante semanas manteniendo su estabilidad (Thekiose et al., 2009). Por otro lado, las enzimas empleadas presentan elevada resistencia a posibles inhibidores presentes en las muestras biológicas (Kaneko et al., 2007; Mori y Notomi, 2008) y pueden tolerar amplios rangos de pH y temperatura (Francois et al., 2011). Todo esto hace posible que el LAMP pueda llevarse a cabo con un mínimo procesamiento de la muestra (Paris et al., 2007) o incluso sin extracción previa de ADN para llevar a cabo la amplificación (Mikita et al., 2014; Modak et al., 2016).

Además, la detección de los productos de amplificación se puede realizar mediante diferentes métodos, destacando la simple detección visual de los resultados (Zhang et al., 2014; Esmatabadi et al., 2015). Los métodos más empleados son: *(i) detección visual mediante turbidez*: durante la amplificación de ADN mediante LAMP se genera una elevada cantidad de pirofosfato de magnesio, que es insoluble y precipita generando turbidez en el medio de reacción (Mori et al., 2001), pudiéndose observar a simple vista (Karanis et al., 2007) o bien el pellet formado tras una breve centrifugación (Le Roux et al., 2009). Este método no necesita de equipamiento o reactivos especiales y permite realizar la reacción en un solo paso. Sin embargo, no permite valorar la sensibilidad con exactitud (Le et al., 2012; Kubota et al., 2008). *(ii) Detección colorimétrica* (Figura8). La detección de los productos amplificados también puede realizarse visualmente utilizando reactivos intercalantes, capaces de interaccionar con el ADN y producir un cambio de color (Wastling et al., 2010). El más empleado es el SYBR® Green I, el cual

produce un cambio de color de naranja (negativo) a verde (positivo) perceptible a simple vista o empleando luz UV (Zhang et al., 2014). El principal problema de este tipo de reactivos es que a determinadas concentraciones pueden inhibir la amplificación y tienen que ser añadidos al final de la misma. Esto implica que deben abrirse los tubos conteniendo los amplicones ya formados, lo que aumenta el riesgo de contaminaciones cruzadas. Otros reactivos utilizados son los llamados no intercalantes, que producen cambio de color por reacción química. Éstos no inhiben el proceso de amplificación y pueden añadirse desde el inicio de la reacción, evitando así la manipulación posterior de los productos amplificados y disminuyendo el riesgo de contaminación. Los más empleados son la calceína (Wastling et al., 2010) y el *Hidroxinaphtol Blue* (HNB) (Goto et al., 2009); últimamente también se han empleado diversos colorantes sensibles al pH (Tanner et al., 2015). El principal inconveniente de estos reactivos es que pueden reducir ligeramente la sensibilidad absoluta de la técnica y el cambio de color producido puede ser muy tenue dificultando la lectura del resultado (Goto et al., 2009; Wastling et al., 2010). *(iii) Electroforesisen en agarosa*: la resolución de los productos de amplificación del LAMP en geles de agarosa permite visualizar un patrón de bandas en escalera característico (Mori y Notomi 2009). Es un método más sensible que los visuales y se propone como el más indicado para la comprobación de posibles resultados colorimétricos dudosos (Zhang et al., 2014). *(iv) Detección a tiempo real:* se puede detectar la turbidez generada por el pirofosfato de magnesio a tiempo real empleando turbidímetros (Mori et al., 2004) o espectofotómetros (Mori et al., 2001) y realizar un análisis cuantitativo. La utilización del turbidímetro es el método más sencillo; sin embargo, la heterogenicidad de las partículas que se forman, su distribución espacial, la redisolución del magnesio o la turbidez que se genera tras el procesamiento de la muestra pueden limitar la lectura (Chuang et al., 2012). Otra forma de detección a tiempo real es utilizando reactivos intercalantes en el ADN que emiten fluorescencia a medida que se genera la amplificación (Taner y Evans, 2014). La detección a tiempo real basada en la fluorescencia es más rápida que la turbidez, pero requiere de reactivos más caros y equipamientos más complejos, convirtiéndola en una técnica de mayor coste (Zhang et al., 2014).

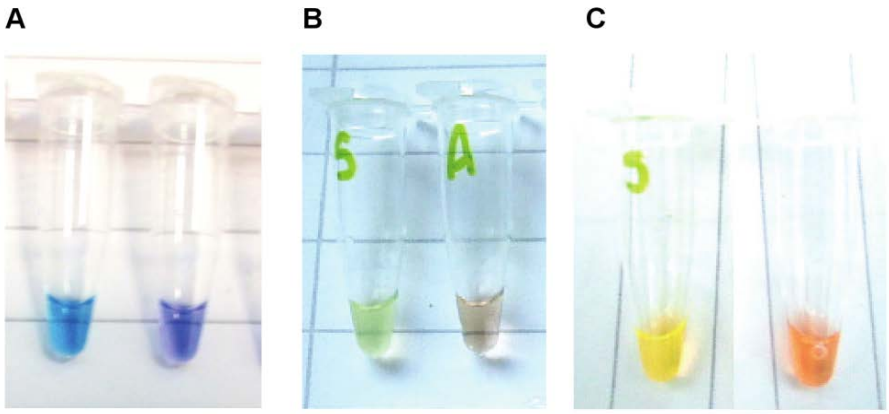

Figura 8. Ejemplos de detección colorimétrica de los resultados en la amplificación tipo LAMP. (A) Utilizando *Hidroxinaphtol Blue*, (B) calceína y (C) SYBR® Green I. En todos los casos, el tubo de la izquierda corresponde al control positivo y el de la derecha al control negativo. Tomado de Wastling et al., 2010.

1.3.1. Aplicación del LAMP como diagnóstico *point-of-care*

Las pruebas de diagnóstico *point-of-care* (*Point of Care Tests*; POCT) son pruebas diagnósticas desarrolladas en el laboratorio pero que se realizan "en o cerca del sitio de atención al paciente", permitiendo un resultado rápido y, potencialmente, la aplicación de un tratamiento temprano. Presentan numerosas ventajas sobre el diagnóstico convencional de laboratorio ya que se pueden realizar en diferentes lugares y dispositivos, no requieren de personal con formación técnica específica y permiten centrarse más en el aspecto clínico de la enfermedad y cuidado de los pacientes en el lugar donde se encuentren (Nichols et al., 2007). Se considera que la técnica LAMP tiene un elevado potencial para su aplicación como POCT en áreas endémicas de NTDs por su facilidad de uso e interpretación de los resultados (Njiru et al., 2012). Sin embargo, todavía se requiere de un reto muy importante para el acercamiento del LAMP como método de diagnóstico molecular a las poblaciones que más lo necesitan. Se requiere trabajar todavía para para proporcionar a la técnica las características que le permitan cumplir con los criterios establecidos para un "verdadero POCT" incluidos en el término ASSURED (Mabey et al., 2004): *Affordable* (asequible), *Sensitive* (sensible), *Specific* (específico), *User-friendly* (intuitivo y fácil de manejar con mínimo entrenamiento), *Rapid and Robust* (rápido y estable), *Equipment-free*

(sin equipamiento accesorio o mínimo), *Delivered* (distribuible a quien lo necesite). De esta forma, se están desarrollando diversas estrategias encaminadas a resolver los inconvenientes que se presentan para mantener la cadena de frío, evitar la extracción de ADN o desarrollar nuevos dispositivos que faciliten su aplicación práctica en el campo.

Para poder prescindir de la cadena de frío hay que estabilizar la mezcla de reacción para evitar su deterioro en el tiempo, permitiendo así el almacenamiento y transporte sin necesidad de refrigeración (Selvam et al., 2013). Los métodos empleados hasta la fecha para estabilizar los componentes de la reacción de LAMP han sido el secado (Hayashida et al., 2015) y la liofilización (Carter et al., 2017). Otra estrategia utilizada para evitar la cadena de frío es realizar modificaciones en las polimerasas empleadas en la amplificación encaminadas a mejorar su estabilidad, como por ejemplo en la *Bst* 2.0 *Warm Start* polimerasa. Esta enzima es un homólogo diseñado *in silico* de la *Bst* polimerasa nativa (*Bst* polimerasa *Large Fragment*) que presenta mayor rendimiento y además posee un aptámero unido de forma reversible que inhibe a la enzima hasta alcanzar los 45ºC. Esto evita la posible amplificación durante la preparación de la mezcla de reacción a temperatura ambiente así como el almacenamiento de de las reacciones sin refrigeración durante horas sin alterar el resultado final (Poole et al., 2012).

Con el objetivo evitar la extracción y purificación del ADN también se están desarrollando modificaciones sobre las enzimas que aumentan su actividad y mejoran su tolerabilidad a distintos inhidores presentes en las muestras biológicas (Jevtuševskaja et al., 2017). Otra estrategia es el diseño de procedimientos simples para la obtención de ADN semipurificado para llevar a cabo la reacción de amplificación, como ocurre ya en el caso del diagnóstico mediante LAMP de la malaria o la tuberculosis (Mitari et al., 2011; Polley et al., 2013). Otro ejemplo es el caso para la detección del virus de la influeza, en el que se encuentra disponible una mezcla de reactivos para la obtención de ADN semipurificado a partir de torundas nasales sin procesamiento previo (Nakaguchi et al., 2011).

Por otro lado, también se han utilizado sistemas portátiles simples para poder prescindir de una fuente eléctrica estable, conocidas como NINA (*Non-Instrumented Nucleic Acid Amplification*) que mantienen la temperatura constante

a 65ºC durante 45 minutos para poder llegar a completar la reacción de amplificación (Mohon et al., 2016). También hay sistemas de detección a tiempo real portátiles con batería que permiten realizar el LAMP en zonas de acceso remoto (Dominguez et al., 2010; Seyrig et al., 2015). Últimamente, además, estos sistemas portátiles se asocian a las nuevas tecnologías y el uso de la telefonía móvil permitiendo acelerar la detección, el análisis y la gestión de los resultados (Damhorst et al., 2015; Priye et al., 2017).

Un dispositivo de detección de la amplificación de gran utilidad para ser empleado como POCT son los llamados *Lateral Flow Dipsticks* (LFD). Es una técnica inmunocromatográfica que emplea anticuerpos específicos contra los productos de amplificación permitiendo reducir el tiempo y el equipamiento necesario para realizar el LAMP (Jaroenram et al., 2009). En los últimos años están cobrando gran relevancia los *Microfluidic cassettes* (Whitesides et al., 2006), unos sistemas capilares que al pasar la muestra a través de ellos son capaces de extraer el ADN y producir la amplificación (Asiello y Baeumner, 2011). Estos sistemas se han asociado con chips capaces de detectar los productos amplificados, recibiendo el nombre de μ-TAS (*μ-Total Analysis System*), o "*lab-on-a-chip*" y son altamente efectivos cuando se asocian a la tecnología LAMP (Abe et al., 2011). Por este motivo algunos autores proponen estos sistemas como la mejor alternativa para la aplicación del LAMP como método de diagnóstico POCT (Notomi et al., 2015).

Finalmente, las mejoras implementadas han permitido el desarrollo de métodos LAMP en formato kit "listo para el uso" o "ready-to-use", como para el diagnóstico de la tuberculosis (Mitarai et al., 2011), la malaria (Hopkins et al., 2013), la tripanosomosis africana humana (Hayashida et al., 2015) o, más recientemente, para detección simultánea de los virus del Dengue, Chikungunya y Zika (Yaren et al., 2017). Estos kits incorporan la mezcla de la reacción ya estabilizada por liofilización o secado y no precisan de extracción previa de ADN. Además, emplean sistemas portátiles para la amplificación y el proceso se completa en tan sólo una hora.

1.3.2. LAMP y helmintos

Hasta la fecha, existen diversos métodos LAMP desarrollados que permiten la detección de ADN de diferentes helmintos y que se recogen en la tabla 5. De entre los descritos, en este apartado revisaremos las características de los métodos LAMP para las enfermedades causadas principalmente por trematodos, entre las que se encuentra la esquistosomosis, que es el objeto principal dede esta tesis doctoral.

Tabla 5. Métodos LAMP desarrollados para la detección de diferentes helmintos.

Grupo	Especie	Referencia
Trematodos	*Amphimerus* sp.	Cevallos et al., 2017
	Clonorchis sinensis	Cai et al., 2010; Chen et al., 2013
	Fasciola hepatica	Ai et al., 2010; Martínez-Valladares et al., 2016
	Fasciola gigantica	Ai et al., 2010
	Opisthorchis viverrini	Le at al., 2012; Arimatsu et al., 2012; Arimatsu et al., 2015
	Paragonimus westermani	Chen et al., 2011
	Schistosoma haematobium	Abbasi et al., 2010; Hamburger et al., 2013
	Schistosoma japonicum	Xu et al., 2010; Kumagai et al., 2010; Wang et al., 2011; Xu et al., 2015; Tong et al., 2015
	Schistosoma mansoni	Abbasi et al., 2010; Hamburger et al., 2013; Song et al., 2015; Caldeira et al., 2017
Cestodos	*Echinococcus granulosus*	Salant et al., 2012; Wassermann et al., 2014; Ni et al., 2014; Ahmed et al., 2016
	Echinococcus multilocularis	Ni et al., 2014
	Taenia solium, T. saginata y *T. asiatica*	Nkouawa et al., 2009; Nkouawa et al., 2010; Nkouawa et al., 2012; Nkouawa 2016
Nematodos	*Angiostrongylus cantonensis*	Liu et al., 2011; Chen at al., 2011
	Ascaris lumbricoides	Shiraho et al., 2016
	Brugia malayi y *B. timori*	Poole et al., 2012; Poole et al., 2017
	Dirofilaria immitis	Aonuma et al., 2009; Aouma et al., 2010; Raele ey al., 2016
	Loa loa	Drame et al., 2014; Fernández-Soto et al., 2014; Poole et al., 2015
	Necator americanus	Mugambi et al., 2015
	Onchocerca volvulus	Alhassan et al., 2014; Poole et al., 2017
	Strongyloides stercoralis	Watts et al., 2014; Fernández-Soto et al., 2016
	Toxocara canis	Machukova et al., 2010; Ozlati et al., 2016
	Trichinella spiralis	Li et al., 2012; Lin et al., 2013
	Wuchereria bancrofti	Takagai et al., 2011; Kouassi et al., 2015; Poole et al., 2017

El conocimiento del genoma de diferentes helmintos en los últimos años (www.sanger.ac.uk/resources/downloads/helminths/) ha facilitado el estudio de secuencias de ADN útiles en el diseño específico de primers para el desarrollo de técnicas moleculares empleadas en el diagnóstico de enfermedades. Esto ha permitido que la mayoría de los métodos LAMP para la detección de trematodos hayan comenzado a describirse a partir del año 2010 y que actualmente muchos se encuentren aún en fase de evaluación.

De forma general, con el fin de mejorar la sensibilidad, los métodos LAMP para trematodos utilizan secuencias repetidas dentro del genoma como las secuencias mitocondriales, ribosomales o secuencias altamente repetidas. En la Tabla 6 se recogen los principales métodos desarrollados hasta la fecha indicando sus principales características, como el ADN diana utilizado para la amplificación, el tiempo de reacción, el uso o no de *loop-primers*, el método de detección de los productos amplificados y la sensibilidad alcanzada.

Así, en el caso de la detección de *O. viverrini,* se ha utilizado una secuencia mitocondrial correspondiente al gen Nad1. (Le et al., 2012). En relación a las secuencias ribosomales, las más empleadas corresponden a las ITS (*Internal Trancribed Spacers)*, como para la detección de *Amphimerus* sp. (Cevallos et al., 2017), *C. sinensis* (Chen et al., 2013), *F. hepatica* (Martínez-Valladares et al., 2016), *O. viverrini* (Arimatsu et al., 2012) y *S. mansoni* (Caldeira et al., 2017). También se ha utilizado la secuencia IGS (*Intergenic Spacer*) para la detección y diferenciación específica de *F. hepatica* y *F. gigantica* (Ai et al., 2010) y la secuencia 28S ribosomal para la detección de *S. japonicum* (Kumagai et al., 2010; Tong et al., 2015). Las secuencias altamente repetidas utilizadas para el diseño de *primers* han sido la Sm1-7 de *S. mansoni*, la secuencia Dra-1 de *S. haematobium* (Abbasi et al., 2010) y la secuencia SjR2 de *S. japonicum* (Xu et al., 2010; Xu et al., 2015).

En cuanto al tiempo empleado para llevar a cabo las reacciones de LAMP en la detección de ADN de trematodos, el rango comprende desde los 35 min para el caso de *C. sinensis* (Cai et al., 2010) hasta los 120 min en el caso de *S. mansoni* y *S. haematobium* (Abbasi et al., 2010; Hamburger et al., 2013). La disminución en el tiempo de reacción se consigue con la utilización de loop-primers en la mezcla de reacción como en el caso de *C. sinensis* (Cai et al., 2010), *O. viverrini* (Arimatsu et

al., 2012; Arimatsu et al., 2015), *P. westermani* (Xu et al., 2010) y *S. mansoni* (Caldeira et al., 2017) (Tabla 6).

El rango de la sensibilidad analítica conseguido en los distinos métodos también es muy amplio, desde la detección de 1 ng de ADN en el caso de *O. viverrini* (Arimatsu et al., 2015) hasta 0,1 fg para *S. mansoni* y *S. haematobium* (Abbasi et al., 2010).

El método de detección de los productos de amplificación más empleado en todos los casos es la detección visual mediante cambio de color tras la adición de SYBR® Green I y posterior electroforesis en gel de agarosa. En todos los casos se observó correlación entre los resultados observados visualmente y los obtenidos tras la electroforesis. Sin embargo, en algunos casos como en la detección de *O. viverrini*, se encontraron diferencias entre la detección visual mediante colorantes y la observación de la turbidez, ya que esta valoración resulta menos sensible (Le et al., 2012) (Tabla 6).

Tabla 6. Características principales de los métodos LAMP actualmente descritos para la detección de trematodos.

Especie de trematodo	ADN diana	Tiempo de reacción (min)	*Loop-primers*	Método de detección	Sensibilidad	Referencia
Amphimerus sp.	ITS-2 ribosomal	60-120	No	SYBR/Gel	1 pg	Cevallos et al., 2017
C. sinensis	Catepsina B3	35	LF y LB	SYBR/Gel	10^{-8} ng/µL	Cai et al., 2010
	ITS-2 ribosomal	60	No	SYBR/Gel	10 fg	Chen et al., 2013
F. hepatica y F. gigantica	IGS ribosomal	45	No	SYBR/Gel	10^{-5} ng	Ai et al., 2010
F. hepatica	ITS-2 ribosomal	60	No	SYBR/Gel	10^{-3} ng/µL	Martínez-Valladares et al., 2016
O.viverrini	Nad1-MIT	70	No	Turbidez/SYBR/SYBR-UV/Gel	1 pg	Le et al., 2012
	ITS-1 ribosomal	60	LB	Turbidez/HNB/SYBR-RT/SYBR-UV	10^{-3} ng/µL	Arimatsu et al., 2012
	OVMS-6	60	LF y LB	HNB	1 ng	Arimatsu et al., 2015
P. westwemani	ITS-2 ribosomal	45	LF y LB	SYBR/Gel	10^{-8} ng/µL	Chen et al., 2011
S. japonicum	SjR2	90	No	SYBR/Gel	0,08 fg	Xu et al., 2010
	28S ribosomal	--	No	Turbidez/Gel	100 fg	Kumagai et al., 2010
	SjR2	--	No	Gel	10^{-4} ng	Wang et al., 2011
	SjR2	90	No	SYBR	--	Xu et al., 2015
	28S ribosomal	60	No	FD kit/Gel	100 fg	Tong et al., 2015
S. mansoni	Sm1-7	90/Chip	No	EvaGreen-RT/On-Chip	0,5 fg (RT)	Song et al., 2015
	ITS	40	LF y LB	Gel	70 fg	Caldeira et al., 2017
S. mansoni y *S. haematobium*	Sm1-7/Dra1	120	No	SYBR-UV/Gel	0,1 fg	Abbasi et al., 2010
	Sm1-7/Dra1	120	No	SYBR/Gel	--	Hamburger et al., 2013

ITS: de las siglas en inglés *Internal Trancribed Spacer*
IGS: de las siglas en inglés *Intergenic Spacer*
LF y LB: *primers loop-forward* y *loop-backward* respectivamente
SYBR: detección visual a simple vista tras añadir SYBR® Green I
SYBR-UV: detección visual con luz ultra violeta tras añadir SYBR® Green I
SYBR-RT: detección a tiempo real empleando SYBR® Green I
Gel: electroforesis en gel de agarosa
FD kit: detección con *Fluorescent detection reagent* del *Loopamp DNA amplification kit*
EvaGreen-RT: detección a tiempo real empleando EvaGreen®
On-Chip: método de detección empleando un *Microfluidic chip*

En relación a la aplicación del LAMP en la detección de trematodos en muestras humanas, los principales trabajos descritos se recogen en la Tabla 7. En la mayoría de los casos se valora el método molecular en relación a los métodos clásicos parasitológicos. Para valoración del LAMP en el diagnóstico de *Amphimerus* sp. y *O. viverrini* se utilizaron muestras de heces de individuos de zona endémica con examen parasitológico previo mediante microscopía. En ambos casos el LAMP detectó un número mayor de casos positivos que los métodos parasitológicos, mostrando una mayor sensibilidad. Los valores diagnósticos obtenidos para el LAMP de sensibilidad y especificidad fueron de 76,67% y 80,77%, respectivamente, en la detección de *Amphimerus* sp. (Cevallos et al., 2017) y de 100% y 61,5% para *O. viverrini* (Arimatsu et al., 2012). La disminución observada en la especificidad se debe a que el LAMP detectó ADN del parásito en muestras que resultaron parasitológicamente negativas previamente.

El LAMP utilizado para la detección de *P. westermani* detectó un total de 17 muestras de esputo y líquido pleural de pacientes con infección confirmada parasitológicamente (Chen et al., 2011). En el caso de *S. japonicum*, dos trabajos han aplicado el LAMP en muestras de suero procedentes de una provincia endémica de esquistosomosis en China, resultando ser más sensible en comparación con las técnicas de PCR, ELISA o IHA (Tabla 7). En la estimación de la especificidad en estos trabajos se emplearon muestras de pacientes residentes en áreas no endémicas de esquistosomosis, obteniéndose una elevada especificidad. Sin embargo, a pesar de ser pacientes de zona no endémica, los métodos basados en la detección de anticuerpos (IHA y ELISA) mostraron reactividad cruzada y una especificidad menor (Xu et al., 2010; Xu et al., 2015).

Tabla 7. Aplicación del método LAMP para la detección de ADN de diferentes trematodos en muestras humanas.

Especie de trematodo	**Tipo muestra**	**Número de muestras**	**Extracción de ADN**	**Sensibilidad**	**Especificidad**	**Referencia**
Amphimerus sp.	Heces	44	*Mini Stool DNA Extraction kit*	LAMP: 76.67%	LAMP: 82.14%	Cevallos et al., 2017
O. viverrini	Heces	50	*QIAamp DNA Mini Stool kit*	LAMP: 100%	LAMP: 61,5%	Arimatsu et al., 2012
P. westermani	Esputo Líquido pleural	17	SDS/proteinasa K y *Wizard SV Genomic DNA Purification System*	LAMP: 100%	----	Chen et al., 2011
S. japonicum	Suero	50	Método *in house*	LAMP: 96,7% PCR: 60%	LAMP: 100% PCR: 100%	Xu et al., 2010
	Suero	152	fenol/cloroformo	LAMP: 95,5% ELISA: 84,6% IHA: 91,8%	LAMP: 100% ELISA: 85,7% IHA: 88,1%	Xu et al., 2015

IHA: hemaglutinación indirecta
Mini Stool DNA Extraction kit (*Macharey-Nagel*)
QIAamp DNA Mini Stool kit (*QIAGEN*)
Wizard SV Genomic DNA Purification System (*Promega*)

El método LAMP también se ha evaluado en la detección de trematodos en muestras de origen animal, bien en infecciones naturales o en modelos experimentales de infección. En infecciones naturales se ha aplicado el LAMP con excelentes resultados para el diagnóstico de la fasciolosis en 16 muestras de heces parasitológicamente positivas de ganado bovino (Ai et al., 2010).

En modelos experimentales de infección, se ha empleado el LAMP para la detección de de *F. hepatica* en ovejas (Martinez-Valladares et al., 2016), *S. mansoni* en ratones (Song et al., 2015) y *S. japonicum* en conejos (Xu et al., 2010) (Xu et al., 2015; Wang et al., 2011), detectándose el ADN del parásito en todos los casos en la fase aguda de la infección, antes de la detección microscópica de huevos de los diferentes parásitos (Song et al., 2015; Martinez-Valladares et al., 2016) e incluso antes que otros métodos de diagnóstico, ya sean inmunológicos (Xu et al., 2015) o moleculares (Wang et al., 2011).

En otros estudios el LAMP demostró su utilidad para la detección de ciertos trematodos en sus correspondientes hospedadores intermediarios. Por ejemplo, para la identificación de *P. westermani* y *C. sinensis* mediante LAMP se emplearon muestras recogidas en el campo de hospedadores intermediarios primarios (caracoles) y secundarios (crustáceos y peces), probando la elevada sensibilidad de la técnica en comparación con métodos parasitológicos (Cai et al., 2010; Chen et al., 2011) y moleculares (PCR) (Chen et al., 2013). En relación a la detección de esquistososmas en sus hospedadores intermediarios, se emplearon infecciones experimentales de caracoles del género *Oncomelania* (para *S. japonicum*), *Bulinus* (para *S. haematobium*) y *Biomphalaria* (para *S. mansoni*) y en todos los casos el LAMP detectó ADN del parásito durante la fase prepatente de la infección, esto es, antes de la emisión de cercarias (Kumagai et al., 2010; Abbasi et al., 2010). También se ha evaluado el LAMP para la detección de las tres especies de esquistosomas mencionadas en caracoles recogidos en condiciones de campo, mostrando ser una técnica rápida, sensible y de fácil aplicación para la identificación de hospedadores intermediarios infectados y determinar así puntos de transmisión activa de la esquistosomosis en una zona determinada (Tong et al., 2015; Hamburger et al., 2013; Caldeira et al., 2017).

Para determinar la efectividad del LAMP como método de detección de ADN tras la aplicación de un tratamiento se han utilizado modelos animales de infección. En el caso de *S. japonicum,* en conejos infectados con 200 cercarias, el LAMP amplificó ADN de parásito en suero hasta la semana 12 post-tratamiento (Xu et al., 2010). Utilizando dosis de infección menores (80 cercarias) en el mismo modelo experimental, el LAMP resultó positivo hasta la octava semana post-tratamiento. Es importante destacar que el nivel de anticuerpos anti-esquistosoma se detectó mediante ELISA y IHA durante 23 semanas post-tratamiento (Xu et al., 2015). En este mismo trabajo, también se evaluó el LAMP como herramienta para valorar la respuesta al tratamiento en pacientes. Se realizó un seguimiento a 47 individuos infectados con *S. japonicum* a los 3, 6 y 9 meses tras el tratamiento. El ADN del parásito no se detectó en suero a los 3 meses en el 31,9%, en el 61,7% a los 6 meses y en el 83% a los 9 meses. Los resultados mediante LAMP resultaron mejores en comparación con IHA y ELISA, ya que a los 9 meses alcanzaron un porcentaje de conversión de 31,9% y 25,5%, respectivamente (Xu et al., 2015). Es necesario realizar más estudios con otras especies de trematodos para determinar la utilidad del LAMP en la valoración de la eficacia del tratamiento y poder ser empleado como diagnóstico tras campañas de quimioterapia preventiva.

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C.II

Capítulo II:
Hipótesis y objetivos

2.1. Hipótesis de trabajo

Actualmente no existe un método de diagnóstico adecuado para la detección de la esquistosomosis, ya que los métodos parasitológicos y serológicos presentan baja sensibilidad y especificidad, y los métodos moleculares (PCR y sus variantes) no se utilizan de forma rutinaria en la práctica clínica debido al alto nivel de especialización y requerimientos técnicos, quedando restringidos a laboratorios de referencia. La tecnología LAMP (*loop-mediated isothermal amplification*) presenta las ventajas de rapidez, simplicidad y bajo coste y se ha utilizado con éxito para el diagnóstico de enfermedades tropicales. Nuestra hipótesis de trabajo se fundamenta en que esta tecnología reúne las características necesarias para convertirse en un método de diagnóstico útil para la esquistosomosis y de fácil aplicación en áreas endémicas de bajos recursos.

2.2. Objetivos

El **objetivo general** de esta tesis doctoral es diseñar, desarrollar y evaluar técnicas de diagnóstico molecular basadas en la amplificación de ADN tipo LAMP en zonas endémicas de esquistosomosis.

Los **objetivos específicos** son:

1. Diseño y desarrollo de un método LAMP para la detección de *Schistosoma mansoni* y evaluación en un modelo experimental murino.
2. Diseño y desarrollo de un método LAMP para la detección de *Schistosoma haematobium* y evaluación en muestras clínicas.
3. Diseño y desarrollo de un método LAMP para la detección de *Schistosoma mansoni* en *Biomphalaria glabrata* infectados experimentalmente.
4. Aplicación del método LAMP para la detección de *Schistosoma haematobium* en una zona de alta transmisión de esquistosomosis en Angola.
5. Aplicación del método LAMP para la detección de *Schistosoma mansoni* en una zona de baja transmisión de esquistosomosis en Brasil.

C.III

Capítulo III:
Artículos de investigación

3.1. Artículo 1

A Loop-Mediated Isothermal Amplification (LAMP) Assay for Early Detection of Schistosoma mansoni in Stool Samples: A Diagnostic Approach in a Murine Model

Fernández-Soto P, **Gandasegui J**, Sánchez Hernández A, López-Abán J, Vicente B, Muro A.

PLoS Negl Trop Dis. 2014; 8(9): e3126.

Resumen

La esquistosomosis intestinal, principalmente debida a *Schistosoma mansoni*, es una de las enfermedades parasitarias más prevalentes en el mundo. Para superar las limitaciones de las técnicas de diagnóstico parasitológicas e inmunológicas en la detección de *S. mansoni*, especialmente en la fase aguda de la enfermedad, es necesario el desarrollo de nuevos métodos de diagnóstico moleculares que presenten una relación coste-efectividad mejor. Una opción prometedora sería la amplificación de ADN tipo LAMP (*loop-mediated isothermal amplification*) que en comparación con las técnicas basadas en la PCR presenta una serie de ventajas, como la simplicidad, la rapidez, el bajo coste y el alto rendimiento diagnóstico. Adicionalmente, como los resultados pueden leerse a simple vista, la técnica presenta un gran potencial para ser aplicada en países en desarrollo.

Se seleccionó una secuencia de ADN correspondiente a un microsatélite mitocondrial para el diseño de *primers* específicos para la amplificación de ADN de *S. mansoni* en muestras de heces. Empleamos un modelo de infección experimental murino para la obtención de muestras de heces y suero semanalmente desde la semana 0 hasta la 8 post-infección. Se llevó a cabo la detección de huevos en heces mediante Kato-Katz y la detección de anticuerpos mediante ELISA para monitorizar la infección. Los *primers* diseñados fueron testados usando un *kit* comercial y una mezcla *in house* para la amplificación mediante LAMP, comparando los resultados entre las dos técnicas. La especificidad se evaluó empleando ADN de 16 parásitos diferentes, incluyendo varias especies de esquistosomas, y no se observó reactividad cruzada. El límite de detección de nuestro LAMP (SmMIT-LAMP) fue de 1 fg de ADN de *S. mansoni*. Cuando se evaluó en las muestras de heces, se detectó ADN a la primera semana post-infección.

Por primera vez, hemos desarrollado un método LAMP eficiente, fácil de realizar, específico y sensible para la detección de *S. mansoni* en la fase prepatente. Este método es potencialmente aplicable y adaptable para el diagnóstico y vigilancia de la enfermedad en áreas endémicas.

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PLOS | NEGLECTED TROPICAL DISEASES

A Loop-Mediated Isothermal Amplification (LAMP) Assay for Early Detection of *Schistosoma mansoni* in Stool Samples: A Diagnostic Approach in a Murine Model

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Abstract

Background: Human schistosomiasis, mainly due to *Schistosoma mansoni* species, is one of the most prevalent parasitic diseases worldwide. To overcome the drawbacks of classical parasitological and serological methods in detecting *S. mansoni* infections, especially in acute stage of the disease, development of cost-effective, simple and rapid molecular methods is still needed for the diagnosis of schistosomiasis. A promising approach is the loop-mediated isothermal amplification (LAMP) technology. Compared to PCR-based assays, LAMP has the advantages of reaction simplicity, rapidity, specificity, cost-effectiveness and higher amplification efficiency. Additionally, as results can be inspected by the naked eye, the technique has great potential for use in low-income countries.

Methodology/Principal findings: A sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region was selected as a target for designing a LAMP-based method to detect *S. mansoni* DNA in stool samples. We used a *S. mansoni* murine model to obtain well defined stool and sera samples from infected mice with *S. mansoni* cercariae. Samples were taken weekly from week 0 to 8 post-infection and the Kato-Katz and ELISA techniques were used for monitoring the infection. Primer set designed were tested using a commercial reaction mixture for LAMP assay and an *in house* mixture to compare results. Specificity of LAMP was tested using 16 DNA samples from different parasites, including several *Schistosoma* species, and no cross-reactions were found. The detection limit of our LAMP assay (SmMIT-LAMP) was 1 fg of *S. mansoni* DNA. When testing stool samples from infected mice the SmMIT-LAMP detected *S. mansoni* DNA as soon as 1 week post-infection.

Conclusions/Significance: We have developed, for the first time, a cost-effective, easy to perform, specific and sensitive LAMP assay for early detection of *S. mansoni* in stool samples. The method is potentially and readily adaptable for field diagnosis and disease surveillance in schistosomiasis-endemic areas.

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Introduction

Schistosomiasis, a disease caused by parasitic worms of several species of genus *Schistosoma*, is one of the 17 neglected tropical diseases (NTDs) recognized by World Health Organization (WHO) [1]. Presently, human schistosomiasis, mainly caused by *Schistosoma mansoni* species, is one of the most widespread of all human parasitic diseases, ranking second only to malaria in terms of its socioeconomic and public health importance in developing countries in tropical and subtropical areas, especially in Sub-Saharan Africa. The disease is endemic in 74 countries infecting more than 200 million people worldwide, with 732 million people at risk of infection in known transmission areas [2], [3], [4]. On a global scale, one of thirty individuals has schistosomiasis [5]. It is also noted that the prevalence of imported schistosomiasis is increasingly a problem in non-endemic areas due to the growing number of international travelers to endemic areas, expatriates and immigrants from endemic countries [6], [7], [8].

Over time, several diagnostic techniques including parasitological and immunological methods have been tested for diagnosis of schistosome infection. As is well known, traditional parasitological methods, such as Kato-Katz assay for counting eggs in feces, are relatively inexpensive and easy to perform providing basic information on prevalence and infection intensity. However, a

Author Summary

Schistosomiasis is one of the most widespread of all human parasitic diseases, *Schistosoma mansoni* being the most important species causing human intestinal schistosomiasis. The diagnosis of the disease is mainly based on parasitological and serological methods, but they are not effective in detecting *S. mansoni* infections in the acute stage of the disease. New diagnostic tools to detect the disease during the first weeks would be desirable, permitting early treatment and preventing the pathology associated with chronic infections. An approach is the loop-mediated isothermal amplification (LAMP) technique, which can amplify DNA with high specificity and sensitivity under isothermal conditions. DNA amplification and reading of results require minimum equipment, thus the technique has great potential for use in diagnosis of neglected tropical diseases. In our study, we developed and evaluated a LAMP assay for the early detection of *S. mansoni* DNA in stool samples from mice experimentally infected with the parasite. The results indicated that our LAMP assay is specific, sensitive and cost-effective in detecting *S. mansoni* DNA in stool samples as soon as one week post-infection, when parasitological and serological methods are not effective. The assay has the potential to be developed further as a field diagnostic tool for use in schistosomiasis-endemic areas.

major limitation of these methods is their lack in sensitivity, especially in low-grade infections, as occurs in areas of low prevalence or in individuals with recent infections [9]. In addition, they are cannot be carried out in the acute phase of schistosomiasis since parasite has not started yet to lay eggs. When parasites cannot be directly detected, the immunological methods are usually applied to patients with schistosomiasis clinical signs. However, serology-based analyses currently continue to present problems, such us obtaining schistosome antigens, inability to discriminate between current or previous infection, high level of cross reactivity as well as persistence of antigens and antibodies after chemotherapy usually causing false positive results [10]. To overcome the shortcomings of both parasitological and immunological diagnostic methods, the development of new, more sensitive and specific molecular diagnostic tools for the diagnosis of schistosomiasis are desirable. In recent years, several studies have reported the application of polymerase chain reaction (PCR)-based assays for high sensible and specific detection of *Schistosoma* spp. DNA in human clinical samples, such as feces [11], [12], [13], [14], [15], sera [11], plasma [16] and urine [17]. Even though these studies have demonstrated that PCR-based technologies provided reliable, specific and sensitive tools, they are not still widely used in low-income countries because highly skilled personnel and expensive cyclers are needed. Therefore, the development of cost-effective, simple and rapid detection methods is still needed for the diagnosis of schistosomiasis.

An interesting alternative to PCR-based technologies is a molecular technique named loop-mediated isothermal amplification (LAMP). This assay is a one-step amplification reaction that amplifies a target DNA with high specificity, efficiency and rapidly under isothermal conditions [18]. LAMP employs a DNA polymerase (*Bst* polymerase) with strand-displacement activity, along with two inner primers (FIP, BIP) an outer primers (F3, B3) which recognize six separate regions within a target DNA. The auto-cycling reactions lead to accumulation of a large amount of the target DNA and other reaction by-products, such as magnesium pyrophosphate, that allow rapid visual detection and real-time measurement of turbidity [19] or visual fluorescence in the presence of fluorescent intercalating dyes [20]. Moreover, as LAMP assay is an isothermal amplification method it does not require an expensive cycler and can be performed in economical heating blocks or water baths. On this basis, simple heating methods, such as chemical heaters or thermal bottles, have been recently designed for removing the dependence upon stable electricity and allowing for LAMP to be conducted at any time in any setting [21], [22], [23], [24]. Thus, LAMP assay has all the characteristics required of realtime assays along with simple operation for easy adaptability to field conditions.

Since the LAMP assay was first reported [18], many LAMP reactions have been developed for molecular detection and diagnostics of bacterial, viral, fungal and parasitic diseases in both animals and plants [25] and their performance observed by comparing to molecular techniques, such as PCR, in order to evaluate the feasibility of LAMP technology [26], [27].

As recently reviewed by Mori et al [28], of the 17 NTDs recognized by WHO, 14 have been studied using LAMP assay, including schistosomiasis caused by *S. japonicum* in experimentally infected rabbits to evaluate the technique for early diagnosis and efficacy of chemotherapy [29], [30]. Other successfully approaches for LAMP assay to be used for *Schistosoma* spp. detection have been mainly focused in field settings for monitoring infected snails with *S. mansoni*, *S. haematobium* [31], [32] and *S. japonicum* [33], [34].

Thus, with the aim to improve in developing new, applicable and cost-effective molecular tools for the diagnosis of schistosomiasis, in our work we have developed a LAMP assay for early specific detection of *S. mansoni* in mice stool samples. In the present study, specific LAMP primer set designed was tested using different reaction mixtures each containing different *Bst* polymerases to compare results and cost-effectiveness. We also evaluated the sensitivity of the LAMP assay in comparison with classical diagnostic techniques, such as Kato-Katz and ELISA. To the best of our knowledge, this is the first report using LAMP assay for early diagnosis of active schistosomiasis in mice stool samples.

Methods

Ethics statement

The study protocol was approved by the institutional research commission of the University of Salamanca. Ethical approval was obtained from the Ethics Committee of the University of Salamanca (protocol approval number 48531), which approved the animal protocol. Animal procedures in this study complied with the Spanish (Real Decreto RD53/2013) and the European Union (European Directive 2010/63/EU) guidelines on animal experimentation for the protection and humane use of laboratory animals and were conducted at the accredited Animal Experimentation Facility (Servicio de Experimentación Animal) of the University of Salamanca (Register number: PAE/SA/001).

Mice and *Schistosoma mansoni* experimental infection

Six to seven-week old female CD1 mice weighing 16–24 g (Charles River Laboratories, Barcelona, Spain) were used in the study as the source for blood and stool samples. Animals were housed at the accredited Animal Experimentation Facility of the University of Salamanca in standard polycarbonate cages and placed in humidity and temperature controlled environment with a 12 hour photoperiod and received sterilized food and water *ad libitum*. Mice were each infected with 200 *S. mansoni* cercariae which were obtained from *Biomphalaria glabrata* snails previously infected with *S. mansoni* miracidia as described elsewhere [35].

The infection was carried out following the methodology previously described by Smithers et al [36]. Uninfected mice (control group) were used as source for negative samples. All mice blood and stool samples were taken weekly from week 0 to week 8 post-infection (p.i.). Animals were monitored regularly by qualified members in animal welfare at the Animal Experimentation Facility of the University of Salamanca. Infected mice were humanely euthanised by intraperitoneal injection with pentobarbital at a 60 mg/Kg dose using 30 g needles at week 8 p.i.

Mice samples and monitoring infection by Kato-Katz and ELISA

A total of 90 stool samples and 90 blood samples were taken from all mice throughout infection. Five stool samples as well as five blood samples were taken weekly and processed from both infected and uninfected mice groups from week 0 to week 8 p.i. Feces weekly obtained from each infected mouse was divided into two portions: one was immediately processed and examined by triplicate for counting eggs using the Kato-Katz technique [37] in a conventional microscope and another was stored at −20°C to be used afterward for DNA extraction for molecular assays. The Kato-Katz technique was used as the *gold standard* assay to predetermine the existence of *S. mansoni* infection in stool samples. Results obtained were expressed as mean±SE. Feces weekly obtained from each non-infected mouse were kept frozen individually until DNA extraction as mentioned below.

After collection of the whole blood from each mouse in a defined time-point p.i., the sera samples were obtained by allowing the blood to clot for 15–30 minutes at room temperature and removing the clot by centrifuging at 1,000–2,000× g for 10 minutes in a refrigerated centrifuge. The resulting supernatants were immediately transferred into a clean tube and stored at −20°C until use for the evaluation of specific humoral immune response by ELISA for IgG detection. Firstly, soluble somatic extracts from adult *S. mansoni* worms (SmAg) were obtained and determined protein concentration as previously described [38]. Briefly, polystyrene microtiter plates (Costar, USA) were coated with 100 µL/well of SmAg at a protein concentration of 5 µg/mL diluted in carbonate buffer (pH 9.6). Diluted serum at 1:100 was added to the wells and incubated for 1 h at 37°C. Horseradish peroxidase rabbit anti-mouse IgG (Sigma, USA) 1:2000 was added. Washes were carried out three times with 200 µL of PBS-Tween 20/well. After incubation for 1 h at 37°C, substrate solution (ortho phenylene diamine+H_2O) was added and the reaction was stopped after 10 min with 3 N H_2SO_4. Sera, tested by duplicate, were considered positive when the OD value exceeded the mean ± 2 SE absorbance of sera from non-infected animals.

DNA extraction for molecular analyses

DNA from mice stool samples. Approximately 220–250 mg of frozen stool samples from each mouse were used for DNA extraction using the NucleoSpin Tissue Kit (Macherey-Nagel, GmbH & Co., Germany) -according to the modified protocol for DNA extraction from stool- following the manufacturers' instructions. DNA samples obtained from feces from non-infected mice were pooled and the resulting mix was treated as a single negative control sample to minimize the number of subsequent reactions.

Parasites DNA samples. *S. mansoni* DNA was extracted from frozen adult worms available in our laboratory using NucleoSpin Tissue (Macherey-Nagel, Germany) following the manufacturers' instructions. The concentration of *S. mansoni* DNA adult worms was measured three times by spectrophotometry using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies) to obtain an average concentration and then diluted with ultrapure water to a final concentration of 0.5 ng/µL. Subsequently, serial 10-fold dilutions from *S. mansoni* DNA thus obtained (0.5 ng/µl) were also prepared with ultrapure water ranging from 0.05 ng/µl to 0.0005 fg/µl (10^{-1} to 10^{-9}) and stored at −20°C until use. DNA thus prepared was used as a positive control in all PCR and LAMP reactions as well as for assessing sensitivity of both assays.

Additionally, to determine the specificity of both PCR and LAMP assays, a total of 16 DNA samples from several helminths and protozoa were included as heterogeneous control samples. Of these samples, DNA from *Fasciola hepatica*, *Loa loa* and *Brugia pahangi* were already accessible in our laboratory. Other DNA samples were generously donated by the following: *Dicrocoelium dendriticum* and *Calicophoron daubneyi*, Y. Manga (CSIC, León, Spain); *Hymenolepis diminuta* and *Taenia taeniformis*, P. Foronda (University of La Laguna, Tenerife, Spain); *Anisakis simplex*, C. Cuéllar (UCM, Madrid, Spain) *Trichinella spiralis*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica*, E. Rodríguez (ISCIII, Madrid, Spain) and *Schistosoma intercalatum*, A. O. Castro (CIBP-INSA, Porto, Portugal). Genomic DNA from adult male and female *Schistosoma haematobium*, Egyptian Strain, NR-31682 and genomic DNA from adult male and female *Schistosoma japonicum*, Chinese Strain, NR-36066 were obtained from the Schistosomiasis Resource Center for distribution by BEI Resources, NIAID, NIH (https://www.beiresources.org/Collection/51/Schistosome-Resource-Centers.aspx).

Concentration of these DNA samples was measured by the same method as described for *S. mansoni* DNA and then also diluted with ultrapure water to a final concentration of 0.5 ng/µL. In order to look for protein contaminations a common purity check by measuring the A_{260}/A_{280} ratio was made for all samples. All these DNA samples were kept at −20°C until use in molecular assays.

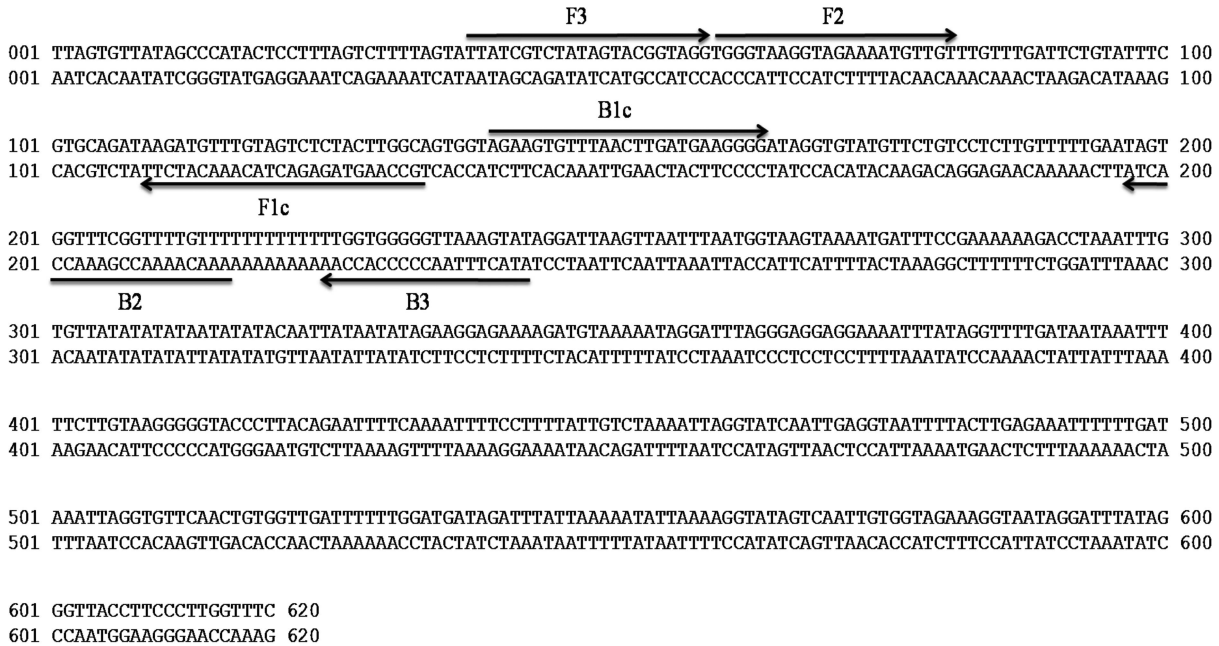
S. mansoni specific LAMP primers design

The first step in primers design was based on literature searches to identify potential sequences of DNA which were suspected to be used in specific detection of *S. mansoni*. Genbank sequences initially considered were tested *in silico* through BLAST searches [39] and alignment analysis. Finally, a 620 base pair (bp) sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region was preferred and retrieved from GenBank (Accession No. L27240) [40] for the design of specific primers. Forward and backward outer primers (F3 and B3) and forward and backward inner primers (FIP: F1c-F2 and BIP: B1c-B2, respectively) were designed using the Primer Explorer V4 software (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/). Several LAMP primer sets were suggested by the software and further refinement in primer design was developed manually based on the criteria described in "A Guide to LAMP primer designing" (http://primerexplorer.jp/e/v4_manual/index.html). Specific LAMP primers sequences finally selected as well as their positions relative to the 620 bp target sequence are shown in Figure 1. All the primers were synthesized by Eurofins MWG Operon.

PCR using outer primers F3 and B3

The outer LAMP primer pair, designated F3 and B3, was initially tested for the amplification of *S. mansoni* DNA by a *touchdown*-PCR to verify whether the correct target was amplified. The PCR assay was conducted in 25 µL reaction mixture containing 2.5 µL of 10× buffer, 1.5 µL of 25 mmol/L $MgCl_2$, 2.5 µL of 2.5 mmol/L dNTPs, 0.5 µL of 100 pmol/L F3 and B3,

A



B

Primers	Length (bp)	Sequence (5'-3')
F3	22	TTATCGTCTATAGTACGGTAGG
B3	19	ATACTTTAACCCCCACCAA
FIP (F1c-F2)	47	GCCAAGTAGAGACTACAAACATCTT-TGGGTAAGGTAGAAAATGTTGT
BIP (B1c-B2)	45	AGAAGTGTTTAACTTGATGAAGGGG-AAACAAAACCGAAACCACTA

Figure 1. Lamp primer set targeting the selected sequence (GenBank Accesion. No. L27240) for mitochondrial *S. mansoni* minisatellite DNA region amplification. (A) The location of the LAMP primers within the selected sequence is shown. Arrows indicate the direction of extension. (B). Sequence of LAMP primers: F3, forward outer primer; B3, reverse outer primer; FIP, forward inner primer (comprising F1c and F2 sequences); BIP, reverse inner primer (comprising B1c and B2 sequences).
doi:10.1371/journal.pntd.0003126.g001

2 U *Taq*-polymerase and 2 µL (1 ng) of DNA template. Initial denaturation was conducted at 94°C for 1 min, followed by a touchdown program for 15 cycles with successive annealing temperature decrements of 1.0°C every 2 cycles. For these 2 cycles, the reaction was denatured at 94°C for 20 s followed by annealing at 58°C–56°C for 20 s and polymerization at 72°C for 30 s. The following 15 cycles of amplification were similar, except that the annealing temperature was 55°C. The final extension was performed at 72°C for 10 min.

The specificity of PCR using outer primers F3 and B3 was also tested with heterogeneous DNA samples from other parasites included in the study. Moreover, the sensitivity of the PCR was also assayed to establish the detection limit of *S. mansoni* DNA with 10-fold serial dilutions prepared as mentioned above. The assay was performed with 2 µL of the diluted template in each case. Negative controls (ultrapure water instead DNA template) were included. The PCR products (3–5 µL) were subjected to 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light.

LAMP assay

Two different reaction mixtures containing the primers designed were used to assess the LAMP assay to compare results. On one hand, the LAMP assay was performed using the *Loopamp DNA amplification Kit* (Eiken Chemicals Co., Tokyo, Japan) following manufacturers' instructions. Briefly, the reaction was carried out with a total of 25 µL reaction mixture containing 12.5 µL of 2× Reaction Mix, 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1 µL of *Bst* DNA polymerase (Large fragment; LF), along with 2 µL of DNA template.

On the other hand, we tried to set up our own LAMP reaction mixture testing another *Bst* polymerase, namely *Bst* 2.0 WarmStart DNA polymerase, as well as different betaine and $MgSO_4$ concentrations instead of those supplied by the commercial kit. Thus, LAMP reactions mixtures (25 µL) contained 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1.4 mM of each dNTP (Bioron), 1× Isothermal Amplification Buffer −20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), betaine (ranging 0.8, 1, 1.2, 1.4 or 1.6 M) (Sigma, USA), supplementary $MgSO_4$ (ranging 2, 4, 6 or 8 mM) (New England Biolabs, UK) and 8 U of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, UK) with 2 µL of template DNA.

To establish the standard protocol for the two LAMP reaction mixtures assayed, different temperatures were tested using a

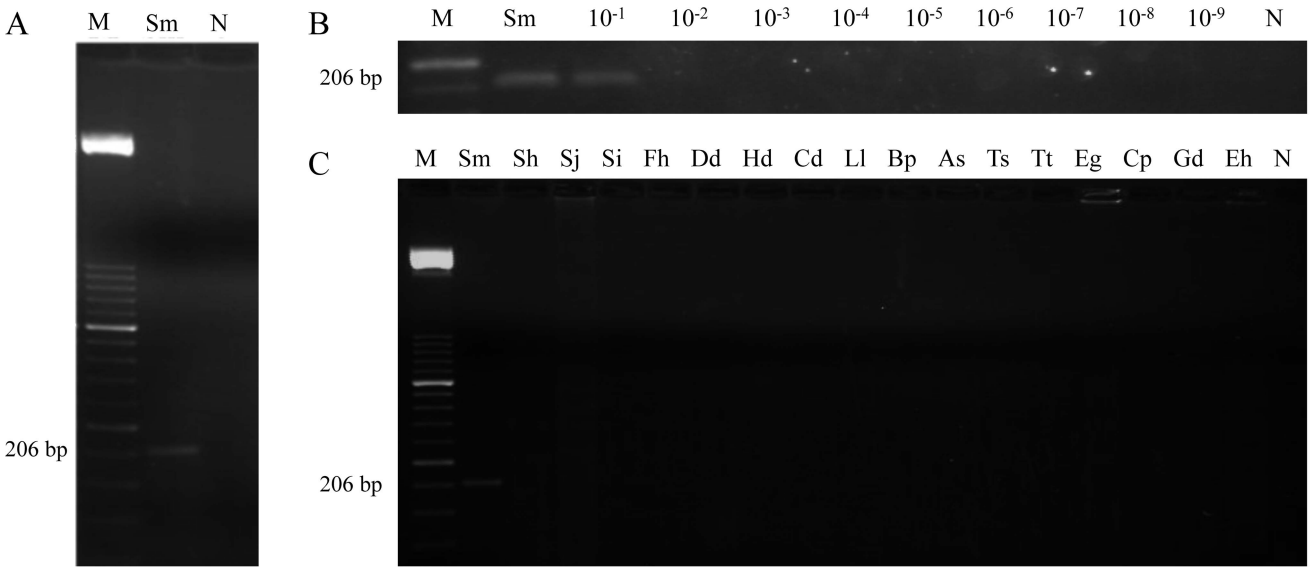


Figure 2. PCR verification, detection limit and specificity using outer primers F3 and B3. (A) PCR verification of expected 206 bp target length amplicon. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sm, *S. mansoni* DNA (1 ng); lane N, negative control (no DNA template). (B) Detection limit of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sm: *S. mansoni* DNA (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions of *S. mansoni* DNA; lane N, negative control (no DNA template). (C) Specificity of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, Sh, Sj, Si, Fh, Dd, Hd, Cd, Ll, Bp, As, Ts, Tt, Eg, Cp, Gd, Eh, *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *Dicrocoelium dendriticum*, *Hymenolepis diminuta*, *Calicophoron daubneyi*, *Loa loa*, *Brugia pahangi*, *Anisakis simplex*, *Trichinella spiralis*, *Taenia taeniformis*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template).
doi:10.1371/journal.pntd.0003126.g002

heating block (K Dry-Bath) set at 61, 63 and 65°C for 60 min and then heated at 80°C for 5 min to terminate the reaction. In each case, the optimal temperature was determined and used in the following tests. Because of the highly sensitivity of LAMP reaction, DNA contamination and carry-over of amplified products were prevented by using sterile tools at all times, performing each step of the analysis in separate work areas and minimizing manipulation of the reaction tubes. Negative controls (ultrapure water or DNA from non-infected stool samples) were included in each LAMP reaction. These controls never amplified.

Detection of LAMP products. After LAMP reaction, white turbidity of the reaction mixture due to the accumulation of magnesium pyrophosphate (a by-product of the reaction) was visually inspected by the naked eye. The LAMP amplification results were also visually detected by adding 2 µL of 1:10 diluted 10,000× concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tubes. Green fluorescence was clearly observed in successful LAMP reaction, whereas it remained original orange in the negative reaction. Additionally, the LAMP reaction products (5 µL aliquots) were also monitored using 2% agarose gel electrophoresis stained with ethidium bromide, visualized under UV light and then photographed using the ultraviolet (UV) image system (Gel documentation system, UVItec, UK).

Specificity and sensitivity of the LAMP assay. The specificity of the LAMP assay using the two different reaction mixtures to amplify only *S. mansoni* DNA was tested against several DNA samples obtained from other parasites used as controls, including *S. haematobium*, *S. japonicum*, *S. intercalatum*, *Fasciola hepatica*, *Loa loa*, *Brugia pahangi*, *Dicrocoelium dendriticum*, *Calicophoron daubneyi*, *Hymenolepis diminuta*, *Taenia taeniformis*, *Anisakis simplex*, *Trichinella spiralis*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica*. To determine the lower detection limit of the LAMP assay, genomic DNA from *S. mansoni* 10-fold serially diluted as mentioned above was subjected to amplification in comparison with a conventional PCR using outer primers F3 and B3.

Evaluation of the LAMP assay. To evaluate the ability of the LAMP assay designed to amplify *S. mansoni* DNA in real samples, we used DNA samples extracted from feces taken weekly from each experimentally infected mouse with 200 *S. mansoni* cercariae. In order to compare results, the *Loopamp DNA amplification Kit* and our *in house* LAMP reaction mixture were tested separately with all infected mice stool samples by duplicate from week 0 to week 8 p.i. In all amplification assays, a negative control (DNA mix from non-infected mice stool samples) was included.

Results

Monitoring of *S. mansoni* infection in mice by Kato-Katz and ELISA

The results obtained using Kato-Katz and indirect ELISA techniques on weekly stool and sera samples, respectively, from infected mice with 200 *S. mansoni* cercariae are showed in Figure S1. After infection, using the Kato-Katz technique we could only detect eggs in feces from week 6 to week 8 p.i. Specific detectable antibody levels could be measurable by ELISA from week 4 p.i. to week 8 p.i.

Specificity and sensitivity of PCR using outer primers

To make sure that the expected target was amplified, a conventional PCR reaction was performed using outer primers F3 and B3 to amplify *S. mansoni* DNA. Then, a 206 bp amplicon was successful obtained (Figure 2A). In order to determine the lower detection limit of the PCR, a 10-fold serial dilution ranging from

10^{-1} to 10^{-9} of *S. mansoni* DNA was amplified. The minimum amount of DNA detectable by PCR was 0.1 ng (Figure 2B). Moreover, when DNA samples from other parasites included in the study were subjected to this PCR assay, amplicons were never obtained (Figure 2C). Additionally, when *in silico* comparisons of the expected 206 bp sequence were carried out using BLASTn searches with the currently available genomes of *S. mansoni*, *S. haematobium* and *S. intercalatum* at Wellcome Trust Sanger Institute web site (http://www.sanger.ac.uk) and *S. japonicum* at GenDB web site (http://www.genedb.org), respectively, the higher homology in alignment length, percentage of identities and E-value were obtained for *S. mansoni*. For *S. intercalatum*, *S. haematobium* and *S. japonicum*, much lower values were found (Table S1).

Setting up LAMP assay

The optimal incubation temperature for LAMP assay using the *Loopamp DNA amplification Kit* tested with the *S. mansoni* primer set was established in a conventional heating block using a range of temperatures (61, 63 and 65°C) for 60 min to optimize the reaction conditions and then heated at 80°C for 5 min to inactivate the enzyme. The LAMP reaction could successfully take place at temperatures of 61°C, 63°C and 65°C -within the temperature range (60–65°C) recommended by the manufacturers'- although better results on agarose gels were obtained when using 63°C for amplification. Thus, the optimal temperature for LAMP using the commercial kit was established at 63°C and used for all the following applications.

To establish the standard protocol for our *in house* LAMP assay using *Bst* 2.0 WarmStart DNA polymerase we also applied a range of temperatures (61, 63 and 65°C) for testing different mixtures containing variable concentrations of betaine (ranging 0.8, 1, 1.2, 1.4 or 1.6 M) combined with supplementary variable concentrations of $MgSO_4$ (ranging 2, 4, 6 or 8 mM). The best amplification results were obtained when the reaction mixture contained 1 M of betaine combined with supplementary 6 mM of $MgSO_4$ (resulting a final concentration of 8 mM $MgSO_4$ in 1× Isothermal Amplification Buffer) and was incubated for 60 min at 63°C in a heating block. Thereby, the reaction mixture, in addition to the specific primer set designed -hereafter SmMIT-LAMP-, was set up as the most suitable and used in all successive LAMP reactions.

Once the most favorable conditions and molecular components were established for the two different LAMP reactions, all positive results could be visually observed by the naked eye by inspecting white turbidity as well as the color change after adding SYBR Green I. Additionally, after electrophoresis on agarose gels a ladder of multiple bands of different sizes could be also observed in positive samples (Figure 3).

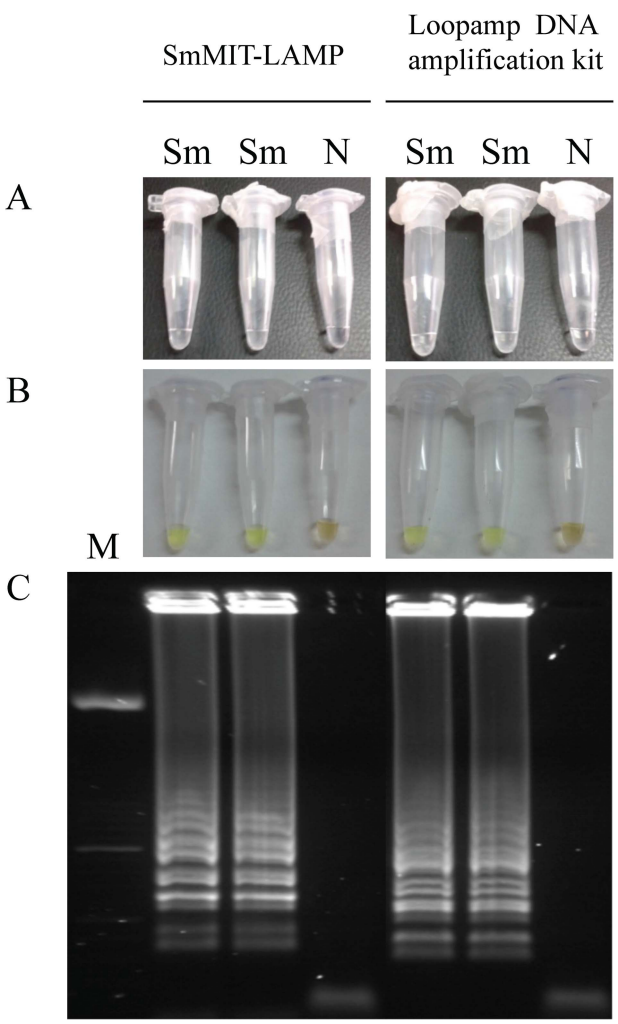


Figure 3. LAMP detection of *S. mansoni* genomic DNA samples using SmMIT-LAMP or the *Loopamp DNA amplification kit* at 63°C for 1 h. (A) The turbidity of the reaction mixture was inspected by the naked eye. (B) The LAMP amplification results were also visually detected by adding the fluorescent dye SYBR Green I to the reaction tubes. A successful LAMP reaction would turn to green; otherwise, it would remain orange (C) LAMP products were also monitored using 2% agarose gel electrophoresis stained with ethidium bromide. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm: *S. mansoni* DNA (1 ng); lane N, negative control (no DNA template).
doi:10.1371/journal.pntd.0003126.g003

Specificity and sensitivity of LAMP assay

To determine the specificity of LAMP assay for *S. mansoni*, 16 additional DNA samples from other parasites were tested for amplification. We obtained identical results using both the *Loopamp DNA amplification Kit* and SmMIT-LAMP reaction mixtures. Thus, a positive result was only obtained using DNA from *S. mansoni* whereas DNA samples from other specimens were not amplified. These results indicate that no cross-amplification was observed with these heterogeneous species in the LAMP assay, demonstrating its high specificity (Figure 4A).

Nevertheless, when sensitivity was evaluated using *S. mansoni* DNA 10-fold serially diluted, the limit of detection of LAMP using the *Loopamp DNA amplification Kit* was 10 fg (Figure 4B), whereas the limit of detection using SmMIT-LAMP was established in 1 fg (Figure 4C). These results showed that sensitivity of the SmMIT-LAMP assay is tenfold higher than that of the LAMP assay by using a standard reaction mixture supplied by the commercial kit. Furthermore, the detection limit of SmMIT-LAMP was 10^5 times more than that previously obtained by PCR (see Figure 2B).

Examination of mice stool samples by LAMP

When testing stool samples from mice infected with *S. mansoni* by the *Loopamp DNA amplification Kit*, we detected positive results continuously from week 2 p.i. to week 8 p.i. in all samples analyzed (Figure 5A). By contrast, when using SmMIT-LAMP for amplification, positive results were continuously obtained in all stool samples from week 1 p.i. to week 8 p.i. (Figure 5B). Therefore, the SmMIT-LAMP assay developed was able to detect *S. mansoni* DNA in stool samples one week earlier in comparison

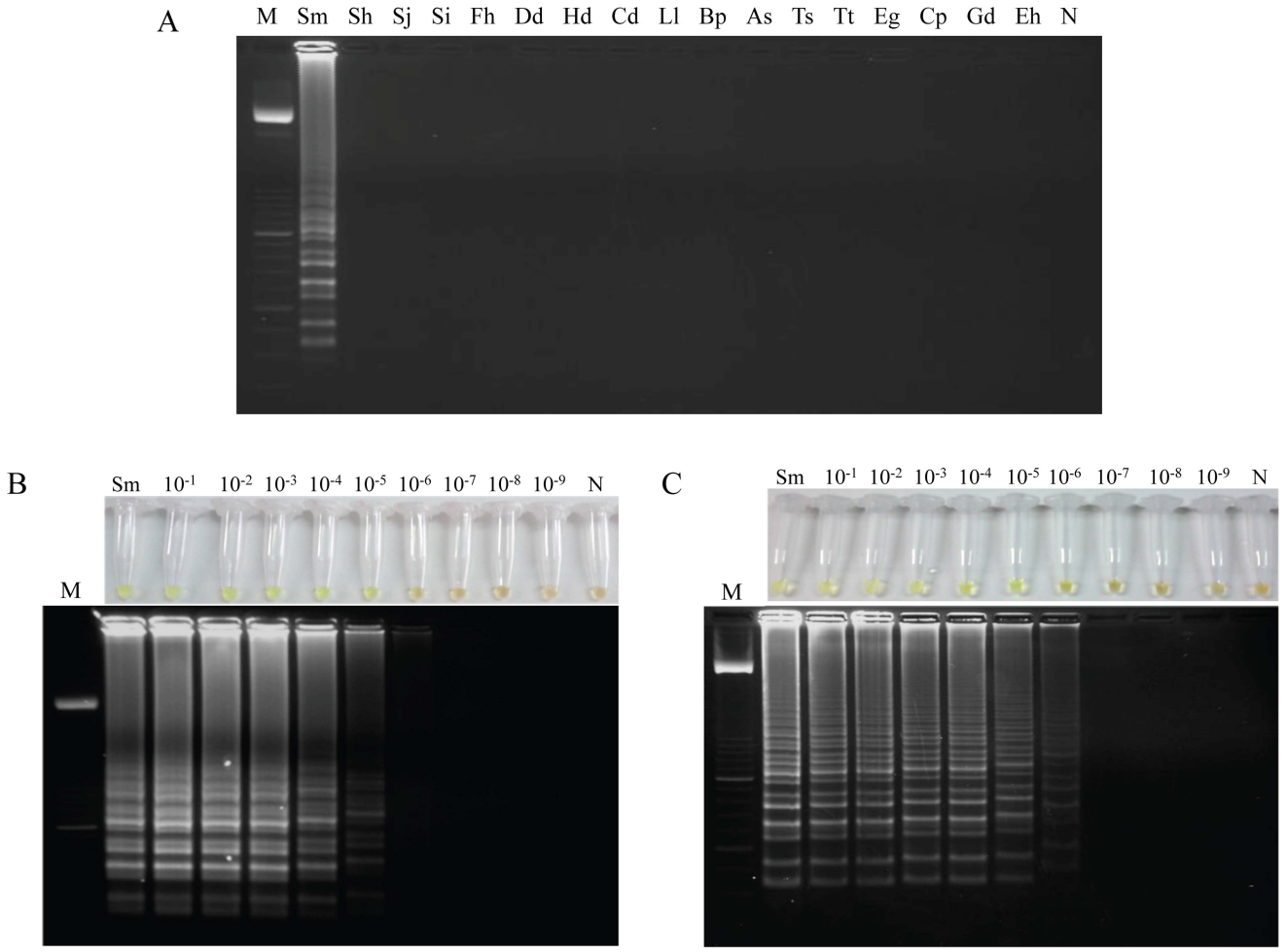


Figure 4. Specificity and sensitivity assessment of the LAMP assay for *S. mansoni*. (A) Specificity assessment performed with SmMIT-LAMP is shown. Identical results were obtained using *Loopamp DNA amplification Kit*. A ladder of multiple bands of different sizes could be only observed in *S. mansoni* DNA sample. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, Sh, Sj, Si, Fh, Dd, Hd, Cd, Ll, Bp, As, Ts, Tt, Eg, Cp, Gd, Eh, *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *Dicrocoelium dendriticum*, *Hymenolepis diminuta*, *Calicophoron daubneyi*, *Loa loa*, *Brugia pahangi*, *Anisakis simplex*, *Trichinella spiralis*, *Taenia taeniformis*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template). (B) Sensitivity assessment performed with the *Loopamp DNA amplification kit* at 63°C for 1 h using serial dilutions of *S. mansoni* genomic DNA by the addition of SYBR Green I (up) or by visualization on agarose gel (down). (C) Sensitivity assessment performed with SmMIT-LAMP at 63°C for 1 h using serial dilutions of *S. mansoni* genomic DNA by the addition of SYBR Green I (up) or by visualization on agarose gel (down). Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm: genomic DNA from *S. mansoni* (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions; lanes N: negative controls (no DNA template).
doi:10.1371/journal.pntd.0003126.g004

to the LAMP assay accomplished with a standard commercial reaction mixture.

Discussion

Human schistosomiasis, caused by several species of the trematode *Schistosoma*, is a major endemic parasitic disease in many tropical regions of Asia, Africa and South America, with *S. mansoni* being the most important species in terms of prevalence, morbidity, mortality and socioeconomic impact [2]. This morbidity and mortality is mainly associated with the chronic stage of infection, when egg deposition followed by granuloma formation in different organs, especially in liver and intestine, occurs. Although the use of praziquantel as chemotherapeutic treatment and control for the disease has a clear effect on morbidity [41], [42], resistance has been already described after repeated mass drug administration [43]. Thus, methods that allow early diagnosis, both in acute and chronic stages, are a prerequisite for effective disease control. Moreover, diagnostic tools able to detect *S. mansoni* infections mainly in acute stage would be of great value permitting early treatment that could prevent the pathology associated with chronic infections.

Currently, the *gold standard* method for diagnosis of *S. mansoni* infections is the Kato-Katz technique to count of parasite eggs excreted in feces because of its low operational costs, practicality and ability to be quantitative. However, this method requires sequential samples and is unable to detect prepatent infection, low levels of infection particularly found in children [44] or infections in individuals with a low worm burden and those in low disease transmission areas [45]. On the other hand, although many of the serology-based analyses developed present greater sensitivity than Kato-Katz technique, they currently continue to present problems in acute infections detection, lack of sensitivity, cross-reactions and false positives usually corresponding to patients who have already

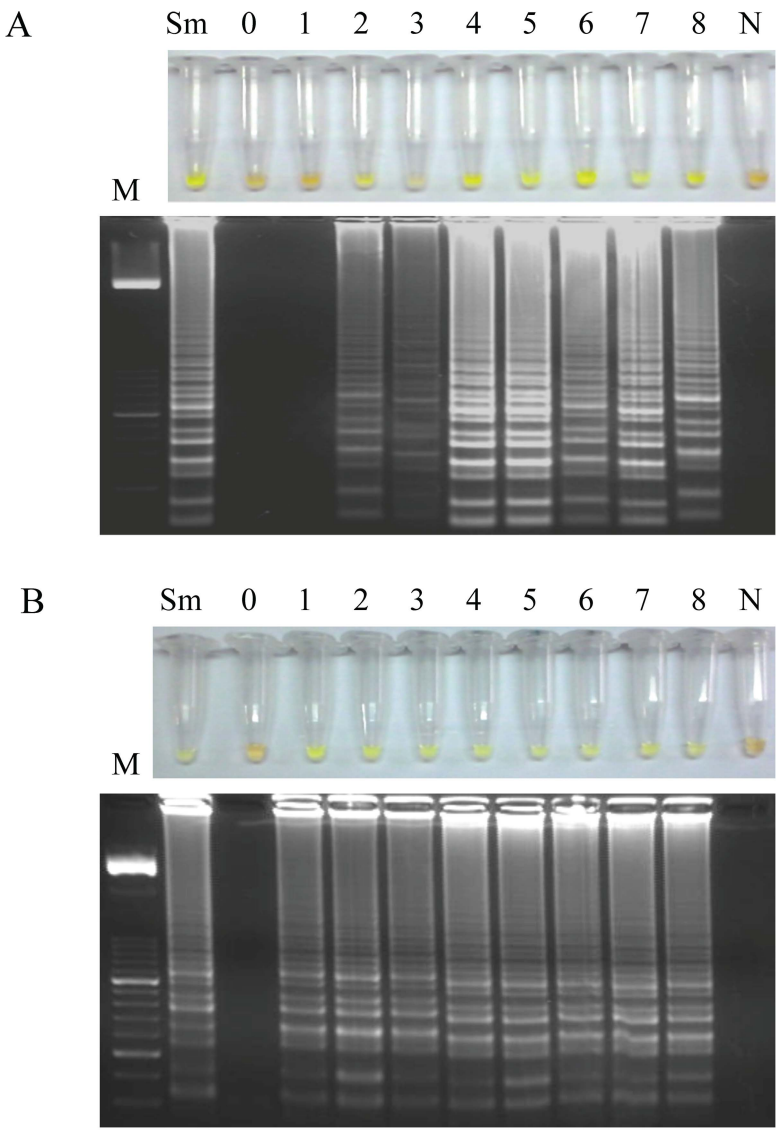


Figure 5. Examination of stool samples weekly obtained from mice infected with 200 *S. mansoni* cercariae. (A) By using the *Loopamp DNA amplification kit*. (B) By using the SmMIT-LAMP developed in this study. Figure shows the results obtained in feces samples weekly obtained from week 0 p.i. to week 8 p.i. from an infected mouse randomly selected. Identical results were obtained in all infected mice. Lanes M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, *S. mansoni* DNA, as positive control (1 ng); lanes 0–8, weeks 0, 1, 2, 3, 4, 5, 6, 7 and 8 p.i., respectively; lanes N; DNA mix from pooled DNA samples obtained from feces from non-infected mice, as negative control.
doi:10.1371/journal.pntd.0003126.g005

eliminated the parasite after efficient chemotherapy [46], [47]. All this together is currently a drawback and a considerable number of schistosomiasis patients can be incorrectly diagnosed. In this scenario, there is a need in developing new specific and more sensitive molecular diagnostic tools easy to perform in field conditions for diagnosis of schistosomiasis due to *S. mansoni*. An interesting and promising approach is the LAMP technology. Compared to PCR-based assays, LAMP has the advantages of reaction simplicity, rapidity, specificity, cost-effective and higher amplification efficiency. Furthermore, since DNA amplification and reading of results require minimum equipment, the technique has great potential for use in low-income countries [26], [27], [28]. These advantages of the technique make it appealing for use in schistosomiasis-endemic regions.

In our study, we used a *S. mansoni* murine model in order to test a new LAMP assay for early diagnosis of schistosomiasis in stool samples. Classical diagnostic techniques, such as Kato-Katz and ELISA were used for monitoring infection. Mice have been shown to be permissive to *S. mansoni* [48] and also widely used in studying dynamics of schistosome infections, including diagnosis [49]. The use of a *S. mansoni* murine model allowed us to collect well-defined stool samples that would otherwise have been difficult to obtain from human patients, such as stool samples from recently acquired infections. Thus, using the Kato-Katz technique we could detect eggs in feces collected from infected mice from week 6 to 8 p.i. When measuring specific detectable antibody levels by ELISA we could detect IgG in infected mice from week 4 p.i. until the end of the experiment. Very similar results were previously obtained by our group in detecting eggs in stool and specific antibodies in sera from mice infected with 200 *S. mansoni* cercariae [50]. As it would be logical to expect, classical diagnostic techniques were not effective to detect the acute stage of infection.

In our work, a 620 bp sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region [40] was selected as a target for designing a LAMP-based method to detect *S. mansoni* DNA. The minisatellite region in the mitochondrial genome of *S. mansoni* seems to be unique to that species and has been already used as a target for PCR-based identification of infected snails [51]. In addition, mitochondrial sequences have some advantages over the more usual nuclear targets for amplification approaches. As each cell contains many mitochondria, multiple copies occur in every cell providing many copies of any mitochondrial DNA target region. Thus, greater sensitivity will be possible if the target sequence is present in high copy number and is highly specific and widely conserved within a particular pathogen species or group [52]. The mito-LAMP strategy has already been successfully developed for detection of several parasites, including *Opisthorchis viverrini* [53], *Trichinella spiralis* [54], *Echinococcus granulosus* [55] *and Plasmodium* spp. or *P. falciparum* specifically [56].

Once the primer set was designed, we attempted to verify the specificity for a 206 bp expected fragment amplification using a conventional PCR performed with the two outers primers to amplify *S. mansoni* DNA. As a result, a correctly sized amplicon was obtained. Additionally, no cross-reactions were found when using DNA as a target from other parasites tested in the study, including several *Schistosoma* species, such as *S. haematobium*, *S. japonicum* or *S. intercalatum*, thereby ensuring high specificity for target amplification. Furthermore, *in silico* comparisons of the expected 206 bp sequence with the *on line* available genomes of *Schistosoma* spp. showed the higher homology in alignment length with *S. mansoni*.

After verifying the specificity of the outer primers by PCR in only *S. mansoni* DNA amplification, we attempted to establish the most suitable reaction mixture for the four specific primers operation in the LAMP assay. To do this, we used a standard reaction mixture supplied by the *Loop Amplification kit* containing *Bst* DNA polymerase LF and an *in house* reaction mixture containing *Bst* 2.0 WarmStart polymerase. The latter, is an *in silico* designed homologue of *Bst* DNA polymerase LF with a reversibly-bound aptamer, which inhibits polymerase activity at temperatures below 45°C. This feature prevent about the possible undesired activity from DNA polymerases during preparation of reaction mixtures at room temperature [57], [58], [59], allowing the preparation as well as storage of the LAMP reactions for hours without changing in the final readout, as recently reported in diagnosis of brugian filariasis by LAMP using *Bst* 2.0 WarmStart polymerase [60]. It should be noted that this feature is a very important advantage in order to perform a LAMP assay in field settings where usually limited resources are found.

Regarding specificity, both LAMP reaction mixtures performed equally well at established optimal incubation temperature and exclusively *S. mansoni* DNA was amplified. However, when sensitivity of the LAMP reaction mixtures were evaluated using *S. mansoni* DNA 10-fold serially diluted, the limit of detection using SmMIT-LAMP resulted tenfold higher than that obtained using the standard reaction mixture supplied by the commercial kit (1 fg *vs*. 10 fg, respectively), thus indicating that SmMIT-LAMP is sensitive enough to detect *S. mansoni* DNA at a very low level. We underline the importance of setting up the best conditions and molecular components for primers set operation in a LAMP assay. Besides, developing an *in house* LAMP assay is much more cost-effective than using more expensive commercial kits when a large number of samples must be tested.

That increased sensitivity achieved using SmMIT-LAMP was subsequently corroborated when weekly stool samples from infected mice were tested using both LAMP reaction mixtures. In this sense, SmMIT-LAMP allowed us to detect *S. mansoni* DNA in all infected mice samples one week earlier than using the LAMP commercial reaction mixture (1 week p.i. *vs*. 2 week p.i., respectively). Therefore, an early diagnosis of active *S. mansoni* infection was possible in stool samples using SmMIT-LAMP as soon as one week p.i. It is also noteworthy that green fluorescence by adding SYBR Green I was clearly observed in all successful LAMP reactions, whereas it remained original orange in the negative reactions. This color inspection by the naked eye is a great advantage of the LAMP technique and may be preferentially used under field conditions in endemic areas without requiring electrophoresis to visualize the amplification results.

In conclusion, the results of our study demonstrated that the established SmMIT-LAMP assay is cost-effective, easy to perform, specific and sensitive enough for early detection of *S. mansoni* DNA in stool samples. Although further research for evaluation of the method for the application in patients' samples is required, the method is potentially and readily adaptable for field diagnosis and disease surveillance in schistosomiasis-endemic areas.

Supporting Information

Figure S1 Monitoring of *S. mansoni* experimental infection in mice by Kato-Katz and ELISA in weekly stool and sera samples, respectively, from weeks 0 to 8 post-infection. Mice were experimentally infected with 200 *S. mansoni* cercariae. X axis represent weeks post-infection. Y axis represent number of *S. mansoni* eggs/g of feces (mean±SE; left) and absorbances of respective sera (OD) read ad 492 nm (mean±SE; right).
(TIFF)

Table S1 BLASTn results comparing the *S. mansoni* expected 206 base pair target amplicon with the whole genome of currently available *Schistosoma* species. *Schistosoma* species, currently genomes versions available, scaffolds, alignment situation, alignment length, identities and E-value are indicated. Results obtained for *S. mansoni, S. haematobium and S. intercalatum* come from Wellcome Trust Sanger Institute web site (http://www.sanger.ac.uk) and results obtained for *S. japonicum* come from GenDB web site (http://www.genedb.org). bp: base pair; %: percentage of identity.
(DOCX)

Checklist S1 Checkmarks for the STARD checklist.
(PDF)

Flow Diagram S1 A diagram showing experimental design and results. p.i. indicates post-infection.
(TIFF)

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Author Contributions

Conceived and designed the experiments: PFS JGA AM JLA. Performed the experiments: JGA PFS ASH BVS. Analyzed the data: PFS JGA AM JLA. Contributed reagents/materials/analysis tools: JGA PFS ASH BVS JLA. Contributed to the writing of the manuscript: PFS JGA AM.

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3.2. Artículo 2

The Rapid-Heat LAMPellet Method: A Potential Diagnostic Method for Human Urogenital Schistosomiasis.

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PLoS Negl Trop Dis. 2015;9(7): e0003963.

Resumen

La esquistosomosis urogenital, producida por *S. haematobium*, es una enfermedad desatendida que afecta alrededor de 112 millones de personas, mayoritariamente en África Subsahariana. La detección microscópica de huevos en orina continua siendo el diagnóstico de referencia. En este trabajo hemos desarrollado un nuevo método LAMP (*loop-mediated isothermal amplification*) para la detección de ADN de *S. haematobium* en muestras de orina de origen humano como una herramienta simple, efectiva, de alto rendimiento y aplicable en el diagnóstico de la esquistosomosis urogenital.

El LAMP diseñado amplifica un fragmento de ADN del espaciador inter-génico ribosomal. La técnica fue evaluada usando una batería de muestras de orina de pacientes infectados previamente sometidas a exámenes parasitológicos. Para su potencial aplicación en condiciones de campo, empleamos diferentes métodos para el procesamiento de la muestra y la obtención del ADN, incluyendo un *kit* comercial, la técnica de NaOH modificada y un simple calentamiento del sedimento urinario, empleando pequeños volúmenes de diferentes fracciones de orina (orina total, sobrenadante y *pellet*). El calentamiento del sedimento urinario fue el método más efectivo para la amplificación de ADN de *S. haematobium* desde muestras de orina, llegando a detectar 1 fg/μL de ADN. Cuando analizamos todas las muestras de orina, los valores diagnósticos de nuestro LAMP alcanzaron una sensibilidad de 100% (95% IC: 81.32%-100%) y una especificidad de 86.67% (95% IC: 75.40%-94.05%), y para la detección microscópica de huevos en orina, una sensibilidad de 69.23% (95% IC: 48.21% -85.63%) y una especificidad de 100% (95% IC: 93.08%-100%).

Se ha desarrollado y evaluado el primer método LAMP para la detección de ADN de *S. haematobium* en muestras de orina de pacientes sin necesidad de un método sofisticado para la obtención del ADN. El procedimiento, llamado *Rapid-Heat LAMPellet method,* presenta potencial para ser aplicado bajo condiciones de campo para el diagnóstico de la esquistosomosis urogenital.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

The Rapid-Heat LAMPellet Method: A Potential Diagnostic Method for Human Urogenital Schistosomiasis

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Abstract

Background

Urogenital schistosomiasis due to *Schistosoma haematobium* is a serious underestimated public health problem affecting 112 million people - particularly in sub-Saharan Africa. Microscopic examination of urine samples to detect parasite eggs still remains as definitive diagnosis. This work was focussed on developing a novel loop-mediated isothermal amplification (LAMP) assay for detection of *S. haematobium* DNA in human urine samples as a high-throughput, simple, accurate and affordable diagnostic tool to use in diagnosis of urogenital schistosomiasis.

Methodology/Principal Findings

A LAMP assay targeting a species specific sequence of *S. haematobium* ribosomal intergenic spacer was designed. The effectiveness of our LAMP was assessed in a number of patients´ urine samples with microscopy confirmed *S. haematobium* infection. For potentially large-scale application in field conditions, different DNA extraction methods, including a commercial kit, a modified NaOH extraction method and a rapid heating method were tested using small volumes of urine fractions (whole urine, supernatants and pellets). The heating of pellets from clinical samples was the most efficient method to obtain good-quality DNA detectable by LAMP. The detection limit of our LAMP was 1 fg/μL of *S. haematobium* DNA in urine samples. When testing all patients´ urine samples included in our study, diagnostic parameters for sensitivity and specificity were calculated for LAMP assay, 100% sensitivity (95% CI: 81.32%-100%) and 86.67% specificity (95% CI: 75.40%-94.05%), and also for microscopy detection of eggs in urine samples, 69.23% sensitivity (95% CI: 48.21% -85.63%) and 100% specificity (95% CI: 93.08%-100%).

Competing Interests: The authors have declared that no competing interests exist.

Conclusions/Significance

We have developed and evaluated, for the first time, a LAMP assay for detection of *S. haematobium* DNA in heated pellets from patients´ urine samples using no complicated requirement procedure for DNA extraction. The procedure has been named the Rapid-Heat LAMPellet method and has the potential to be developed further as a field diagnostic tool for use in urogenital schistosomiasis-endemic areas.

Author Summary

Human schistosomiasis is a disease caused by several species of parasitic worms of the genus *Schistosoma* that is affecting 200 million people, especially in sub-Saharan Africa. Most people are infected with *Schistosoma haematobium*, the species that causes urogenital schistosomiasis and also bladder cancer in many chronic infections. The definitive diagnostic test is based on microscopic examination of urine samples to detect parasite eggs. This method has low sensitivity, high day-to-day variability and cannot be carried out in the acute phase of the disease since the parasite has not started yet to lay eggs. New high-throughput diagnostic tools would be desirable, permitting early treatment and preventing the pathology associated with chronic infections. An interesting approach is the loop-mediated isothermal amplification (LAMP) technique because of its simplicity in operation and potential use in clinical diagnosis and surveillance of infectious diseases. In this study, we developed and evaluated a LAMP assay for detection of *S. haematobium* DNA in patients´ urine samples using heated pellets with no complicated requirement procedure for DNA extraction, namely the Rapid-Heat LAMPellet method. This is a new, easy, rapid and cost-effective LAMP method that should prove useful for mass screening in limited-resource settings in urogenital schistosomiasis-endemic areas.

Introduction

Human schistosomiasis, a parasitic freshwater snail transmitted disease caused by several species of genus *Schistosoma* trematode worms, is one of the 17 neglected tropical diseases (NTDs) considered by World Health Organization (WHO) [1]. It is estimated that 732 million persons are at risk of infection worldwide and over 200 million people are infected with this disease in 74 different countries, especially in sub-Saharan Africa [2–4], where both associated morbidity and mortality are a significant barrier to social and economic development [5–7]. It must be also observed that the prevalence of imported schistosomiasis is increasingly a problem in non-endemic areas due to the growing number of international travellers to endemic areas, expatriates and immigrants from endemic countries [8–10]. Although humans are mainly infected by five species of schistosomes, namely *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi*, and *S. intercalatum*, the main burden of disease in sub-Saharan Africa is usually attributed to two species referred to as the major human schistosomes: *S. mansoni*, causing hepatic and intestinal schistosomiasis and *S. haematobium*, the chief cause of urogenital schistosomiasis [3].

More people are infected with *S. haematobium* than with the other schistosomes; it is estimated that 112 million people suffer from urogenital schistosomiasis [11–14]. The infection typically results in haematuria, anaemia, dysuria and genital and urinary tract lesions, but in

severe cases it may also lead to kidney damage. It is well known that the deposition of *S. haematobium* eggs eventually leds to squamous cell carcinoma of the bladder in many chronically infected individuals [15, 16] the International Agency for Cancer Research (IACR) in association with WHO classified *S. haematobium* as a Group 1 biological carcinogen [17]. Moreover, most of women infected with *S. haematobium* suffer from female genital schistosomiasis of the lower genital tract [13]; which impairs fertility [18] and also increases susceptibility of the woman to HIV [19].

For the diagnosis of urogenital schistosomiasis, the *gold standard* remains microscopic detection of excreted ova in urine samples [20] after using either sedimentation/centrifugation or filtration methods [21]. These conventional methods are inexpensive, easy to perform under field conditions and relatively rapid. However, parasitological diagnosis has classically low sensitivity, especially in low-grade infections and may be affected by day-to-day variability in egg excretion, often missing diagnosis by microscopy [22, 23]. In addition, egg count-based criteria cannot be carried out in the acute phase of the disease since the parasite have not yet started to produce eggs. The collection of a larger number of urine samples per individual on consecutive days instead of a single one may increase the sensitivity of microscopic detection, but is more expensive and also time-consuming [23]. Identifying blood in the urine-micro or macrohaematuria- has been widely and successfully used as a good indicator of *S. haematobium* infection, mainly in a high prevalence situation. However, haematuria is a nonspecific symptom of urogenital schistosomiasis in areas of low endemicity and can be incorrectly estimated depending on the infection prevalence in an area [24, 25]. Antibody-based assays are useful to confirm *S. haematobium* infections, but do not distinguish active infection from past exposure, and so low sensitivity and specificity results frequently occur. Moreover, antibody tests are usually negative during acute symptomatic urogenital schistosomiasis. On the other hand, assays that detect circulating antigens seem very promising in the early phase of infection but still lack sensitivity in the diagnosis of light infections [20, 26, 27].

To overcome the drawbacks of both classical parasitological and immunological diagnostic methods, the development of new, more sensitive and specific molecular diagnostic tools for the diagnosis of urogenital schistosomiasis are desirable and still needed. In recent years, several studies have reported the utility of polymerase chain reaction (PCR)-based assays for sensitive and specific detection of *S. haematobium* DNA in human urine [28–30] and serum [31] samples. However, the PCR-based technologies are not widely used in low-income *S. haematobium* endemic countries because skilled operators and costly equipment are needed.

In this way, the loop-mediated isothermal amplification (LAMP)assay [32] offers a field-friendly alternative to PCR-based technologies as it is less time consuming than PCR and can be performed using a simple heating block or water bath, with results read by the naked eye under natural or UV light [33, 34]. Additionally, LAMP reagents can be storage at room temperature for weeks [35], the reaction shows low susceptibility to typical inhibitory compounds occurring in samples [36–38], its robustness against variation of reaction conditions such as pH and temperature has been described [39] and it can operate with minimal handling and processing of DNA samples for amplification [40], [41–43], or even without prior DNA extraction [36]. Thereby, considering these salient advantages over most DNA-based amplification tests, LAMP technology shows a potential use in clinical diagnosis and surveillance of infectious diseases, particularly under field conditions for most NTDs [44, 45].

Several successful approaches for LAMP assay for *Schistosoma* spp. detection have been recently reported in laboratory settings using experimentally infected animals, such as *S. japonicum* in rabbits [46, 47] or *S. mansoni* in mice [48], as well as in field settings for monitoring infected snails with *S. mansoni*, *S. haematobium* [49, 50] and *S. japonicum* [51, 52]. Additionally, a LAMP to detect *S. japonicum* in human sera has been also reported [53].

Thus, with the aim to develop new, applicable and cost-effective molecular tools for the diagnosis of urogenital schistosomiasis, in our work we have developed a new sensitive and specific LAMP assay for detection of *S. haematobium* in human urine samples. In this study, the effectiveness of the LAMP assay was evaluated in a number of patients´ urine samples with parasitological proven infection with *S. haematobium*. Different fractions of urine samples (whole urine, supernatants and pellets) as well as different methods for DNA extraction were used to compare results and cost-effectiveness. To the best of our knowledge, this is the first report using LAMP assay for detection of *S. haematobium* in human urine samples.

Methods

Ethics statement

Human urine samples used in this study were obtained as part of public health activities at Hospital Universitario Insular, Las Palmas de Gran Canaria, Spain. Later, samples were sent and stored at CIETUS, University of Salamanca, Spain, for further analyses. Human urine samples were not collected specifically for this study and all were obtained under written informed consent and coded and tested as anonymous samples. Participation of healthy urine donors for obtaining simulated artificial urine samples was voluntary. All participants were given detailed explanations about the aims, procedures and possible benefit of the study. The study protocol was approved by the institutional research commission of the University of Salamanca. Ethical approval was obtained from the Ethics Committee of the University of Salamanca (protocol approval no. 48531).

Urine samples collection

Patients´ urine samples. A total of 94 human urine samples were selected from a set of samples collected from patients attending during May 2002 to April 2009 at Hospital Universitario Insular, Las Palmas de Gran Canaria, Spain, as part of public health diagnostic activities. All these patients were suggested to several parasitological diagnostic tests for suspected infectious diseases by specialized technicians according to standard routine laboratory procedures. Among the 94 human urine samples selected, a number of 78 were obtained from Sub-Saharan immigrants with a microscopy-confirmed infection of 39 samples with: *S. haematobium* (n = 18), *S. mansoni* (n = 7), several helminths (n = 9)-counting four infections with hookworms, two with *Strongyloides stercoralis*, two with *Trichuris trichiura*, one with *Enterobius vermicularis* and one mixed infection with *Loa loa*, *Mansonella perstans* and *T. trihiura*-, and other infectious agents (n = 5)-including protozoa (*Plasmodium falciparum*, *Giardia duodenalis*, *Trichomonas vaginalis)*, bacteria *(Chlamidia trachomatis)* and virus (hepatitis B virus; HBV). A set of urine samples from patients with eosinophilia without a confirmed diagnosis (n = 15) as well as a set of urine samples from patients without either eosinophilia and no apparent disease (n = 24) were also selected. Additionally, urine samples from healthy non-endemic individuals (n = 16) were included to use in the study as negative controls samples. All patients´ urine samples were collected using sterile plastic containers and a volume of approximately 10 mL each was stored at -80°C without the addition of any preservative or chemical until sending to CIETUS, Salamanca, Spain, for further molecular analyses.

Artificial urine samples. Fresh urine was collected from healthy staff donors with no history of travel to endemic area of schistosomiasis. The collected urine was divided into aliquots of 100 μL each and then artificially spiked with 2 μL of 10-fold serially diluted *S. haematobium* DNA ranging from 50 ng/μL to 0.5 atg/μL, thus resulting in a set of simulated urine samples with a final parasite DNA concentration ranging from 1 ng/μL to 0.01 atg/μL. Once prepared,

these urine samples were stored at -20°C until further use for DNA extraction and to test the sensitivity of the LAMP assay.

DNA extraction optimization

Parasites DNA samples. Genomic DNA from adult male and female *S. haematobium*, Egiptian Strain, NR-31682, was obtained from the Schistosomiasis Resource Center for distribution by BEI Resources, NIAID, NIH (https://www.beiresources.org/Collection/51/Schistosome-Resource-Centers.aspx). The original supplied *S. haematobium* DNA concentration (100 ng/μL) was confirmed by measuring in a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies) and then was diluted with ultrapure water to a final concentration of 50 ng/μL. Subsequently, serial 10-fold dilutions were also prepared with ultrapure water ranging from 5 ng/μL to 0.5 atg/μL and stored at -20°C until used. DNA thus prepared was used as positive control in all PCR and LAMP reactions and also to assess sensitivity of both assays. In addition, *S. haematobium* DNA was used to prepare the artificial urine samples as mentioned above.

To determine the specificity of both PCR and LAMP assays, a panel of 20 DNA samples from several other helminths and protozoa were used as heterogeneous control samples including, *S. mansoni*, *S. japonicum*, *S. bovis*, *Fasciola hepatica*, *Loa loa*, *Brugia pahangi*, *Strongyloides venezuelensis*, *Dicrocoelium dendriticum*, *Calicophoron daubneyi*, *Hymenolepis diminuta*, *Taenia taeniformis*, *Anisakis simplex*, *Trichinella spiralis*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia intestinalis*, *Entamoeba histolytica*, *Plasmodium vivax*, *P. ovale* and *P. malariae*. Concentration of these DNA samples was also measured by using a Nanodrop ND-100 and then also diluted with ultrapure water to a final concentration of 0.5 ng/μL. All these DNA samples were kept at -20°C until use in molecular assays.

Patients´ urine samples processing. Three different methods for DNA extraction were evaluated from eighteen patients´ urine samples with parasitologically confirmed *S. haematobium* infections, to obtain DNA to be used as a template in later LAMP amplification: a commercially available DNA extraction kit, a modified hot sodium hydroxide (NaOH) extraction method and a rapid heating urine sample method. Additionally, these methods were tested with different sets of aliquots obtained from each urine sample to compare results, including whole urine, urine supernatant and urinary sediment (pellets). These sets of aliquots were prepared as follows. After thawing, three whole urine aliquots of 100 μL, as well as three whole urine aliquots of 2 mL were taken from each urine sample in new clean 2 mL microcentrifuge tubes. Later, aliquots of 2 mL were centrifuged at 5000 rpm for 5 min at room temperature (RT) to pellet the urinary sediment (insoluble fraction of urine) and maintain the supernatant (soluble fraction of urine). Then, a volume of 100 μL was recovered from the supernatant and transferred to a new clean test tube. Excess supernatant was discarded but maintaining a minimal volume of 100 μL to resuspend the urinary sediment (pellet) at the bottom of the tube. In this way, from each patient´s urine sample we finally obtained three aliquots of 100 μL containing whole urine, three aliquots of 100 μL containing supernatant and three aliquots of 100 μL containing resuspended pellet. Afterwards, each type of these aliquots-whole, supernatant and pellet- was used to obtain DNA by using the three different extraction methods assayed.

DNA extraction

In the first procedure for DNA extraction we used the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK) following the manufacturers´ instructions. DNA samples thus obtained were stored at -20°C until use in LAMP reactions.

In the second procedure, we used the hot NaOH extraction method [54] with minimal modifications in the standard protocol by adding sodium docecyl sulfate (SDS) to ensure disruption of the *S. haematobium* eggs to release the DNA. Briefly, an equal volume of a 50 mM NaOH solution containing 0.1% of SDS was added to urine aliquots of 100 μl and then heated at 95°C for 30 min. Subsequently, the tubes were centrifugated at 5000 rpm for 5 min and a volume of 50 μL of supernatant was recovered in a new clean tube and mixed with an equal volume of a 1 M Tris-HCl solution at pH 8.0. Each new solution thus obtained was stored at -20°C until further use as template in LAMP assays.

In the third procedure,-named the "Rapid-Heat LAMP method"-, each aliquot of whole urine, supernatant and pellet obtained from each urine sample was heated at 95°C for 15–20 min and then briefly spun to pellet the debris. After this, 2 μL of the supernatant were used immediately as template for each LAMP reaction. The remaining volume of each sample was stored at -20°C. To obtain DNA to be used as template in LAMP reactions to test the remaining 76 clinical urine samples included in the study, we firstly obtained the urinary sediment (pellet) as already indicated and, subsequently, the Rapid-Heat LAMP method was applied.

Artificial urine samples DNA obtaining. DNA was extracted from simulated artificial urine samples by using both the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK) following manufacturers´ instructions and the in house procedure, the Rapid-Heat LAMP method.

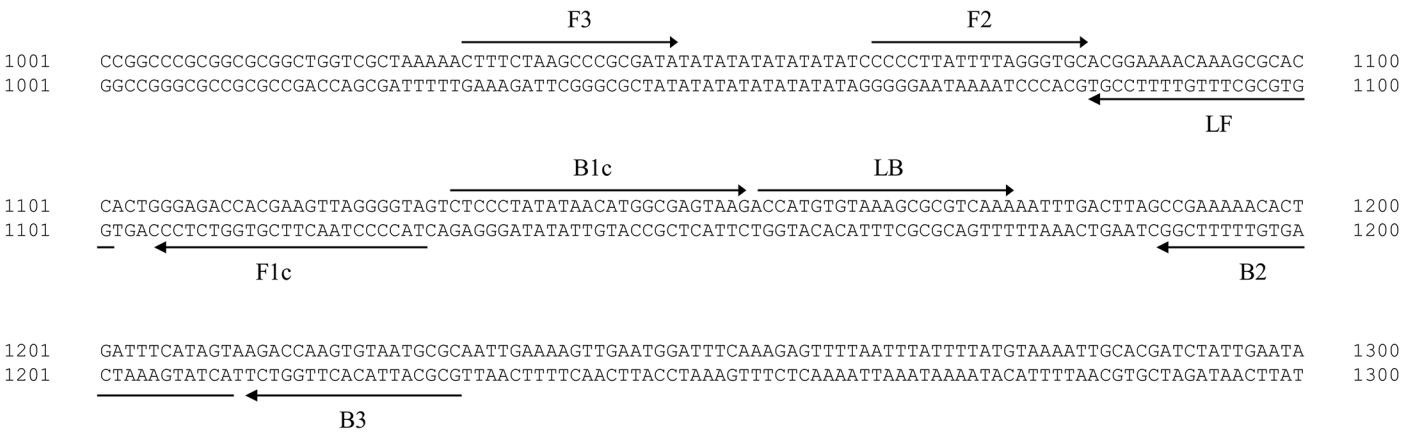
S. haematobium LAMP primer design

A set of six oligonucleotide primers were used for the LAMP assay, targeting eight regions in the 2522 base pair (bp) sequence of *S. haematobium* ribosomal intergenic spacer (IGS) DNA retrieved from GenBank (Accession No. AJ223838) [55]. The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), and loop forward (LF) and backward (LB) primers were designed using the online Primer Explorer V4 software (Eiken Chemical Co. Ltd, Tokyo, Japan; http://primerexplorer.jp/elamp4.0.0/index.html) according to the general criteria described by Notomi et al. [32] and finally selected based on the criteria described in "A Guide to LAMP primer designing" (http://primerexplorer.jp/e/v4_manual/index.html). The location and nucleotide sequences of the six primers are shown in Fig 1. All the primers were of HPLC grade (Thermo Fisher Scientific Inc., Madrid, Spain). To confirm the specificity for the designed primers in annealing exclusively with the *S. haematobium* DNA correct target sequence, a BLASTN local search and alignment analysis [56] was carried out in different online databases against currently available nucleotide sequences for other organisms (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) as well as specifically against human, murine (Ensembl; http://www.ensembl.org/Multi/Tools/Blast) and other related *Schistosoma* species genomes (Sanger Institute; http://www.sanger.ac.uk/resources/software/blast/).

PCR using outer primers F3 and B3

The outer LAMP primer pair, designated F3 and B3, was initially tested for the amplification of *S. haematobium* DNA by a touchdown-PCR (TD-PCR) to verify whether the correct target was amplified. The PCR assay was conducted in 25 μL reaction mixture containing 2.5 μL of 10x buffer, 1.5 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2.5 mmol/L dNTPs, 0.5 μL of 100 pmol/L F3 and B3, 2 U *Taq*-polymerase and 2 μL (1 ng) of DNA template. Conditions for TD-PCR amplification were as follows: an initial denaturation was conducted at 94°C for 1 min, followed by a touchdown program for 15 cycles with successive annealing temperature decrements of 1.0°C every 2 cycles. For these 2 cycles, the reaction was denatured at 94°C for 20 s followed by annealing at 58°C-55°C for 20 s and polymerization at 72°C for 30 s. The following

A



B

Primers	Length (bp)	Sequence (5′-3′)
F3	18	CTTTCTAAGCCCGCGATA
B3	18	GCGCATTACACTTGGTCT
FIP (F1c-F2)	41	TACCCCTAACTTCGTGGTCTCC-CCCCCTTATTTTAGGGTGC
BIP (B1c-B2)	48	CTCCCTATATAACATGGCGAGTAAG-ACTATGAAATCAGTGTTTTTCGG
LF	19	GGTGCGCTTTGTTTTCCGT
LB	22	ACCATGTGTAAAGCGCGTCAAA

Fig 1. Lamp primer set targeting the selected sequence (GenBank Accession No. AJ223838) for ribosomal intergenic spacer *S. haematobium* DNA region amplification. (A) The location of the LAMP primers within the selected sequence is shown. Arrows indicate the direction of extension. (B). Sequence of LAMP primers: F3, forward outer primer; B3, reverse outer primer; FIP, forward inner primer (comprising F1c and F2 sequences); BIP, reverse inner primer (comprising B1c and B2 sequences); LF (loop forward primer); LB (loop backward primer).

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15 cycles of amplification were similar, except that the annealing temperature was 54°C. A final extension was performed at 72°C for 10 min.

The specificity of PCR using outer primers F3 and B3 was also tested with 20 heterogeneous DNA samples from other parasites included in the study. The sensitivity of the PCR was also assayed to establish the detection limit of *S. haematobium* DNA with 10-fold serial dilutions ranging from 0.5 ng/μL to 0.5 atg/μL prepared as mentioned above. The assays were performed with 2 μL of the diluted template in each case, thus resulting a final concentration of DNA ranging from 1 ng/μL to 1 atg/μL. Negative controls (ultrapure water instead of DNA template) were included in each run. The PCR products (5–10 μL) were subjected to 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light.

LAMP reaction

To evaluate the LAMP primer set designed in *S. haematobium* DNA amplification, we set up the reaction mixture using *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, UK) combined with different betaine (Sigma, USA) and $MgSO_4$ (New England Biolabs, UK) concentrations. Thus, LAMP reactions mixtures (25 μL) contained 1.6 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 primers, 0.4 μM of each LB and LF primers, 1.4 mM of each

dNTP (Bioron), 1x Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), betaine (ranging 0.8, 1 or 1.2 M), supplementary $MgSO_4$ (ranging 4, 6 or 8 mM) and 8 U of *Bst* 2.0 WarmStart DNA polymerase with 2 μL of template DNA. To establish the standard protocol for LAMP reactions mixtures assayed, a range of temperatures (61, 63 and 65°C) was tested in a heating block for 30, 50 or 60 min and then heated at 80°C for 5–10 min to inactivate the enzyme and thus to terminate the reaction. Then, both optimal temperature and incubation time were determined and used in the following tests. Positive (*S. haematobium* DNA) and negative (no DNA template) controls were always included in each LAMP assay.

Analysis of LAMP products. Firstly, when possible, turbidity caused by the accumulation of magnesium pyrophosphate (a by-product of the reaction) was visually inspected by the naked eyes. The positive amplification results were also visually detected by adding 2 μL of 1:10 diluted 10,000x concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tubes. Green fluorescence was clearly observed in a successful LAMP reaction, whereas it remained original orange in the negative one. After the LAMP reactions, 3–5 μL of each product were used for 2% agarose gel electrophoresis stained with ethidium bromide. A GelDoc imaging system (UVItec, UK) was used to observe the band patterns. The samples were considered positive if they showed a characteristic ladder-like band pattern.

Evaluation of LAMP assay. To evaluate the LAMP assay, we used the serially diluted artificial samples and also the patients´ urine samples with parasitological confirmed *S. haematobium* infection. The patients´ urine samples were tested by LAMP after being processed in different ways (as whole urine, supernatant and pellet) to test the most successful method for DNA extraction to be used as template as previously described. Once the most favorable urine fraction and DNA extraction method were established, LAMP assay was used to test twice all the human urine samples included in our study.

Specificity and sensitivity of the LAMP assay. The specificity of the LAMP assay to amplify only *S. haematobium* DNA was tested against a panel of 20 DNA samples obtained from other parasites used as controls, as mentioned above. On the other hand, in order to determine the lower detection limit of the LAMP assay, genomic DNA from *S. haematobium* 10-fold serially diluted-ranging from 0.5 ng/μL to 0.5 atg/μL, as mentioned above- was subjected to amplification. Moreover, the sensitivity was also assayed with the simulated urine samples artificially spiked with the same dilutions after DNA extraction by using the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK) and the Rapid-Heat LAMP method.

Statistical analysis

To estimate the accuracy of the LAMP assay as a diagnostic test, the percentages of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using the MedCalc statistical program version 15.2.2 (MedCalc Software, Ostende, Belgium) according to the software instruction manual (www.medcalc.org).

Results

Sensitivity and specificity of PCR using outer primers

To confirm that the expected target was amplified, a PCR reaction was performed using outer primers F3 and B3 to amplify *S. haematobium* DNA. Thus, a 199 bp amplicon was successful obtained (Fig 2A). In order to determine the lower detection limit of the PCR reaction, a 10-fold serial dilution ranging from 10^{-1} to 10^{-9} of *S. haematobium* DNA was amplified. The minimum amount of DNA detectable by PCR using outer primers was 1 ng (Fig 2B).

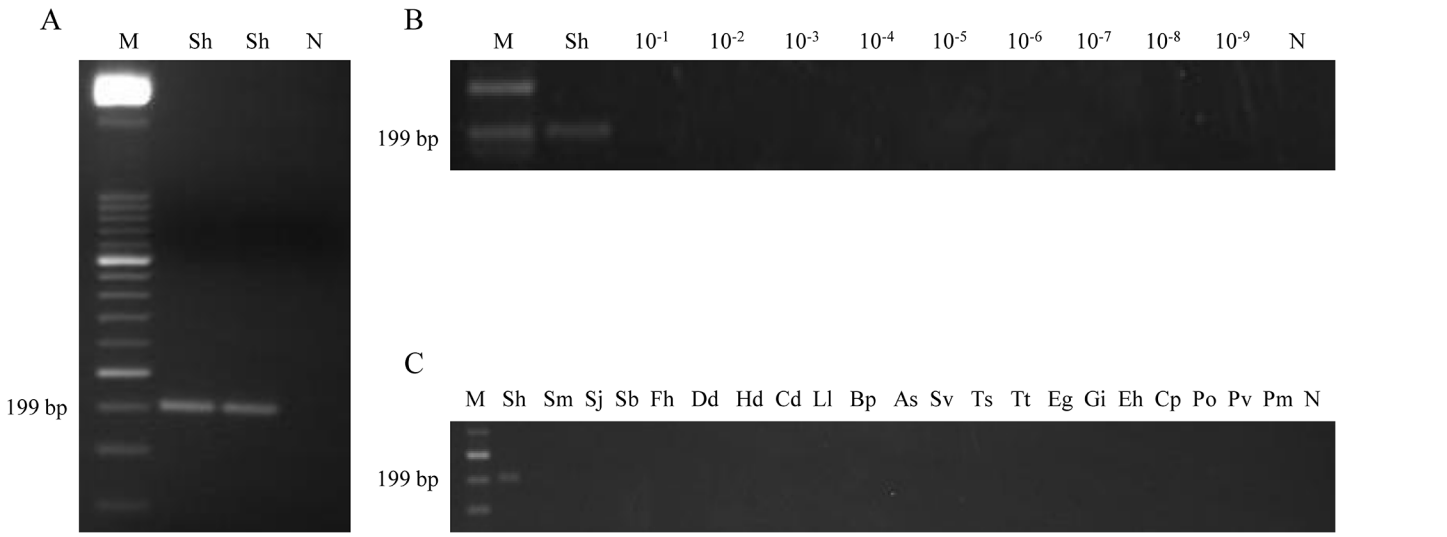

Fig 2. PCR verification, detection limit and specificity using outer primers F3 and B3. (A) PCR verification of expected 199 bp target length amplicon. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sh, *S. haematobium* DNA (1 ng); lane N, negative control (no DNA template). (B) Detection limit of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sh: *S. haematobium* DNA (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions of *S. haematobium* DNA; lane N, negative control (no DNA template). (C) Specificity of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh, Sm, Sj, Sb, Fh, Dd, Hd, Cd, Ll, Bp, As, Sv, Ts, Tt, Eg, Gi, Eh, Cp, Po, Pv, Pm, *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. bovis*, *Fasciola hepatica*, *Dicrocoelium dendriticum*, *Hymenolepis diminuta*, *Calicophoron daubneyi*, *Loa loa*, *Brugia pahangi*, *Anisakis simplex*, *Strongyloides venezuelensis*, *Trichinella spiralis*, *Taenia taeniformis*, *Echinococcus granulosus*, *Giardia intestinalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Plasmodium ovale*, *P. vivax* and *P. malariae* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template).

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According to specificity, when DNA samples obtained from other parasites included in the study were subjected to this PCR assay, amplicons were never amplified (Fig 2C).

Setting up LAMP assay

To establish a standard procedure for the LAMP assay we used the *Bst* 2.0 WarmStart DNA polymerase applying a range of temperatures (61, 63 and 65°C) for testing different mixtures containing variable concentrations of betaine (ranging 0.8, 1 or 1.2 M) combined with supplementary variable concentrations of MgSO4 (ranging 4, 6 or 8 mM) in a heating block for 30, 50 and 60 min. The best amplification results were obtained when the reaction mixture contained 1 M of betaine combined with supplementary 6 mM of MgSO4 (resulting a final concentration of 8 mM MgSO4 in 1x Isothermal Amplification Buffer) and was incubated for 50 min at 63°C in a heating block (Fig 3A). Once the most favourable conditions and molecular components were established for the LAMP assay, all positive results in subsequent reactions could be clearly visually observed by the naked eye by inspecting the colour change after adding SYBR Green I as well as the typical ladder of multiple bands after electrophoresis on agarose gels.

Specificity and sensitivity of LAMP assay

To determine the specificity of the primers designed, a panel of 20 DNA samples from other parasites were subjected to the LAMP assay. As shown in Fig 3B, only LAMP products were amplified when *S. haematobium* DNA was used as template and no false positive amplification was observed, thus indicating the high specificity of the established LAMP assay.

Regarding to the sensitivity of the LAMP assay, a 10-fold serial dilution of *S. haematobium* genomic DNA was amplified by LAMP. The results indicated that the detection limit for the LAMP reaction was 100 fg (Fig 3C). This suggested that the LAMP assay is 10^4 times more

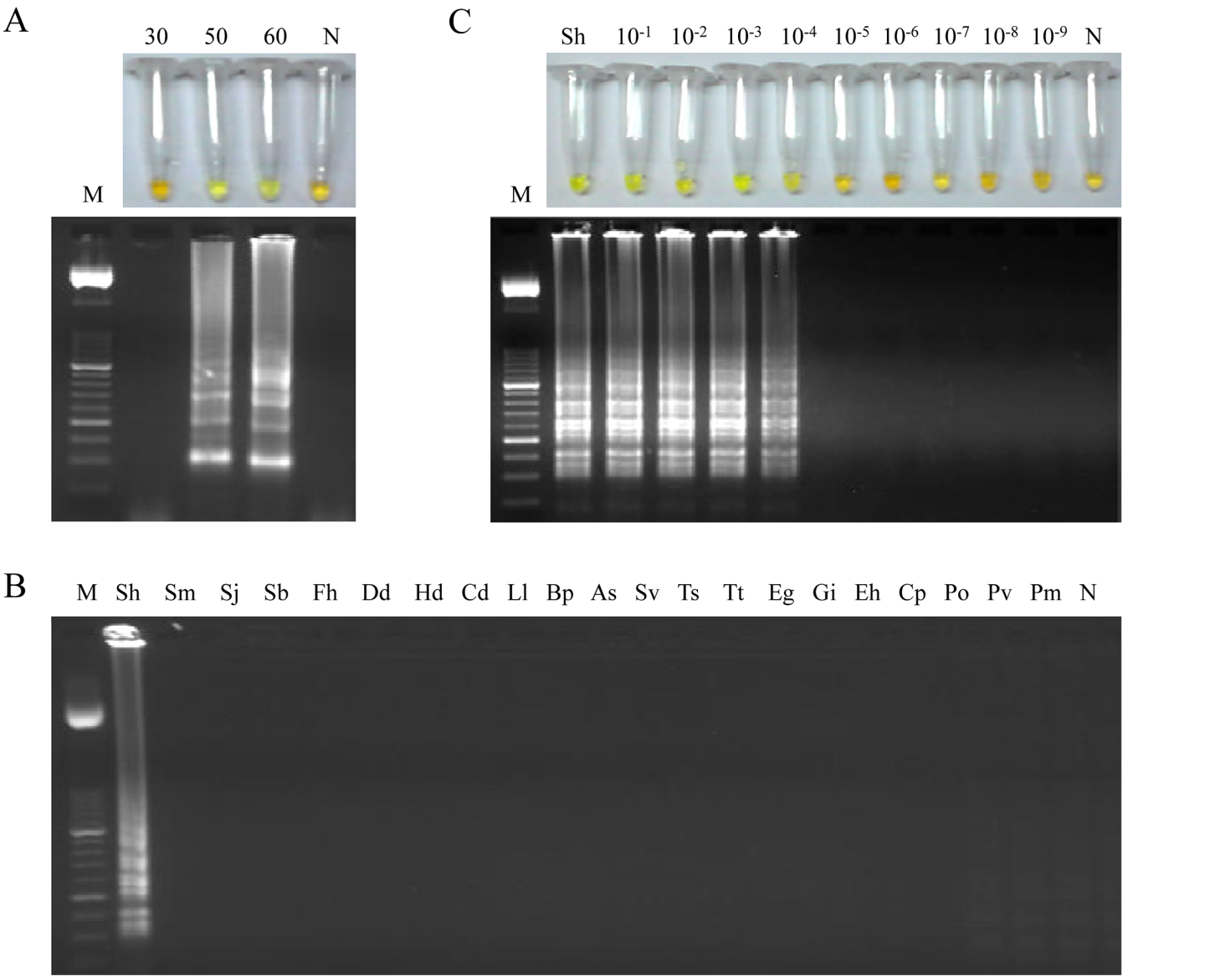

Fig 3. Setting up LAMP assay. (A) LAMP amplification results obtained at different incubation times (30, 50 and 60 min) tested in a heating block by the addition of SYBR Green I (up) or by visualization on agarose gel (down). Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes 30, 50, 60, amplification results of *S. haematobium* DNA (1 ng) for 30, 50 and 60 minutes of incubation time, respectively. (B) Specificity of the LAMP assay for *S. haematobium*. A ladder of multiple bands of different sizes could be only observed in *S. haematobium* DNA sample. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh, Sm, Sj, Sb, Fh, Dd, Hd, Cd, Ll, Bp, As, Sv, Ts, Tt, Eg, Gi, Eh, Cp, Po, Pv and Pm, *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. bovis*, *Fasciola hepatica*, *Dicrocoelium dendriticum*, *Hymenolepis diminuta*, *Calicophoron daubneyi*, *Loa loa*, *Brugia pahangi*, *Anisakis simplex*, *Strongyloides venezuelensis*, *Trichinella spiralis*, *Taenia taeniformis*, *Echinococcus granulosus*, *Giardia intestinalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Plasmodium ovale*, *P. vivax* and *P. malariae* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template). (C) Sensitivity assessment performed with LAMP at 63°C for 50 min using serial dilutions of *S. haematobium* genomic DNA. Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions; lane N: negative controls (no DNA template).

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sensitive than the PCR using outer primers F3 and B3 (see Fig 2B). On the other hand, the sensitivity of LAMP assay in simulated fresh human urine samples artificially contaminated with DNA from *S. haematobium* was also examined. In this case, the detection limit of LAMP assay was 10 fg/μL when performing the DNA extraction with the commercial kit (Fig 4A), whereas

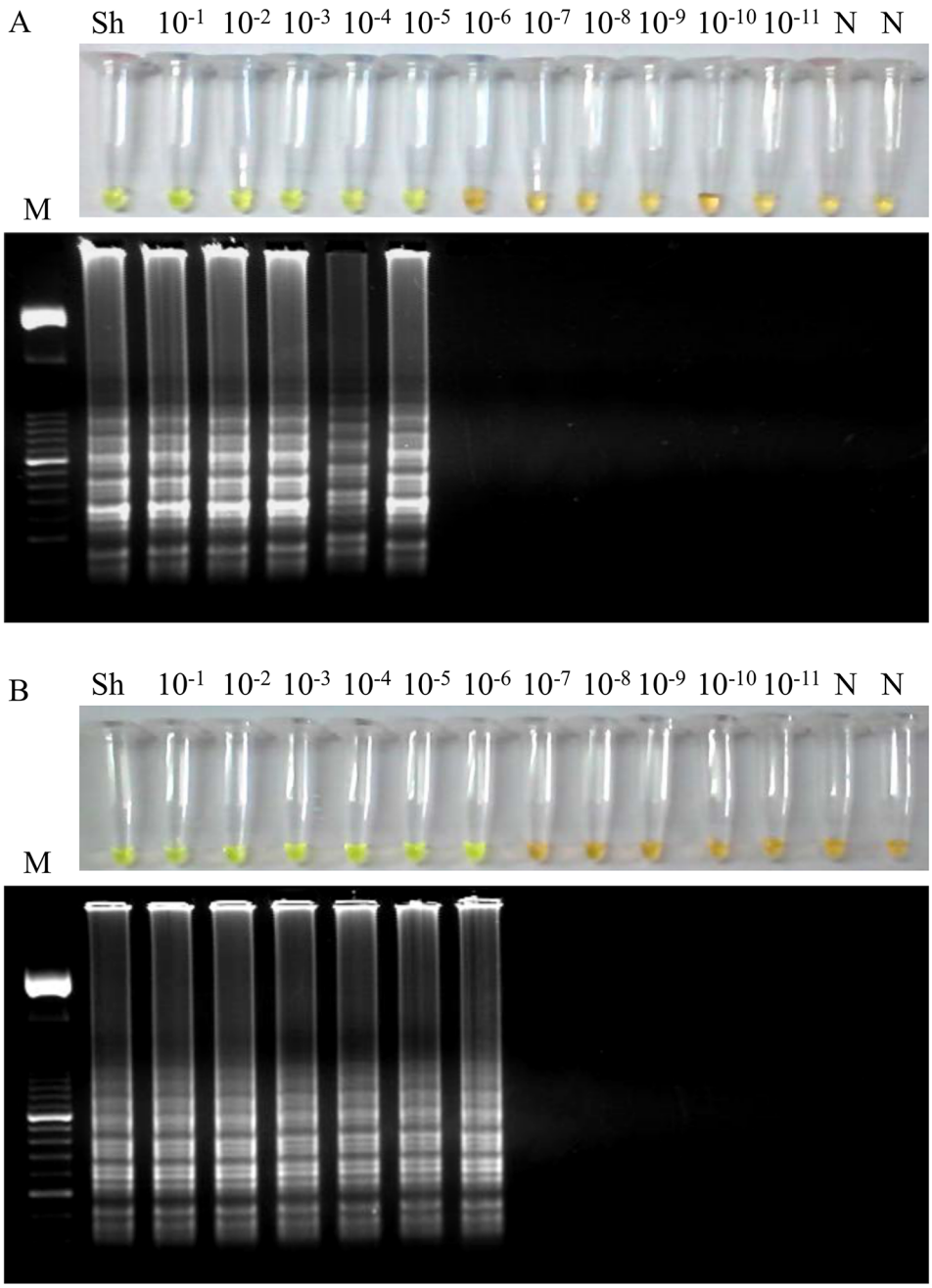

Fig 4. Sensitivity of the LAMP assay in simulated human urine samples artificially contaminated with DNA from *S. haematobium*. (A) Sensitivity assessment of LAMP when performing the DNA extraction with the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK) from serial dilutions of *S. haematobium* genomic DNA. (B) Sensitivity assessment of LAMP when performing the DNA extraction with a simple heating method from serial dilutions of *S. haematobium* genomic DNA. Lanes M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes 10^{-1}–10^{-11}: 10-fold serially dilutions; lanes N: negative controls (no DNA template).

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the detection limit was established in 1 fg/μL using the Rapid-Heat LAMPellet method for DNA extraction (Fig 4B).

LAMP tests in confirmed *S. haematobium*-positive patients´ urine samples

Comparative LAMP results obtained when testing aliquots of whole urine, supernatants and pellets from patients´ urine samples with parasitological confirmed *S. haematobium* infection after using the three different DNA extraction methods attempted in our study are shown in Figs 5, 6 and 7, respectively.

In LAMP tests using a starting volume of whole patients´ urine samples of 100 μL/each we obtained 15/18 positive results when performing DNA extraction using the i-genomic Urine DNA Extraction Mini Kit (Fig 5A), 11/18 when using the NaOH/SDS extraction method (Fig 5B) and 12/18 when the Rapid-Heat LAMP method was applied (Fig 5C).

In LAMP tests for supernatant fraction of patients´ urine samples we obtained only 3/18 positive results when performing DNA extraction using the i-genomic Urine DNA Extraction Mini Kit (Fig 6A), 4/18 when using the NaOH/SDS extraction method (Fig 6B) and 9/18 when the Rapid-Heat LAMP method was applied (Fig 6C).

Finally, in LAMP tests for the urinary sediment (pellet) obtained from the urine samples we obtained 17/18 positive results when performing DNA extraction using the i-genomic Urine DNA Extraction Mini Kit (Fig 7A), 15/18 when using the NaOH/SDS extraction method (Fig 7B) and a total of 18/18 when the Rapid-Heat LAMP method was applied (Fig 7C). Thus, in general, the higher effectiveness in LAMP amplification of *S. haematobium* DNA in patients´ urine samples was obtained when the urinary sediment (pellet) was used for DNA extraction; moreover, the simple Rapid-Heat LAMP method provided the best results of the three methods assayed for extracting DNA detectable by LAMP. Thereby, the minimal pellet obtained from urine samples, in addition to the Rapid-Heat LAMP method for DNA detection-hereafter "Rapid-Heat LAMPellet method"-, was set up as the most advantageous procedure to be used in successive LAMP reactions to detect *S. haematobium* DNA in urine samples and to test all the clinical samples included in our study.

Rapid-Heat LAMPellet method in clinical sample collection

The results of all 94 patients´ urine samples evaluated by duplicated for *S. haematobium* DNA detection by using the Rapid-Heat LAMPellet method are presented in Table 1. We obtained LAMP positive results in 18/18 confirmed *S. haematobium* infected urine samples, in 1/9 urine samples with other helminths species confirmed infections (specifically a patient infected with a "hookworm"), in 1/5 urine samples with other agents confirmed infections (specifically a patient infected with *Trichomonas vaginalis*), in 1/15 urine samples from patients with eosinophilia without a confirmed diagnosis and, finally, in 5/24 urine samples from patients without either eosinophilia and none apparent disease. The seven parasitological *S. mansoni*-positive urine samples as well as the 16 urine samples from healthy non-endemic donors (used as negative controls samples) were all negative by LAMP. All positive results could be visually observed in tubes by color change after adding SYBR Green I and also after electrophoresis on agarose gels as a ladder of multiple bands of different sizes (S1 Fig). Considering the results obtained, diagnostic parameters for sensitivity and specificity were calculated for our LAMP assay, 100% sensitivity and 86.67% specificity, and also for microscopy detection of eggs in urine samples, 69.23% sensitivity and 100% specificity. The PPV and NPV for both LAMP assay and microscopy were also calculated; all statistic data obtained are showed in Table 1.

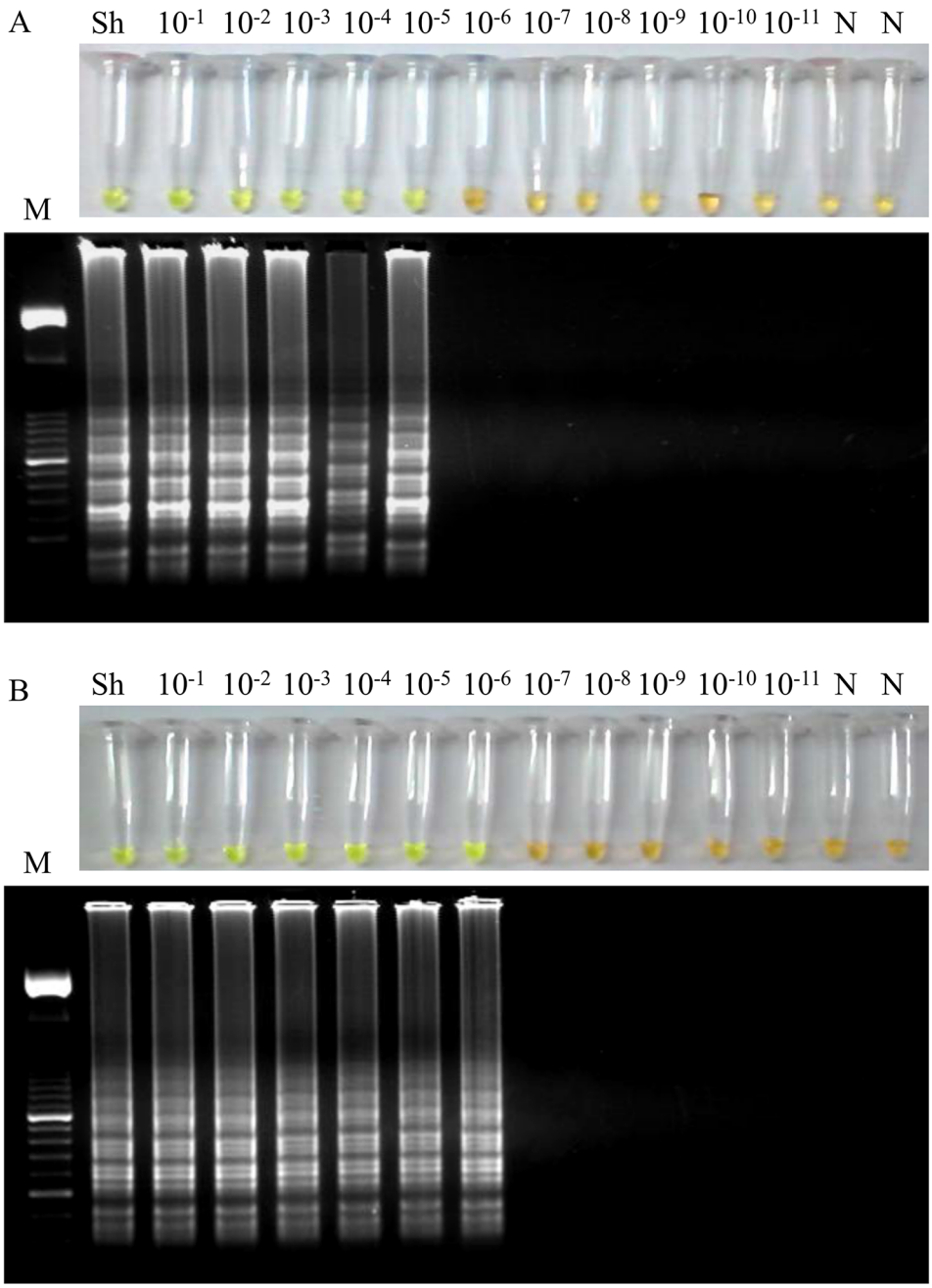

Fig 5. Examination of aliquots of whole urine from *S. haematobium*-positive patients´ urine samples by LAMP. Figure shows the LAMP results (up, by color change; down, by agarose electrophoresis) when using aliquots of 100 μL of whole urine to obtain DNA as template by using (A) the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK); (B) the heating NaOH-SDS method and (C) the rapid heating method. Lanes M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes 1–18: *S. haematobium*-positive samples; lanes N: negative controls (no DNA template).

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Discussion

Urogenital schistosomiasis due to S. *haematobium* remains a serious underestimated public health problem, particularly in sub-Saharan Africa. Frequency of urogenital schistosomiasis in

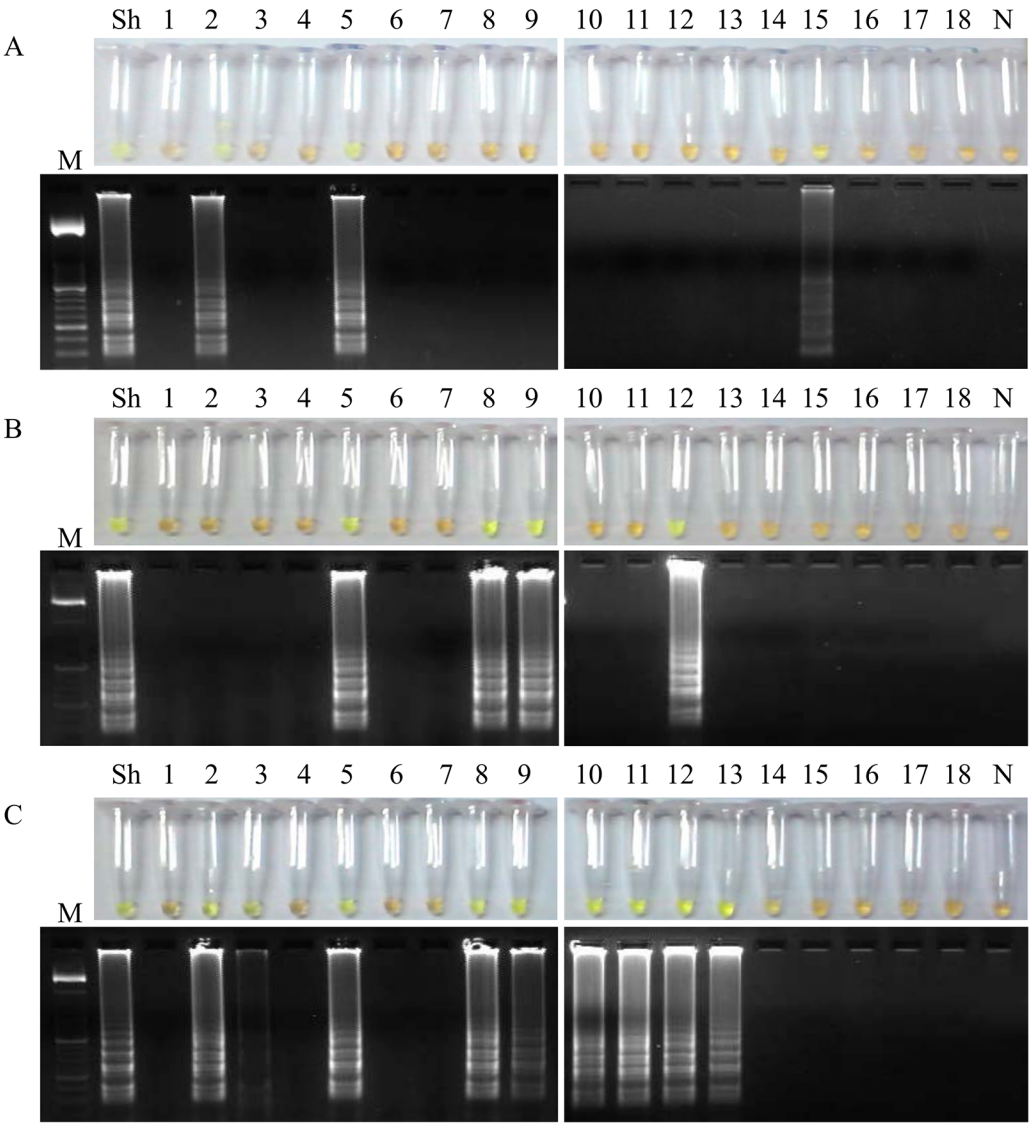

Fig 6. Examination of aliquots of supernatants from *S. haematobium*-positive patients´ urine samples by LAMP. Figure shows the LAMP results (up, by color change; down, by agarose electrophoresis) when using aliquots of 100 μL of supernatants to obtain DNA as template by using (A) the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK); (B) the heating NaOH-SDS method and (C) the rapid heating method. Lanes M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes 1–18: *S. haematobium*-positive samples; lanes N: negative controls (no DNA template).

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travellers, expatriates and migrants is in the same range to that of intestinal schistosomiasis due to *S. mansoni* [57]. As there is no vaccine to protect against schistosomal infection, mass praziquantel treatment of populations at risk of infection is being conducted routinely in many endemic areas; however, reinfections rapidly occur because of recurrent direct contact with water infected with parasites [58]. Considering the current problems of parasitological, serological and molecular methods in detecting schistosomal infections [59], new, simple, accurate and affordable diagnostic tools are essential for providing specific treatment and for maximizing the success of control of urogenital schistosomiasis in endemic areas; as well as for monitoring drug effectiveness.

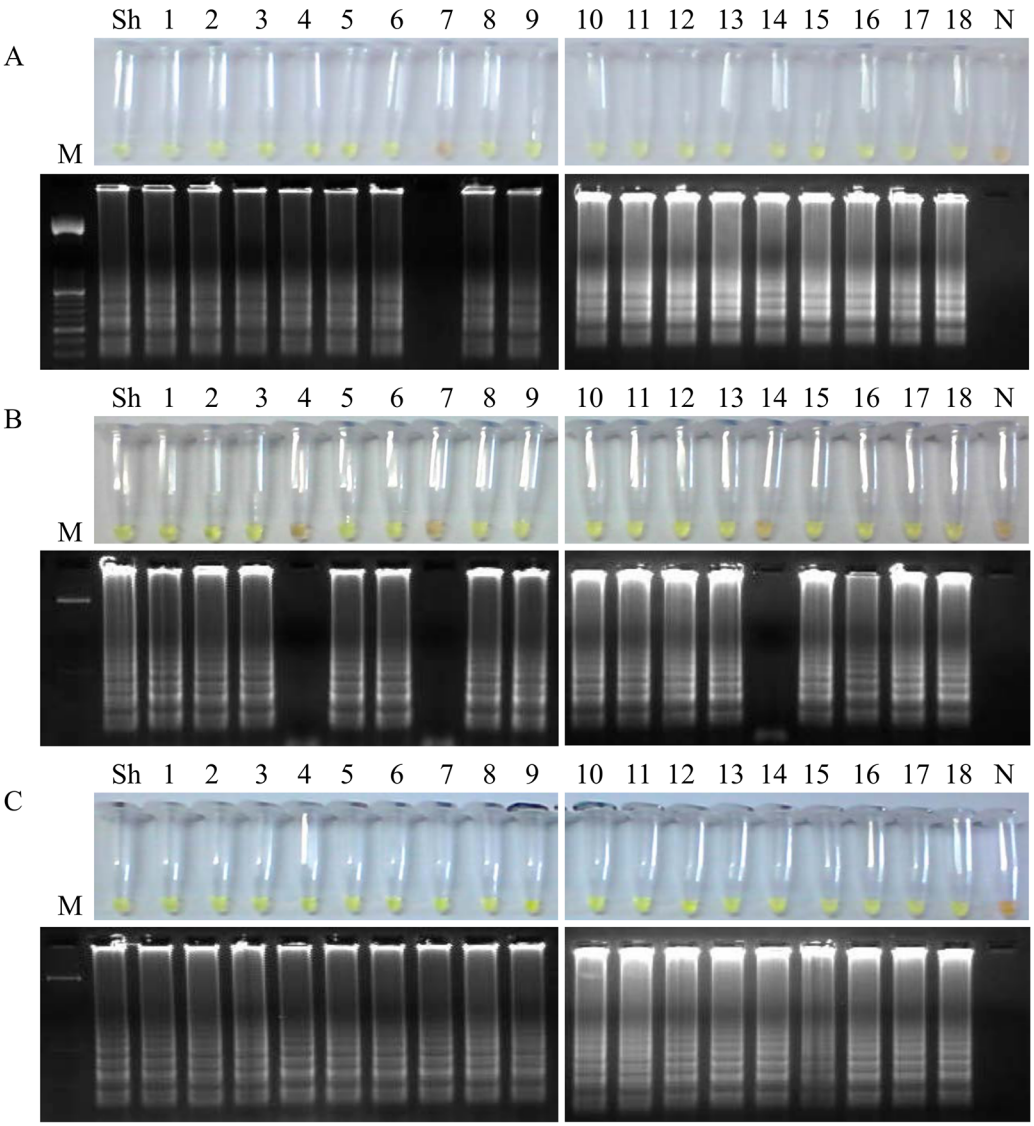

Fig 7. Examination of aliquots of urinary sediment (pellets) from *S. haematobium*-positive patients´ urine samples by LAMP. Figure shows the LAMP results (up, by color change; down, by agarose electrophoresis) when using aliquots of 100 μL of pellets to obtain DNA as template by using (A) the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK); (B) the heating NaOH-SDS method and (C) the rapid heating method-the rapid-heat LAMPellet method-. Lanes M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes 1–18: *S. haematobium*-positive samples; lanes N: negative controls (no DNA template).

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Table 1. Estimation of sensitivity, specificity, predictive values and likelihood ratios by Rapid-Heat LAMPellet method against standard parasitological test (microscopy) for current study for identifying *Schistosoma haematobium* infection in patients´ urine samples.

Diagnostic test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Rapid-Heat LAMPellet	100% (81.32%-100%)	86.67% (75.40%-94.05%)	63.23% (48.21%-85.63%)	100% (93.08%-100%)
Microscopy	69.23% (48.21%-85.63%)	100% (93.08%-100%)	100% (81.32%-100%)	86.67% (75.40%-94.05%)

PPV, Positive Predictive Value

NPV, Negative Predictive Value

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Point-of-care tests are being developed as economic evaluation diagnostic technologies for infectious diseases control strategies as they are easy to use and interpret, require minimal laboratory infrastructure, are cost-effective, reduce patient waiting time and potentially therefore reduce loss to follow-up, and may have comparable or higher sensitivity to microscopy [60]. The LAMP technology-as a DNA amplification method- combines rapidity, simplicity and high specificity [32] and has a wide range of possible applications, including point-of-care testing in developing countries [61, 62]. We have developed a LAMP assay for rapid, sensitive, specific and cost-effective detection of *S. haematobium* in human urine samples, even in the absence of parasites eggs in excreta, as a basis for a potential field diagnostic tool for use in schistosomiasis endemic areas. Besides its excellent performance, the most striking results of this study are the simplicity to perform the whole process without requiring DNA extraction from a small volume of starting urine to get the urinary sediment (pellet) to carry out the molecular analysis. We have named this simple procedure the "Rapid-Heat LAMPellet method".

To accomplish its development, we designed a specific set of six primers targeting eight regions in a species specific sequence of *S. haematobium* ribosomal IGS [55]. The ribosomal IGS regions within *Schistosoma* species generally contain unique sequence motifs which are specific to that group of organisms. In addition, the IGS target locus has been already used for successful detection of *Schistosoma* spp. infection in freshwater snails by real-time PCR and oligochromatographic dipstick rapid technology (PCR-OC) [63]. Several other advantages of these sequences to be use in molecular studies have been already reported elsewhere [55, 64].

Once the primer set was designed, *in silico* comparisons of the expected 199 bp sequence with the on line available genomes showed the higher homology in alignment length with *S. haematobium* and no cross-reaction was found, specifically with *S. mansoni*; this result is especially important as these two species are the main schistosomes producing co-infections in most areas of sub-Saharan Africa [58]. Specificity results obtained in *in silico* were later verified by PCR using outer primers F3-B3.

After this, we attempted to establish the most suitable reaction mixture for the six specific primers in the LAMP assay. We used the *Bst* polymerase 2.0 WarmStart as this warm-start version has several advantages compared to wild-type *Bst* DNA polymerase large fragment, such as faster in obtaining amplification signals [65] and increased stability at room temperature [66]. These features are important when testing a large number of samples under field conditions in endemic areas where limited resources for the maintenance of a cold chain exists. As the LAMP reaction might be facilitated by the addition of loop primers [67] our LAMP assay designed was accelerated by the addition of a pair of loop primers, thus allowing to amplify successfully *S. haematobium* DNA in only 50 min, whereas a previously described LAMP assay to amplify *S. haematobium* DNA in freshwater snails takes 120 min to complete the reaction [49, 50].

The specificity of the LAMP assay was determined using a panel of heterogeneous control DNA samples of a number of parasites. The assay specifically produced typical ladder patterns from the target sequence only for *S. haematobium* DNA. The sensitivity of the LAMP resulted 10^4 times greater than that of PCR using outer primers (100 fg *vs*. 10^6 fg or 1ng, respectively). It is usually considered that LAMP is highly sensitive compared to conventional PCR methods and other studies also found a higher sensitivity when comparing LAMP results in contrast to PCR in amplification of DNA from *Schistosoma* species, including *S. japonicum* [47], *S. haematobium* and *S. mansoni* [49, 48].

The effectiveness of our LAMP assay was assessed in patients´ urine samples with confirmed *S. haematobium* infection by microscopic examination. Bearing in mind a potential easy and cost-effective large-scale application in field conditions, we evaluated different DNA

extraction methods for their ability to isolate DNA from small volumes of different fractions of human urine samples, including whole urine, urine supernatant and urinary sediment (pellet) to compare results. A simple, quick and economically DNA extraction method for use in combination with small volumes of clinical urine specimens could greatly reduce the infrastructure requirements of collecting, handling, storing and processing the patients´ samples in schistosomiasis endemic areas where limited resources exist.

The three different DNA extraction methods tested in our work were much more efficient in extracting detectable DNA by LAMP when using aliquots of whole urine and pellets than supernatants. This seems to be logical since after centrifugation to remove and retain supernatants, both potential free *S. haematobium* DNA and parasite eggs-and therefore containing DNA- found in patients´ urine samples should be concentrated at the bottom of the tube, thus improving the sensitivity of the DNA molecular detection methods, as previously described [68]. When using the pellets, the simple rapid-heating method allowed us to obtain a very good-quality detectable DNA that did not compromise LAMP amplification and all the *S. haematobium*-positive urine samples tested were successfully amplified.

The consistent results in DNA obtained from aliquots of whole urine and pellets when applying a commercial kit may be due to the well-known effectiveness of this procedure to isolate genomic DNA from urine samples suitable for further molecular analyses [69]. Urine specimens contain many inhibitors which may interfere in DNA amplification [70], so removing inhibitors as much as possible by using a kit is convenient to ensure that DNA will be subsequently efficiently amplified. However, since this procedure could be very expensive to use when a large number of samples must be tested, an inexpensive and simple rapid-heating method is much more advantageous. It is also known that DNA purification from samples could be omitted in LAMP reactions, since LAMP assays have shown a significant tolerance to inhibitor substances derived from a number of biological samples [71], [72], [73]. Additionally, other LAMP assays with high sensitivity and no complicate requirement procedure for DNA extraction have been developed for molecular detection and diagnostic of bacterial [74] and parasitic [75] diseases in urine samples. Moreover, a simple heating DNA obtaining method has been also successfully applied with other clinical samples, such us blood [41] and swaps [42] in LAMP amplification of both *Plasmodium* and *Leishmania* species nucleic acids, respectively.

To really establish the sensitivity of our LAMP assay in urine samples that most closely resembled the patients´ urine specimens analyzed, we used a panel of simulated human urine samples artificially spiked with *S. haematobium* genomic DNA. For these samples, to extract DNA as template in LAMP we used both the commercial kit and the rapid-heat methods since these procedures showed the highest efficiency to obtain detectable DNA by LAMP in *S. haematobium*-positive clinical samples. After extracting DNA with the commercial kit, LAMP detection limit resulted tenfold higher than that obtained using *S. haematobium* genomic DNA 10-fold serially diluted without DNA extraction (10 fg *vs*. 100 fg, respectively). Unexpectedly, when heating the simulated samples, we obtained a limit of detection tenfold higher than that obtained when using purified DNA samples by the commercial kit (corresponding to 1 fg *vs*. 10 fg, respectively). An increased sensitivity has been also reported when using crude DNA extraction methods compared with a commercial method (i.e. DNazol) for template preparation from the pellets or supernatants of nasopharyngeal aspirates for LAMP detection of adenovirus [76]. Thus, the sensitivity value of 1 fg was considered as the lower limit of the detection threshold of the LAMP assay in detecting *S. haematobium* DNA in human urine samples. By reference, as *S. mansoni* genome contains approximately 580 fg of DNA [77], theoretically our LAMP assay would detect *S. haematobium* diluted DNA in urine samples corresponding to less than the equivalent to a single parasite cell. Such sensitivity is a feature of great

value to overcome the difficulties of detecting urogenital schistosomiasis in areas of low transmission or in individual cases with a very low worm burden.

Then, taking into account both the high sensitivity and the good-quality detectable *S. haematobium* DNA by LAMP in easy to obtain and handling heated pellets from clinical urine samples, we tested the remaining 76 specimens included in our study by the Rapid-Heat LAMPellet method. We obtained negative results by LAMP in all parasitologically *S. mansoni*-positive urine samples tested (corroborating again that no cross-reaction with that schistosome species occurs) and also in urine samples from healthy non-endemic donors used as negative controls. Nevertheless, eight LAMP positive results were obtained when testing patients´ urine samples from other groups which were formerly microscopy-confirmed as *S. haematobium*-negative. It may be rational to consider that those eight LAMP positive results are truly *S. haematobium*-infected samples which were undetected in the microscopic analysis since this method is very low sensitive, especially in low-grade infections and high day-to-day variable. Regarding the two LAMP positive results in patients´ urine samples with other microscopy-confirmed infectious diseases (i.e. hookworm and *T. vaginalis*), it is not uncommon to find co-infections of *S. haematobium* with other organisms such as bacteria, protozoa and helminths, including the hookworms [78]. It is unlikely that this result is due to a cross-reaction with hookworm since we obtained LAMP negative results in other three patients´ urine samples with microscopy-confirmed infection with this geohelminth. One eosinophilic without confirmed diagnosis patient as well as five non-eosinophilic without apparent pathologic disease individuals had *S. haematobium*-positive results by LAMP. The presence of absence of eosinophils is usually used as a biomarker for helminthic infections, including schistosomiasis [79]; however, it is not predictive of *Schistosoma* species infection and may generate inconsistent results [80]. Thus, application of our LAMP method may improve the identification of cases with low-intensity infections as well as in cases which did not pass eggs in urine samples, thus revealing infections in people frequently presumed to be uninfected. Finally, although all patients´ urine samples were tested in duplicate with the same result, it would be very interesting to know how reproducible the technique is when testing in field settings as well.

In conclusion, we have demonstrated that simply rapid-heating urinary pellets for good-quality DNA extraction was effective for use in LAMP assays with regard of detecting *S. haematobium* in clinical urine samples. This procedure has been named the Rapid-Heat LAMPellet method and it would be well-suited to diagnose urogenital schistosomiasis in resource-limited endemic regions because of its rapidity, easy handling, cost-effectiveness and both high detection specificity and sensitivity. The next step for refining the assay by conducting a field evaluation in an endemic setting should be desirable.

Supporting Information

S1 Checklist. STARD checklist.
(PDF)

S1 Fig. Examination of the patients´ urine samples by the Rapid-Heat LAMPellet method. Figure shows the LAMP results (up, by color change; down, by agarose electrophoresis) when testing clinical urine samples from different groups of patients included in our study by using heated pellet following by the specific LAMP assay for *S. haematobium* DNA detection. (A) Urine samples from patients with confirmed infection with several helminths. (B) Urine samples from patients with confirmed infection with different infectious agents (protozoa, bacteria and virus). (C) Urine samples from patients with eosinophilia but not confirmed diagnosis. (D) Urine samples from patients without either eosinophilia and none apparent disease. (E) Urine samples from patients with confirmed *S. mansoni* infection. (F) Urine samples from

healthy non-endemic individuals (negative controls). Lanes M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sh: genomic DNA from *S. haematobium* (1 ng); lanes numbered, number of urine samples included in each group of patients.
(TIFF)

S1 Flowchart. A diagram showing experimental design and results.
(TIFF)

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Author Contributions

Conceived and designed the experiments: JG PFS AM JLA. Performed the experiments: JG PFS CCR. Analyzed the data: JG PFS AM JLA. Contributed reagents/materials/analysis tools: CCR JLPA BV. Wrote the paper: JG PFS AM.

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3.3. Artículo 3

Biompha-LAMP: A New Rapid Loop-Mediated Isothermal Amplification Assay for Detecting Schistosoma mansoni in Biomphalaria glabrata Snail Host.

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PLoS Negl Trop Dis. 2016;10(12): e0005225.

Resumen

La esquistosomosis continúa siendo una de las enfermedades parasitarias más comunes, afectando alrededor de 200 millones de personas en el mundo. *Schistosoma mansoni* es la especie más importante causante de la esquistosomosis intestinal y los caracoles de agua dulce del género *Biomphalaria* son ampliamente conocidos por su papel como hospedador intermediario del parásito. El desarrollo de nuevas técnicas moleculares para la detección a gran escala de caracoles infectados para determinar focos de transmisión de la enfermedad se considera un punto importante para los programas de control de la esquistosomosis. Este trabajo está enfocado al desarrollo y evaluación de un nuevo método LAMP, combinado con un método simple para la obtención del ADN, para la detección de *S. mansoni* en caracoles infectados como herramienta para ser aplicada en condiciones de campo.

El método LAMP se diseñó empleando un juego completo de 6 *primers* que amplificaban un fragmento de ADN del espaciador inter-génico 28S-18S ribosomal. El límite de detección de la técnica fue de 0.1 fg de ADN realizando la incubación durante 50 min. Posteriormente, el LAMP fue evaluado detectando ADN *de S. mansoni* en caracoles de la especia *B. glabrata* infectados experimentalmente con diferentes niveles de infección: en la fase prepatente (antes de la emisión de cercarias), en infecciones leves (usando caracoles expuestos a un número bajo de miracidios) y en la detección de caracoles infectados en muestras en forma de *pool* (dentro de un grupo de caracoles no infectados). La obtención del ADN se llevó a cabo empleando un *kit* comercial o con un simple calentamiento con NaOH. Finalmente, detectamos ADN de *S. mansoni* en todos los grupos de infección empleando un método fácil y rápido para extraer el ADN.

Nuestro LAMP, llamado Biompha-LAMP, es sensible, específico, rápido y potencialmente adaptable como una herramienta efectiva para el control de caracoles infectados con *S. mansoni*, ya sea individualmente o en forma de *pool*. Este método podría ser aplicable en estudios a gran escala para la vigilancia y monitorización de sitios de transmisión en áreas endémicas de esquistosomosis.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Biompha-LAMP: A New Rapid Loop-Mediated Isothermal Amplification Assay for Detecting *Schistosoma mansoni* in *Biomphalaria glabrata* Snail Host

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Abstract

Background

Schistosomiasis remains one of the most common endemic parasitic diseases affecting over 230 million people worlwide. *Schistosoma mansoni* is the main species causing intestinal and hepatic schistosomiasis and the fresh water pulmonate snails of the genus *Biomphalaria* are best known for their role as intermediate hosts of the parasite. The development of new molecular monitoring assays for large-scale screening of snails from transmission sites to detect the presence of schistosomes is an important point to consider for snail control interventions related to schistosomiasis elimination. Our work was focussed on developing and evaluating a new LAMP assay combined with a simple DNA extraction method to detect *S. mansoni* in experimentally infected snails as a diagnostic tool for field conditions.

Methodology/Principal findings

A LAMP assay using a set of six primers targeting a sequence of *S. mansoni* ribosomal intergenic spacer 28S-18S rRNA was designed. The detection limit of the LAMP assay was 0.1 fg of *S. mansoni* DNA at 63˚C for 50 minutes. LAMP was evaluated by examining *S. mansoni* DNA in *B. glabrata* snails experimentally exposed to miracidia at different times post-exposure: early prepatent period (before cercarial shedding), light infections (snails exposed to a low number of miracidia) and detection of infected snails in pooled samples (within a group of uninfected snails). DNA for LAMP assays was obtained by using a commercial DNA extraction kit or a simple heat NaOH extraction method. We detected *S. mansoni* DNA in all groups of snails by using no complicated requirement procedure for DNA obtaining.

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Competing Interests: The authors have declared that no competing interests exist.

Conclusions/Significance

Our LAMP assay, named *Biompha*-LAMP, is specific, sensitive, rapid and potentially adaptable as a cost-effective method for screening of intermediate hosts infected with *S. mansoni* in both individual snails and pooled samples. The assay could be suitable for large-scale field surveys for schistosomes control campaigns in endemic areas.

Author Summary

Schistosoma mansoni is the main species causing intestinal and hepatic schistosomiasis worldwide and the snails of the genus *Biomphalaria* are best known for their role as intermediate hosts of the parasite. Molecular xenomonitoring for large-scale screening of snails from transmission sites to detect the presence of schistosomes is an important point to consider for snail control interventions related to schistosomiasis elimination. In our study, we have developed a new simple rapid LAMP assay to detect *S. mansoni* in *Biomphalaria glabrata* snails under different situations of infection: early prepatent period, light or low-grade infections and in snails pooled samples. Besides, a simple and rapid method for DNA extraction from snails' tissues was successfully used. This LAMP assay (named *Biompha*-LAMP) could be potentially useful for large-scale screening in searching infected snails with *S. mansoni* in field applicable conditions.

Introduction

Human schistosomiasis continues to be one of the most important neglected tropical diseases affecting over 230 million people worldwide. *Schistosoma mansoni* is the main species causing hepatic and intestinal schistosomiasis in Sub-Saharan Africa and solely in South America [1–4]. The fresh water snails of genus *Biomphalaria* act as the parasite´s intermediate host which are able to produce a constant output of hundreds or even thousands of cercariae for months [5]. The cercarial emission from infected snails is the route of infection for humans including those who may have been successfully treated in a control program. By themselves, preventive chemotherapy campaigns using mass drug administration have shown not to limit transmission in high-risk areas [6, 7]. The distribution and prevalence of the disease are determined, to a large extent, by the presence or absence of *Biomphalaria* snails [8]. In addition to health education, safe water supplies, adequate sanitation and environmental management, a snail control would also reduce transmission of human infection and is necessary for a schistosomiasis comprehensive control program [7, 9]. Among different known monitoring approaches for surveillance of active sites for snail-to-human transmission, the detection of cercarial shedding by infected snails after exposure of the specimens to light during 1-24h has been the most traditionally and widely method used [10]. This method has significant limitations to detect the parasite, especially during the prepatent period of snail infections (non-shedding), in low-grade infections and also due to the aborted development of schistosomes in snails [11]. The dissection of snails to detect sporocysts of schistosomes during the prepatent period is a hard task and often unsuccessful because of its tiny size and also, the lack of experienced personnel for accurate identification of infection [12]. Besides, differentiation in cercariae morphology between *S. mansoni* and other trematodes species sometimes may be difficult [13]. All this

produces an underestimation of the true prevalence and incidence of infection by schistosomes in snail populations.

To overcome these limitations in detecting infected snails, molecular xenomonitoring (the detection of parasite DNA or RNA in snails using molecular-based assays) is a great alternative allowing analysis of pooled snails samples and also offering greater efficiency and sensitivity than dissection of snail tissues, especially when large numbers of specimens must be examined. In recent years, several molecular monitoring polymerase chain reaction (PCR)-based assays have been developed for *S. mansoni* detection in snails, such us conventional PCR [14, 15], nested-PCR [16], multiplex-PCR [17] and real time-PCR [18]. All these studies have demonstrated better results in detecting the parasite than conventional methods but the lack of resources is a major barrier to apply in endemic countries for schistosomiasis because of the highly techniques requirements and skilled personnel. As a potential alternative for molecular xenomonitoring snail sampling adaptable to field conditions could be the loop-mediated isothermal amplification (LAMP) assay [19], a powerful simple and rapid nucleic acid amplification technique with a wide range of possible applications including point-of-care testing in resource-poor settings (such in developing countries) and rapid testing of environmental samples [20].

Several LAMP-based assays have already been reported for the detection of schistosomal DNA in samples from animals in laboratory settings, such as *S. japonicum* in rabbits [21, 22] or *S. mansoni* in mice [23, 24] as well as from both human urine and serum samples for detection of *S. haematobium* [25] and *S. japonicum* [26], respectively. Additionally, other LAMP assays have been described in order to provide a rapid and effective method to detect schistosomal DNA in field-collected intermediate host snails, including *S. japonicum* [27], *S. haematobium* and *S. mansoni* [10] and potentially later adaptation in a large-scale screening of snails pooled samples to be used as method for snails control [28, 29]. The "development of inexpensive, field-applicable diagnostic assays for the large-scale screening of individual or pooled snails from transmission sites to detect the presence of schistosomes" has been listed as an important point to consider in an agenda for snail control interventions related to schistosomiasis elimination [30].

However, for *S. mansoni*, no LAMP assay has been evaluated yet to. In our study, we have developed a new simple rapid LAMP assay to detect *S. mansoni* in *Biomphalaria glabrata* snails under different situations of infection: early prepatent period, light or low-grade infections and in snails pooled samples. Besides, a simple, rapid and economic method for DNA extraction from snails´ tissues was successfully used. The LAMP assay presented here could be potentially useful for large-scale screening in searching infected snails with *S. mansoni* in field applicable conditions.

Methods

Ethics statement

Animal procedures complied with the Spanish (Real Decreto RD53/2013) and the European Union (European Directive 2010/63/EU) regulations on animal experimentation for the protection and human use of laboratory animals. Experiments were conducted at the accredited Animal Experimentation Facility of the University of Salamanca (Register number: PAE/SA/001). Procedures were approved by the Ethics Committee of the University of Salamanca (protocol approval number 48531).

S. mansoni maintenance and snails infections

S. mansoni (LE strain) was maintained routinely by passage through *Biomphalaria glabrata* snails and 4-to-6-week old male CD1 mice (Charles River, Criffa S.A., Barcelona, Spain) at

University of Salamanca. Eight weeks after infection mice were humanely euthanized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) plus heparin (2 IU/mL) and the liver was removed and minced to obtain eggs. Purified eggs were put into water to hatch the miracidia for experimental infection of snails. Snails were exposed individually to 9 miracidia in 6-well plates. After 30–40 days, cercariae were shed from infected snails by exposure to light within 60 min at room temperature. Using this routine procedure, a number of *B. glabrata* snails were exposed to different numbers of miracidia in order to detect subsequently *S. mansoni* DNA at different times post-exposure (p.e.) simulating different conditions such as: *i)* detection of infected snails in the early prepatent period (before cercarial shedding), *ii)* detection of light infections (snails exposed to a low number of miracidia) and *iii)* detection of infected snails in pooled samples (within a broad group of snails), as described below. A scheme of the different snails infections carried out in the study is showed in fig 1.

Prepatent period (Fig 1A). A total of twenty snails (n = 20) were individually placed into a 24-well polystyrene plate and exposed to 9 miracidia each; next, they were divided into two groups of 10 snails: group 1 and group 2. A specimen from each group was sacrificed every day during the first week (days 1–7), and later on day 14, 21 and 28 p.e.. After crushing, soft tissues from snails belonging to group 1 were immediately extracted from the shells by using a fine needle and storage individually at -20˚C until DNA extraction. Snails from group 2 were sacrificed by immersion in pure ethanol and then preserved for a week until extraction of the soft tissues from the shells by using a fine needle; then, the excess ethanol solution was removed by drying on filter paper and promptly processed for DNA obtaining.

Light infections (Fig 1B). A total of twelve snails (n = 12) were also individually placed into a 24-well polystyrene plate and divided into two groups (group 1 and group 2) of 6 specimens each. Three snails of each group of 6 specimens were exposed to a single miracidium and the other 3 were exposed to 4 miracidia. All specimens were sacrificed at 24 h p.e.. Snails from group 1 were crushed and soft tissues were immediately extracted from the shells by using a fine needle and preserved at -20˚C until DNA extraction. Snails from group 2 were sacrificed by immersion in ethanol and subsequently preserved for a week until extraction of the soft tissues from the shells by using a fine needle; then, the excess ethanol solution was removed by drying on filter paper and promptly processed for DNA obtaining.

Pooled samples (Fig 1C). A total of 6 snails -3 previously exposed to a single miracidium and 3 previously exposed to 9 miracidia and also showing cercarial shedding after 40 days p.e.- were placed individually into 50 mL sterile conical tubes together with 5, 10 and 20 uninfected snails, respectively. After 24h, all the pooled samples were crushed for soft tissues extraction by using a fine needle and promptly processed.

DNA obtaining

Snails and miracidia DNA extraction. DNA from snails for LAMP assays was obtained by using two different methods: *i)* a commercial DNA extraction kit (NucleoSpin Tissue; Macherey-Nagel, Germany) for snails' tissues preserved at -20˚C following the manufacturers' instructions and, *ii)* a heat sodium hydroxide (NaOH) extraction method [28] for snails´ tissues preserved in absolute ethanol. Briefly, in the heat NaOH extraction method, for processing individual snails a volume of 200 μL of a 50 mM NaOH solution was added and then heated at 95˚C for 30 min. Subsequently, the tubes were centrifugated at 5000 rpm for 5 min and a volume of 50 μL of supernatant was recovered in a new clean tube and mixed with an equal volume of a 1 M Tris-HCl (pH 8.0) solution. When preparing pooled samples for DNA extraction, a greater volume of NaOH was used (10 mL instead 200 μL) due to the larger amount of snails' tissues to be digested. Each new solution thus obtained was stored at -20˚C

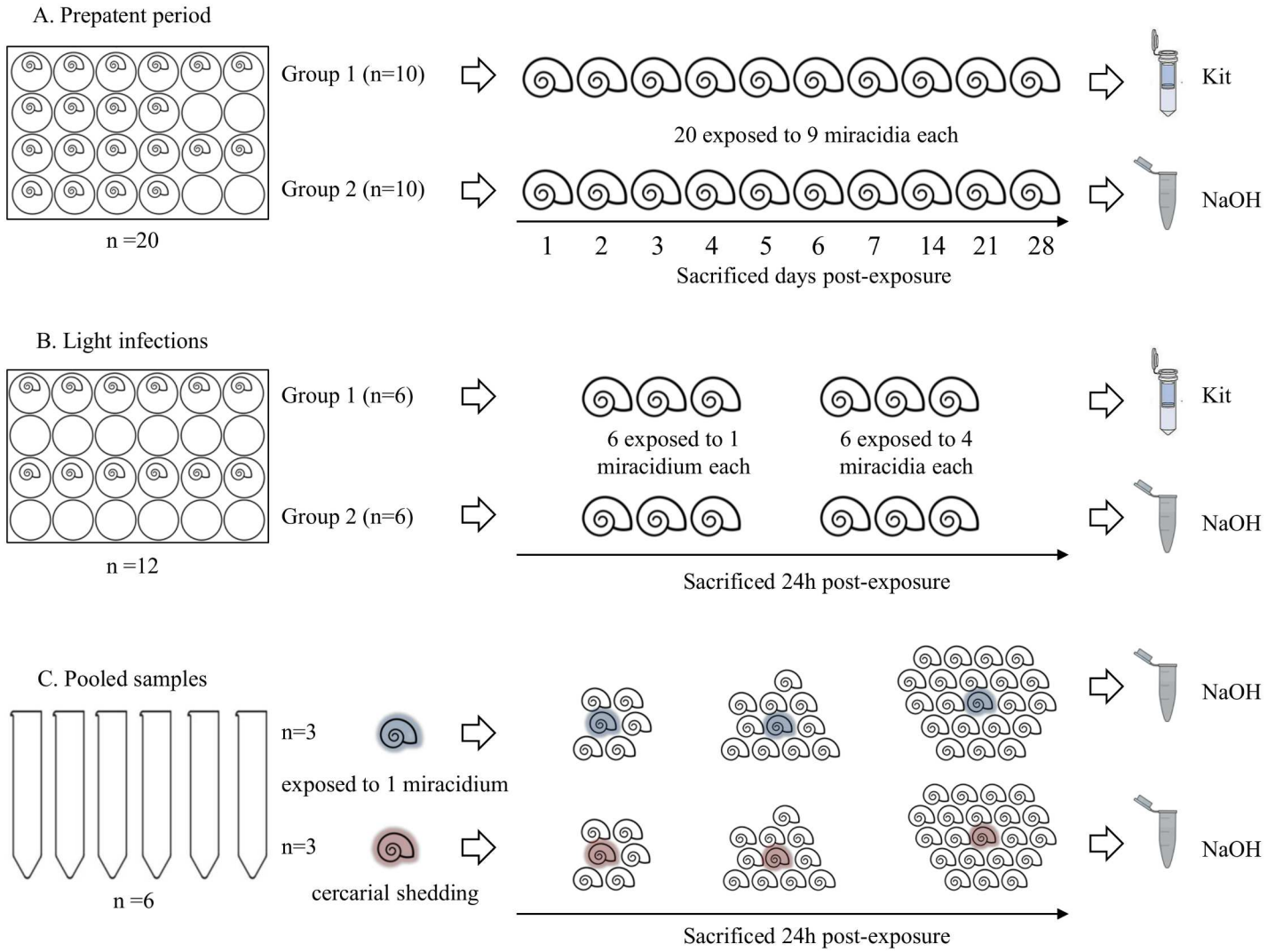

Fig 1. Scheme of experimentally infected snails in this study. (A) Prepatent period. (B) Light infections. (C) Pooled samples. Kit and NaOH, indicate the commercial kit or the heat NaOH extraction method for DNA obtaining, respectively. Number or snails used in each infection, exposition of snails to miracidium/miracidia, snails showing cercarial shedding and sacrificed days post-exposure are indicated by a text in figure.

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until further use as template in LAMP assays. DNA from two separate miracidia to be used as template control for LAMP assays was obtained by the two different mentioned methods. DNA from 2 uninfected snails and 2 snails with confirmed cercarial shedding to be used as additional negative and positive controls, respectively, were also obtained by the two different DNA extraction methods used.

Parasites DNA extraction. DNA from *S. mansoni* frozen adult worms available in our laboratory was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturers' instructions and prepared to a final concentration of 0.5 ng/μL. Then, DNA was 10-fold serially diluted (ranging from 0.05 ng/μL to 0.5 atg/μL) and stored at -20˚C until further use. DNA thus prepared was used as a template control in LAMP reactions as well as for assessing sensitivity.

To determine the specificity of LAMP assay, DNA from other several trematodes requiring snails as intermediate hosts in their life cycle with vertebrates were used as heterogeneous

control samples, including *Schistosoma haematobium* and *S. intercalatum* (affecting people), *Schistosoma bovis* and *Dicrocoelium dendriticum* (affecting cattle) and *Schistosoma japonicum* and *Fasciola hepatica* (affecting people and/or cattle). These DNA samples were also diluted to a final concentration of 0.5 ng/μL and kept frozen until use.

S. mansoni LAMP primer design

A 3022 base pair (bp) sequence corresponding to the ribosomal intergenic spacer 28S-18S ribosomal RNA gene [31] was retrieved from GenBank (Accesion no. AJ223842) for further studies. Specificity for *S. mansoni* was tested *in silico* through BLAST alignment analysis [32], as well as searches and comparisons in available online genomes databases for *Schistosoma* spp. (*e.g.* SchistoDB; http://schistodb.net/schisto/). A 284 bp unique region for *S. mansoni* was selected and used for LAMP primer design using the Primer Explorer v4 software (http://primerexplorer.jp/e/). A set of six primers -including a forward outer primer (F3), a reverse outer primer (B3), a forward inner primer (FIP), a backward inner primer (BIP), a loop forward primer (LF) and a loop backward primer (LB)- was selected based on the criteria described in "A guide to LAMP primer designing" (http://primerexplorer.jp/e/v4_manual/index.html). LAMP primers sequences and their positions in the selected target for *S. mansoni* are shown in fig 2.

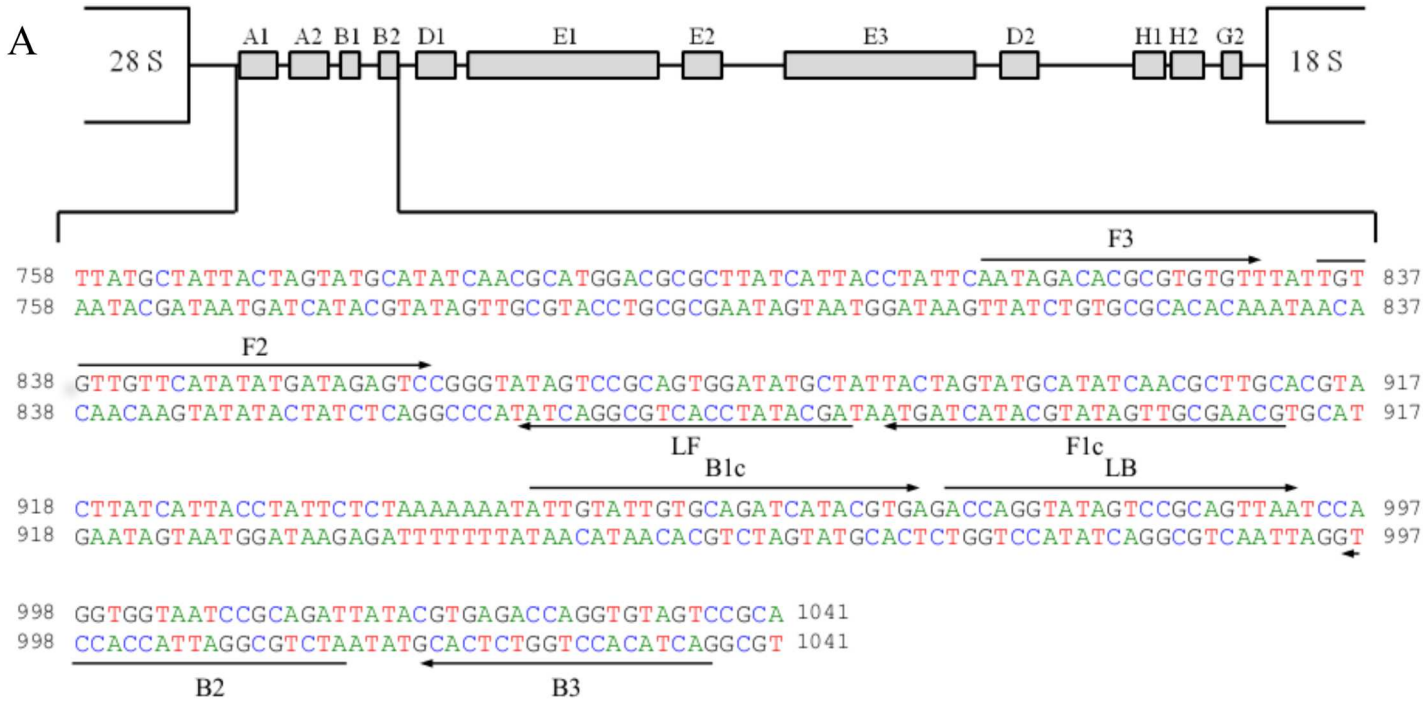
PCR using outer primers F3 and B3

The outer LAMP primer pair (F3 and B3) was initially tested to verify the correct amplification of the selected target of *S. mansoni* DNA by a touchdown PCR (TD-PCR). Briefly, the PCR F3-B3 assay was conducted in 25 μL reaction mixture containing 2.5 μL of 10x buffer, 1.5 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2.5 mmol/L dNTPs, 0.5 μL of 100 pmol/L F3 and B3, 2 U *Taq*-polymerase and 2 μL (10 ng) of DNA template. Initial denaturation was conducted at 94˚C for 1 min, followed by a touchdown program for 15 cycles with successive annealing temperature decrements of 1.0˚C (from 57˚C to 52˚C) every 2 cycles. Subsequently, the specificity and sensitivity of PCR F3-B3 were tested using 2 μL of heterogeneous DNA samples included in the study and 2 μL of *S. mansoni* DNA 10-fold serially diluted, respectively, prepared as mentioned above. Negative controls (ultrapure water) were always also included. The PCR products (3–5 μL) were subjected to 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light.

Setting up LAMP assay

The LAMP reactions were carried out in a final volume of 25 μL containing 1.6 μM of each of the FIP and BIP primers, 0.2 μM of the F3 and B3 primers, 0.4 μM of the LF and LB primers, 1x Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), 1 M betaine, 6 mM supplementary $MgSO_4$ and 8 U of *Bst* 2.0 DNA polymerase with 2 μL of template DNA. To establish the optimal reaction time for LAMP assay amplifying the minimum amount of *S. mansoni* DNA using the set of six primers, three different assays were carried out adding different amounts of *S. mansoni* DNA (1 ng, 1 pg and 1 fg, respectively) and varying the incubation time at 63˚C for 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, followed by 5–10 min to 80˚C to terminate the reaction. The optimal reaction time was determinate and used in all the following tests. The amplification results were visually detected by adding 2 μL of 1:10 diluted 10.000X concentration fluorescent dye SYBR Green I (Invitrogen) an also on a 1.5% agarose gel electrophoresis stained with ethidium bromide. LAMP sensitivity and specificity

A



B

Primer	Length (bp)	Sequence (5'-3')
F3	18	AATAGACACGCGTGTGTT
B3	18	GACTACACCTGGTCTCAC
FIP (F1c-F2)	50	GCAAGCGTTGATATGCATACTAGTA-TGTGTTGTTCATATATGATAGAGTC
BIP (B1c-B2)	43	ATTGTATTGTGCAGATCATACGTGA-ATCTGCGGATTACCACCT
LF	21	AGCATATCCACTGCGGACTAT
LB	22	ACCAGGTATAGTCCGCAGTTAA

Fig 2. LAMP primer set targeting the selected sequence (GenBank Accesion. No. AJ223842) for ribosomal intergenic spacer 28S-18S ribosomal RNA gene *S. mansoni* DNA region amplification. (A) The location of the LAMP primers within the selected sequence is shown. Arrows indicate the direction of extension. (B) Sequence of LAMP primers: F3, forward outer primer; B3, reverse outer primer; FIP, forward inner primer (comprising F1c and F2 sequences); BIP, reverse inner primer (comprising B1c and B2 sequences); LF, loop forward primer; LB, loop reverse primer.

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were determinate using genomic DNA from *S. mansoni* 10-fold serially diluted and other heterogeneous DNA samples from other parasites, respectively, as mentioned above.

Results

PCR F3-B3 sensitivity and specificity

The *in silico* 225 bp expected amplicon was successfully amplified when using PCR F3-B3 (Fig 3A). The minimum amount of DNA detectable was 0.01 ng (Fig 3B). When DNA samples from other parasites included in the study were subjected to this PCR assay, amplicons were never obtained (Fig 3C).

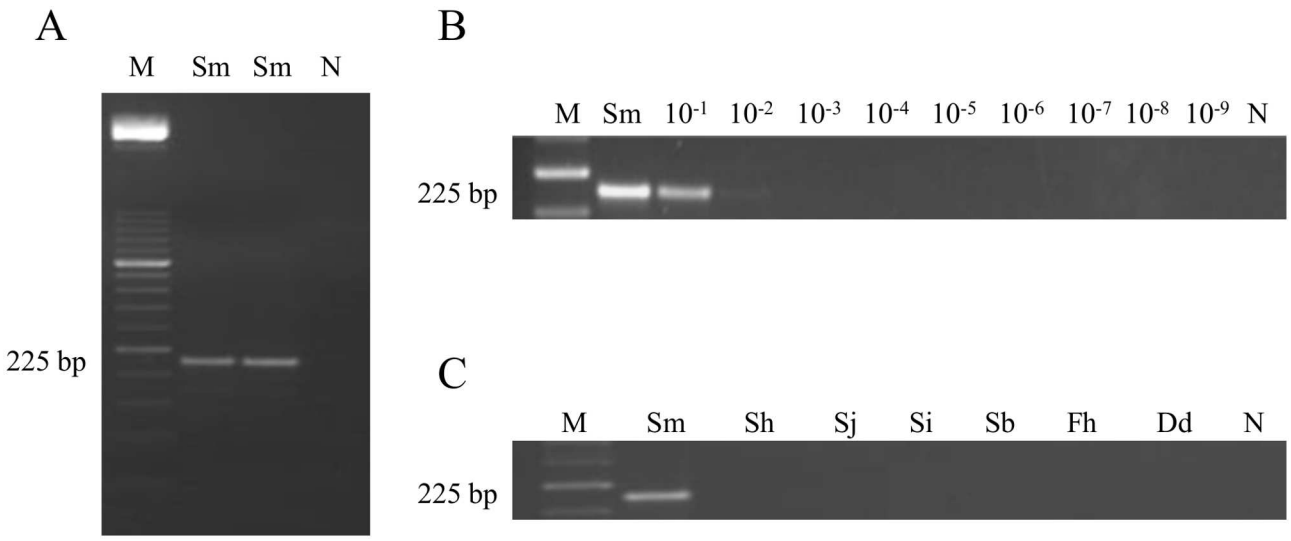

Fig 3. PCR verification, detection limit and specificity using outer primers F3 and B3. (A) PCR verification of expected 225 bp target length amplicon. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, *S. mansoni* DNA (1 ng); lane N, negative control (no DNA template). (B) Detection limit of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sm: *S. mansoni* DNA (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions of *S. mansoni* DNA; lane N, negative control (no DNA template). (C) Specificity of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, Sh, Sj, Si, Sb, Fh, Dd, *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *S. bovis*, *Fasciola hepatica* and *Dicrocoelium dendriticum* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template).

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Setting up the LAMP assay: *Biompha*-LAMP

When using 1 ng (Fig 4A), 1 pg (Fig 4B) or 1 fg (Fig 4C) of *S. mansoni* DNA as template for LAMP assay at 63˚C, we obtained positive results as soon as 20 min, 30 min and 50 min, respectively. All amplification results were clearly visualized by naked eye after adding the fluorescent dye as well as on agarose gel electrophoresis showing the typical ladder-like pattern. Afterwards, we evaluated the sensitivity of the LAMP assay at 63˚C for 50 min by using *S. mansoni* DNA 10-fold serially diluted. The limit of detection was 0.1 fg (Fig 4D), showing that LAMP assay is 10^5 fold higher than PCR F3-B3. Regarding specificity, the LAMP assay was positive only for *S. mansoni* and no positive DNA products were observed when other species were used as templates (Fig 4E). Thereby, the LAMP assay at 63˚C for 50 min was set up as the most suitable to test all the DNA samples from *B. glabrata* snails included in the study and hereinafter was namely *Biompha*-LAMP.

Application of LAMP in snails samples: *Biompha*-LAMP analysis

The results obtained in *Biompha*-LAMP assays to detect *S. mansoni* DNA in snail samples from the different experimental snails infections carried out in the study are showed in fig 5. We detected *S. mansoni* DNA in all infected snails tested before cercarial shedding at different days p.e. regardless of the method used for DNA extraction, thus is, the commercial kit (Fig 5A) or the heat NaOH extraction method (Fig 5B). We also obtained *Biompha*-LAMP positive results in those snails previously exposed to a low number of miracidia (one or four) using both commercial kit or the heat NaOH extraction method for DNA obtaining (Fig 5C and 5D, respectively). We did not obtain positive results in pooled samples containing snails previously exposed to a single miracidium and processed by the heat NaOH extraction method (Fig 5E). However, we obtained *Biompha*-LAMP positive results in pooled samples containing snails

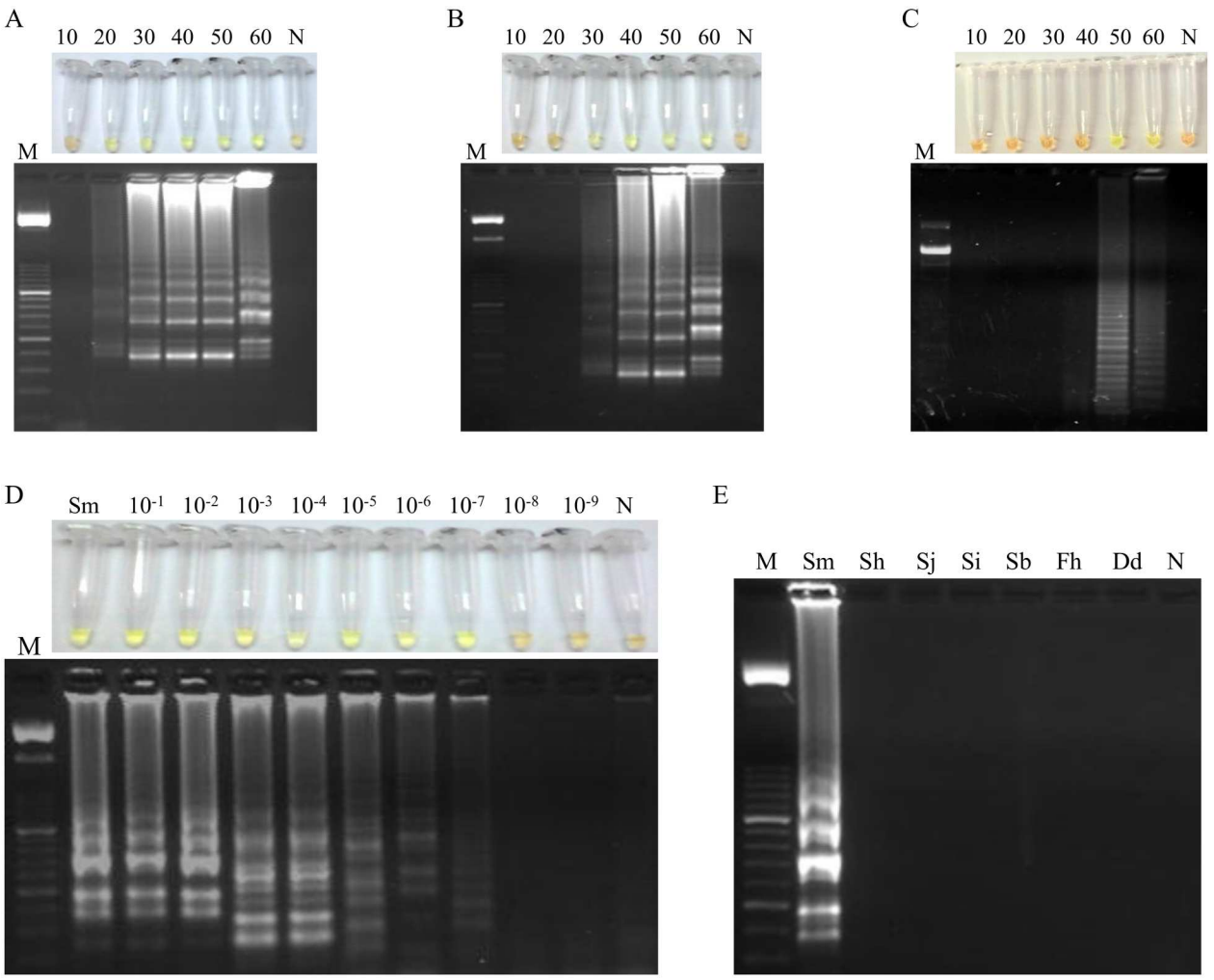

Fig 4. Setting up LAMP assay. LAMP amplification results using (A) 1 ng, (B) 1 pg and (C) 1 fg of *S. mansoni* DNA obtained at different incubation times (10, 20, 30, 40, 50 and 60 min) tested in a heating block by the addition of SYBR Green I (top) or by visualization on agarose gel (bottom). Lanes M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes N: negative control (no DNA template). (D) Sensitivity assessment performed with LAMP at 63˚C for 50 min using serial dilutions of *S. mansoni* genomic DNA. Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sm: genomic DNA from *S. mansoni* (1 ng); lanes 10^{-1}–10^{-9}: 10-fold serially dilutions; lane N: negative control (no DNA template). (E) Specificity of the LAMP assay for *S. mansoni*. A ladder of multiple bands of different sizes could be only observed in *S. mansoni* DNA sample. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, Sh, Sj, Si, Sb, Fh and Dd, *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *S. bovis*, *Fasciola hepatica* and *Dicrocoelium dendriticum* DNA samples (1 ng/each), respectively; lane N, negative control (no DNA template).

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with confirmed cercarial shedding (Fig 5F). In all *Biompha*-LAMP assays, positive controls included (DNA from *S. mansoni* adult worms, miracidia or infected snails) showed an amplification product whereas negative controls (distilled water as template or DNA from uninfected snails) never amplified.

Discussion

B. glabrata, as an intermediate snail host for *S. mansoni*, plays a crucial role in both multiplication and transmission of schistosomes. Thus, snail control interventions are considered a priority and still needed for the interruption of schistosomiasis transmission. The early detection of prepatently infected *B. glabrata* snails using simple, sensitive and inexpensive molecular

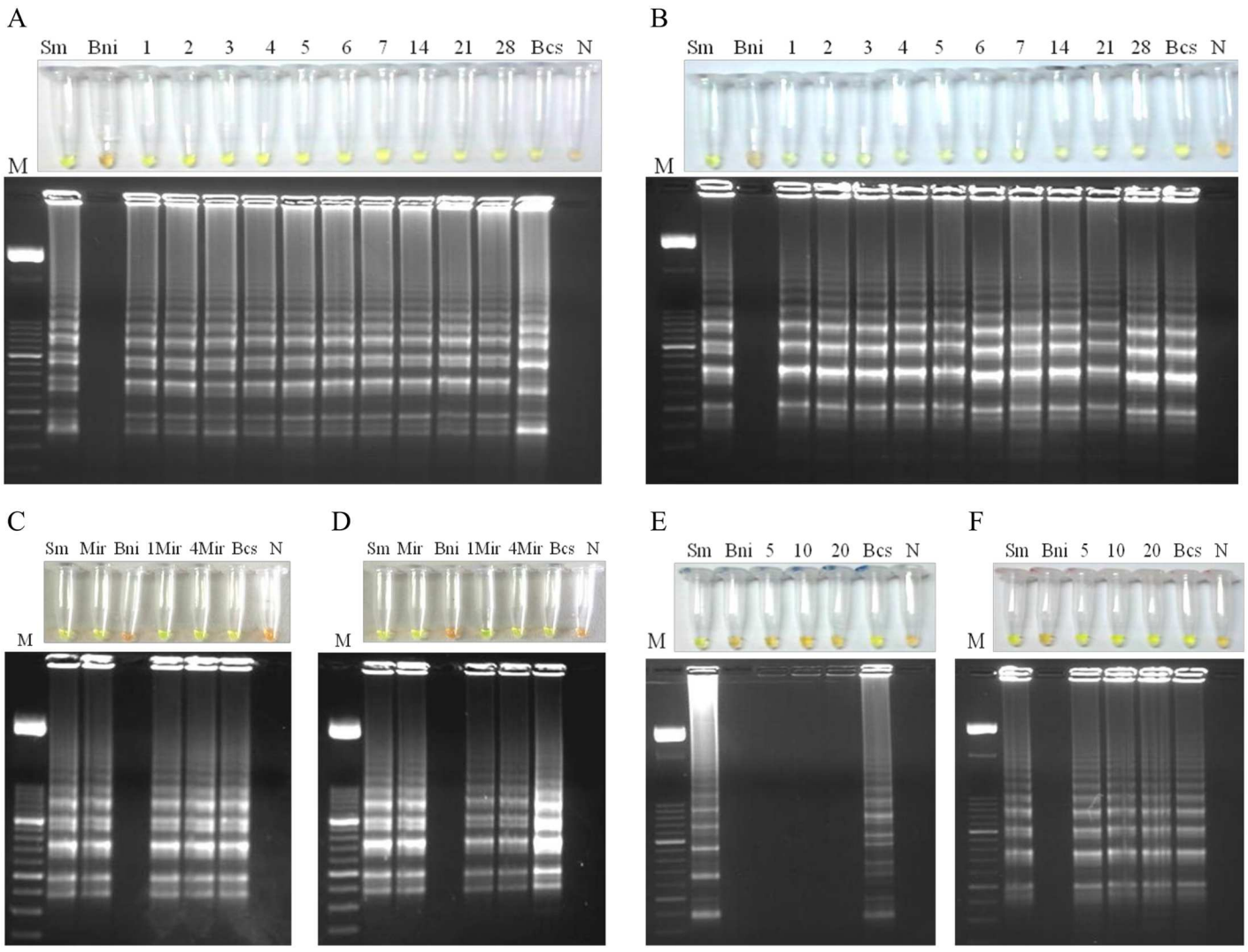

Fig 5. Application of LAMP in snails samples: *Biompha*-LAMP analysis. (A) and (B) Analysis of snails before cercarial shedding at different days post-exposure to 9 miracidia each using a commercial kit or the heat NaOH extraction method for DNA obtaining, respectively. Lanes M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane Sm, *Schistosoma mansoni* DNA (1 ng); lanes Bni, *Biomphalaria glabrata* DNA non infected; Lanes 1–7, 14, 21 and 28, days post-exposure to miracidia; lanes Bcs, *Biomphalaria glabrata* DNA with cercarial shedding; lanes N: negative control (no DNA template). (C) and (D) Analysis of snails exposed to one or four miracidia at 24h post-exposure using a commercial kit or the heat NaOH extraction method, respectively. Lanes M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, *Schistosoma mansoni* DNA (1 ng); lanes Mir; DNA obtained from one miracidium; lanes Bni, *Biomphalaria glabrata* DNA non infected; lanes 1Mir and 4Mir, DNA obtained from snails exposed to one or 4 miracidia, respectively; lanes Bcs, *Biomphalaria glabrata* DNA with cercarial shedding; lanes N: negative control (no DNA template). (E) and (F) Analysis of pooled samples containing snails previously exposed to 1 miracidium or with confirmed cercarial shedding, respectively, using the heat NaOH extraction method. Lanes M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes Sm, *Schistosoma mansoni* DNA (1 ng); lanes Bni, *Biomphalaria glabrata* DNA non infected; lanes 5, 10 and 20, DNA obtained from pooled samples containing one snail exposed to 1 miracidium or with confirmed cercarial shedding together with 5, 10 or 20 uninfected snails; lanes Bcs, *Biomphalaria glabrata* DNA with cercarial shedding; lanes N: negative control (no DNA template).

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methods to detect *S. mansoni* DNA sequences in snails would be very helpful in rapid evaluation of the potential risk of transmission in suspected areas of schistosomiasis and would also provide for more effective disease control measures. In this sense, LAMP assay has become a most suitable tool than PCR-based methods for rapid molecular monitoring of vectors [33] and intermediate snails hosts of several parasites, including schistosomes [34–36] because of

its operational simplicity, less time-consuming and versatility of visual detection readout options for field application [20].

In this work, we have developed and evaluated a rapid, sensitive and specific LAMP assay combined with a simple and economic DNA extraction method to detect experimentally infected snails with *S. mansoni*. This methodology could be potentially suitable for monitoring of infected snails in endemic areas of schistosomiasis with basic laboratory facilities.

To design the specific set of six primers for our LAMP assay, an intergenic spacer (IGS) of the large subunit (28S) ribosomal RNA gene was selected [31]. Ribosomal genes within *Schistosoma* species are known to be multi-copy (over 80–137 copies are estimated) and tandemly repeated within *S. mansoni* genome [37]. Therefore, using a repetitive selected portion of the genome as target for amplification might greatly increase LAMP sensitivity. Additionally, the ribosomal IGS frequently contain specific sequence motifs, thus allowing differentiation of *Schistosoma* species and also avoiding cross-reactions with other target organisms such as the intermediate snail hosts. Furthermore, a section of the ribosomal IGS of both *S. haematobium* and *S. mansoni* has previously been successfully used for molecular detection of schistosomes DNA in freshwater snails by using either RT-PCR or oligochromatographic dipstick assay (PCR-OC) [18]. Nevertheless, in terms of potential use of this new LAMP assay in field conditions, an additional validation using other DNA samples from other *Biomphalaria* species and also other *Schistosoma* species should be formerly tested.

After verifying the operation, sensitivity and specificity of PCR F3-B3 in amplification of the *in silico* expected fragment of 225 bp of *S. mansoni* IGS 28S rRNA gene, we attempted to set up the best conditions for primers set operation in the LAMP reaction. The design of our LAMP assay included a pair of loop primers (LF and LB), which it has been reported to accelerate the LAMP reaction speed and then reducing the reaction time to about 30 min [38]. When testing different reaction times, we obtained amplification of 1 ng and 1 pg of *S. mansoni* DNA at 63˚C in just only 20 min and 30 min, respectively, as was confirmed by naked eye and electrophoresis. We also obtained amplification of such a small amount as 1 fg of *S. mansoni* DNA when the reaction was incubated at 63˚C for 50 min. According to this result, both this temperature and reaction time were selected to establish the limit of detection of the LAMP assay, which finally resulted in 10^5 times higher than that obtained by PCR F3-B3 (0.1 fg *vs*. 10 pg, respectively). Thus, the value of 0.1 fg was considered as the lower limit of the detection threshold of our LAMP assay in detecting *S. mansoni* genomic DNA. It has been reported that a number of 10 *S. mansoni* miracidia yield 0.45 ng of genomic DNA [16] and also that *S. mansoni* genome contains approximately 580 fg of DNA [39]. Then, theoretically our LAMP assay would detect *S. mansoni* DNA corresponding to less than the equivalent to one single miracidium or a single parasite cell. A high sensitivity has also been previously reported when using other LAMP assays to detect schistosomes in infected snails [10, 29], but a long time of 120 min was required to complete the reaction, whereas our LAMP assay (*Biompha*-LAMP) takes only 50 min to obtain the same limit of DNA detection.

The applicability and effectiveness of our *Biompha*-LAMP assay in detecting laboratory-infected snails with *S. mansoni* could be assessed on a number of *Biomphalaria* specimens in a very early prepatent period (as soon as one day after miracidial exposure) as well as in low-grade infections (snails infected with only 4 miracidia or even in monomiracidial infections) regardless of the method used for DNA extraction. Our results were consistent with those previously reported in detecting infected snails from prepatent period by molecular methods, such as PCR [14, 16] and other LAMP assays [10, 29]. This feature is of a great value since at 24 hours p.e. sporocysts has not yet undergone germinal cell division [40] and besides that, not all miracidia subsequently complete the infection and develop until cercariae [41]. Thus, *Biompha*-LAMP could be a good alternative to parasitological methods in detecting trace amounts

of *S. mansoni* DNA present in low-infected snails in low-transmission areas of schistosomiasis. An additional advantage is that not complicate or expensive procedure for snails DNA extraction is required because a simple and economical heat NaOH method is just enough to obtain a quality DNA for LAMP amplification. In this sense, another successful LAMP assays to detect schistosomes DNA without requiring a purified nucleic acid have been already reported [25, 29].

In recent years, the large-scale molecular screening of pooled field-collected snails in transmission areas of schistosomiasis has been reported as a simple and efficient tool for snails surveillance [28, 29]. For potential applications of our *Biompha*-LAMP in such setting, we tested in laboratory conditions several different size crushed pooled samples. When a snail previously exposed to a single miracidium was placed and crushed together with other 5, 10 or 20 uninfected snails no amplification was obtained. Probably, the greater volume of NaOH used to crush a greater amount of soft tissues for snails DNA extraction diluted in excess DNA concentration to be detected, since a single snail exposed to a single miracidium was previously individually found to be LAMP positive. When testing pooled samples containing a single snail showing cercarial shedding together with other several uninfected snails, a LAMP positive result was always obtained. This is consistent with the fact that a high number of cercariae increase the total amount of *S. mansoni* DNA in pooled samples.

In conclusion, the current study has demonstrated that our new designed *Biompha*-LAMP assay is specific, sensitive, rapid and could be a potentially adaptable cost-effective diagnostic method for screening of intermediate hosts infected with *S. mansoni* in both individual snails and pooled samples. Moreover, the rapidity of the reactions including loop primers shows that *Biompha*-LAMP is suitable for large-scale field surveys for schistosomes control campaigns in endemic areas.

Author Contributions

Investigation: JG PFS AM.

Methodology: JG PFS JHG JLA BV AM.

Resources: BV JHG JLA.

Visualization: JG PFS AM.

Writing – original draft: JG PFS AM.

Writing – review & editing: JG PFS AM.

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3.4. Artículo 4

Field and laboratory comparative evaluation of a LAMP assay for diagnosis of urogenital schistosomiasis in Cubal, Central Angola.

Gandasegui J, Fernández-Soto P, Dacal E, Rodríguez E, Yepes E, Aznar Ruiz de Alegría ML, Espasa M, Ninda A, Bocanegra C, Salvador F, Sulleiro E, Moreno M, Muro A.

PLoS Negl Trop Dis. 2017. Submitted.

Resumen

El método de filtración de orina y el empleo de tiras reactivas para detectar la microhematuria son dos técnicas ampliamente usadas para el diagnóstico de la esquistosomosis urogenital en zonas de bajos recursos. Se sabe que estos métodos presentan una serie de limitaciones, especialmente la baja sensibilidad. En un trabajo previo, nuestro grupo diseñó un método LAMP (*loop-mediated isothermal amplification*) para el diagnóstico sensible y específico de *S. haematobium* en muestras de orina. El objetivo de este trabajo es evaluar la utilidad de nuestro método LAMP para el diagnóstico de la esquistosomosis urogenital en una zona endémica en Cubal, Angola central. Además, por primera vez, la reproducibilidad de la técnica fue evaluada en un laboratorio de referencia.

Un total de 172 muestras de orina recogidas en pacientes de edad escolar fueron analizadas para la detección de microhematuria, examen microscópico y detección molecular mediante LAMP. Las muestras sufrieron un almacenaje inadecuado en un laboratorio mal equipado. El LAMP en campo se llevó a cabo con y sin extracción previa de ADN en muestras de orina y los resultados se leyeron visualmente mediante la detección de turbidez y mediante cambio de color. Un total de 83/172 (48.30%) fueron microhematuria positivas, en 87/172 (50.60%) se detectaron huevos de *S. haematobium* mediante microscopía y 127/172 (73.83%) resultaron positivas en la detección de ADN de *S. haematobium* mediante LAMP. Además, la relación de los resultados entre LAMP y microscopía, y LAMP y microhematuria fue estadísticamente significativa (p=0.0002 y p=0.0009, respectivamente). Cuando las muestras de ADN purificado se reanalizaron en un laboratorio correctamente equipado en España y empleando la misma metodología que en el campo, la reproducibilidad general alcanzó un 72.10%.

La sencillez, versatilidad y la buena relación coste-efectividad demostrada por nuestro método LAMP cuando se aplicó en campo junto con la consistente reproducibilidad en un laboratorio de referencia, sugiere que esta técnica podría ofrecer un método de diagnóstico molecular para la esquistosomosis urogenital en zonas endémicas remotas y de bajos recursos.

PLOS Neglected Tropical Diseases

Field and laboratory comparative evaluation of a LAMP assay for diagnosis of urogenital schistosomiasis in Cubal, Central Angola.

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Abstract:	Background: Direct microscopy of filtered urine and reagent strip tests to identify microhaematuria are widely used for diagnosis of urogenital schistosomiasis in poor resource settings. These methods are known to have limitations with regard to sensitivity. In a previous work we designed a LAMP (loop-mediated isothermal amplification) assay for the sensible and specific detection of Schistosoma haematobium DNA in urine samples. This work aimed to assess the utility of our LAMP for diagnosis of urogenital schistosomiasis in an endemic area in Cubal, Central Angola. In addition, the reproducibility of the assay was evaluated in a reference laboratory for the first time. Methodology/Principal findings: A total of 172 urine samples from school age children were tested for microhaematuria, microscopic detection of S. haematobium eggs and

	molecular DNA detection by LAMP assay. Urine samples suffered from inadequate storage conditions in a poorly equipped laboratory. Field-LAMP tests were performed with and without prior DNA extraction from urine samples and the results visually detected by turbidity and also by color change. In total, 83/172 (48.30%) were positive for microhaematuria, 87/172 (50.60%) were microscopy positive for S. haematobium eggs detection and, 127/172 (73.83%) showed LAMP positive results for detecting S. haematobium-specific DNA. MacNemar's test showed a statistical significant relation between LAMP results and microscopy-detected S. haematobium infections (p-value 0.0002) and microhaematuria (p-value 0.0009), respectively. When purified DNA samples were analyzed again in a well-equipped laboratory in Spain using the same LAMP methodology, the overall reproducibility achieved 72.10%. Conclusions/Significance: The easily, cost-effectiveness and feasibility demonstrated by our LAMP assay in field conditions joined with the remarkable reproducibility in a reference laboratory, strongly suggests that our LAMP assay could provide an effective test for molecular diagnosis of urogenital schistosomiasis in disease-endemic remote areas.

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Field and laboratory comparative evaluation of a LAMP assay for diagnosis of urogenital schistosomiasis in Cubal, Central Angola.

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Abstract

Background: Direct microscopy of filtered urine and reagent strip tests to identify microhaematuria are widely used for diagnosis of urogenital schistosomiasis in poor resource settings. These methods are known to have limitations with regard to sensitivity. In a previous work we designed a LAMP (*loop-mediated isothermal amplification*) assay for the sensible and specific detection of *Schistosoma haematobium* DNA in urine samples. This work aimed to assess the utility of our LAMP for diagnosis of urogenital schistosomiasis in an endemic area in Cubal, Central Angola. In addition, the reproducibility of the assay was evaluated in a reference laboratory for the first time.

Methodology/Principal findings: A total of 172 urine samples from school age children were tested for microhaematuria, microscopic detection of *S. haematobium* eggs and molecular DNA detection by LAMP assay. Urine samples suffered from inadequate storage conditions in a poorly equipped laboratory. Field-LAMP tests were performed with and without prior DNA extraction from urine samples and the results visually detected by turbidity and also by color change. In total, 83/172 (48.30%) were positive for microhaematuria, 87/172 (50.60%) were microscopy positive for *S. haematobium* eggs detection and, 127/172 (73.83%) showed LAMP positive results for detecting *S. haematobium*-

specific DNA. MacNemar's test showed a statistical significant relation between LAMP results and microscopy-detected *S. haematobium* infections (p-value 0.0002) and microhaematuria (p-value 0.0009), respectively. When purified DNA samples were analyzed again in a well-equipped laboratory in Spain using the same LAMP methodology, the overall reproducibility achieved 72.10%.

Conclusions/Significance: The easily, cost-effectiveness and feasibility demonstrated by our LAMP assay in field conditions joined with the remarkable reproducibility in a reference laboratory, strongly suggests that our LAMP assay could provide an effective test for molecular diagnosis of urogenital schistosomiasis in disease-endemic remote areas.

Author Summary

Urogenital schistosomiasis, caused by the trematode flatworm *Schistosoma haematobium*, is the most prevalent form of schistosomiasis in humans, affecting 112 million people mainly in Africa and in the Middle East and emerging in Mediterranean Europe. Chronic infection with *S. haematobium* has been linked with carcinoma of the bladder and increased risk for HIV infection. Direct microscopy of filtered urine remains as the reference for diagnosis and reagent strip tests can also be used to identify microhaematuria in urine as a diagnostic indicator for *S. haematobium* infection. Both methods lack in sensitivity and new molecular diagnostic tools that can improve the sensitivity would be desirable. Currently, LAMP (*loop-mediated isothermal amplification*) technology shows all the characteristics required of a molecular assay with simple operation for potential use in diagnosis of infectious diseases in the field conditions. We recently described a novel LAMP assay to detect *S. haematobium* specific DNA in urine samples with no complicated requirement procedure for DNA extraction for molecular analysis. Here, we use our LAMP assay in a low-income urogenital schistomomiasis-endemic area using purified and non-purified DNA from urine samples for molecular analysis in comparison to microscopy and microhaematuria. Besides, this is the first time that the reproducibility of a LAMP assay was also evaluated in a reference laboratory to compare results. A very reasonable agreement between field-based and laboratory-based test results was obtained considering the long time spent in an inadequate storage of the urine samples under field conditions and shipping. The easily, cost-effectiveness and feasibility demonstrated by our LAMP assay in field conditions joined with the remarkable reproducibility in a reference laboratory could provide an effective molecular tool for diagnosis of urogenital schistosomiasis in disease-endemic remote areas.

Introduction

Urogenital schistosomiasis is a chronic, parasitic disease caused by the blood fluke *Schistosoma haematobium* affecting 112 million people with the highest prevalence living in sub-Saharan Africa, Middle East and emerging in Mediterranean Europe [1-3]. The most common clinical presentations are haematuria, anaemia, dysuria and genital and urinary tract lesions, but in severe cases it may also lead to kidney damage [4]. In chronic stages of the disease, the deposition of *S. haematobium* eggs eventually allowed to squamous cell carcinoma of the bladder [5]. Moreover, urogenital schistosomiasis has also been shown to increase the risk of sexually transmitted diseases, including HIV infection, particularly in female genital schistosomiasis [6,7]. In endemic countries, association between morbidity and mortality due to *S. haematobium* entails a significant barrier to economic and social development [8].

The microscopic identification of *S. haematobium* excreted ova in filtered urine samples remains the 'gold standard' method for diagnosis of urogenital schistosomiasis, mostly under field conditions [9]. However, microscopy of filtered urine lacks sensitivity, especially in low-grade infections, leading to an underestimation of the true prevalence of the disease [10]. Moreover, egg count-based criteria cannot be carried out in the acute phase of the disease since the parasites have not yet started to produce eggs. The detection of microhaematuria using reagent strips has been widely used as a rapid diagnostic indicator for *S. haematobium* infection, especially in high-transmission areas [11-13]. However, microhaematuria should not be considered as definitive diagnostic as it is a non-specific sign of urogenital schistosomiasis [4]. Antiboby-based diagnostic tests are useful tools for diagnosing urogenital schistosomiasis in several specific circumstances, but they suffer from many drawbacks and their application is very limited [14,15]. On the other hand, circulating antigen-based detection methods have been developed to diagnose active schistosomiasis with moderate to good reliability in detecting some schistosomes species, but poor accuracy for detecting *S. haematobium* [16].

Several molecular PCR-based methods have been used as alternatives to microscopy and serology for diagnosis urogenital schistosomiasis [17,18]. These methods not only require expensive automated thermal cycler and associated PCR kits but also an acceptable level of training and infrastructure, which does not exist in many low-income countries. A good alternative could be the loop-mediated isothermal amplification (LAMP) assay, a powerful nucleic acid amplification technique which combines rapidity, simplicity, and high specificity [19]. At present, LAMP technology has all the characteristics required of a real-time assay along with simple operation for potential use in the clinical diagnosis of infectious diseases, including point-of-care testing under field conditions in developing countries [22-22]. Additionally, a number of LAMP assays have already been

successfully used for detecting DNA from several schistosomes species, including *S. japonicum, S. mansoni and S. haematobium* [23-32].

The first LAMP assay for high sensitive and specific detection of *S. haematobium* in human urine samples was recently reported by our group [33]. In that work, we demonstrated that simply rapid-heating urinary pellets for good-quality DNA extraction -the Rapid-Heat LAMPellet method- was effective for use in LAMP assays with regard of detecting *S. haematobiun* in a number of patients' urine samples with proven infection with the parasite. Now, the aim of this study was to evaluate that previously described LAMP assay in field diagnosis in a low-income urogenital schistosomiasis-endemic area. In addition, this is the first time that the reproducibility of the LAMP assay was also evaluated in a reference laboratory to compare results.

Methods

Ethics statement

The study was approved by the Ethical Review Board of the Vall d'Hebron University Hospital (Barcelona, Spain) and by the local institutions. Ethical approval was also obtained from the Ethics Committee of the University of Salamanca (protocol approval no. 48531). Participation in the study was voluntary and with prior parental consent. We excluded children whose parents or legal guardians objected to their participation. All relevant authorities (village chiefs, school teachers and headmasters) were informed about the purpose and procedures of the study. Written informed consent was obtained from all parents or children guardian and all students with confirmed infection received appropriate treatment. Procedures were performed in accordance with the ethical standards laid down in the Declaration of Helsinki as revised in 2013.

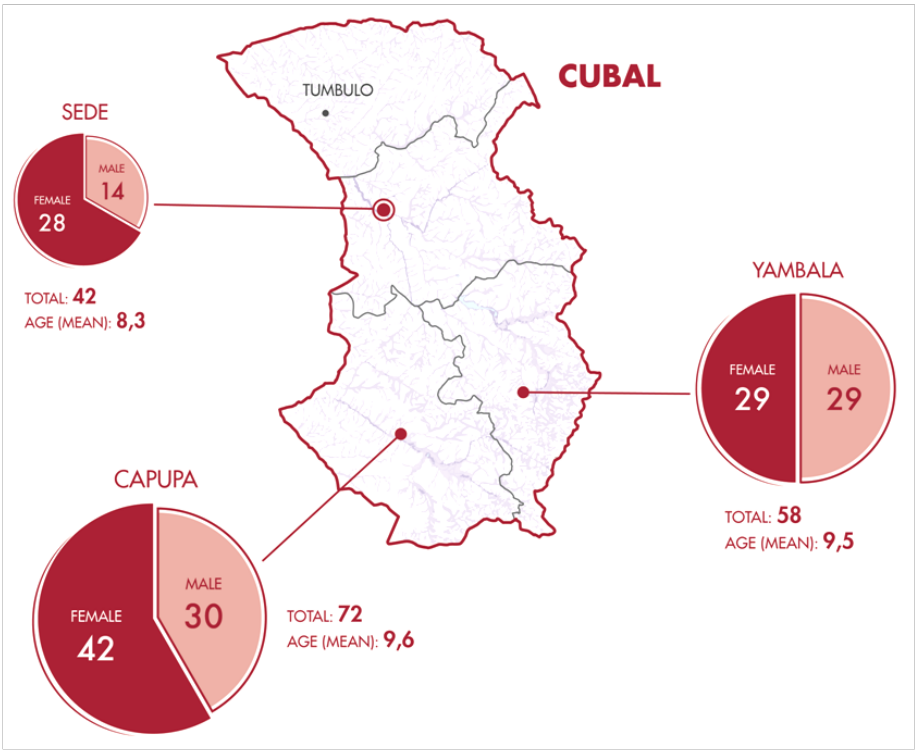
Study area, population and samples collection

The study was conducted between February and July 2015 in the city council of Cubal, Benguela Province, western-central Angola, Africa. The city council of Cubal is formed by Cubal Sede and three communities namely Yambala, Capupa and Tumbulo with an overall estimated population of 322.000 with 151.000 (47%) children under 15 [34]. The study population included a total of 252 school-age children ranging 9-10 years old recruited in 10 different schools of Cubal: 2 located in Cubal Sede, 4 in Yambala and 4 in Capupa. Scholars were randomly selected by one of consecutive pairs, in alphabetical order. Participation in the study was voluntary and with prior parental consent. Gender, age, school and community were recorded in a questionnaire.

Urine samples were requested from all children finally included in the study and collected *in situ* during daily classes between 10:00-14:00h for optimum *S. haematobium* egg passage [35]. From each participant, a single individual sample

of 15 mL was collected in a sterile container and then transported to the laboratory of the Hospital Nossa Senhora da Paz, Cubal Sede, for further processing. Unfortunately, several samples (80/252; 31.74%) were excluded from the study due to a microscopy unrealized (54), an incorrect maintenance (16) or lack of information during storing and shipping (10). Thus, a total of 172 urine samples were finally included and tested in the study. Geographical location and demographic information are summarized in Fig 1A.

A



B

	Microscopy positive; n=87	Microscopy negative; n=85	p value
Age: years, median ± SD	9.62 ± 2.3	8.90 ± 2.4	n.s.
Sex: male/female	39/48	34/51	n.s.
Microhaematuria (%)	62 (71.70)	21 (24.71)	< 0.0001

Fig 1. Map of the study area and population. (A) Geographical location and demographic information. (B) Microscopy and microhaematuria results regarding age and gender.

Urine samples processing and analysis

Microhaematuria and *S. haematobium* eggs detection. First, urine samples were screening for microhaematuria by using reagent strips (COMBI-SCREEN®, Analyticon Biotechnologies, Germany). Then, a volume of 2 mL was reserved and stored at -20ºC for further DNA extraction for molecular analyses. The remaining volume of each urine sample (13 mL, approximately) was processed using the sedimentation by gravity method [36]. Next, all sediments obtained were

examined for schistosome eggs under a microscope by qualified technicians and blinded to the microhaematuria results. No quantitative urine analysis was performed. The parasitological results were finally referred in terms of presence or absence of eggs in urine.

DNA obtaining

Aliquots of 2 mL of frozen urine samples were used for DNA obtaining for molecular analysis. After thawing, each aliquot of 2 mL was divided into two new clean microcentrifuge tubes both containing 1 mL. Then, new aliquots of 1 mL were centrifuged at 5000 rpm for 5 min at room temperature (RT) to pellet the urinary sediment. Excess supernatant was discarded but maintaining a minimal volume of 100 μL to resuspend the urinary pellet at the bottom of the tube. In this way, from each urine sample we finally obtained two aliquots of 100 μL. For each one, a different DNA extraction method was performed as follows. For one of them we used the i-genomic Urine DNA Extraction Mini Kit (Intron Biotechnology, UK) following the manufacturers' instructions. Later, 2 μL of purified DNA thus obtained were used as template for LAMP amplifications. For the other, the resuspended urinary sediment was heated at 95ºC for 15 min and then briefly spun to pellet the debris. After this, 2 μL of the supernatant were used immediately as template for each LAMP reaction without any purification procedure.

LAMP assay.

Both purified and unpurified DNA obtained from each of the two aliquots of all urine samples were analyzed by conventional LAMP assay and the "Rapid-Heat LAMPellet method", respectively, following the same protocol previously described in Gandasegui et al (2015) [33]. Briefly, the reaction mixtures (25 μL) contained 1.6 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 primers, 0.4 μM of each LB and LF primers, 1.4 mM of each dNTP, 1x Isothermal Amplification Buffer - 20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20-, 1 M betaine, 6 mM supplementary $MgSO_4$ and 8 U of *Bst* 2.0 Warm Start DNA polymerase with 2 μL of template (purified DNA or heated urine). Reaction was incubated for 60 min at 63ºC in a heating block and heated at 80ºC for 5-10 min to terminate the reaction.

When the LAMP reactions were finished, turbidity caused by the accumulation of magnesium pyrophosphate was visually inspected by naked eye after a brief centrifugation. The positive amplification results were also visually detected by adding 2 μL of 1:10 diluted 10.000X concentration SYBR® Green I dye to the reaction tubes. Green fluorescence was clearly observed in positive LAMP reactions, whereas it remained original orange in negative reactions. Testing for LAMP were blinded to both microscopy and microhaematuria results.

After competing all analysis, samples (purified DNA and crude urine) were stored at -20ºC until they were sent in October 2015 to the Center for Research in

Tropical Diseases of the University of Salamanca (CIETUS, Salamanca, Spain). Urine samples suffered from an inadequate storage conditions in a poorly equipped laboratory. The samples were kept frozen whenever possible due to periodically power outages at the Hospital Nossa Senhora da Paz. Additionally, it was not possible to keep the cold chain for shipping the samples to Spain.

Once at our reference laboratory, all 172 urine samples were reanalyzed by LAMP using the same procedures as in endemic area to assess the reproducibility of the technique. Results were also checked by turbidity, color change and, additionally, by electrophoresis to corroborate the colorimetric results.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software package (version 6, GraphPad Software, Inc., San Diego, CA, USA; https://www.graphpad.com). Standard statistical tests were used to analyze the groups, including Chi-square and Fisher's exact test (two-sided). Comparison of LAMP results with those obtained by microscopy and microhematuria were analyzed by McNemar's test for matched pairs. Comparisons were considered significant at a P value < 0.05.

Results

Microhaematuria and microscopy

Using strip tests, microscopic haematuria was detected in a total of 83/172 (48.30%) urine samples. *S. haematobium* eggs were detected in a total of 87/172 samples, resulting in an overall prevalence of infection of 50.58% with no statistically significant associations between age or gender (Fig 1B). Besides, 62 of the 87 microscopy-positive samples and 21 of the 85 microscopy-negative samples, respectively, tested positive for microhaematuria. The occurrence of microhaematuria was significantly ($p<0.0001$) associated with the presence of *S. haematobium* eggs in urine.

LAMP assay screening under field conditions

The LAMP results obtained after screening the urine samples under field conditions using both purified and non-purified DNA in comparison to microscopy results are showed in Fig 2. Using purified DNA (Fig 2.A), LAMP-positive results were visually detected by turbidity (LAMP-T+) in a total of 124/172 (72.10%) samples. The number of positive results increased to 127/172 (73.83%) after adding SYBR® Green I (LAMP-SYBR+). Using non-purified DNA as template for the Rapid-Heat LAMPellet method (Fig 2.B) we visually detected a total of 98/172 (56.97%) positive samples by turbidity (RHE-T+). In this case, the number of positive results increased to 109/172 (63.37%) after adding SYBR® Greeen I (RHE-SYBR+). Regardless of the quality of the DNA for amplification, in all samples showing a white pellet a change in color was also obtained when adding SYBR®

Green I. The color-change method offered more positive results than turbidity, especially when using non-purified DNA as template in LAMP reactions.

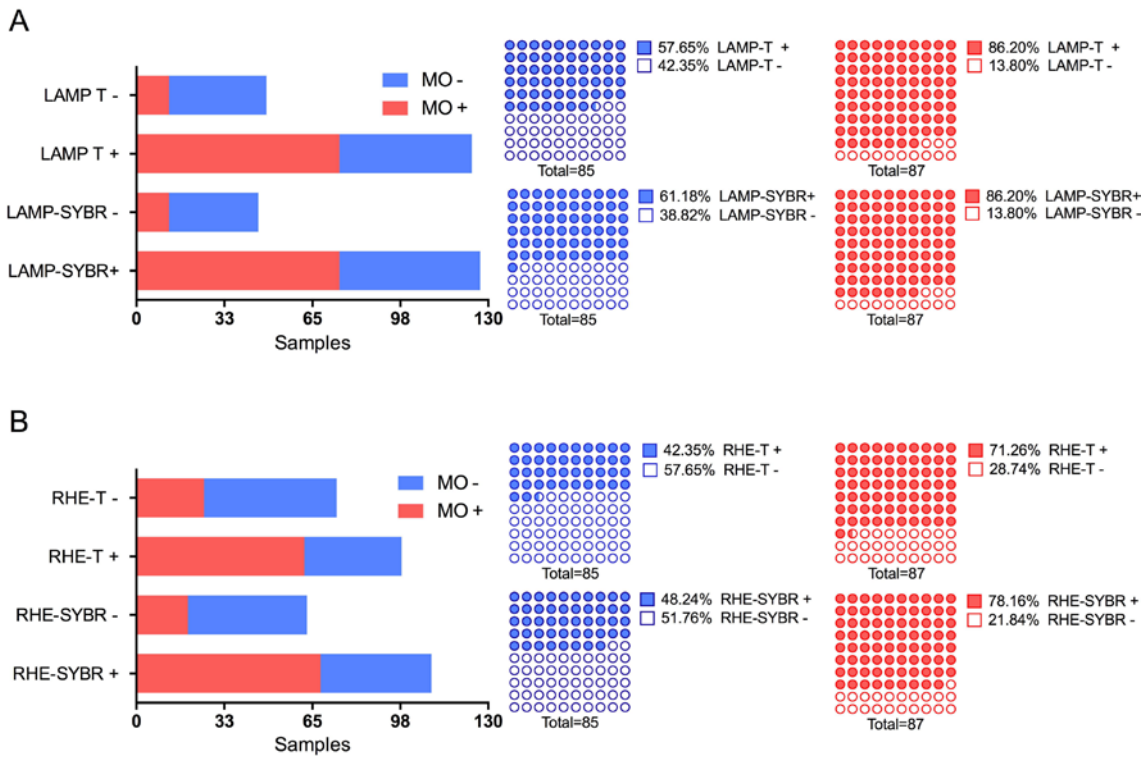


Fig 2. Results obtained by LAMP assays of urine samples under field conditions using purified and non-purified DNA in comparison to microscopy. (A) LAMP assay using purified DNA. Blue and red color show microscopy negative (MO-) and positive (MO+) samples for *S. haematobium*. Graphics bars show the number of LAMP negative (LAMP T-) and positive (LAMP T+) results obtained by turbidity and negative (LAMP-SYBR-) and positive (LAMP-SYBR+) adding SYBR® Green, respectively. Circles show the percentage of LAMP positive and negative samples in comparison to microscopy positive and negative samples. (B) LAMP assay using non-purified DNA: Rapid-Heat LAMPellet method (RHE). Blue and red color show microscopy negative (MO-) and positive (MO+) samples for *S. haematobium*. Graphics bars show the number of LAMP negative (RHE-T-) and positive (RHE-T+) results obtained by turbidity and negative (RHE-SYBR-) and positive (RHE-SYBR+) adding SYBR® Green, respectively. Circles show the percentage of LAMP positive and negative samples in comparison to microscopy positive and negative samples.

The LAMP results were compared with the urine microscopy findings as the reference standard. Of the total of 87 microscopy-positive urine samples, 75 (86.20%) resulted LAMP-positive when testing with purified DNA and revealed with SYBR® Green I (LAMP-SYBR+) and 68 (78.16%) when testing with non-purified DNA by the Rapid-Heat LAMPellet method (RHE-SYBR+). Of the total of 85 microscopy-negative urine samples, 53 (61.18%) resulted LAMP-positive when testing with purified DNA and revealed with SYBR® Green I (LAMP-SYBR+) and 41

(48.24%) when testing non-purified DNA by the Rapid-Heat LAMPellet method (RHE-SYBR+).

The LAMP results were also compared with microhaematuria tests. The overlaps of all resulting microscopy, microhaematuria and LAMP assays are shown using Venn diagrams in Fig 3. Up to 56 of the 62 urine samples with both microscopy and microhematuria positive results were LAMP-positive (Fig 3.A). It is noteworthy that up 37 urine samples with both microscopy and microhematuria negative results were also LAMP-positive. On the other hand, a total of 27 urine samples were negative for all three detection tests applied (Fig 3.B). MacNemar's test showed a statistical significant relation between LAMP results and microscopy-detected *S. haematobium* infections (p-value 0.0002) and microhematuria (p-value 0.0009), respectively.

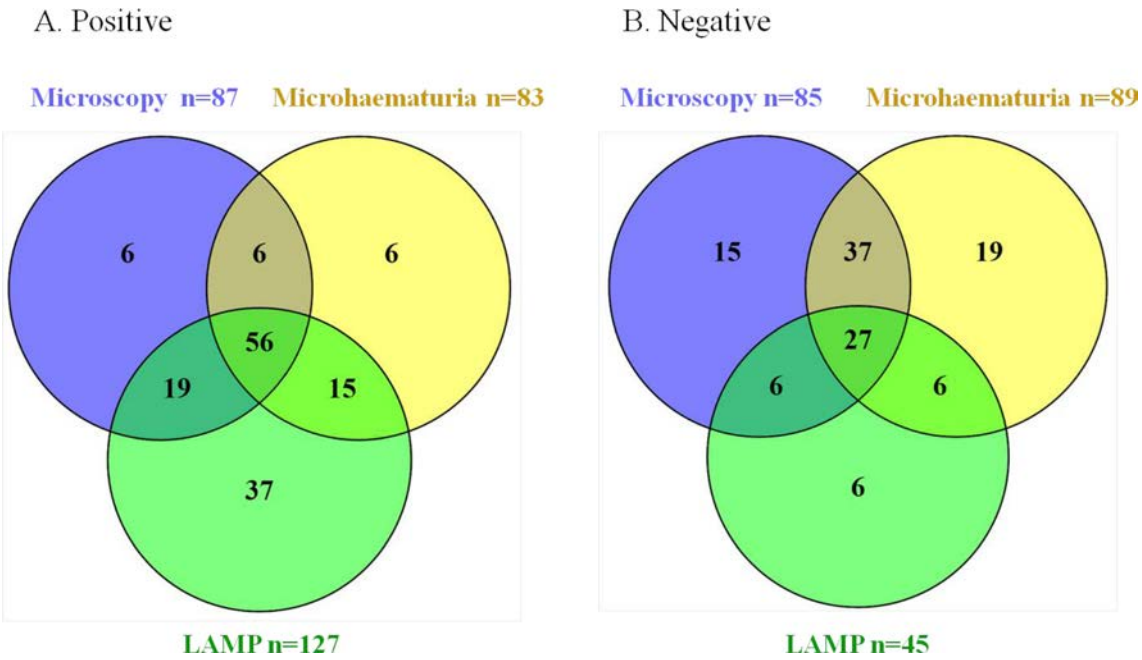


Fig 3. Venn diagram for 3-way comparison of microscopy, microhaematuria and LAMP results. (A) Venn diagrams showing positive results. (B) Venn diagrams showing negative results.

LAMP assay screening at a reference laboratory

Comparison of the LAMP results obtained at a reference laboratory with those previously obtained under field conditions is shown in Fig 4. When performing the LAMP assays in our laboratory, results were repeated in a total of 124/172 (72.09%) purified DNA samples, counting coincidences in positive (96/127; 75.59%) and negative (28/45; 62.22%) results (Fig 4A). When non-purified DNA was used as template for the Rapid Heat-LAMPellet method, results were repeated in 85/172 (49.41%), counting coincidences in positive (51/109; 46.78%) and in

negative (34/63; 53.96%) results (Fig 4B). At laboratory, in both LAMP methods, all positive results visually detected by turbidity, after adding SYBR® Green I and also by electrophoresis were coincident. As an example, Fig 5 shows the correspondence between colorimetric and agarose results in a number of samples analyzed using purified DNA in the laboratory. Regardless of the quality of the DNA for amplification, the highest number of matches when repeating the LAMP assays -counting positive and negative coincidences- occurred in those samples with a previously microscopy or microhaematuria positive result when testing under field conditions. Data comparing the LAMP assay and the Rapid-Heat LAMPellet method with microscopy and microhaematuria results are collected in S1 Table and S2 Table, respectively.

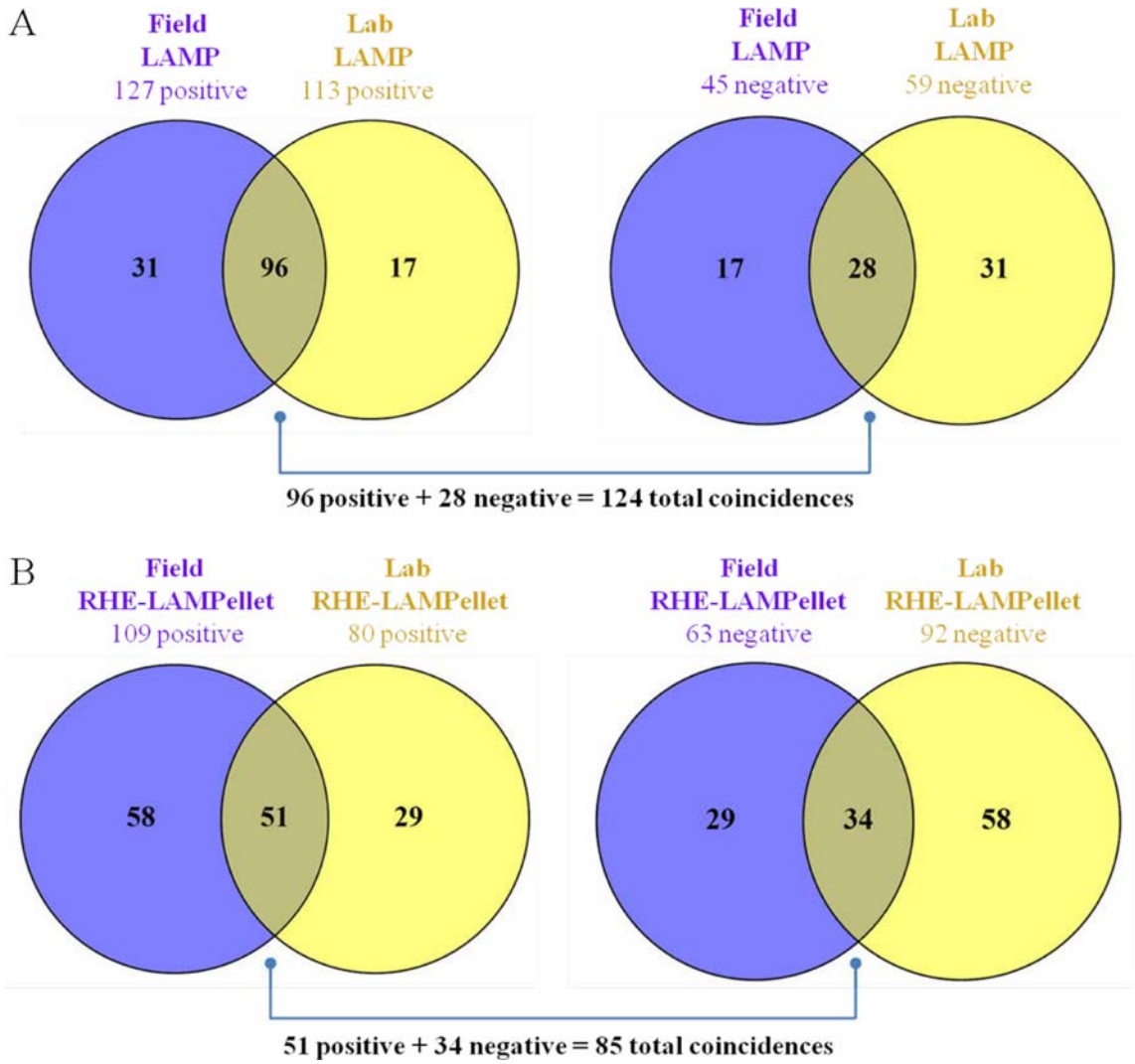


Fig 4. Comparison of the field-tests and lab-tests results obtained by LAMP and the Rapid-Heat LAMPellet method. (A) Overlaps between positive and negative LAMP results showing total coincidences. (B) Overlaps between negative positive and negative RHE-LAMPellet showing total coincidences.

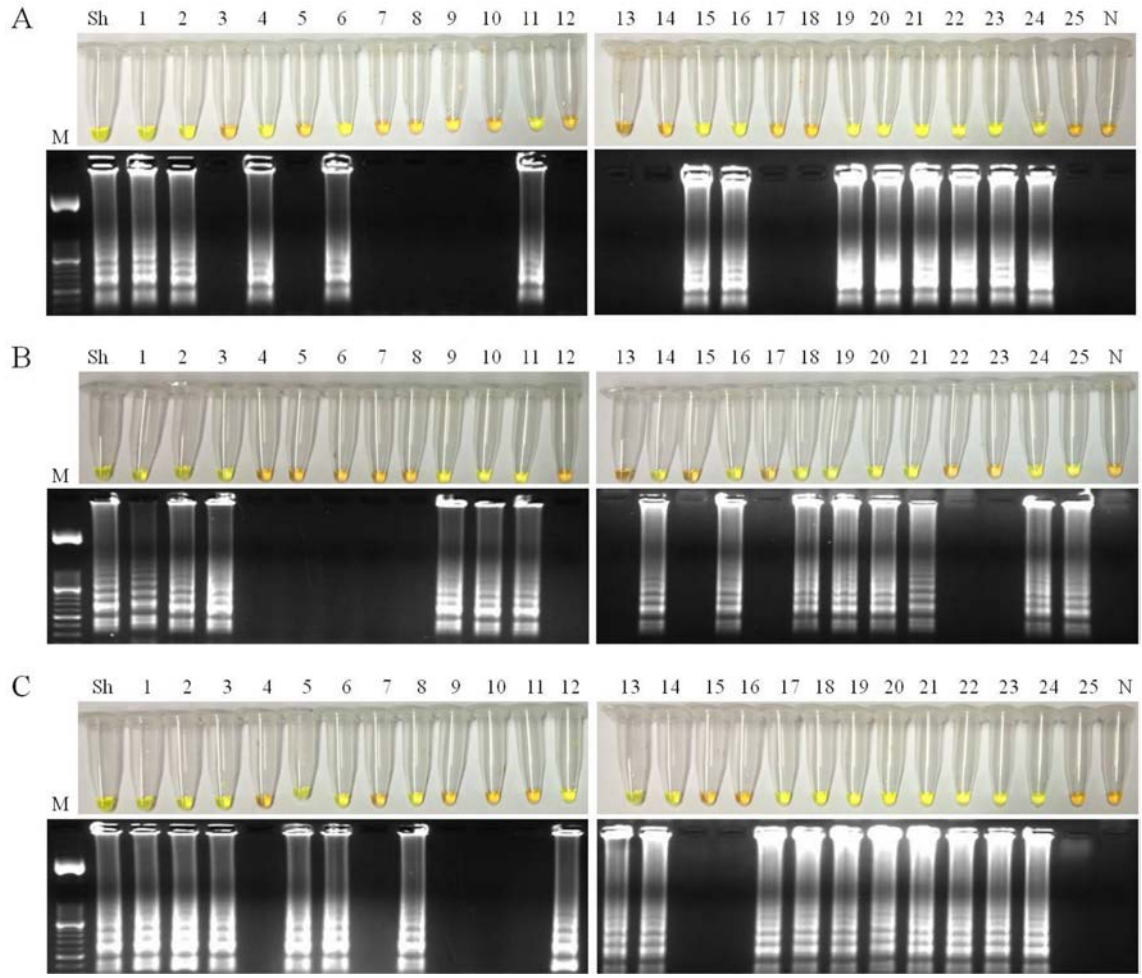


Fig 5. Examination of patients' urine samples by LAMP assay using purified DNA as template for amplification at a reference laboratory. (A) Twenty-five samples from Cubal Sede, (B) twenty-five samples from Capupa and (C) twenty-five samples from Yambala. Lanes M, molecular DNA ladder; lanes Sh, positive control (*S. haematobium* genomic DNA); lanes 1-25, patients' samples; lanes N, negativo control (water, no DNA).

Discussion

Direct microscopy of filtered urine remains as the 'gold standard' for diagnosis of urogenital schistosomiasis due to its overall accessibility and cost-effectiveness [37]. Reagent strip tests can also be used as a relatively inexpensive option to identify microhaematuria in urine as a diagnostic indicator for *S. haematobium* infection [13]. However, both methods have limitations with regard to sensitivity and the optimal diagnostic test for an urogenital schistosomiasis-endemic region will likely depend on the local intensity of infection [38]. PCR-based methods have been proven to be good tools to improve diagnostic accuracy for urogenital schistosomiasis, but hardly used for clinical diagnosis in endemic areas because of the technical limitations in low-income countries. Besides, cross-laboratory

comparability is difficult and the molecular methods are restricted to a few reference laboratories [39]. Current research is developing alternatives to enable rapid diagnosis with minimal training including new rapid antigen-based tests, microfiltration technology and DNA-based methods [40]. An enhancement in DNA-based methods is the LAMP technology, which has been advocated as a low cost genetic analysis tool for resource poor settings [41]. Therefore, in this study, with the aim of improving the molecular diagnostic testing for urogenital schistosomiasis in field conditions, we evaluated our previously described LAMP assay to detect *S. haematobium* DNA firstly in a low-income schistosomiasis-endemic area and later in a reference laboratory in order to assess the feasibility and reproducibility of the assay.

In this work, the prevalence of *S. haematobium* infection was 50.58% and 48.30% as diagnosed using direct microscopy and urine strip tests, respectively. These values resulted slightly lower than those reported in a recent study in Cubal (61% and 52%, respectively) and no statistically significant association was also found in relation to age or gender, thus supporting the idea of a high transmission for *S. haematobium* in Cubal and surroundings [42]. However, in our study, when we tested the urine samples by LAMP assay using purified DNA, the overall prevalence increased significantly reaching 73.83%. As known, diagnosis of *S. haematobium* infection using microscopy may miss cases especially during light infection [43] but molecular methods, including PCR [44, 45] and LAMP [46], have been reported to improve sensitivity in diagnosis of schistosomiasis. Our LAMP assay could detect not only DNA from *S. haematobium* eggs but also transrenal DNA from breakdown products of the parasite thus increasing sensitivity in the diagnosis. In this way, up to 23.25% more infections were detected by LAMP when comparing to microscopy (73.73% *vs.* 50.58%) in a single urine sample/subject. Additionally, it is important to note that *S. haematobium* DNA was detected by LAMP using a minimal starting processing volume of 100 μL for extraction instead 13 mL used for microscopy. Thus, LAMP assay using purified DNA obtained from urine samples appears to be much more sensitive than the urine microscopy findings used commonly as the 'gold standard' test for urogenital schistosomiasis. Moreover, the LAMP results were easily visualized by turbidity by the naked eye up to 72.10% of the samples tested. This is a great advantage in low-income areas compared to other DNA-based molecular methods.

The total prevalence initially obtained subsequently slightly decreased to 63.37% when crude urine samples were used as template in the 'Rapid-Heat LAMPellet method' for *S. haematobium* DNA amplification and color change displayed. Even so, this value was higher to that obtained by microscopy (50.58%) and also by the urine dipstick tests (48.30%). In addition to our LAMP assay for *S. haematobium*, other several studies have shown that the LAMP reaction can be performed on crude samples for direct detection of pathogens [47-49], since LAMP is more tolerant to inhibitory substances in biological samples than other DNA-based

amplification methods [50]. However, various polymerases with strand displacement activity –as the *Bst* polymerase 2.0 Warm Start used here- may be susceptible to various inhibitors in urine samples, thus slightly decreasing up 10% in sensitivity [51]. In this sense, it would be desirable to carry out additional studies using other commercially available polymerases in order to potentially increase inhibitor resistance and thus improving the sensitivity of the LAMP assay when using crude urine samples. Obviously, a higher sensitivity would be optimal for the Rapid-Heat LAMPellet method in field conditions. Notwithstanding, this molecular approach could be a very useful option in molecular diagnostic of urogenital schistosomiasis in areas where the provision of DNA extraction can be particularly expensive and impracticable. The method could be also potentially applied as a population-based estimator of urogenital schistosomiasis prevalence as well as urine dipstick test for microhaematuria [52]. In this case, the Rapid-Heat LAMPellet method would be species-specific for *S. haematobium* detection whereas microhaematuria is kwon to be a non-specific sign of urogenital schistosomiasis [4]. As noted elsewhere, with the use of more sensitive diagnostics many heme-positive egg-negative subjects in endemic areas could be detected as *S. haematobium*-infected [13].

In the reference laboratory study, we obtained 72.09% of total matches when analyzing again purified DNA by LAMP. We think that this outcome shows very reasonable agreement between field-based and laboratory-based test results considering the long time spent in an inadequate storage of the samples under field conditions. The repeated freezing and thawing as well as the lack of a cold chain for a long time for shipment to our laboratory may have decisively contributed to a potential degradation of DNA thus decreasing sensitivity in LAMP assay and, therefore, the number of matching results in amplification. This option would be more relevant in the case of comparison of using crude urine samples as template for LAMP assay since only 49.51% of coincidences were obtained at lab. It has been reported that urinary DNA deteriorates very quickly as a result of variation in urine specimen storage, handling and preservation [53-55] thus affecting to the performance of successful molecular amplification assays [56]. In this sense, most reports still use LAMP for the detection of DNA already extracted and purified from biological samples. It could be also possible that several other factors could lead to an apparent loss of LAMP efficiency, such as bacterial and/or fungal contamination during the storage of samples. Further studies are needed in order to improve the stability of urinary DNA in fresh urine sediment to be used in LAMP assays without a prior DNA extraction method. This would represent a great promise as an easy and cost-effectiveness molecular diagnostic field-test not only for urogenital schistosomiasis, but also for other neglected tropical diseases.

Finally, it is very important to note that regardless of the quality of the DNA used as template for amplification, the highest number of matches between field-LAMP test and laboratory-LAMP test occurred in those samples with a microscopy

positive finding (truly infections) and/or microhaematuria positive finding (very probable infections). This outcome reinforce the reliability of our LAMP assay for *S. haematobium* DNA detection in urine samples.

Interlaboratory comparisons are needed to determine the repeatability and reproducibility of analytical methods to be standardized [57-59]. In our study, we have compared, for the first time, the reproducibility of a LAMP assay testing the same samples in two very different settings: in a resource-poor laboratory in a schistosomiasis-endemic area and in a well-equipped modern laboratory. The easily, cost-effectiveness and feasibility demonstrated by our LAMP assay in field conditions joined with the remarkable reproducibility in a reference laboratory, strongly suggests that our LAMP assay designed for specific detection of *S. haematobium* DNA, could provide an effective test for molecular diagnosis of urogenital schistosomiasis in disease-endemic remote areas.

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S1 Table. Data comparing LAMP assay with microscopy and microhaematuria.

	LAMP +			LAMP -			
Tests	Field	Lab	Coincidences (%)	Field	Lab	Coincidences (%)	Total coincidences (%)
MO + n=87	75	68	61/75 (81.33)	12	19	5/12 (41.66)	66/87 (75.86)
MO – n=85	52	44	34/52 (65.38)	33	41	23/33 (69.69)	57/85 (67.06)
MH + n=83	71	66	62/71 (87.32)	12	17	8/12 (66.67)	70/83 (84.34)
MH – n=89	56	46	33/56 (58.93)	33	43	20/33 (60.60)	53/89 (59.55)
Total n=172	127	113	96/127 (75.59)	45	59	28/45 (62.22)	124/172 (72.09)

MO+, MO- : microscopy positive and negative
MH+, MH-: microhaematuria positive and negative

S2 Table. Data comparing Rapid-Heat LAMPellet method with microscopy and microhaematuria.

	RHE-LAMPellet +			RHE-LAMPellet -			
Tests	Field	Lab	Coincidences (%)	Field	Lab	Coincidences (%)	Total coincidences (%)
MO + n=87	68	48	37/68 (54.41)	19	39	8/19 (42.10)	45/87 (51.72)
MO – n=85	41	32	14/41 (34.14)	44	53	26/44 (59.09)	40/85 (47.05)
MH + n=83	61	48	38/61 (62.29)	22	35	12/22 (54.54)	50/83 (62.24)
MH – n=89	48	32	13/48 (27.08)	41	57	22/41 (53.65)	35/89 (39.32)
Total n=172	109	80	51/109 (46.78)	63	92	34/63 (53.96)	85/172 (49.41)

MO+, MO- : microscopy positive and negative
MH+, MH-: microhaematuria positive and negative

3.5. Artículo 5

A field survey using LAMP assay for detection of Schistosoma mansoni in a low-transmission area of schistosomiasis in Umbuzeiro, Brazil: assessment in human and snail samples.

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Resumen

La esquistosomosis es una enfermedad parasitaria de gran importancia en Brasil, siendo *Schistosoma mansoni* la única especie que afecta a humanos y *Biomphalaria straminea* uno de los hospedadores intermediarios del parásito. Se ha utilizado una *nested*-PCR basada en una secuencia mitocondrial específica de *S. mansoni* como método de referencia en estudios epidemiológicos para la identificación de caracoles infectados. Actualmente, se sabe que la amplificación de ADN mediante LAMP es mucho más eficiente que la técnica de PCR. El objetivo de este trabajo es determinar la utilidad de un método LAMP previamente descrito, SmMIT-LAMP, para la detección de *S. mansoni* en muestras humanas y de caracoles en un área de baja transmisión en el municipio de Umbuzeiro, Paraíba, en el nordeste de Brasil.

Se recogieron un total de 427 muestras de heces entre junio-julio de 2016, mostrando una prevalencia de 3.04% (13/427) mediante la técnica de Kato-Katz. También se identificaron 1175 caracoles como *Biomphalaria straminea,* recogiéndose de 14 criaderos diferentes y distribuidos en 46 muestras en forma de *pool*. El ADN de las muestras de heces y de caracoles se extrajo siguiendo el método de fenol/cloroformo. Cuando se aplicó SmMIT-LAMP, 46/162 (30.24%) muestras de heces resultaron positivas, incluyendo 12/13 (92.31%) previamente positivas por Kato-Katz y 37/149 (24.83%) previamente negativas por Kato-Katz. Mediante *nested*-PCR, únicamente 1/46 muestras de caracoles en forma de *pool* resultó positiva. Mediante SmMIT-LAMP, la misma muestra también fue positiva y otra muestra de otro criadero también resultó positiva. Los datos obtenidos en muestras humanas y de caracoles han sido utilizados para crear mapas de riesgo de esquistosomosis mediante la estimación de la densidad de kernel.

Este es el primer estudio en el que se evaluó el LAMP en muestras de heces y caracoles en un área de baja transmisión. SmMIT-LAMP es más eficiente en la detección de *S. mansoni* en comparación con Kato-Katz en heces y *nested*-PCR en caracoles. SmMIT-LAMP es una herramienta útil para identificar focos de transmisión y estimar el riesgo de infección de la esquistosomosis.

A field survey using LAMP assay for detection of *Schistosoma mansoni* in a low-transmission area of schistosomiasis in Umbuzeiro, Brazil: assessment in human and snail samples.

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Abstract

Background. In Brazil, schistosomiasis is a parasitic disease of public health relevance, mainly in poor areas where *Schistosoma mansoni* is the only human species encountered and *Biomphalaria straminea* is one of the intermediate host snails. A nested-PCR based on a specific mitochondrial *S. mansoni* minisatellite DNA region has been successfully developed and applied as a reference method in Brazil for *S. mansoni* detection, mainly in host snails for epidemiological studies. Nowadays, the amplification efficiency of LAMP is known to be higher than PCR. The present work aimed to assess the utility of our previously described SmMIT-LAMP assay for *S. mansoni* detection in human stool and snail samples in a low-transmission area of schistosomiasis in the municipality of Umbuzeiro, Paraíba State, Northeast Region of Brazil.

Methodology/Principal findings. A total of 427 human stool samples were collected during June-July 2016 in the municipality of Umbuzeiro and an overall prevalence of 3.04% (13/427) resulted positive by duplicate Kato-Katz thick smear. A total of 1175 snails identified as *Biomphalaria straminea* were collected from 14 breeding sites along the Paraíba riverbank and distributed in 46 pools. DNA from human stool samples and pooled snails was extracted using the phenol/chloroform method. When performing the SmMIT-LAMP assay a total of 49/162 (30.24%) stool samples resulted positive, including 12/13 (92.31%) previously resulting Kato-Katz positive and 37/149 (24.83%) previously Kato-Katz negative. By nested-PCR, only 1/46 pooled DNA snail samples was positive. By SmMIT-LAMP assay, the same sample also resulted positive and another one more was positive from a different breeding site. Data of human and snail surveys

were used to build risk maps of schistosomiasis incidence using kernel density analysis.

Conclusions/Significance. This is the first study in which a LAMP assay was evaluated in both human stool and snail samples from a low-transmission schistosomiasis-endemic area. Our SmMIT-LAMP proved to be much more efficient in detection of *S. mansoni* in comparison to the 'gold standard' Kato-Katz method in human stool samples and the reference molecular nested-PCR in snails. The SmMIT-LAMP has demonstrated to be a molecular useful tool to identify foci of transmission in order to build risk maps of schistosomiasis.

Introduction

Schistosomiasis has been a public health problem in Brazil for decades with 4-6 million people infected with *Schistosoma mansoni* and around 25 million at risk of infection [1]. Nineteen of the twenty-six federal states of Brazil are the most affected by the disease, especially in the northeastern region of the country. Special program for schistosomiasis control was implemented more than 40 years ago, decreasing prevalence, morbidity, and mortality over the past years [2]. Nevertheless, parasitological or immunological test are not effective for detecting *S. mansoni* infection in low prevalence areas and polymerase chain reaction (PCR)-based molecular diagnostic methods have been successfully developed and applied in endemic areas of schistosomiasis in Brazil [3-7]. However, they are not still widely used in low-income countries due to the highly technical requirements and skilled personnel making them unviable for routine application in field conditions.

Snails of genus *Biomphalaria* are best known for their role as intermediate hosts of the S*chistosoma mansoni* which are able to produce hundreds or thousands of cercariae for months. Detection of cercarial shedding by infected snails after exposure of the specimens to light has been the most traditionally method used to detect active sites for snail-to-human transmission [8]. This technique has several disadvantages: non-shedding of snail during the prepatent period, lack of experienced personnel for identification acute infection, and difficulty in differentiating the morphology of the cercariae between trematodes species. To avoid these limitations, the detection of *S. mansoni* DNA in snail has been a good option offering greater sensitivity than classical methods with the advantage of detecting parasite of pooled snail samples. Therefore, several PCR-based assays have been developed to detect snails infected with *S. mansoni* [9, 10]. One of the most used has been a nested-PCR for monitoring *S. mansoni*-infected *Biomphalaria* spp. and has been approved as reference test to identify active foci of schistosomiasis transmission in Brazil [11]. However, PCR-based techniques are difficult to be applied in endemic areas of schistosomiasis because of the highly techniques requirements and skilled personnel is required.

Loop-mediated isothermal amplification (LAMP) technology was discovered seventeen-years ago [12], being a powerful tool to apply as point-of-care testing in resource-poor settings [13]. LAMP assay has been developed for molecular detection and diagnostics of several Neglected Tropical Diseases (NTDs) and applied mainly in NTDs produced by protozoa as human African trypanosomiasis and leishmaniasis [14, 15]. Additionally, LAMP assays have already been successfully described for detecting NTDs produced by helminth parasites, including filariasis, soil-transmitted helminthiases and foodborne trematodiases [16-21]. Recently, several molecular monitoring LAMP-based assays have been also developed for the detection of schistosomes [22-26]. In a previous work, a 620 bp sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region was selected as a target for designing a LAMP-based method to detect *S. mansoni* DNA. This technique, called SmMIT-LAMP, was developed by our research group allowed us to detect *S. mansoni* DNA testing stool samples from infected mice [27].

Thus, with the aim to apply SmMIT-LAMP as a cost-effective molecular tool for the detection of *S. mansoni* in field applicable conditions, in this study we assess SmMIT-LAMP in human and snail samples collected in an endemic area of Brazil. Moreover, the results obtained by Kato-Katz analysis of human stool samples and nested-PCR performed in snails will be compared with the SmMIT-LAMP assay. It is the first time that a LAMP-based method is used to identify transmission foci and to evaluate the epidemiological risk of acquiring schistosomiasis.

Methods

Ethic statement

The study was approved by the Aggeu Magalhães Research Center Ethics Committee (protocol approval no. CAAE 56338916.6.0000.5190). Participation in the study was voluntary and prior parental consent. Participants were given detailed explanations about the aims, procedures and possible benefits of the study. Written informed consent was obtained from all subjects prior to the collection of biological samples for parasitological and molecular evaluation. Parents or guardians of children who participated in the study provided written informed consent on the child's behalf. All participants with confirmed infection received appropriate treatment. All samples were coded and treated anonymously.

Study area, population and mapping

The study was conducted during June and July 2016 in the municipality of Umbuzeiro, located in the Agreste region of Paraíba State in the Northeast Region of Brazil (Fig 1). The municipality of Umbuzeiro covers an area of 181,327 km^2 and has a population of 9,300 inhabitants (51.28 inhabitants/km^2) with 3,986 and 5,314 people living in urban and rural areas, respectively, at 2010 census [28]. This location was chosen for the study because it is a known rural area with a low-endemicity of schistosomiasis and there were no records of mass treatment of the

population within the last ten years. Moreover, this municipality is crossed by the Paraíba River, the only hydrographic basin in the region and the population has work and leisure activities centered on the river.

The locality was georeferenced by means of the global positioning system (GPS) technology, using a GPS receiver (Garmin, model eTrex) with minimum accuracy of 10 meters, configured in the Universal Transverse Mercator (UTM) projection Datum SIRGAS 2000. Using the TrackMaker Pro software, the GPS receiver data was transferred to a computer, making it possible to save files (map, case distribution, breeding sites and foci) in format that were used in the spatial data analysis, which was done by means of the ArcGis software and 10.1. The shapefiles of Brazil, Paraíba and Umbuzeiro were obtained at both open access websites IBGE (http://mapas.ibge.gov.br/bases-e-referenciais/bases-cartograficas/malhas-digitais.html) and the Digital Elevation Model satellite images from Geo Catálogo MMA (http://geocatalogo.mma.gov.br/index.jsp).

Collection of samples.

Human stool sampling and parasitological tests. A total of 427 participants from 127 households were included in the study. The average household size was 3.36 people per household. Participants, including 199 males (46.60%) and 228 females (53.39%) with a median age of 29.81 (range 1-91; SD: 20.83) were registered and recruited from door to door for the parasitological survey. Each participant was given a parasitological flask for stool collection. Samples were collected on a second visit the following morning. A single stool sample was individually obtained from each participant.

After collection, samples were transported to the Schistosomiasis Laboratory and Reference Service of CPqAM/Fiocruz for parasitological screening by Kato-Katz technique [29]. Duplicate Kato-Katz thick smear slides were prepared from each stool sample for the detection of *S. mansoni* eggs by well-trained technicians. After preparation of slides, the remaining samples were kept at -20ºC until further DNA extraction for molecular analysis as described below.

Snail sampling and processing. To determine the snail collection locations a survey along the Paraíba riverbank was carried out according to the presence of *Biomphalaria* snails and the use of the river for leisure, labor activities or crossing path as the main epidemiological criteria. *Biomphalaria* snails were *in situ* sorted out based on shell characteristics. Up to 14 snail breeding sites were located and selected for the study. In each breeding site, snails were collected using scoops and tweezers for 15 min and placed into properly labeled moistened ventilated plastic tubs for later transportation to the Schistosomiasis Laboratory and Reference Service of CPqAM/Fiocruz.

A number of specimens from each breeding site were selected at random for species confirmation using standard taxonomic identification keys [30]**.** In order to identify the snails breeding sites as potential transmission foci for schistosomiasis,

all the snails were exposed to artificial light to shed the cercariae in case they were infected [31]. Then, snails were divided into 46 batches containing a maximum number of 30 snails/pool for easy handling and processing maintaining their identification according to the different 14 breeding sites. Afterwards, snails were storage at -20ºC until further DNA extraction for molecular analysis.

DNA obtaining and molecular analysis.

DNA obtaining. Human stool samples (5-10 g/each) and pools of snails (4-30 speciments/batch) were used for DNA extraction using an adapted phenol/chloroform method [32]. Briefly, the feces or the whole snails -including the shell and the soft parts- were homogenized in 10 mL of lysis solution (10mM NaCl, 0.5% SDS, 25 mM EDTA, 10 mM Tris-HCl, pH 8.0). Following a brief centrifugation (5000 rpm), the supernatant was extracted with phenol/chloroform and precipitated with isopropanol. The pellet was resuspended in 1 mL of TE buffer. Then, 2 µL of DNA purified from stool or snail pooled samples were used for molecular analysis.

Two-step nested PCR for snails analysis. A nested PCR was performed using two pairs of primers in two sequential reactions as previously described by Melo et al., (2006) [11] with modifications. Briefly, 50 pmol of outer primers (Schfo19 and Unvfo2) were used in the first PCR, and 50 pmols of internal primers (Schfo17 and Schre16) were used in the second PCR. Two microlitres of the product of the first PCR was used as template for the second PCR. The mixtures for both PCR reactions were prepared containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP 50 pmol of each primer and 2.5 units of Taq DNA polymerase (Amersham Pharmacia Biotech, USA). In the first reaction, program was run for 30 cycles, consisting of denaturation at 92 ºC for 30 s, annealing at 65 ºC for 1 min and extension at 72 ºC for 1 min. In the second PCR, program was the same, with the exception of annealing temperature at 58 ºC. Several positive (*S. mansoni* DNA) and negative (no template) controls were included in each PCR run. PCR products (5 µL) were detected in 2% agarose gels and photographed over an UV light system.

SmMIT-LAMP for human stool samples and snails analysis

All the human stool and pooled snails samples were tested using the reaction mixture and specific primer set for LAMP assay –SmMIT-LAMP- previously established by Fernández-Soto et al., (2014) [27]. The SmMIT-LAMP method amplifies a specific sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region (GenBank Acc. No. L27240). Briefly, the reaction was carried out with a total of 25 µL reaction mixture containing 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1.4 mM of each dNTP (Intron), 1x Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), 1 M betaine (Sigma, USA), supplementary 6 mM of $MgSO_4$ (New England Biolabs, UK) and 8 U of

Bst 2.0 WarmStart DNA polymerase (New England Biolabs, UK) with 2 µL of template DNA. Reaction tubes were placed in an economic heating block at a constant temperature of 63ºC for 60-90 min and then heated at 80ºC for 5 min to stop the reaction. In all SmMIT-LAMP trials positive *(S. mansoni* DNA) and negative (water instead DNA) controls were always included.

The LAMP-positive results could be visually inspected by the naked eye by color change after adding 2 µL of 1:10 diluted 10,000x concentration fluorescent dye SYBR® Green I to the reaction tubes. Green fluorescence was clearly observed in successful LAMP reaction, whereas it remained original orange in the negative reaction. To avoid as much as possible the potential risk of cross-contamination with amplified products, all tubes were briefly centrifuged and carefully opened before adding the fluorescent dye.

Spatial data analysis

Data of human and snails surveys and results of parasitological and molecular analysis were used to build risk maps. Based on the number of snails collected in each station a thematic map demonstrating the abundance of snails in the breeding sites and foci of transmission was built. A kernel density analysis (kernel intensity estimator) was also performed to draw a risk map of schistosomiasis incidence according to the diagnostic methods used for detection. Kernel density estimation (KDE) is a statistical technique of interpolation, nonparametric method, which produces a continuous surface (cluster) of the density calculated at all locations for visual identification of hotspots without changing their local characteristics [33]. The parameters used to kernel analysis were the method data classification "equal interval" and bandwidth method defined using an adaptive beam as more appropriate for analyzing local studies [34]. The area unit was defined in m^2 and the kernel spatial resolution in 10 meters.

Statistical methods

Statistical analyses were performed using GraphPad Prism software package (version 6, GraphPad Software, Inc., San Diego, CA, USA; https://www.graphpad.com). Comparison of LAMP results with those obtained by microscopy were analyzed by McNemar's test for matched pairs. Comparisons were considered significant at a p-value < 0.05. The diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the SmMIT-LAMP and the Kato-Katz method using the MedCalc statistical program version 15.2.2 (MedCalc Software, Ostende, Belgium) according to the software instruction manual (www.medcalc.org).

Results

Parasitological analysis by Kato-Katz

A total of 13/427 (3.04%) human stool samples were positive by duplicate Kato-Katz thick smear, including samples obtained from 5 males and 8 females (median age 45; range 14-90; SD 22.76). In all Kato-Katz positive slides, the *S. mansoni* eggs count was very low, as well as the number of egg per gram of feces (EPG) with an average from 12 to 180. Up to 4/13 positive slides were negative at least in one of the two analyses (S1 Table).

Spatial distribution of parasitological positive cases is represented in Fig 1A. All Kato-Katz positive cases were detected in a zone located at the northeast of the study area (Fig 1B). In that zone, a total of 162 samples had been previously collected in the parasitological survey, counting the 13 Kato-Katz positive samples obtained and 149 Kato-Katz negative samples. These 162 samples were further subjected to molecular analysis by LAMP assay as described below.

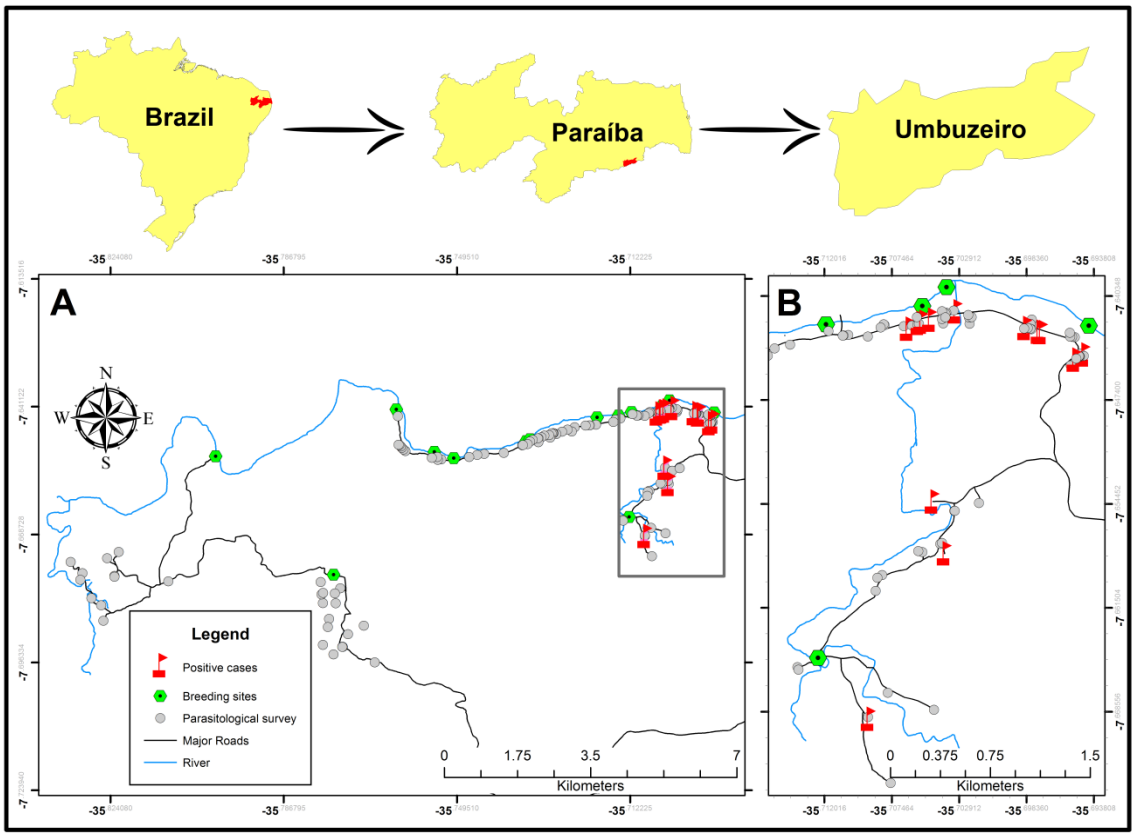

Fig 1. Map of the study area in the municipality of Umbuzeiro, located in the Agreste region of Paraíba State in the Northeast Region of Brazil. (A) Distribution of households included in parasitological survey (grey points); Kato-Katz positive samples (red flags) and breeding sites of *Biomphalaria straminea* (green points). (B) Enlargement of the area of the study where Kato-Katz positive results were obtained.

SmMIT-LAMP analysis of human stool samples

The SmMIT-LAMP results obtained after testing the stool samples in comparison to Kato-Katz results are showed in Fig 2. When performing the SmMIT-LAMP assay a total of 49/162 (30.24%) stool samples resulted positive, including up to 12 of the 13 (92.31%) previously resulting Kato-Katz positive and, additionally, 37/149 (24.83%) previously Kato-Katz negative.

MacNemar's test showed a statistical significant relation between LAMP results and microscopy-detected *S. haematobium* infections (p-value<0.0001). Considering the microscopy findings by Kato-Katz as the reference standard, the following diagnostic parameters were calculated for the SmMIT-LAMP in this study: 92.86% sensitivity (95% CI: 66013% -99.82%); 80.11% specificity (95% CI: 73.64% - 85.59%); 26.00 % positive predicted value (95% CI: 20.28% -32.67%) and 99.33% negative predicted value (95% CI: 95.75% -99.90%).

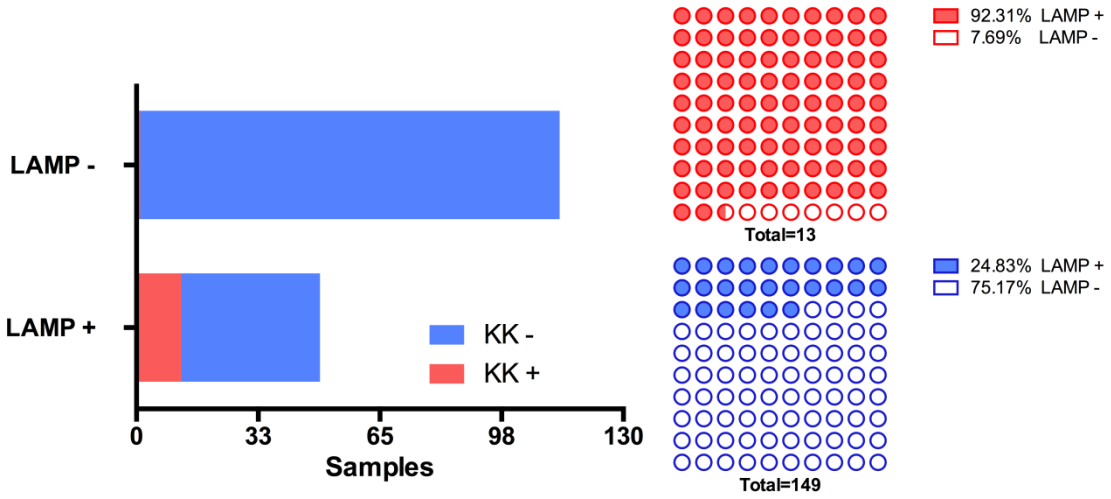

Fig 2. Results obtained by LAMP assay in comparison to Kato-Katz. Blue and red color shows negative (KK-) and positive (KK+) samples by Kato-Katz. Graphics bars show the number of LAMP positive (LAMP+) and negative (LAMP-) samples. Circles show the percentage of samples LAMP positive and negative in comparison to Kato-Katz positive and negative samples.

Snail identification, cercarial shedding, nested PCR and SmMIT-LAMP analysis.

A total of 1175 snails were collected with an average number of specimens per breeding site of 83.92 (range 4-370; SD: 109.44). All snails were identified as *Biomphalaria straminea*. None of the snails examined by exposure to artificial light for cercariae to emerge was identify as infected.

When testing by nested-PCR the 46 pooled DNA snails samples, only one pool resulted positive. By SmMIT-LAMP assay, the same pooled snails sample also resulted positive and another pool was positive from a different breeding site.

Geographical distribution of breeding sites where pooled snails samples resulted positive by molecular assays is shown in Fig 3. In these two points the abundance of snails was the largest of the survey. Additionally, the two pooled snail positive samples were located in the same area where the highest number of microscopy-positive results were previously detected. According to this, two potential foci of schistosomiasis transmission were identified in the study area.

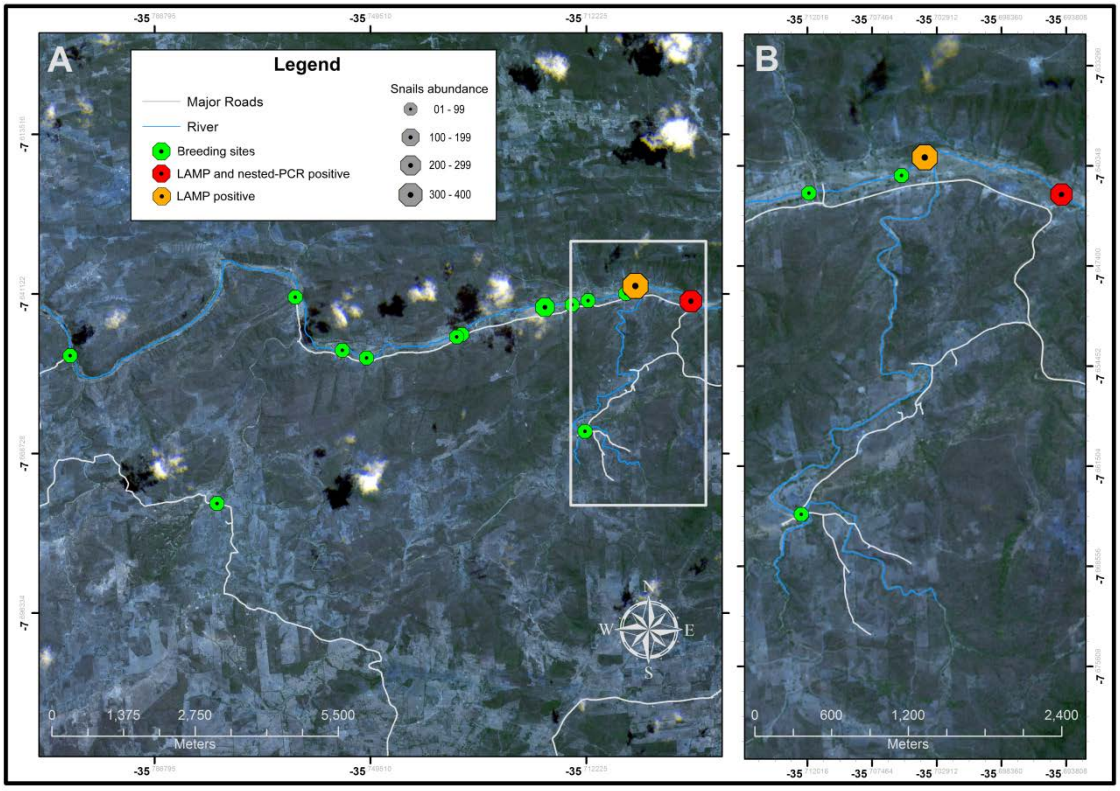

Fig 3. Geographical distribution of breeding sites showing positive pooled snails samples by molecular assays. (A) Breeding sites and identification of potential foci of transmission of *Schistosoma mansoni* using LAMP and nested-PCR (red point) and only LAMP assay (orange point). The abundance of snails is also indicated (grey points). (B) Enlargement of the area of the study where molecular assays positive results were obtained.

Risk of schistosomiasis infection

The distribution of both microscopy and SmMIT-LAMP positive cases by households included in the study and the risk maps of schistosomiasis infection generated with the kernel density method are shown in Fig 4. Only one positive result per household was detected when using the Kato-Katz technique (Fig 4A) whereas up to 5 positive results per household could be obtained when using the SmMIT-LAMP assay (Fig 4B). The potential risk of schistosomiasis transmission considering the results obtained when testing by Kato-Katz (Fig 4C) and SmMIT-LAMP (Fig 4D) is also represented. Two foci of schistosomiasis transmission were

located at the breeding sites where pooled snails samples resulted positive by molecular assays.

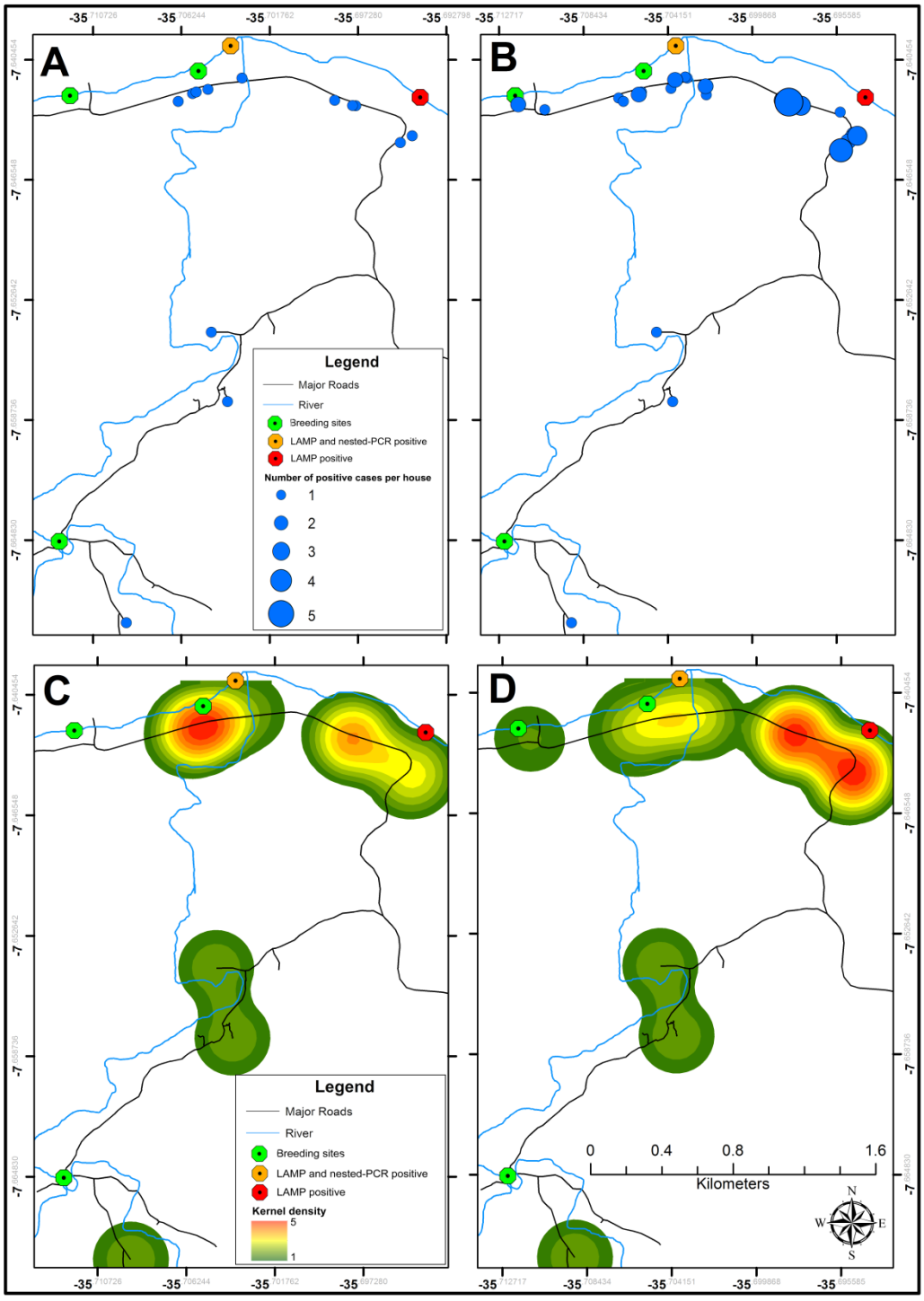

Fig 4. Risk maps of the schistosomiasis incidence according to Kato-Katz and SmMIT-LAMP results. (A) Distribution of cases by households including in the parasitological survey using Kato-Katz (B) Distribution of cases by household using LAMP assay; (C) Kernel risk map of the occurrence of cases by Kato-Katz method; (D) Kernel risk map of the occurrence of cases by the LAMP assay.

Discussion

Our study was conducted in a known low prevalence area of schistosomiasis in Brazil. Kato-Katz results obtained in the population survey corroborated previous results published from the study area [35]. Only 13 stool samples (3.04%) resulted microscopically positive including up to 10 samples with light infections (1-99 EPG) and, additionally, 4/13 positive cases presented absence of *S. mansoni* eggs at least in one of the two slides examined microscopically. These data are in line with the known low sensitivity of the Kato-Katz technique for diagnosing schistosomiasis in areas of low prevalence and parasite load [4, 36].

Molecular assays rise as a potential alternative to traditional parasitological methods in situations were highly sensitive diagnostic test are needed [37]. In this context, we tried to evaluate our SmMIT-LAMP assay in an area where the Kato-Katz previously showed a low sensitivity. When performing the SmMIT-LAMP a higher number of positive samples were detected with an overall prevalence of 30.24%. Moreover, of the Kato-Katz positive samples up to 92.31% were LAMP-positive, thus indicating a high sensitivity of the technique. Thus, our SmMIT-LAMP seems to be much more sensitive than microscopy eggs detection used commonly as the classical reference test for intestinal schistosomiasis. In addition, *S. mansoni* DNA for LAMP analysis was extracted using a cost-effective phenol/cloroform method without compromising the sensitivity of the technique. A number of LAMP assays have been previously reported for parasites detection with a minimal DNA extraction requirement, including schistosomes [38, 39]. Moreover, the SmMIT-LAMP results were easily visualized by color change by the naked eye. This is a great advantage for epidemiological surveys in low-income areas compared to other DNA-based molecular methods.

Biomphalaria straminea was the solely species identified as intermediate host in this studied area. This finding is according with a previous malacological survey in this region [40]. Among the three species of host snails for *S. mansoni* in Brazil, *B. straminea* is considered to be the most epidemiologically important species because of its wide geographic distribution [41]. Besides, *B. straminea* are naturally more resistant to *S. mansoni* infection than other snail species [42]. Thus, a number of epidemiological studies have demonstrated the utility of the nested-PCR method for *S. mansoni* detection in pooled *B. straminea* samples when the parasitological assays have not been effective [11, 40]. In our work, using this nested-PCR as reference one pooled sample of *B. straminea* was detected but, by contrast, no one was positive by classical cercarial shedding tests.

In order to test our SmMIT-LAMP in detection of *S. mansoni* in *B. straminea*, we analyzed the pooled snail samples to compare results with nested-PCR assay. The SmMIT-LAMP assay was originally designed on a sequence corresponding to a specific mitochondrial *S. mansoni* minisatellite DNA region [43]. This sequence was also previously used for designed a specific PCR-based method for detection of *S.*

mansoni with no cross-reaction with other Brazilian trematodes which have as intermediate hosts snails of genus *Biomphalaria* [10]. When applying the two molecular methods for snail samples screening two breeding sites were identified as active foci of schistosomiasis transmission including one detected by both nested-PCR and SmMIT-LAMP, and one more by SmMIT-LAMP. These two foci of transmission were located in the same zone of the study area where the Kato-Katz positive human cases were detected. In recent years, the large-scale screening by LAMP of pooled field-collected snails in transmission areas of schistosomiasis has been reported as a simple and efficient tool for snails surveillance, including *S. mansoni* in Brazil [44, 26]. In our study, the SmMIT-LAMP assay was applied for the first time to evaluate the *S. mansoni* infection not only in pooled field-collected snails but also in human stool samples.

Data obtained in SmMIT-LAMP and Kato-Katz tests were used to create Kernel density-based maps of risk of schistosomiasis. The Kernel density has previously been used to build maps of risk for several helminthiases, including schistosomiasis [45-47]. The risk areas obtained resulted to be closely located to the snail breeding sites identified as foci of schistosomiasis transmission by the SmMIT-LAMP and nested-PCR. Precisely, in those breeding sites the greatest contact between the population and the river was observed for work activities (extraction of sand from the river), domestic activities (washing clothes and dishes), and leisure activities (fishing and children's recreation). All these activities are known to be associated with transmission of schistosomiasis [48, 49]**.** In addition, this area is commonly used by the inhabitants as a route for crossing the river further increasing the risk of infection.

In summary, this is the first study in which a LAMP assay was evaluated in both human stool and snail samples from a low-transmission schistosomiasis-endemic area. Our SmMIT-LAMP proved to be much more efficient in detection of *S. mansoni* in comparison to the 'gold standard' Kato-Katz method in human stool samples and the reference molecular nested-PCR in snails. Moreover, SmMIT-LAMP has demonstrated to be a molecular useful tool to identify foci of transmission in order to build risk maps of schistosomiasis.

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S1 Table. Positive samples detected by duplicate Kato-Katz thick smear. The table shows the results obtained in the first (KKT-1) and the second (KKT-2) Kato-Katz technique and the mean of eggs per grams (EPG) of feces for each sample.

No. sample	KKT- 1	KKT-2	EPG
1	3	3	72
2	0	1	12
3	1	2	36
4	4	8	144
5	6	7	156
6	1	1	24
7	9	6	180
8	1	0	12
9	1	1	24
10	1	0	12
11	0	1	12
12	2	1	36
13	5	1	72

C.IV

Capítulo IV: Conclusiones

1. Se han desarrollado tres nuevos métodos moleculares basados en la tecnología LAMP denominados SmMIT-LAMP, Sh-LAMP y Biompha-LAMP, útiles para la detección de *S. mansoni*, *S. haematobium* y caracoles infectados con *S. mansoni.*

2. La aplicación de SmMIT-LAMP en modelo experimental murino permite detectar ADN de *S. mansoni* en la fase aguda de la infección y sirve para el diagnóstico de la esquistosomosis en áreas de baja transmisión tanto en muestras humanas como en caracoles infectados con el parásito.

3. La aplicación de Sh-LAMP es útil para la detección de *S. haematobium* en muestras de orina sin extracción de ADN en áreas endémicas de alta transmisión.

4. La aplicación de Biompha-LAMP permite identificar ADN de *S. mansoni* en caracoles infectados experimentalmente con un solo miracidio, siendo una herramienta útil para futuros estudios epidemiológicos.

5. La aplicación de SmMIT-LAMP y Sh-LAMP en condiciones de campo ha demostrado su alto rendimiento diagnóstico y su potencial aplicación como técnicas de diagnóstico *point-of-care*.

6. La comparación de la técnica LAMP en zona endémica y en laboratorio de referencia indica la reproducibilidad de los métodos y la garantía de su aplicación con éxito.

1. *Nós desenvolvemos três novos métodos moleculares baseados na tecnologia LAMP chamado SmMIT-LAMP, Sh-LAMP e Biompha-LAMP, útil para a detecção de S. mansoni, S. haematobium e caracóis infectados com S. mansoni.*

2. *A aplicação de SmMIT LAMP num modelo experimental murino detectou ADN de S. mansoni na fase aguda da infecção e foi util para o diagnóstico de esquistossomose em áreas de baixa transmissão tanto em amostras humanas e caracíos infectados com o parasita .*

3. *A aplicação de Sh-LAMP é útil para a detecção de S. haematobium em amostras de urina sem extracção de ADN em áreas endémicas de transmissão elevada.*

4. *A aplicação de Biompha-LAMP identifica ADN de S. mansoni em caracóis infectados experimentalmente infectados com um miracidio, sendo uma ferramenta útil para futuros estudos epidemiológicos.*

5. *A aplicação de SmMIT-LAMP e Sh-LAMP em condições de campo demonstrou seu alto desempenho diagnóstico e seu potencial aplicação como técnicas de diagnóstico ponto-of-care.*

6. *A comparaçao da técnica LAMP em área endêmica e laboratório de referência indica a reprodutibilidade dos métodos e garantir a sua implementação bem sucedida.*

An.

Anexo metodológico

En este apartado se describe con más detalle la metodología realizada para la tecnología LAMP.

1. Procesamiento de la muestra y obtención del ADN

En los trabajos presentados en la Tesis Doctoral se obtuvo ADN a partir de parásitos, hospedadores intermediarios (caracoles) y muestras de heces y orina procedentes de personas o de ratones infectados experimentalmente con *S. mansoni*. En la Tabla 8 se indican los principales métodos empleados en función del tipo de muestra.

Tabla 8. Principales métodos de extracción de ADN.

Tipo de muestra	Kit de extracción de ADN	Método *in house*
Parásitos	*NucleoSpin Tissue DNA extraction kit (Macherey-Nagel)*	Fenol/Cloroformo
Caracoles	*NucleoSpin Tissue DNA extraction kit (Macherey-Nagel)*	NaOH/Tris-Cl Fenol/Cloroformo
Heces	*NucleoSpin Tissue DNA extraction kit (Macherey-Nagel)*	Fenol/Cloroformo
Orina	*i-genomic Urine DNA extraction kit (iNtRON Biotechnology)*	NaOH/Tris-Cl Calentamiento rápido del *pellet* urinario

Aplicación de *NucleoSpin Tissue DNA extraction kit* en muestras de tejidos

- *Procedimiento*

1. Cortar aproximadamente 25 mg de tejido en pequeños trozos y añadir 180 µL de *buffer* T1 y 25 µL de proteinasa K previamente reconstituida. Incubar a 56ºC hasta completar la lisis (1-3 horas aproximadamente). Agitar regularmente. En el caso de resultar más cómodo, las muestras se pueden dejar incubando toda la noche.
2. Agitar las muestras y añadir 200 µL de *buffer* B3, agitar e incubar a 70ºC durante 10 minutos. Añadir 210 µL de etanol absoluto y mezclar.
3. Para cada muestra, colocar una columna de extracción en un tubo colector y cargar la muestra. Centrifugar a 11.000 rpm durante 1 minuto. Al finalizar, se descarta el tubo colector y se coloca la columna en un nuevo tubo.

4. Añadir 500 µL de *buffer* BW, centrifugar a 11.000 rpm durante 1 minuto, desechar el volumen que ha pasado a través de la columna y colocar de nuevo la columna en el tubo colector.
5. Añadir 600 µL de *buffer* B5, centrifugar a 11.000 rpm durante 1 minuto, desechar el volumen que ha pasado a través de la columna y colocar de nuevo la columna en el tubo colector.
6. Centrifugar a 11.000 rpm durante 1 minuto para secar la membrana de la columna de extracción. Colocar la columna en un tubo de 1,5 mL.
7. Añadir *buffer* B5 previamente calentado a 70ºC, incubar 3 minutos a temperatura ambiente y centrifugar a 11.000 rpm durante 1 minuto. Para eluir el ADN puede usarse volúmenes diferentes del *buffer* B5. Para obtener una concentración alta de ADN, se añaden 60 µL de *buffer* B5. Para que se produzca un elevado rendimiento, añadir 100 µL de Buffer B5, eluir y repetir el procedimiento.

Aplicación de *NucleoSpin Tissue DNA extraction kit* en muestras de heces

- *Procedimiento*

1. Tomar 250 mg de muestra, añadir 1 mL de *buffer* TE y agitar hasta conseguir resuspender la muestra por completo. Centrifugar 15 minutos a 4.000 rpm. Desechar el sobrenadante.
2. Resuspender el *pellet* en 0,2-1 mL de *buffer* T1, tomar 200 µL y llevarlos a un tubo nuevo de 1,5 mL. Añadir 25 µL de proteinasa K previamente reconstituida e incubar a 56ºC hasta completar la lisis (1-3 horas aproximadamente). En el caso de resultar más cómodo, las muestras se pueden dejar incubando toda la noche.
3. Continuar desde el paso 2 del apartado anterior (*NucleoSpin Tissue DNA extraction kit* en muestras de tejidos). En las heces, especialmente en las de ratón, muchas partículas no se digieren por competo. Es importante no coger partículas sólidas antes de transferirlo a la columna, ya que pueden bloquear el paso de los distintos *buffers* y dificultar la extracción. Si fuera necesario, se puede realizar una centrifugación corta antes de transferirlo a la columna.

Aplicación de *i-genomic Urine DNA extraction kit* en muestras de orina

- *Procedimiento*

1. Coger un volumen de orina determinado y centrifugar a 4.000 rpm durante 15 minutos. El volumen de orina inicial puede variar. El kit aconseja comenzar con el mayor volumen posible. En este trabajo usamos 2 mL y 1,5 mL. Tras la centrifugación, desechar el sobrenadante hasta dejar 100 µL del *pellet* urinario y orina. Posteriormente resuspender el *pellet*.
2. Añadir 200 µL de *buffer* UG, 20 µL de proteinasa K y 5 µL de RNasa a la muestra. Mezclar e incubar a 65 ºC durante 15 minutos. Agitar regularmente. El tiempo de incubación puede alargarse hasta los 30 minutos para conseguir una mejor lisis.
3. Tras la incubación, añadir 250 µL de *buffer* UB y mezclar invirtiendo los tubos. A continuación, añadir 250 µL de EtOH al 80 % y mezclar invirtiendo los tubos. En estos dos pasos es importante no agitar fuertemente la mezcla para conservar la integridad del ADN.
4. Transferir la mezcla a la columna de extracción y centrifugar a 13.000 rpm durante 1 minuto, desechar el tubo colector y colocar la columna en un nuevo tubo.
5. Añadir 700 µL de *buffer* UWA, centrifugar a 13.000 rpm durante 1 minuto, desechar el volumen que ha pasado a través de la columna y colocar de nuevo la columna en el tubo colector.
6. Añadir 700 µL de *buffer* UWB, centrifugar a 13.000 rpm durante 1 minuto, desechar el volumen que ha pasado a través de la columna y colocar de nuevo la columna en el tubo colector.
7. Centrifugar a 13.000 rpm durante 1 minuto para secar la membrana de la columna de extracción. Colocar la columna en un tubo de 1,5 mL.
8. Añadir 50 µL de *buffer* UE, incubar 3 minutos a temperatura ambiente y centrifugar a 13.000 rpm durante 1 minuto.

Fenol/Cloroformo

- *Procedimiento*:

1. En el caso de la muestra de heces, se toman entre 5 y 10 g de muestra y se homogeniza en la solución de lisis en un tubo de 10 mL en proporciones de 1,2 mL por cada gramo de heces. Si la extracción se está realizando desde muestras

en forma de *pool* de caracoles infectados y no infectados, se trituran todos los caracoles juntos (conchas y tejidos blandos conjuntamente) y se añade solución de lisis en las mismas proporciones que en el caso anterior. Se agita bien la muestra y se lleva a incubar a 60ºC durante 1-3 horas. Es conveniente agitar la mezcla regularmente.

2. Una vez finalizada la digestión de los tejidos, se centrifuga la mezcla a 5000 rpm durante 3-5 minutos y se pasa al sobrenadante a un nuevo tubo de 10 mL.
3. A continuación, se añade fenol en proporciones 1:1, se agita y se lleva de nuevo a centrifugar bajo las mismas condiciones que en el caso anterior. En este caso se habrán separado dos fases claramente diferenciadas; en la parte inferior del tubo estará el fenol y en la parte superior la solución con el ADN. Repetir una vez más
4. Tomamos la fase superior, la llevamos a un tubo nuevo y añadimos una mezcla al 50% de fenol y cloroformo en proporciones 1:1. Llevamos a centrifugar bajo las mismas condiciones. Repetir una vez más.
5. Tomamos la fase superior y la llevamos a un tubo nuevo. Esta vez añadimos cloroformo en proporciones 1:1 y llevamos a centrifugar. Tras esta centrifugación, tomamos de nuevo la fase superior. Repetir una vez más.
6. Llevamos el sobrenadante a un tubo nuevo y añadimos alcohol isopropílico para que se produzca la precipitación del ADN. Dejamos la mezcla en el congelador a -20ºC durante 15-30 minutos.
7. Centrifugamos a 5000 rpm durante 7 minutos, desechamos el sobrenadante y lavamos el precipitado con etanol al 70% dos veces.
8. Dejamos secar al aire y resuspendemos el ADN en 1 mL de TE a 42ºC durante 20 minutos.

NaOH/Tris-Cl

Este método de extracción de ADN es muy versátil y con ciertas modificaciones se ha podido aplicar tanto en muestras de orina como en muestras de caracoles, ya sea individualmente o en forma de *pool*.

- *Procedimiento*:

1. En el caso de la orina, se centrifugan 2 mL a 4000 rpm durante 15 minutos. Desechamos el sobrenadante hasta dejar 100 µL del *pellet* urinario con la orina.

Si se quiere extraer el ADN de caracoles individualmente, se aplastan entre dos portas y ayudándonos de una aguja se extraen los tejidos blandos y se pasan a un tubo de 1,5 mL. En el caso de un *pool* de caracoles, se colocan todos juntos en un tubo de 50 mL y se trituran las partes duras y las partes blandas conjuntamente.

2. En el caso de la orina, se añaden 100 µL una solución de NaOH 50 mM al 0,1% de SDS para favorecer la rotura de los huevos. En los caracoles individuales, se añaden 200 µL de NaOH 50 mM; y para las muestras en *pool*, 10 mL de NaOH 50 mM debido a la mayor cantidad de tejidos para ser digeridos.
3. Se incuba la mezcla a 95ºC durante 30 minutos. Es importante cerrar los tubos ayudándonos de papel de *parafilm*, ya que cuando se calientan a alta temperatura pueden abrirse por sí solos. A continuación, se centrifuga a 5000 rpm durante 5 minutos.
4. Tras la centrifugación, se toman 100 µL del sobrenadante y se neutralizan con un volumen igual de Tris-Cl 1 M (pH 8). Se añaden 2 µL directamente a la reacción.

Calentamiento rápido del sedimento urinario

En este caso, más que un método de obtención del ADN, esta técnica es básicamente un procesamiento simple de la orina antes de ser añadida a la reacción.

- *Procedimiento*

1. Se colocan 2 mL de la muestra en un tubo del volumen correspondiente y se centrifuga a 4000 rpm durante 15 minutos.
2. Se desecha el sobrenadante hasta dejar 100 µL del *pellet* urinario con la orina. Se resuspende y se lleva a incubar al termobloque a 95ºC durante 15-20 minutos.
3. Tras la incubación, se centrifuga breve a alta velocidad para precipitar las partículas sólidas.
4. Se toman 2 µL del sobrenadante y se añaden directamente a la reacción.

2. Selección de secuencias y diseño de *primers*

La selección de secuencias y diseño de *primers* para el LAMP es uno de los puntos críticos de la técnica. No sólo van a determinar la sensibilidad y la especificidad final, si no que al poseer un sistema de *primers* tan complejo, muy pocas secuencias son susceptibles de ser empleadas. Las características principales que se van a estudiar para la selección de una secuencia son:

- Grado de repetición en el genoma: esta característica es muy importante, ya que si una secuencia está altamente repetida en el genoma la probabilidad de detectarla será mucho mayor que si sólo existe una copia.
- Grado de especificidad: es necesario realizar un estudio de especificidad, valorando la reactividad cruzada con otras especies afines, tipo de hospedador donde se quiere aplicar, diseñar una técnica a nivel de género, etc.
- Grado de conservación: es importante que la secuencia no presente variaciones frecuentes de un individuo a otro, o de una cepa a otra, con el fin de que amplifique ADN del parásito en todos los casos.
- Grado de estudio de la secuencia: es mejor que sea una secuencia que se haya estudiado en profundidad y su composición nucleotídica se haya corregido varias veces.

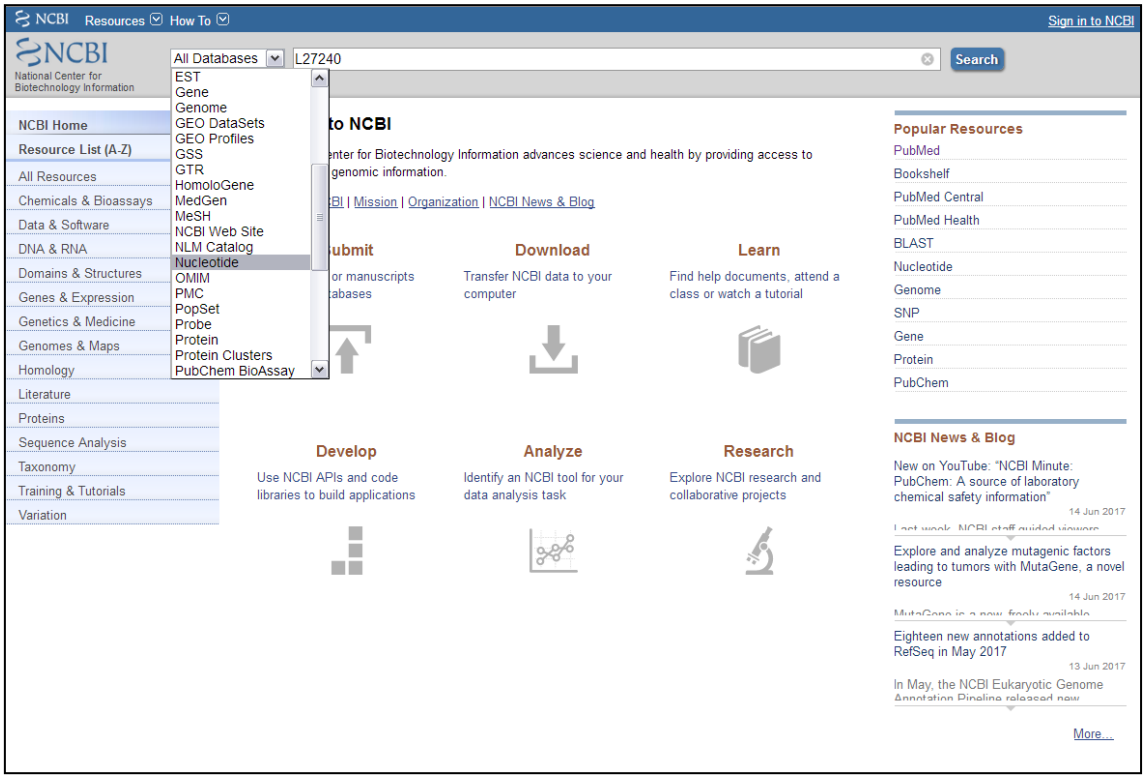
Atendiendo a estas características, se deben buscar secuencias de ADN mitocondriales, secuencias ribosomales y secuencias repetitivas. Hay una gran multitud de bases de datos con secuencias de ADN, las más utilizadas en este trabajo han sido PubMed, NCBI, Gene DB, *50 Helminth Genomes Initiative* (Welcome Trust Sanger Institute) y Ensembl.

Una vez que se ha seleccionado la secuencia, se descargará y se guardará en formato ".fasta" para que posteriormente pueda manejarse con la mayoría de los programas de edición de secuencias. En este caso, el programa empleado es BioEdit, que posee multitud de herramientas adicionales para realizar un estudio detallado de las secuencias. Para el diseño de *primers* para el LAMP, el *software* más utilizado es el que se encuentra disponible de forma gratuita en la web de Eiken Chemical Co.: PrimerExplorer. Es un *software* muy intuitivo, pero con ciertas limitaciones, como por ejemplo, que no se pueden introducir secuencias menores

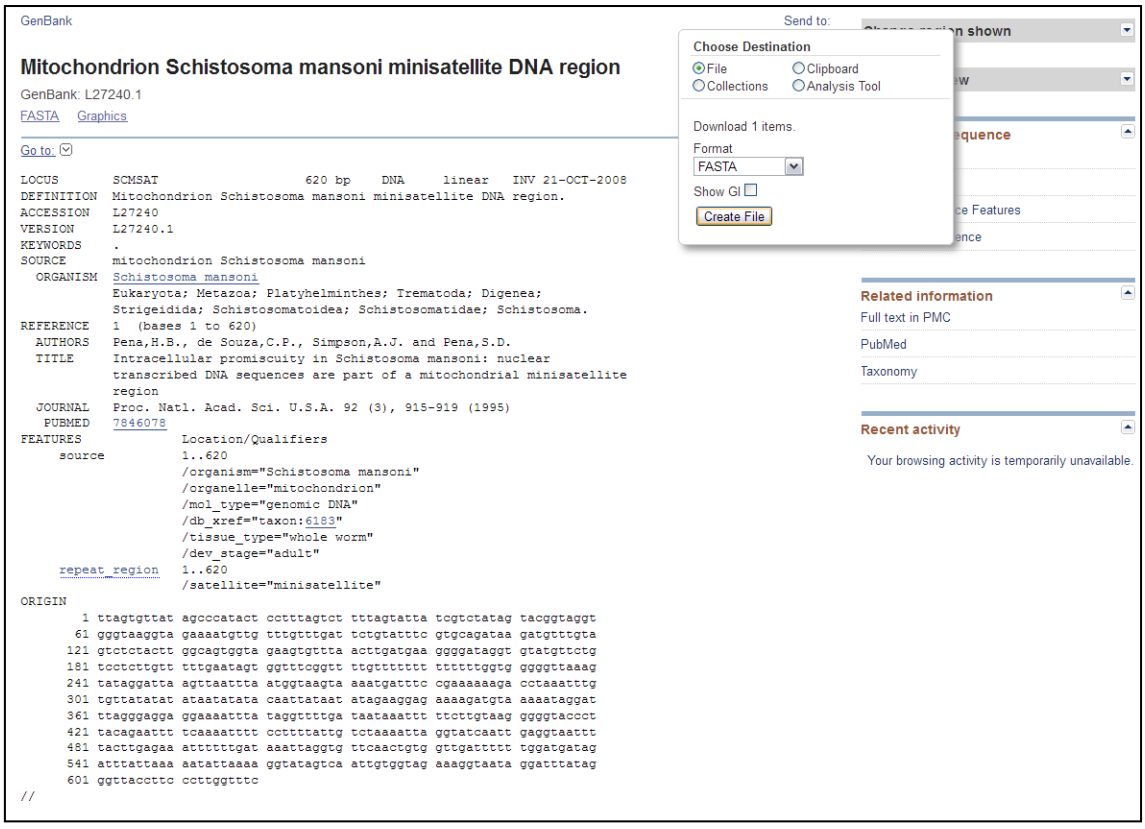
de 200 pb o mayores de 2000 pb. La selección se realizará atendiendo a los criterios presentes en el manual "*A Guide to LAMP primer designing*", disponible en la página web del *software*. Una vez seleccionados los *primers*, es recomendable volver a comprobar la especificidad, pero esta vez únicamente del amplicón.

Ejemplo gráfico: diseño de primers para la técnica SmMIT-LAMP

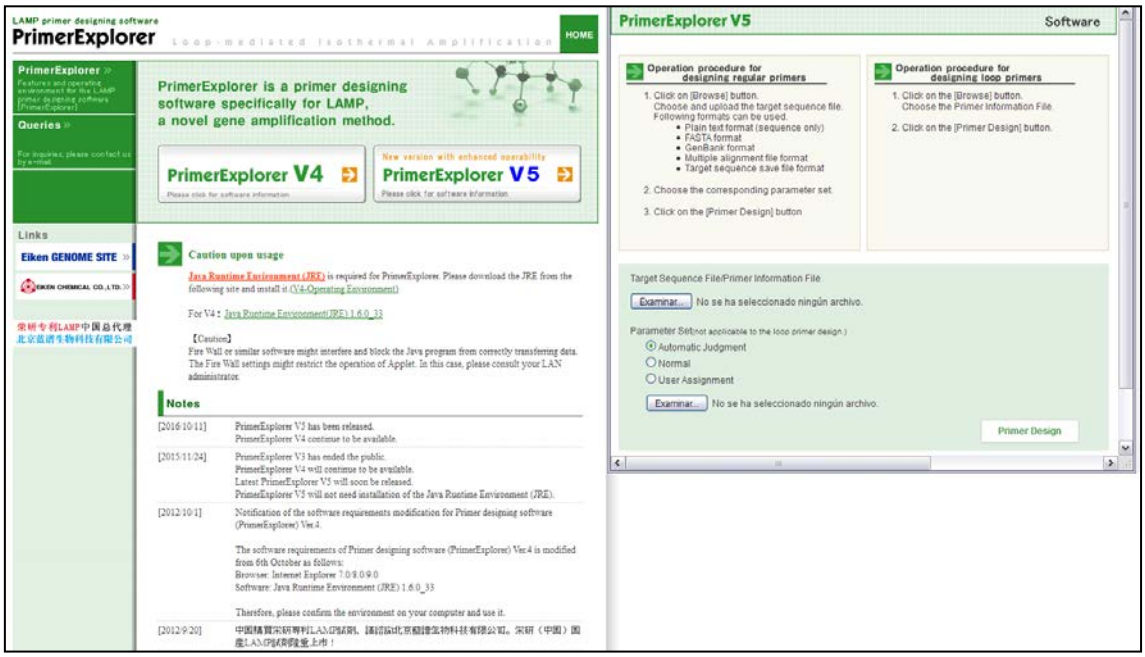
Para el diseño de *primers* de la técnica SmMIT-LAMP, lo primero que se hizo fue realizar una revisión bibliográfica en PubMed para la búsqueda de secuencias de ADN que previamente se hubiesen utilizado en técnicas moleculares de amplificación. Se encontró una secuencia correspondiente a un microsatélite mitocondrial cuyo código de GenBank era L27240. A continuación procedimos a descargar la secuencia. Como se indica en la imagen, es necesario ir a la página web de NCBI (https://www.ncbi.nlm.nih.gov/), seleccionar la base de datos Nucleotide e introducir el código de GenBank en la barra de búsqueda.



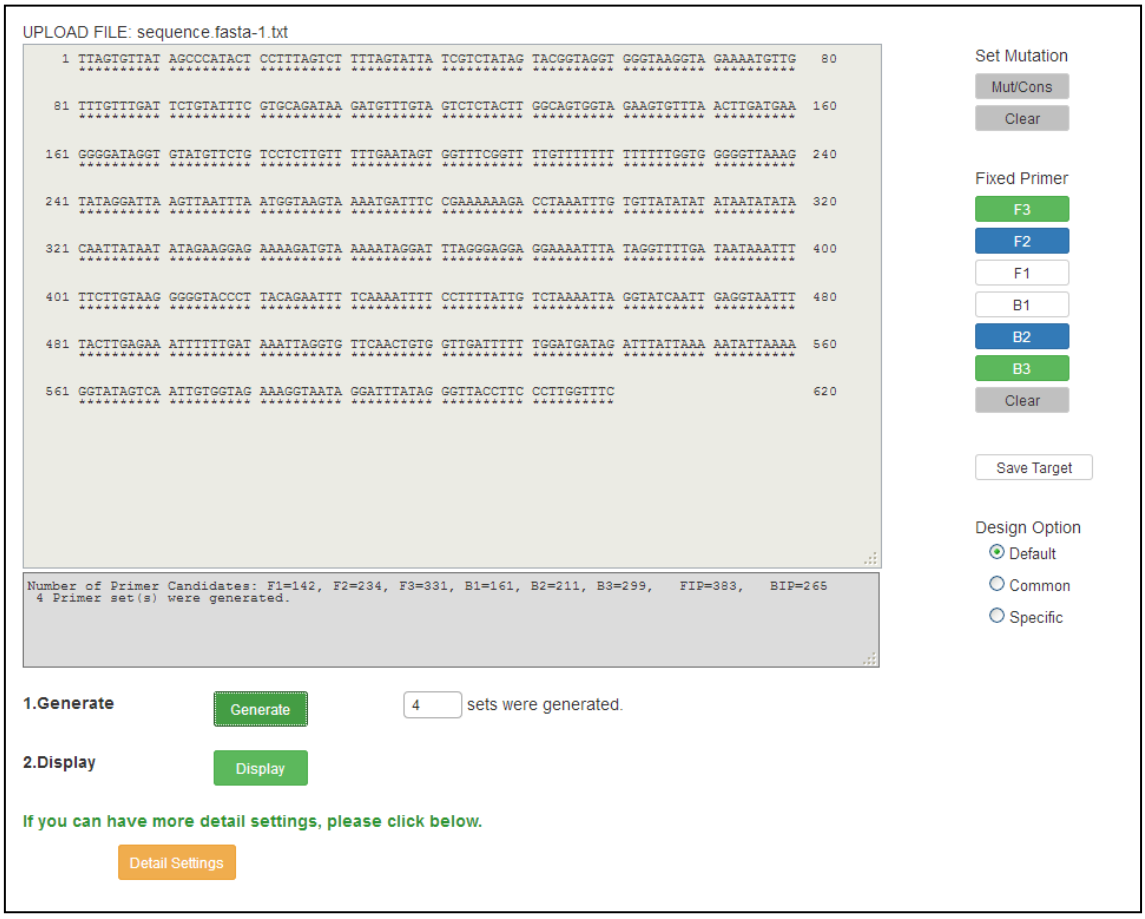
Al realizar la búsqueda, aparecerá una página con toda la información correspondiente a la secuencia (código del GenBank, publicación a la que está asociada, organismo al que pertenece, la secuencia de ADN, etc...). Para descargar la secuencia será necesario hacer clic en la palabra *send to*, y al seleccionar el formato, elegiremos *FASTA*, ya que es el formato más habitual para el manejo de secuencias.



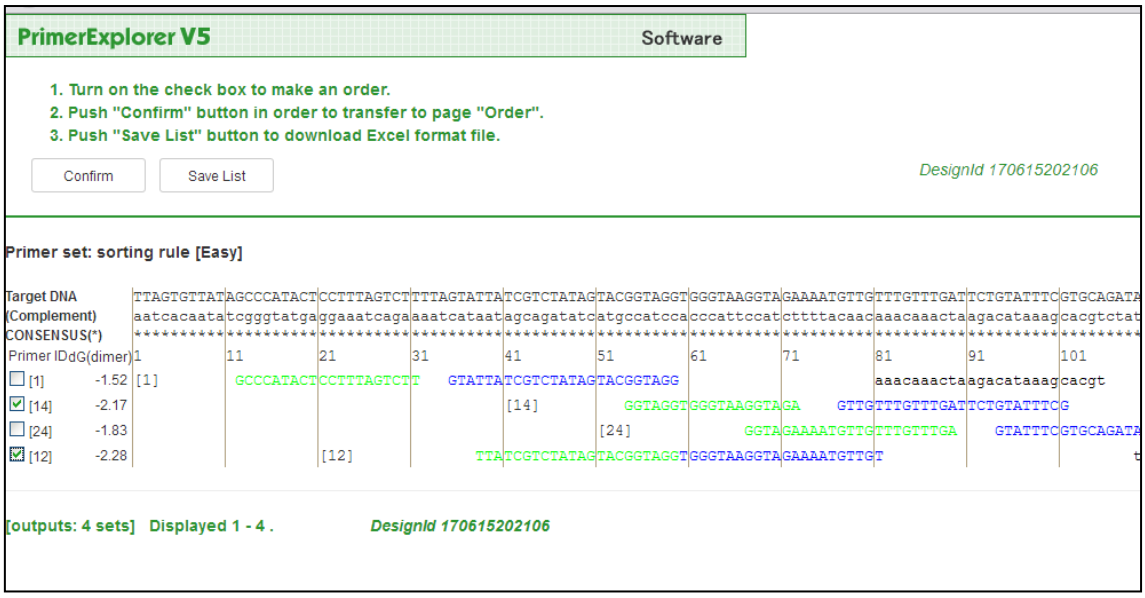
Una vez descargada, podremos visualizarla y editarla empleando el programa BioEdit. En este caso, introduciremos la secuencia directamente en el *software* PrimerExplorer V5 (https://primerexplorer.jp/e/). Haremos clic en la versión 5 y se abrirá una ventana emergente para subir nuestra secuencia al programa y comenzar el diseño y selección de los *primers*. Para ello, en *Examinar*, seleccionamos nuestra secuencia desde donde la hayamos guardado y posteriormente haremos *clic* en *Primer design.*



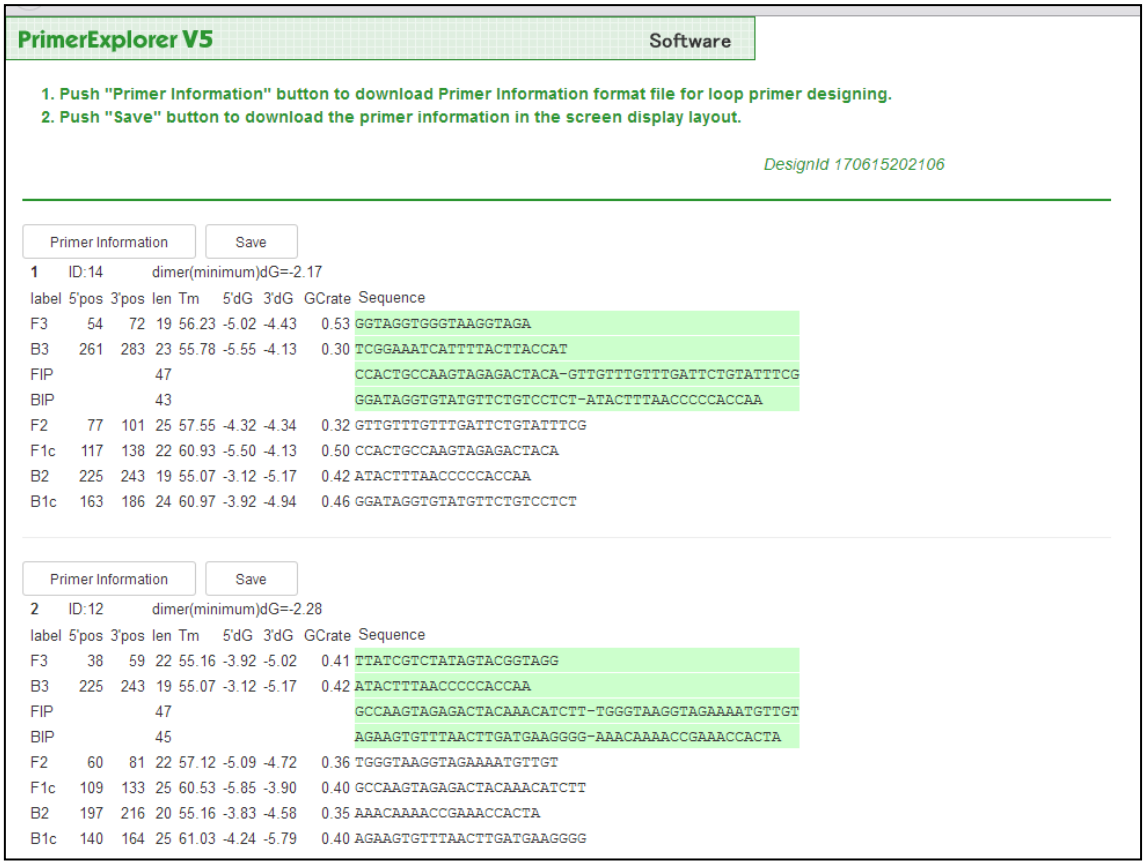
Es importante tener Java actualizado y añadir la página de PrimerExplorer V5 como excepción de seguridad, ya que si no el explorador bloqueará el programa. Una vez dentro del *software*, se pueden modificar las condiciones estándar de diseño en *Detail settings*; sin embargo, esto no es aconsejable si de forma espontánea se generan *primers*. Para ello, haremos *clic* en el botón *Generate* y el programa te dirá directamente cuántos juegos de cebadores se han diseñado. En caso afirmativo, pincharemos en el botón *Display* para ver de forma detallada los juegos de *primers*, la energía libre de Gibbs total de cada juego y donde anillan en la secuencia. En este caso se generan 4 juegos de *primers* distintos.



En la siguiente página podremos seleccionar los juegos de *primers* que nos interesen. Como norma general, desecharemos los cebadores que presenten una energía libre de Gibbs por encima de -2,00. Seleccionaremos estos *primers* y a continuación haremos *clic* en C*onfirm* para ver detalladamente las características de cada cebador.



En la siguiente página vienen detalladlas las características de los cebadores. La selección definitiva del juego de *primers* se hará atendiendo a los criterios descritos en el manual *A Guide to LAMP primer designing*, disponible en la página web del *software*.



PrimerExplorer V5 Software

1. Push "Primer Information" button to download Primer Information format file for loop primer designing.
2. Push "Save" button to download the primer information in the screen display layout.

DesignId 170615202106

Primer Information | Save

1 ID:14 dimer(minimum)dG=-2.17

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	54	72	19	56.23	-5.02	-4.43	0.53	GGTAGGTGGGTAAGGTAGA
B3	261	283	23	55.78	-5.55	-4.13	0.30	TCGGAAATCATTTTACTTACCAT
FIP			47					CCACTGCCAAGTAGAGACTACA-GTTGTTTGTTTGATTCTGTATTTCG
BIP			43					GGATAGGTGTATGTTCTGTCCTCT-ATACTTTAACCCCCACCAA
F2	77	101	25	57.55	-4.32	-4.34	0.32	GTTGTTTGTTTGATTCTGTATTTCG
F1c	117	138	22	60.93	-5.50	-4.13	0.50	CCACTGCCAAGTAGAGACTACA
B2	225	243	19	55.07	-3.12	-5.17	0.42	ATACTTTAACCCCCACCAA
B1c	163	186	24	60.97	-3.92	-4.94	0.46	GGATAGGTGTATGTTCTGTCCTCT

Primer Information | Save

2 ID:12 dimer(minimum)dG=-2.28

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	38	59	22	55.16	-3.92	-5.02	0.41	TTATCGTCTATAGTACGGTAGG
B3	225	243	19	55.07	-3.12	-5.17	0.42	ATACTTTAACCCCCACCAA
FIP			47					GCCAAGTAGAGACTACAAACATCTT-TGGGTAAGGTAGAAAATGTTGT
BIP			45					AGAAGTGTTTAACTTGATGAAGGGG-AAACAAAACCGAAACCACTA
F2	60	81	22	57.12	-5.09	-4.72	0.36	TGGGTAAGGTAGAAAATGTTGT
F1c	109	133	25	60.53	-5.85	-3.90	0.40	GCCAAGTAGAGACTACAAACATCTT
B2	197	216	20	55.16	-3.83	-4.58	0.35	AAACAAAACCGAAACCACTA
B1c	140	164	25	61.03	-4.24	-5.79	0.40	AGAAGTGTTTAACTTGATGAAGGGG

Desde esta página podremos anotar la secuencia de nucleótidos de cada cebador para posteriormente ordenar su síntesis.

3. Amplificación de ADN de *S. mansoni*: SmMIT-LAMP

Antes de comenzar a trabajar con el LAMP, es necesario limpiar con etanol al 70% todo el material que se vaya a utilizar, así como todas las áreas de trabajo. Dado que es una técnica de amplificación de ADN, es necesario trabajar en áreas totalmente separadas para cada procedimiento: la *Máster-Mix* se preparará en la cabina de flujo laminar, el ADN se añadirá en un la zona de extracción y la manipulación de los productos amplificados se realizará en la zona de amplificación.

Las características detalladas de los *primers* empleados en esta técnica se recogen en la Tabla 9.

Tabla 9. Características de los primers del método SmMIT-LAMP

dG total=-2.28								
Primer	5'p	3'p	L	Tm	5'dG	3'dG	GC (%)	Secuencia
F3	38	59	22	55.16	-3.92	-5.02	0.41	TTATCGTCTATAGTACGGTAGG
B3	225	243	19	55.07	-3.12	-5.17	0.42	ATACTTTAACCCCCACCAA
FIP			47					GCCAAGTAGAGACTACAAACATCTT-TGGGTAAGGTAGAAAATGTTGT
BIP			45					AGAAGTGTTTAACTTGATGAAGGGG-AAACAAAACCGAAACCACTA
F2	60	81	22	57.12	-5.09	-4.72	0.36	TGGGTAAGGTAGAAAATGTTGT
F1c	109	133	25	60.53	-5.85	-3.90	0.40	GCCAAGTAGAGACTACAAACATCTT
B2	197	216	20	55.16	-3.83	-4.58	0.35	AAACAAAACCGAAACCACTA
B1c	140	164	25	61.03	-4.24	-5.79	0.40	AGAAGTGTTTAACTTGATGAAGGGG

dG: energía libre de Gibbs
5'p/3'p: posición de anillamiento en el ADN diana
L: longitud
Tm: temperatura de melting
CG(%): porcentaje de G+C

- *Reactivos para realizar el método SmMIT- LAMP*

– Água ultrapura
– Betaína (5M)
– $MgSO_4$ (100mM)
– *Isothermal amplification buffer* (10x)
– dNTPs (10mM *each*/40 mM)
– Primer FIP (100pmol/µL)
– Primer BIP (100pmol/µL)

- Primer F3 (10pmol/µL)
- Primer B3 (10pmol/µL)
- *Bst* 2.0 *WarmStart* polimerasa (8U/µL)
- ADN previamente extraído
- SYBR® Green I 10.000x en DMSO (dilución 1:10)

- *Procedimiento*:

La preparación de la *Master-Mix* se realizará en la cabina de flujo laminar previamente descontaminada e irradiada con luz ultravioleta durante 15 minutos. Es importante que ningún material de trabajo se introduzca en la cabina sin ser descontaminado previamente. El material que se utiliza en la cabina es exclusivo y no puede sacarse ni emplearse para otra actividad. La fórmula para preparar la *Master-Mix* se detalla en la Tabla 10.

Tabla 10. Fórmula para preparar la Máster-Mix del método SmMIT-LAMP: reactivos y su concentración inicial, cantidad añadida y concentración final

Reactivos (Concentración inicial)	**Cantidad (µL)**	**Concentración final**
Água ultrapura	7,7	NA
Betaína (5M)	5	1 M
$MgSO_4$ (100mM)	1,5	8 mM
Buffer (10x)	2,5	1x
dNTPs (40 mM)	3,5	5,6 mM
Primer FIP (100pmol/µL)	0,4	40 pmol
Primer BIP (100pmol/µL)	0,4	40 pmol
Primer F3 (10pmol/µL)	0,5	5 pmol
Primer B3 (10pmol/µL)	0,5	5 pmol
Bst polimerasa (8U/µL)	1	8 U

NA: no aplicable

Una vez preparada la *Master-Mix*, colocaremos los tubos de 0,2 ó 0,5 mL en un soporte adecuado y refrigerado, y agregaremos 23 µL de *Master-Mix*. Se cierran los tubos y se llevan a la zona de extracción.

En la zona de extracción se procede a añadir la muestra

1. Control negativo: 2 µL de agua ultrapura
2. Muestras: 2 µL de ADN previamente extraído
3. Control positivo: 2 µL de ADN genómico

Cerramos bien todos los tubos y los llevamos al lugar donde se vaya a realizar la amplificación (termociclador, termobloque, baño de agua o estufa)

Antes de empezar a preparar la reacción es importante haber llevado a la temperatura de incubación el equipamiento que vaya a utilizarse. Incubamos los tubos a 63 ºC durante 60 min y posteriormente 5-10 minutos a 80 ºC para inactivar la enzima y finalizar la reacción. Una vez finalizada la amplificación se podrá observar la turbidez a simple vista. Con el fin de evitar contaminaciones y para ayudar a observar la turbidez, se realizará una centrifugación rápida de todos los tubos. En el caso de ser positivos, se formará un precipitado blanquecino en el fondo del tubo. Si se desea realizar la detección mediante cambio de color, se añade a cada tubo 2 µL de la dilución 1:10 de SYBR® Green I 10.000x en DMSO. Si la reacción es positiva se producirá un cambio de color de naranja a verde. Se pueden monitorizar los resultados realizando una electroforesis en gel de agarosa al 1,5% observando el típico patrón de bandas en escalera de ADN característico del LAMP.

4. Amplificación de ADN de *S. haematobium:* Sh-LAMP

El procedimiento para llevar a cabo la amplificación mediante LAMP de ADN *de S. haematobium* sigue prácticamente los mismos pasos que en el caso anterior, con alguna excepción. A continuación explicaremos las principales diferencias. Los *primers* utilizados se detallan en la Tabla 11.

Tabla 11. Características de los *primers* empleados para la amplificación de ADN de *S. haematobium*.

dG total=-2.00								
Primer	5'p	3'p	L	Tm	5'dG	3'dG	GCr(%)	Secuencia
F3	480	497	18	55.62	-3.85	-5.16	0.50	CTTTCTAAGCCCGCGATA
B3	662	679	18	56.81	-6.97	-5.25	0.50	GCGCATTACACTTGGTCT
FIP			41					TACCCCTAACTTCGTGGTCTCC-CCCCCTTATTTTAGGGTGC
BIP			48					CTCCCTATATAACATGGCGAGTAAG-ACTATGAAATCAGTGTTTTTCGG
F2	513	531	19	56.95	-6.63	-6.85	0.53	CCCCCTTATTTTAGGGTGC
F1c	555	576	22	62.77	-5.53	-5.20	0.55	TACCCCTAACTTCGTGGTCTCC
B2	638	660	23	57.04	-3.62	-5.30	0.35	ACTATGAAATCAGTGTTTTTCGG
B1c	579	603	25	60.08	-5.53	-3.57	0.44	CTCCCTATATAACATGGCGAGTAAG
LF	532	550	19	61.36	-7.18	-5.69	0.53	GGTGCGCTTTGTTTTCCGT
LB	604	625	22	63.23	-5.05	-4.18	0.45	ACCATGTGTAAAGCGCGTCAAA

dG: energía libre de Gibbs
5'p/3'p: posición de anillamiento en el ADN diana
L: longitud
Tm: temperatura de melting
CG(%): porcentaje de G+C

La fórmula de la *Master-Mix* también es diferente, ya que en este caso se emplean dos *loop-primers* para llevar a cabo la reacción (Tabla 12).

Tabla 12. Fórmula para preparar la *Master-Mix* para amplificar ADN de *S. haematobium* mediante LAMP: reactivos y su concentración inicial, cantidad añadida y concentración final.

Reactivos (Concentración inicial)	**Cantidad (µL)**	**Concentración final**
Água ultrapura	5,7	NA
Betaína (5M)	5	1 M
$MgSO_4$ (100mM)	1,5	8 mM
Buffer (10x)	2,5	1x
dNTPs (40 mM)	3,5	5,6 mM
Primer FIP (100pmol/µL)	0,4	40 pmol
Primer BIP (100pmol/µL)	0,4	40 pmol
Primer F3 (10pmol/µL)	0,5	5 pmol
Primer B3 (10pmol/µL)	0,5	5 pmol
Primer LF (10pmol/µL)	1	10 pmol
Primer LB (10pmol/µL)	1	10 pmol
Bst polimerasa (8U/µL)	1	8 U

NA: no aplicable

En este caso, la incubación de la reacción durará 50 minutos, manteniendo la temperatura a 63ºC y posteriormente 5-10 minutos a 80ºC para inactivar la enzima y finalizar la reacción.

5. Amplificación de ADN de *S. mansoni* en caracoles: Biompha-LAMP

El procedimiento para llevar a cabo la reacción, la fórmula de la *Master-Mix* y el tiempo de incubación es el mismo que en caso de la amplificación de ADN de *S. haematobium*; con la salvedad de la secuencia de los *primers* empleados (Tabla 13).

Tabla 13. Características de los *primers* empleados en el método Biompha-LAMP

dG total=-2.32								
Primer	5'p	3'p	L	Tm	5'dG	3'dG	GC(%)	Secuencia
F3	345	362	18	56.01	-2.98	-4.72	0.44	AATAGACACGCGTGTGTT
B3	551	568	18	55.08	-4.08	-4.76	0.56	GACTACACCTGGTCTCAC
FIP			50					GCAAGCGTTGATATGCATACTAGTA-TGTGTTGTTCATATATGATAGAGTC
BIP			43					ATTGTATTGTGCAGATCATACGTGA-ATCTGCGGATTACCACCT
F2	366	390	25	56.13	-4.72	-4.59	0.32	TGTGTTGTTCATATATGATAGAGTC
F1c	419	443	25	60.92	-6.25	-3.15	0.40	GCAAGCGTTGATATGCATACTAGTA
B2	528	545	18	57.02	-5.14	-5.84	0.50	ATCTGCGGATTACCACCT
B1c	477	501	25	60.63	-3.29	-5.74	0.36	ATTGTATTGTGCAGATCATACGTGA
LF	396	416	21	60.59	-4.37	-3.47	0.48	AGCATATCCACTGCGGACTAT
LB	503	524	22	60.49	-5.84	-3.24	0.45	ACCAGGTATAGTCCGCAGTTAA

dG: energía libre de Gibbs
5'p/3'p: posición de anillamiento en el ADN diana
Lon: longitud
Tm: temperatura de melting
CG(%): porcentaje de G+C

Ap.

Apéndice

1. Otros artículos de investigación

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Strong-LAMP: A LAMP Assay for *Strongyloides* spp. Detection in Stool and Urine Samples. Towards the Diagnosis of Human Strongyloidiasis Starting from a Rodent Model

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Abstract

Background

Strongyloides stercoralis, the chief causative agent of human strongyloidiasis, is a nematode globally distributed but mainly endemic in tropical and subtropical regions. Chronic infection is often clinically asymptomatic but it can result in severe hyperinfection syndrome or disseminated strongyloidiasis in immunocompromised patients. There is a great diversity of techniques used in diagnosing the disease, but definitive diagnosis is accomplished by parasitological examination of stool samples for morphological identification of parasite. Until now, no molecular method has been tested in urine samples as an alternative to stool samples for diagnosing strongyloidiasis. This study aimed to evaluate the use of a new molecular LAMP assay in a well-established Wistar rat experimental infection model using both stool and, for the first time, urine samples. The LAMP assay was also clinically evaluated in patients´ stool samples.

Methodology/Principal Findings

Stool and urine samples were obtained daily during a 28-day period from rats infected subcutaneously with different infective third-stage larvae doses of *S*. *venezuelensis*. The dynamics of parasite infection was determined by daily counting the number of eggs per gram of feces from day 1 to 28 post-infection. A set of primers for LAMP assay based on a DNA partial sequence in the 18S rRNA gene from *S*. *venezuelensis* was designed. The set up LAMP assay (namely, Strong-LAMP) allowed the sensitive detection of *S*. *venezuelensis*

2. Trabajos presentados en congresos

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Sociedad Española de Medicina Tropical y Salud Internacional

VIII CONGRESO NACIONAL DE LA SOCIEDAD ESPAÑOLA DE MEDICINA TROPICALY SALUD INTERNACIONAL (SEMTSI)

CERTIFICADO DE PARTICIPACIÓN

El Presidente del Comité Organizador del VIII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI 2013)

CERTIFICA QUE:

Javier Gandasegui, Pedro Fernández-Soto, Julio López-Abán, Juan Hernández, Belén Vicente, José Rojas-Caraballo, Cordero Miguel Cordero, Antonio Muro

han realizado la presentación de la comunicación con título:

APLICACIÓN DE LA TÉCNICA LAMP PARA LA DETECCIÓN MOLECULAR DE *Schistosoma mansoni* EN MODELO EXPERIMENTAL MURINO.

Durante la celebración del VIII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional, celebrado en Murcia del 6 al 8 de noviembre de 2013.

Y para que conste se expide el presente certificado, en Murcia, a 8 de noviembre de 2013.

Manuel Segovia Hernández

Congreso Reconocido de Interés Científico-Sanitario por la Consejería de Sanidad y Política Social de la Región de Murcia

XIX

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Pedro Fernández Soto

Ha presentado la comunicación oral:

"Diseño y puesta a punto de un método LAMP para la detección sensible y específica de Schistosoma haematobium en muestras de orina."

Gandasegui J, Fernández-Soto P, Vicente B, López-Abán J, Muro A

en el **XIX Congreso de la Sociedad Española de Parasitología y II Encuentro Internacional de Parasitólogos de España, Francia, Italia y Portugal.**

Vitoria-Gasteiz, 25 de julio de 2015

Secretaria Comité Organizador

Prof. Dra. Idoia Postigo

Sociedad Española de Parasitología

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XIX

CONGRESO DE LA SOCIEDAD ESPAÑOLA DE PARASITOLOGÍA

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VITORIA GASTEIZ!

Vitoria-Gasteiz, 23, 24 y 25 de Julio de 2015

Pedro Fernández Soto

Ha presentado la comunicación oral:

"Diagnóstico de la esquistosomosis urogenital: aproximación mediante el Rapid-Heat LAMPellet method."

Gandasegui J, Fernández-Soto P, Carranza C, Pérez-Arellano JL, Vicente B, López-Abán J, Muro A

en el **XIX Congreso de la Sociedad Española de Parasitología y II Encuentro Internacional de Parasitólogos de España, Francia, Italia y Portugal.**

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Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

CERTIFICA QUE:

C. Martín, P. Fernández-Soto, J. Gandasegui, J. Hernández, J. López-Abán, B. Vicente, A. Muro

han presentado la comunicación **Presentación Oral** con título:

VALORACIÓN DE LA TÉCNICA SM-MIT-LAMP PARA LA DETECCIÓN MOLECULAR DE SCHISTOSOMA MANSONI EN MUESTRAS DE ORINA.

en el IX Congreso Nacional de la Sociedad Española de Medicina Tropical y Salud Internacional
celebrado en Calpe (Alicante), del 22 al 24 de octubre de 2015

Y para que así conste a todos los efectos, firma la presente

CERTIFICACIÓN,

en Calpe, a 24 de octubre de 2015

Dr. Francisco Giménez Sánchez

Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

Congreso reconocido de Interés Sanitario por Subsecretaria del Ministerio de Sanidad, Servicios Sociales e Igualdad

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22-24 octubre 2015

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Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

CERTIFICA QUE:

J. Gandasegui, E. Dacal, P. Fernández-Soto, E. Rodríguez, J.M. Saugar, F. Salvador, I. Molina, B. Vicente, J. López-Abán, A. Muro

han presentado la comunicación **Presentación Oral** con título:

EVALUACIÓN PRELIMINAR DE LA TÉCNICA LAMP PARA EL DIAGNÓSTICO DE LA ESQUISTOSOMOSIS UROGENITAL EN ZONA ENDÉMICA DE ÁFRICA: APROXIMACIÓN A LAS CONDICIONES DE CAMPO EN EL MUNICIPIO DE CUBAL, ANGOLA.

en el IX Congreso Nacional de la Sociedad Española de Medicina Tropical y Salud Internacional
celebrado en Calpe (Alicante), del 22 al 24 de octubre de 2015

Y para que así conste a todos los efectos, firma la presente

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J. Gandasegui, P. Fernández-Soto, J. Hernández, J. López-Abán, B. Vicente, A. Muro

han presentado la comunicación **Presentación Oral** con título:

BIOMPHA-LAMP: UN MÉTODO LAMP PARA LA DETECCIÓN DE SCHISTOSOMA MANSONI EN CARACOLES.

en el IX Congreso Nacional de la Sociedad Española de Medicina Tropical y Salud Internacional
celebrado en Calpe (Alicante), del 22 al 24 de octubre de 2015

Y para que así conste a todos los efectos, firma la presente

CERTIFICACIÓN,

en Calpe, a 24 de octubre de 2015

Dr. Francisco Giménez Sánchez

Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

Congreso reconocido de Interés Sanitario por Subsecretaria del Ministerio de Sanidad, Servicios Sociales e Igualdad

IX CONGRESO NACIONAL DE LA SOCIEDAD ESPAÑOLA DE MEDICINA TROPICAL Y SALUD INTERNACIONAL

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semtsi

Calpe-Alicante

22-24 octubre 2015

FRANCISCO GIMÉNEZ SÁNCHEZ

Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

CERTIFICA QUE:

P. Fernández-Soto, A. Sánchez, J. Gandasegui, C. Bajo, J. López-Abán, J.M. Saugar, E. Rodríguez, B. Vicente, A. Muro

han presentado la comunicación **Presentación Oral** con título:

STRONG-LAMP: UN NUEVO MÉTODO DE DETECCIÓN MOLECULAR DE STRONGYLOIDES SPP. EN MUESTRAS DE HECES Y ORINA. AVANZANDO EN EL DIAGNÓSTICO DE LA ESTRONGILOIDOSIS HUMANA.

en el IX Congreso Nacional de la Sociedad Española de Medicina Tropical y Salud Internacional
celebrado en Calpe (Alicante), del 22 al 24 de octubre de 2015

Y para que así conste a todos los efectos, firma la presente

CERTIFICACIÓN,

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Dr. Francisco Giménez Sánchez

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Congreso reconocido de Interés Sanitario por Subsecretaria del Ministerio de Sanidad, Servicios Sociales e Igualdad

CERTIFICADO DE PARTICIPACIÓN

El Presidente del Comité Organizador del **XX Congreso SEIMC 2016**, en nombre de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica

CERTIFICA QUE:

P. Toribo Medina, K. Colmenares, B. Barriga, M.L. Aznar, G. Echaniz, A. Felipe Torres, C. Bocanegra, T. López, M. Moreno, L. Domingas, G. Estevao, E. Dacal, J. Gandasegui, C. Puig, I. Molina, M. Carvalho, C. Llor, F.M. Salvador Velez

han realizado la presentación ORAL de la comunicación con título:

"Valoración del programa de cribaje de contactos de tuberculosis activa en un área rural de Angola."

en el XX Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica, celebrado en Barcelona, del 26 al 28 de mayo de 2016.

Y para que conste se expide el presente certificado en Barcelona a 28 de mayo de 2016.

Dr. José María Miró Meda
Presidente del Comité Organizador

ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

Congress Secretariat
26th ECCMID 2016
Kenes International
Rue François-Versonnex 7
1207 Geneva, Switzerland
secretariat@eccmid.org

Scientific Secretariat
26th ECCMID 2016
c/o ESCMID Executive Office
P.O. Box 214
4010 Basel
Switzerland
eccmid@escmid.org
www.escmid.org

April, 20, 2016

To whom it may concern:

PRESENTATION CONFIRMATION AT ECCMID 2016

We hereby confirm that the following abstract has been submitted, accepted and presented at the 26th ECCMID Congress, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Amsterdam, Netherlands April 9 – 12, 2016.

Title: Outbreak of severe cases of malaria in a rural area of Angola

Abstract Authors: R. Pocinho, G. Echániz, R. Nazário Leão, E. Dacal, J. Gandasegui, N. Maliengue, M. Moreno, M. Aznar

Presenter: Rita Pocinho (Lisboa/ Portugal)

Session Title: Parasitic disease epidemiology

Presentation Type: ePoster Viewing

Presentation Number: EV0817

Yours sincerely,

Carla Seiler
Scientific Programme Coordinator

3. Estancias en el extranjero

Hospital Nossa Senhora da Paz
Missão Católica Cubal. S/n.
CP 690 Benguela
Tfne. 924506835
NIF 7111002210
hnsp.stj@gmail.com

Certificado

Mª TERESA LÓPEZ GARCIA, Directora Clinica do Hospital Nossa Senhora da Paz em Cubal, certifica que o **Sr. Javier Gandasegui Arahuetes**, esteve nesta Instituição a realizar o seu trabalho no serviço de **Laboratório** e em outros serviços, para alem de seu grande contributo na formação do pessoal de **Parasitologia** e ditos serviços com excelente, disponibilidade e aproveitamento, no período de 03 de Julho á 17 de Setembro de 2015.

Cubal, aos 16 de Setembro de 2015

Hospital Diocesano
Nossa Senhora da Paz
Missão Católica Cubal

A Directora Clinica

Mª TERESA LÓPEZ GARCÍA

Recife, 16 de setembro de 2016

DECLARAÇÃO

Declaro para os devidos fins que **Javier Gandasegui Arahuetes, passaporte nº AAJ611565,** realizou estágio doutoral no **Laboratório e Serviço de Referência em Esquistossomose,** Centro de Pesquisas Aggeu Magalhães – Fiocruz/PE, durante o período de 3 meses (segunda quinzena de Junho a primeira quinzena de Setembro/2016).

O doutorando participou do projeto de pesquisa intitulado: Avaliação do teste diagnóstico LAMP (Técnica de Amplificação Isotérmica de DNA em Alça) na detecção de infecções por *Schistosoma mansoni* em hospedeiros definitivos (homem) e intermediários (caramujos *Biomphalaria sp*). Durante sua estadia desenvolveu atividades de campo (inquérito parasitológico e malacológico) e laboratório (extração de DNA com método *in house* e utilização do LAMP em amostras oriendas de áreas endêmicas para esquistossomose).

Sem mais para o momento, coloco-me a disposição para qualquer esclarecimento.

Atenciosamente,

Elainne Christine de Souza Gomes
Pesquisadora em Saúde Pública
Mat Siape 1545194
CPqAM / Fiocruz

cism
centro de investigação em saúde de manhiça

A quem interessar

Manhiça de 15 de Maio de 2017

N/Ref.CISM/0334/05/17

Para os devidos efeitos e fins julgados convenientes se declara que JAVIER GANDASEGUI ARAHUETES Licenciado em Farmácia Barcelona, mestrado em doenças tropicais e candidato ao título de doutoramento em saúde e desenvolvimento nos trópicos, de nacionalidade Espanhola, esteve a realizar um estágio formativo de 26 de Janeiro a 10 de Maio de 2017 no âmbito do programa de colaboração entre o Instituto de Saúde Global de Barcelona (ISGLOBAL) e o Centro de Investigação em Saúde da Manhiça (CISM), Moçambique.
Como parte integrante da sua rotação, Javier teve a oportunidade de conhecer as actividades de pesquisa e de apoio a pesquisa realizadas no CISM através dos departamentos de Demografia, Laboratório, Centro de dados, Clinica, Logística, Recursos humanos e a Unidade de Formação e Comunicação (nesta unidade foi feita a apresentação geral do CISM, importância da comunicação num Centro de pesquisa e a apresentação dos diferentes programas de formação), rotação pelo Hospital Distrital Da Manhiça e pela área de Coordenação Científica.

A nível da demografia teve oportunidade de fazer visita a área de estudo junto com inquiridores e supervisores de campo para perceber como funciona o sistema de vigilância demográfica e no Laboratório teve oportunidade de conhecer de forma detalhada as unidades de Biologia molecular, hematologia, imunologia, bacteriologia, parasitologia, garantia de qualidade, armazém e laboratório auxiliar.

Sob a supervisão do Dr Helio Mucavele e de Dr Jose Munoz (ambos investigadores de CISM e ISGLOBAL respectivamente) esteve envolvido em alguns projectos tais como o da iniciativa de eliminação da Malaria no Sul de Mocambique participando nas últimas campanhas de Administraçao Massiva de Medicamento (MDA) no distrito de Magude e participação no projecto ECOHEMA (sorologia específica para a detecção do esquistosomosse para amostras colhidas numa das areas de estudo do CISM (Ilha Josina Machel); aplicação do LAMP para a detecção de *Schistosoma mansoni* e *Schistosoma haematobium* em amostras de fezes e urina colhidas e a aplicação do LAMP para a detecção de *Strongyloides stercoralis* em amostras de urina colhidas dentro da área de Manhiça sede.

Por outro lado realizou algumas formações teoricas e práticas com a duração de 4 horas cada sobre a amplificação de ADN por LAMP e facilitou uma sessao cientifica dentro das actividades do CISM.

O CISM é gerido pela fundação Manhiça entidade que tem por fim realizar e prover actividade no campo de saúde e desenvolvimento científico e tecnológico, visando atender ás necessidades do país e desenvolver a capacitação nacional nessas áreas.

Dr. Eusébio Macete
Director

Eng.ª Teresa Eduarda Machai
Responsável de Formação

CP1929 – Moçambique – Tel/Fax (+258) 21810002 – Tel. (+258) 21810181 – Cel. (+258) 823168530 – www.manhica.org

4. Premios de investigación

VNiVERSiDAD D SALAMANCA
CAMPUS DE EXCELENCIA INTERNACIONAL

800 AÑOS
1218 - 2018

FACULTAD DE FARMACIA
DECANATO

Campus Unamuno, s/n 37007.Salamanca
Tel.: +34 923 29 4522 Fax:+34 923 29 45 15
www.usal.es/farmacia dec.ffa@usal.es

Dª ANA-CELIA ALONSO GONZÁLEZ, Secretaria de la Facultad de Farmacia de la Universidad de Salamanca,

CERTIFICA QUE:

según consta en la documentación de este Centro, **D. Javier Gandasegui Arahuetes**, estudiante de Doctorado con NIF 70257266-V, recibió el "Premio de Investigación Inmaculada 2015" como primer firmante del artículo "***The Rapid-Heat LAMPellet Method: A Potential Diagnostic Method for Human Urogenital Schistosomiasis***".

Los demás firmantes del artículo son D. Pedro Fernández-Soto, Dña. Cristina Carranza-Rodríguez, D. José Luis Pérez-Arellano, Dña. Belén Vicente, D. Julio López-Abán y D. Antonio Muro.

Y para que conste a los efectos oportunos, y a petición del interesado, expido el presente certificado, con el Vº Bº del Decano, en Salamanca a catorce de marzo de dos mil dieciséis.

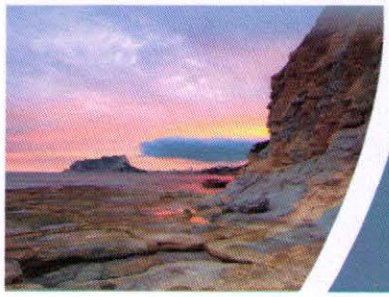
Vº Bº

EL DECANO

UNIVERSIDAD DE SALAMANCA DECANATO FACULTAD DE FARMACIA

Fdo.: Antonio Muro Álvarez

LA SECRETARIA



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Calpe-Alicante

22-24 Octubre 2015

FRANCISCO GIMÉNEZ SÁNCHEZ

Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

CERTIFICA

Que la Comunicación titulada

EVALUACIÓN PRELIMINAR DE LA TÉCNICA LAMP PARA EL DIAGNÓSTICO DE LA ESQUISTOSOMOSIS UROGENITAL EN ZONA ENDÉMICA DE ÁFRICA: APROXIMACIÓN A LAS CONDICIONES DE CAMPO EN EL MUNICIPIO DE CUBAL, ANGOLA

Gandasegui, Javier (1); Dacal, Elena (2); Fernández-Soto, Pedro (1); Rodríguez, Esperanza (2); Saugar, José María (2); Salvador, Fernando (3); Molina, Israel (3); Vicente, Belén (1); López-Abán, Julio (1); Muro, Antonio (1).

(1) CIETUS, Salamanca; (2) ISCIII, Madrid; (3) PROSICS, Barcelona.

ha recibido un premio a la mejor comunicación dentro del IX Congreso Nacional de la Sociedad Española de Medicina Tropical y Salud Internacional celebrado en Calpe (Alicante), del 22 al 24 de octubre de 2015

Y para que así conste a todos los efectos, firma la presente

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Presidente de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)

Congreso reconocido de Interés Sanitario por Subsecretaria del Ministerio de Sanidad, Servicios Sociales e Igualdad

ACADEMIA DE FARMACIA DE CASTILLA Y LEÓN

Premio LABOCAR

Accésit

RAPID-HEAT LAMPELLET METHOD: UN NUEVO MÉTODO BASADO EN LA TECNOLOGÍA DE AMPLIFICACIÓN DE ADN TIPO LAMP (LOOP-MEDIATED ISOTHERMAL AMPLIFICATION) PARA EL DIAGNÓSTICO DE LA ESQUISTOSOMOSIS UROGENITAL

Javier Gandasegui
Pedro Fernández-Soto
Cristina Carranza Rodríguez
José Luís Pérez Arellano
Belén Vicente Santiago
Julio López-Abán

Salamanca, 3 de febrero de 2017

El Presidente

La Secretaria