Tecnología LAMP para el diagnóstico molecular adaptado de enfermedades infecciosas

Tesis Doctoral

Juan García-Bernalt Diego

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Departamento de Biología Animal, Parasitología,
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Juan García-Bernalt Diego

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TESIS DOCTORAL POR COMPENDIO DE PUBLICACIONES

Se presenta este documento de Tesis Doctoral por compendio de publicaciones para optar al título de Doctor por la Universidad de Salamanca. Se aportan para su evaluación un capítulo de libro aceptado para su publicación, dos revisiones científicas publicadas y otra en proceso de publicación, cuatro artículos de investigación publicados y un modelo de utilidad. Los artículos científicos aquí recogidos han sido publicados en revistas científicas indexadas en el *Journal Citation Reports* dentro del primer (Q1) o segundo (Q2) cuartil. En conjunto, adquieren una adecuada relevancia, originalidad y excelencia, siendo el doctorando el primer autor en todos ellos.

1. Capítulo de libro:

Capítulo: 37. Nucleic acid diagnostics
Libro: Handbook of Molecular Biotechnology
Editorial: CRC Press
Año: 2023
Autores: **Juan García-Bernalt Diego**, Pedro Fernández-Soto, Antonio Muro
Afiliaciones: Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS), Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España.
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2. Primer artículo de revisión:

Título: LAMP in neglected tropical diseases: a focus on parasites.
Revista: Diagnostics (Basel)
Año: 2021
Autores: **Juan García-Bernalt Diego**, Pedro Fernández-Soto, Antonio Muro
Afiliaciones: Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación

en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS), Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España.
DOI: https://doi.org/10.3390/diagnostics11030521
Indicadores de calidad: Factor de impacto: 3,992; Q2 en Medicina, General e Interna.

3. Segundo artículo de revisión:

Título: Loop-mediated isothermal amplification in schistosomiasis
Revista: Journal of Clinical Medicine
Año: 2021
Autores: **Juan García-Bernalt Diego**, Pedro Fernández-Soto, Begoña Febrer-Sendra, Beatriz Crego-Vicente y Antonio Muro.
Afiliaciones: Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS), Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España.
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Indicadores de calidad: Factor de impacto: 4,964; Q2 en Medicina, General e Interna.

4. Tercer artículo de revisión:

Título: The future of point-of-care nucleic acid amplification diagnostics after COVID-19: time to walk the walk.
Revista: International Journal of Molecular Sciences
Año: Publicación prevista en 2022
Autores: **Juan García-Bernalt Diego**, Pedro Fernández-Soto, Antonio Muro
Afiliaciones: Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS), Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España.
Estatus: *Under review*
Indicadores de calidad: Factor de impacto: 6,208. Q1 en Bioquímica y Biología molecular; Q2 en Química, Multidisciplinar.

5. Primer artículo de investigación

Título: Progress in loop-mediated isothermal amplification assay for detection of *Schistosoma mansoni* DNA: towards a ready-to-use test.
Revista: Scientific Reports
Año: 2019
Autores: **Juan García-Bernalt Diego**, Pedro Fernández-Soto, Beatriz Crego-Vicente, Sergio Alonso-Castrillejo, Begoña Febrer-Sendra, Ana Gómez-Sánchez, Belén Vicente, Julio López-Abán y Antonio Muro.
Afiliaciones: Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS), Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España.
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Indicadores de calidad: Factor de impacto: 3,998. Q1 en Ciencias multidisciplinares.

6. Modelo de utilidad

Título: Dispositivo de diagnóstico de enfermedades infecciosas
Identificador: ES 1 259 239 U
Año: 2020
Inventores: Antonio Muro Álvarez, Pedro Fernández-Soto, Julio López-Abán, Belén Vicente Santiago, Moncef Belhassen García, **Juan García-Bernalt Diego**, Juan Manuel Corchado Rodríguez, Roberto Casado Vara, Fernando Prieta Pintado y Sara Rodríguez González.
Entidad emisora: Oficina Española de Patentes y Marcas

7. Segundo artículo de investigación

Título: A Simple, Affordable, Rapid, Stabilized, Colorimetric, Versatile RT-LAMP Assay to Detect SARS-CoV-2
Revista: Diagnostics (Basel)
Año: 2021
Autores: **Juan García-Bernalt Diego**[1], Pedro Fernández-Soto[1], Marta Domínguez-Gil[2], Moncef Belhassen-García[1,3], Juan Luis Muñoz Bellido[4], Antonio Muro[1].
Afiliaciones: [1]Grupo de Investigación en Enfermedades Infecciosas y Tropicales (e-INTRO), Instituto de Investigación Biomédica de Salamanca–Centro de investigación en Enfermedades Tropicales de la Universidad de Salamanca (IBSAL–CIETUS),

Facultad de Farmacia, Universidad de Salamanca, 37007, Salamanca, España. [2]Servicio de microbiología, Hospital universitario Río Hortega, 47012, Valladolid, España. [3]Servicio de medicina interna, sección de enfermedades infecciosas, Complejo Asistencial Universitario de Salamanca, Universidad de Salamanca, 37007, Salamanca, España. [4]Servicio de microbiología y parasitología, Complejo Asistencial Universitario de Salamanca, Universidad de Salamanca, 37007, Salamanca, España.

DOI: https://doi.org/10.3390/diagnostics11030438

Indicadores de calidad: Factor de impacto: 3,992; Q2 en Medicina, General e Interna.

8. Tercer artículo de investigación

Título: Detection of SARS-CoV-2 RNA in Urine by RT-LAMP: A Very Rare Finding.

Revista: Journal of Clinical Medicine

Año: 2022

Autores: **Juan García-Bernalt Diego[1]**, Pedro Fernández-Soto[1], Juan Luis Muñoz-Bellido[2], Begoña Febrer-Sendra[1], Beatriz Crego-Vicente[1], Cristina Carbonell[1,3], Amparo López-Bernús[1,3], Miguel Marcos[3], Moncef Belhassen-García[1,3], Muro A[1].

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DOI: https://doi.org/10.3390/jcm11010158

Indicadores de calidad: Factor de impacto: 4,964; Q2 en Medicina, General e Interna.

9. Cuarto artículo de investigación

Título: SMART-LAMP: A smartphone-operated handheld device for real-time colorimetric point-of-care diagnosis of infectious diseases via loop-mediated isothermal amplification.

Revista: Biosensors (Basel)

Año: 2022

Autores: **Juan García-Bernalt Diego**[1], Pedro Fernández-Soto[1], Sergio Márquez-Sánchez[2,3], Daniel Santos Santos[2], Begoña Febrer-Sendra[1], Beatriz Crego-Vicente[1], Juan Luis Muñoz-Bellido[4], Moncef Belhassen-García[5], Juan Manuel Corchado[2,3] Rodríguez y Antonio Muro[1].

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DOI: https://doi.org/10.3390/bios12060424

Indicadores de calidad: Factor de impacto: 5.743. Q1 en Química, Analítica. Q1 en Instrumentos e Instrumentación. Q2 en Nanociencia y nanotecnología.

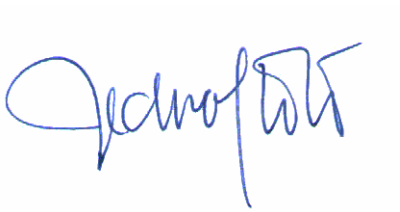
CERTIFICACIÓN

Prof. Dr. Antonio Muro Álvarez, Catedrático de Parasitología del Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola de la Universidad de Salamanca, y Prof. Dr. Pedro Fernández Soto, Profesor Titular de Parasitología del Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola de la Universidad de Salamanca.

Certifican:

*que la Tesis Doctoral "**Tecnología LAMP para el diagnóstico molecular adaptado de enfermedades infecciosas**", 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 **Juan García-Bernalt Diego**, Graduado en Biotecnología por la Universidad de Salamanca, 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 dentro del programa de doctorado Salud y Desarrollo en los Trópicos. Reúne, a nuestro juicio, originalidad y contenidos suficientes, por lo que autorizamos su presentación para ser evaluada.*

Y para que así conste, a efectos legales, expiden el presente certificado en Salamanca, a 27 de octubre de 2022



Fdo. Dr. Antonio Muro Álvarez

Fdo. Dr. Pedro Fernández-Soto

A mi abuela, Manolita.

Lo esencial es no perder la orientación.
Siempre pendiente de la brújula,
siguió guiando a sus hombres hacia el norte invisible,
hasta que lograron salir de la región encantada.

Gabriel García Márquez

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– *Unity is variety and variety in unity is the supreme law of the universe.* –
Isaac Newton

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A mi gran familia grande, a los que están y a los que ya se fueron. A mi abuela Manolita, por su curiosidad infinita, mi hermano Bernardo, mis tías y tíos, primos y primas, a mi madrina Mamen. A mis amigos, mis hermanos, Pisonero y Pérez, que creyeron conmigo en la responsabilidad de investigar y de divulgar, a Daniel, Rodrigo Santos, Rodrigo Manjón, Emilio, Javi y toda una larga lista que les sigue.

A todos ¡Gracias!

ÍNDICE

RESUMEN

Históricamente, el progreso en el diagnóstico de enfermedades infecciosas se ha visto limitado por los requerimientos técnicos y estructurales que presentan muchas de las metodologías utilizadas. Así, tecnologías con elevada sensibilidad y especificidad, como la amplificación de ácidos nucleicos, han quedado restringidas a laboratorios de referencia con importantes infraestructuras. Por ello, infecciones endémicas en países en vías de desarrollo son las que más han sufrido este problema diagnóstico. La carencia de sistemas apropiados para la detección de patógenos ha llevado en muchas ocasiones a un sobre o infra tratamiento, a una falta de este e incluso a un tratamiento innecesario o hasta dañino. Ha resultado también en una distorsión de los datos epidemiológicos de numerosas enfermedades infecciosas.

Las técnicas de amplificación isotérmica de ácidos nucleicos se desarrollaron con la promesa de llevar al campo el diagnóstico molecular realizado en el laboratorio. La amplificación isotérmica mediada por bucle (LAMP, del inglés *Loop-mediated isothermal amplification*) es hoy día la más utilizada entre todas ellas. Sin embargo, tras dos décadas desde su presentación, éste y otros métodos isotérmicos siguen siendo infrecuentes en la práctica clínica diaria.

En este contexto, nos planteamos como **hipótesis** de esta Tesis Doctoral la necesidad de adaptar esta tecnología y combinarla con otras que permitan almacenar y transportar los reactivos de reacción necesarios, sin requerir una cadena de frío. También consideramos preciso disponer de dispositivos pequeños, portátiles y autónomos para llevar a cabo la reacción. Además, la amplificación convendría poder monitorizarla a tiempo real y todos los datos generados ser gestionados cómodamente a través de un dispositivo inteligente.

Esta hipótesis se basó en un análisis bibliográfico detallado que se presenta como **introducción**. En primer lugar, se definen las generalidades de los diferentes diagnósticos basados en ácidos nucleicos, desde la PCR hasta las distintas técnicas de amplificación isotérmica y los diferentes análisis post-amplificación. Una vez establecido este marco teórico, se describen en profundidad las aplicaciones de la tecnología LAMP en las enfermedades tropicales desatendidas (NTDs, del inglés *Neglected Tropical Diseases*), con especial atención a la esquistosomosis y en la infección por SARS-CoV-2. En estas revisiones se destacan las fortalezas de los estudios presentados hasta la fecha y también sus limitaciones. Se establece así una hoja de ruta

para las aportaciones que este trabajo de investigación puede hacer al campo de estudio.

De esta manera, propusimos como **objetivo general** la adaptación de la tecnología LAMP para su validación como método molecular en el diagnóstico de las enfermedades infecciosas. Para ello, se definen diversos objetivos específicos que se van alcanzando en los diferentes artículos de investigación presentados en esta Tesis Doctoral.

El **primer objetivo específico** se basaba en desarrollar y aplicar un método de estabilización sencillo que permitiera almacenar reactivos LAMP listos para el uso a temperatura ambiente. Para ello, utilizamos el método SmMIT-LAMP, previamente publicado por nuestro grupo para la detección de ADN de *Schistosoma mansoni*. Con un simple proceso de desecación, obtenemos reactivos listos para el uso y estables a temperatura ambiente durante varias semanas.

Como **segundo objetivo específico** nos planteamos diseñar y desarrollar un dispositivo portátil y una aplicación móvil, con el fin de realizar reacciones a tiempo real de amplificación isotérmica y analizar y almacenar los resultados obtenidos. Este dispositivo se materializa en el SMART-LAMP, que fue registrado como modelo de utilidad concedido en la Oficina Española de Patentes y marcas bajo el título "Dispositivo de diagnóstico de enfermedades tropicales desatendidas".

Durante la realización de esta Tesis Doctoral, estalló la pandemia provocada por SARS-CoV-2. El desarrollo de métodos de diagnóstico rápidos, sensibles y precisos se convirtió en una tarea de imperiosa necesidad en los primeros compases de la misma. Por ello, establecimos como **tercer objetivo específico** el diseño de diferentes ensayos RT-LAMP (*Reverse transcription*–LAMP) para la detección del virus y su evaluación en diversas muestras clínicas. Describimos ocho ensayos RT-LAMP diferentes para la detección de SARS-CoV-2. Además, realizamos un análisis comparativo de estos en términos de cinética, sensibilidad y especificidad y finalmente seleccionamos los mejores para su posterior aplicación en muestras clínicas. Se observó una alta sensibilidad y especificidad en muestras de exudados nasofaríngeos. Sin embargo, se comprobó que no eran útiles en muestras de orina para la detección de ARN del virus. Por otra parte, se realizaron mejoras sobre el protocolo de estabilización de los reactivos, incrementándose a dos meses su estabilidad a temperatura ambiente.

El **cuarto objetivo específico** tuvo como finalidad evaluar la utilidad diagnóstica del dispositivo portátil SMART-LAMP. Para ello, diseñamos un estudio

piloto en el que combinamos el uso del dispositivo con el método de estabilización de reactivos desarrollado, obteniendo un sistema portátil y fácil de utilizar en zonas de escasa infraestructura. Se ensayó para la detección de *Schistosoma mansoni*, *S. haematobium*, *Strongyloides* spp. y SARS-CoV-2, obteniendo elevados valores predictivos. Los resultados fueron comparables con los obtenidos por RT-qPCR y con un dispositivo comercial de amplificación isotérmica.

En **conclusión**, el trabajo que aquí se presenta muestra: *(i)* el desarrollo de una metodología sencilla y rápida de estabilización de reactivos LAMP, que permite su almacenamiento a temperatura ambiente en un formato listo para el uso; *(ii)* el diseño y desarrollo de un dispositivo portátil (SMART-LAMP) que permite la realización a tiempo real de reacciones de amplificación isotérmica, controlado a través de una aplicación móvil; *(iii)* la puesta a punto de un RT-LAMP para la detección de SARS-CoV-2 con altos valores de sensibilidad y especificidad; *(iv)* la aplicación del SMART-LAMP para diferentes ensayos LAMP, demostrando su excelente rendimiento diagnóstico, con resultados comparables a los obtenidos con técnicas moleculares de referencia (RT-qPCR) y otros dispositivos comerciales de amplificación isotérmica.

SUMMARY

Historically, progress in the diagnosis of infectious diseases has been limited by technical and structural requirements of many of the methodologies used. Thus, highly sensitive and specific technologies, such as nucleic acid amplification, have been restricted to reference laboratories with important infrastructures. As a result, endemic infections in developing countries have suffered the most from this diagnostic problem. The lack of appropriate systems for pathogen detection has often led to over- or undertreatment, lack of treatment and even unnecessary or even harmful treatment. It has also resulted in a distortion of epidemiological data for numerous infectious diseases.

Nucleic acid isothermal amplification techniques were developed with the promise of bringing molecular diagnostics performed in the laboratory to the field. Loop-mediated isothermal amplification (LAMP) is today the most widely used of these techniques. However, two decades after its presentation, this and other isothermal methods are still infrequent in daily clinical practice.

In this context, we propose as a ***hypothesis*** *in this PhD Thesis the need to adapt this technology and combine it with others that allow storage and transport of the necessary reaction reagents, without a cold chain. We also consider it necessary to have small, portable and autonomous devices to carry out the reaction. In addition, amplification should be monitored in real time and all the data generated should be conveniently managed through an intelligent device.*

This hypothesis was based on a detailed bibliographic analysis, which is presented as an ***introduction****. First, the generalities of the different nucleic acid-based diagnostics are defined, from PCR to the different isothermal amplification techniques and the different post-amplification analyses. Once this theoretical framework is established, the applications of LAMP technology in the different infections studied are described in depth: Neglected Tropical Diseases (NTDs), with special attention to schistosomiasis and SARS-CoV-2 infection. These reviews highlight the strengths of the studies presented to date and their limitations. Thus, we establish a roadmap for the contributions of our research can make to the field of study.*

Thus, we proposed as a ***general objective*** *the adaptation of LAMP technology for its validation as a molecular method in the diagnosis of infectious diseases. For this purpose, several specific objectives are defined, which are achieved in the different research articles presented in this Doctoral Thesis.*

*The **first specific objective** was focused on the development and application of a simple stabilization method to store ready-to-use LAMP reagents at room temperature. For this purpose, we used the SmMIT-LAMP method, previously published by our group for the detection of Schistosoma mansoni DNA. With a simple desiccation process, we obtain ready-to-use reagents that are stable at room temperature for several weeks.*

*As a **second specific objective**, we set out to design and develop a portable device and a mobile application, in order to perform real-time isothermal amplification reactions, and to analyze and store the results obtained. This device is materialized in the SMART-LAMP, which was registered as a utility model granted at the Spanish Patent and Trademark Office under the title "Device for the diagnosis of neglected tropical diseases".*

*During this Doctoral Thesis, the SARS-CoV-2 pandemic emerged. The development of rapid, sensitive and accurate diagnostic methods became an urgent need in the early stages of the pandemic. Therefore, we set as a **third specific objective** the design of different RT-LAMP (Reverse transcription-LAMP) assays for the detection of the virus and their evaluation in various clinical samples. We described eight different RT-LAMP assays for the detection of SARS-CoV-2. In addition, we performed a comparative analysis of these in terms of kinetics, sensitivity and specificity, and finally selected the best ones for further application in clinical samples. This analysis in nasopharyngeal exudates demonstrated high sensitivity and specificity in the designed RT-LAMPs. It was also tested in urine samples showing that they were not useful for the detection of the virus's RNA. Additionally, improvements were made on the reagent stabilization protocol, increasing its stability at room temperature to over two months.*

*The **fourth specific objective** was to evaluate the diagnostic utility of the SMART-LAMP device. To this end, we designed a pilot study in which we combined the portable device with the reagent stabilization method developed, obtaining a portable system that is easy to use in areas with limited infrastructure. It was tested against four pathogens (Schistosoma mansoni, S. haematobium, Strongyloides* spp. *and SARS-CoV-2) obtaining high predictive values. The results were comparable with those obtained by RT-qPCR and with a commercial isothermal amplification device.*

*In **conclusion**, the work presented here shows: (i) the development of a simple and rapid methodology for the stabilization of LAMP reagents, which allows their storage at room temperature in a ready-to-use format; (ii) the design and development of a portable device (SMART-LAMP) that allows the real-time performance of isothermal amplification reactions, controlled through a mobile application; (iii) the development of an RT-LAMP for the detection of SARS-CoV-2 in nasopharyngeal samples, showing high sensitivity and*

specificity values; (iv) the application of the SMART-LAMP for different LAMP assays, demonstrating its excellent diagnostic performance, with results comparable to those obtained with gold standard molecular techniques (RT-qPCR) and other commercial isothermal amplification devices.

CAPÍTULO I

Introducción

1.1. Contexto

La prevalencia, distribución, control y manejo de las enfermedades infecciosas depende en gran medida de las condiciones socioeconómicas, culturales y ambientales que las acompañan. En comunidades empobrecidas, especialmente en países en vías de desarrollo, una amplia mayoría de sus habitantes viven en ambientes de superpoblación, con falta de agua potable o condiciones inadecuadas de higiene, generando así un caldo de cultivo idóneo para la adquisición y transmisión de enfermedades[1].

A este respecto, la Organización de las Naciones Unidas (ONU) define el estado de desarrollo de un país en base a su Índice de Desarrollo Humano (HDI, del inglés *Human Development Index*). Este indicador tiene en cuenta numerosos factores para definir la longevidad y calidad de vida de los habitantes de una región, su educación y sus estándares de vida. En una escala de 0 a 1, se clasifica como país en vías de desarrollo todo aquel que no alcanza una puntuación de 0,80. Según el *Human Development Report 2021-2022*, en este año todavía se encuentran por debajo de ese umbral 125 países[2].

A pesar de esta elevada cifra, el HDI global continúa incrementándose, aunque de manera desigual entre regiones. Una medida de ese progreso es la reducción en morbilidad causada por enfermedades, contabilizada en DALYs (del inglés *Disability Adjusted Life-Years*). El guarismo, en números absolutos, se ha mantenido más o menos constante a lo largo de los últimos treinta años, pero al ajustarlo a la expansión y envejecimiento poblacional, se observa una clara tendencia descendente. Entre las diez causas principales de ese descenso, cinco son consecuencia directa de un mejor manejo de algunas enfermedades infecciosas: infecciones de las vías respiratorias bajas, sarampión, tétanos, malaria (todas ellas afectan principalmente a niños) y tuberculosis (afecta principalmente a adultos). Asimismo, se incluyen entre las causas principales enfermedades diarreicas, que también pueden estar provocadas por agentes infecciosos[3]. A pesar de esta reducción generalizada, la carga que ejercen las enfermedades infecciosas sigue siendo desproporcionadamente superior en los países con menores recursos, con una fuerte correlación ($R = 0.87$, $p < 2{,}2 \times 10^{-16}$; vía correlación de Pearson) entre una mayor morbilidad y un menor producto interior bruto per cápita (Figura 1).

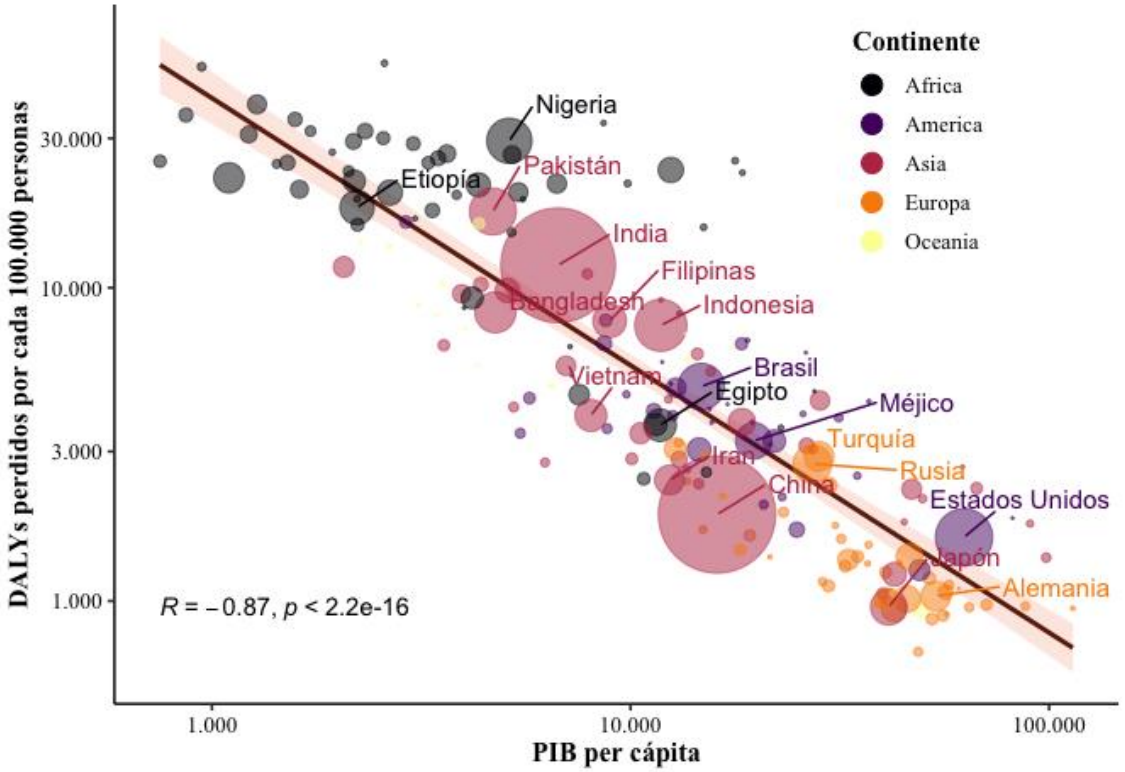

Figura 1. Correlación entre la morbilidad de las enfermedades infecciosas y el producto interior bruto per cápita. Gráfico de burbujas. El tamaño de cada burbuja representa la población de cada país. Representados aparecen 186 de los 194 países del mundo. Solo se muestran los nombres de países con una población superior a 75 millones de habitantes. *R* y *p*-valor obtenidos mediante correlación de Pearson entre las variables de DALYs perdidos por cada 100.000 personas y producto interior bruto (PIB) per cápita. Datos del año 2019, tomados de https://ourworldindata.org/burden-of-disease.

En las regiones tropicales y subtropicales del planeta, donde se concentran la amplia mayoría de países en vías de desarrollo, hay cuatro infecciones o grupos de infecciones principales: VIH, malaria, tuberculosis y enfermedades tropicales desatendidas (NTDs, del inglés *Neglected Tropical Diseases*)[4]. Estas últimas fueron definidas en el año 2005 por los científicos David H. Molyneux, Peter J. Hotez y Alan Fenwick como "condiciones que promueven la pobreza y, a menudo, estigmatizan, teniendo lugar principalmente en áreas rurales de países en vías de desarrollo"[5]. Inicialmente, las NTDs incluían 13 entidades, aunque a día de hoy se consideran ya 20, englobando infecciones causadas por parásitos, virus, bacterias y hongos, así como envenenamientos por mordedura de serpiente[6] (Figura 2). Las NTDs, la malaria, VIH y tuberculosis han decrecido notablemente en prevalencia en los últimos años[7–10].

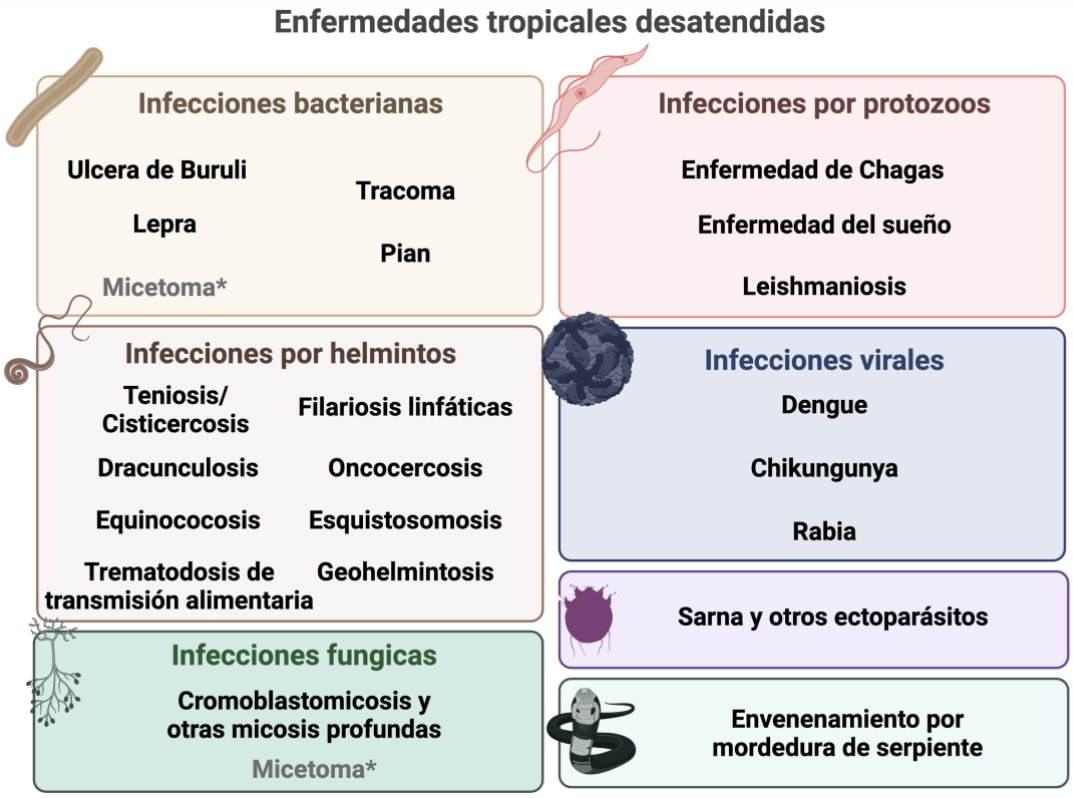

Figura 2. Enfermedades tropicales desatendidas agrupadas por etiología. *El micetoma puede tener tanto etiología bacteriana como fúngica. Figura creada con Biorender.com

Para afrontar la lucha contra estas infecciones se necesita de numerosos actores y mecanismos políticos, sociales, científicos y sanitarios; muchos de los cuales quedan fuera del alcance de este trabajo. A nivel científico, hace ya veinte años, un grupo de expertos en bioética encabezado por el filósofo Peter A. Singer, definió los diez retos más importantes a los que se enfrentaba la biotecnología para mejorar la salud en los países en vías de desarrollo. El primero de ellos se refería a la adaptación de métodos moleculares para la realización de diagnósticos simples y asequibles para la detección de enfermedades infecciosas[11], que será el foco de esta Tesis Doctoral. A pesar del importante progreso de este campo desde entonces, los países en vías de desarrollo presentan todavía carencias evidentes, especialmente en instalaciones básicas de atención primaria, donde la disponibilidad de diagnósticos esenciales no alcanza el 20%[12]. La Organización mundial de la Salud (OMS), en su hoja de ruta 2021-2030 contra las NTDs[13], destaca la necesidad de mejorar en estas técnicas tanto para acelerar la eliminación de enfermedades como la tripanosomosis africana o la lepra, como para reducir la morbilidad de otras como la leishmaniosis visceral, oncocercosis o loasis, o simplemente para reducir u optimizar los costes de programas contra las filariosis linfáticas y la esquistosomosis. Además, el resto de NTDs carece de diagnósticos

adecuados o necesitan mejoras importantes para alcanzar los objetivos propuestos para el año 2030.

El 12 de diciembre de 2019, se detectaron en la ciudad de Wuhan, China, los primeros casos de neumonía causados por el virus que posteriormente conoceríamos como SARS-CoV-2 (del inglés *severe acute respiratory syndrome coronavirus 2*)[14]. Esta infección sería clasificada como pandemia por la OMS el 11 de marzo de 2020. Casi tres años después, las cifras de la pandemia de COVID-19 siguen incrementándose. A octubre de 2022, hay confirmados más de 610 millones de casos y más de 6,5 millones de muertes[15], aunque se estima que las cifras reales podrían ser más bien cercanas a los 20 millones de fallecidos[16].

Esta terrible emergencia mundial necesitó en sus inicios del desarrollo de herramientas para su control y diagnóstico. En este contexto, y dada la experiencia que nuestro grupo de investigación ha adquirido en el desarrollo de tecnologías de diagnóstico isotérmicas tipo LAMP para su uso contra las NTDs, establecimos también como objetivo prioritario de este trabajo el diseño y adaptación de esta tecnología para la detección de SARS-CoV-2.

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1.2

Nucleic acid diagnostics

Chapter 37

Juan García-Bernalt Diego, Pedro Fernández-Soto, Antonio Muro

CRC Press's Handbook of Molecular Biotechnology

In press, 2023

RESUMEN

El marco teórico que encuadra esta Tesis Doctoral es el del diagnóstico molecular o diagnóstico basado en ácidos nucleicos. Este campo cuenta ya con unos 70 años de historia, aunque su evolución se ha acelerado con la llegada del siglo XXI. Este impulso se ha visto más reforzado con los avances que han venido impuestos para dar una respuesta eficaz a la pandemia de COVID-19.

Así, el capítulo "*Nucleic acid diagnostics*", incluido en esta sección, desglosa conceptos teóricos y también prácticos de la puesta a punto de ensayos basados en la amplificación de ácidos nucleicos para la identificación de agentes infecciosos. El documento hace un recorrido metodológico a lo largo del procedimiento de "un diagnóstico molecular" que comienza discutiendo la selección, principales características y limitaciones de las diferentes muestras biológicas. Continúa con los principios de la extracción y purificación de ácidos nucleicos, resaltando consideraciones particulares para el ARN. También se discute la toma de muestra, su transporte o su almacenamiento. A continuación, se indican las técnicas de amplificación de ácidos nucleicos, tomando como referencia la reacción en cadena de la polimerasa o PCR (del inglés *Polymerase chain reaction*) y comparándola con otros métodos derivados de esta, como RT-PCR (del inglés, *Reverse transcription*–PCR), PCR cuantitativa (qPCR, del inglés *quantitative*–PCR) o PCR digital (dPCR, del inglés *digital*–PCR). A su vez, todas estas metodologías se contraponen con técnicas de amplificación isotérmica, prestando especial atención a la técnica LAMP (del inglés *Loop-mediated isothermal amplification*), elemento central de esta Tesis Doctoral. Sin olvidar otras como NASBA (del inglés, *Nucleic acid sequence based amplification*), SDA (del inglés, *Strand displacement amplification*), HDA (del inglés, *Helicase dependent amplification*), RPA (del inglés, *Recombinase polymerase amplification*) o EXPAR (del inglés, *Exponential amplification reaction*). Finalmente se describen los análisis post-amplificación, tanto análisis de curva de anillamiento como secuenciación dirigida y masiva, así como los adelantos más recientes en métodos de detección de ácidos nucleicos basados en CRISPR (del inglés, *Clustered regularly interspaced short palindromic repeats*).

Todo este contenido teórico-práctico, necesario para poder afrontar los retos planteados en este trabajo, desemboca en la pregunta central: ¿Qué requisitos ha de cumplir un diagnóstico molecular para poder ser aplicado en condiciones de recursos

limitados? Si bien es evidente que el diagnóstico basado en ácidos nucleicos ha significado un antes y un después en la manera de detectar infecciones en la clínica y los laboratorios de referencia, su impacto sólo puede calificarse como residual para muchas infecciones endémicas de países tropicales y subtropicales, así como en el diagnóstico a pie de paciente o *point-of-care* (POC). Se abre en este punto un interesante debate sobre la selección de los métodos más apropiados para este tipo de aplicaciones. También, la incorporación de nuevas tecnologías y avances científicos complementarios al propio método de amplificación que son necesarios para que estas técnicas puedan cumplir su objetivo final: el traslado del laboratorio al campo. Todo ello adaptado a las particularidades específicas de cada infección.

En conclusión, este capítulo pretende poner de manifiesto la necesidad de desarrollar y aumentar la disponibilidad de herramientas de diagnóstico portátiles, rápidas y fáciles de usar, con una mínima pérdida de sensibilidad o especificidad, para hacer frente a situaciones de emergencia o de pobreza o la reciente pandemia de COVID-19, la malaria o las NTDs. Para lograr este objetivo final es necesario un esfuerzo concertado de la comunidad científica, la industria, las autoridades sanitarias y los responsables políticos.

1.3

LAMP in Neglected Tropical Diseases: A focus on parasites

Juan García-Bernalt Diego, Pedro Fernández-Soto, Antonio Muro

Diagnostics (Basel). 2021; 15(3):521

RESUMEN

En esta sección se incluye el artículo "*LAMP in Neglected Tropical Diseases: a focus on parasites*" centrado en el uso de la tecnología LAMP para el diagnóstico de NTDs causadas por parásitos. Estas enfermedades siguen representando un grave problema de Salud Pública en regiones tropicales y subtropicales. Aunque se han realizado numerosos esfuerzos dirigidos a su control y manejo, muchos factores han limitado el éxito de estas intervenciones. Entre ellos, cabe destacar la limitada capacidad diagnóstica, derivada de la dificultad que presenta trasladar a zonas de escasos recursos métodos específicos, rápidos y asequibles. Es particularmente complicado adaptar técnicas basadas en la PCR a estas zonas, ya que los requerimientos de infraestructura resultan inaccesibles. En este contexto, las técnicas de amplificación isotérmica, específicamente la técnica LAMP, se consideran desde hace años como una alternativa prometedora, generándose un gran interés en el desarrollo de esta tecnología dirigida a las diferentes NTDs causadas por parásitos.

De los trabajos analizados en esta revisión, pudimos extraer varias conclusiones que guiaron nuestro posterior estudio, reflejado en los artículos de investigación incluidos más adelante. Hasta la fecha, se ha generado abundante literatura científica centrada en el diagnóstico de NTDs mediante LAMP. De hecho, para todas las enfermedades parasitarias incluidas en el panel de las NTDs se dispone de al menos un ensayo LAMP. El primer test se presentó tan solo tres años después del desarrollo de esta tecnología y su uso se ha extendido rápidamente.

El estudio de la técnica LAMP se ha realizado desde diversos enfoques. En primer lugar, se ha evaluado la selección de secuencias diana. Estas regiones muestran una gran similitud con las regiones escogidas para la PCR, entre las que abundan secuencias repetitivas de los genomas de los parásitos, especialmente ribosómicas y mitocondriales. También se ha evaluado su utilidad en estudios de xenomonitorización en vectores y de detección de la infección de hospedadores intermediarios infectados, especialmente en zonas de baja prevalencia, donde la elevada sensibilidad de la tecnología LAMP puede ayudar a una mejor toma de decisiones. Los estudios en modelos animales, aunque menos abundantes, han permitido caracterizar las muestras más adecuadas para el diagnóstico, así como la efectividad de la técnica con diferentes dosis y tiempos de infección. Aunque hay estudios con muestras clínicas disponibles para la mayoría de los parásitos causantes de NTDs, estos tienen limitaciones importantes en el tamaño muestral, en la

comparación con otros diagnósticos o en su aplicación a diferentes grupos poblacionales. Además, ha sido poco utilizada para la evaluación de la eficacia post-tratamiento. La escasez de estos trabajos puesta de manifiesto en esta revisión ha motivado uno de los proyectos más recientes de nuestro laboratorio.

Finalmente, la conclusión más significativa durante la realización de este trabajo de revisión es que algunos de los ensayos de amplificación LAMP presentes en la literatura cumplen prácticamente todos los requisitos planteados en el criterio REASSURED (del inglés, *Real-time connectivity*, *Ease of specimen collection*, *Affordable*, *Sensitive*, *Specific*, *User-friendly*, *Rapid and Robust*, *Equipment-free*, *Deliverable to end users*). Por tanto, serían buenos candidatos para ser utilizados como métodos de diagnóstico a pie de paciente en zonas endémicas. Sin embargo, ninguno de ellos ha tenido un impacto real en la práctica diagnóstica rutinaria de estas enfermedades. Los puntos más débiles de los tests disponibles son: (*i*) la dificultad de obtención y procesamiento de la muestra, (*ii*) la necesidad de equipamiento complejo para llevarse a cabo, (*iii*) la falta de conexión a tiempo real y (*iv*) su limitada aplicación en el diagnóstico de aquellas personas que más lo necesitan. Es por ello, que estos cuatro puntos van a ser la principal diana a la que se dirigirán nuestros esfuerzos en el trabajo de investigación de esta Tesis Doctoral.

diagnostics

MDPI

Review

LAMP in Neglected Tropical Diseases: A Focus on Parasites

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Abstract: Neglected Tropical Diseases (NTDs), particularly those caused by parasites, remain a major Public Health problem in tropical and subtropical regions, with 10% of the world population being infected. Their management and control have been traditionally hampered, among other factors, by the difficulty to deploy rapid, specific, and affordable diagnostic tools in low resource settings. This is especially true for complex PCR-based methods. Isothermal nucleic acid amplification techniques, particularly loop-mediated isothermal amplification (LAMP), appeared in the early 21st century as an alternative to PCR, allowing for a much more affordable molecular diagnostic. Here, we present the status of LAMP assays development in parasite-caused NTDs. We address the progress made in different research applications of the technique: xenomonitoring, epidemiological studies, work in animal models and clinical application both for diagnosis and evaluation of treatment success. Finally, we try to shed a light on the improvements needed to achieve a true point-of-care test and the future perspectives in this field.

Keywords: LAMP; neglected tropical diseases; parasites; point-of-care diagnostic

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1. Neglected Tropical Diseases Caused by Parasites: The Diagnostic Limitation

In 2005, the "Neglected Tropical Diseases" (NTDs) concept was defined by researchers David H. Molyneux, Peter J. Hotez and Alan Fenwick. They grouped thirteen infectious diseases, caused by bacteria and parasites, that fitted a common definition: "A poverty-promoting and often stigmatizing condition occurring primarily in rural areas of low income countries" [1]. Since then, the World Health Organization (WHO) has updated that list to twenty conditions caused by bacteria, viruses, parasites, and snake envenoming affecting some of the world's poorest communities, predominantly in Africa, Asia, and the Americas. Those living without adequate sanitation and in close contact with infectious vectors, domestic animals and livestock are worst affected (https://www.who.int/neglected_diseases/diseases/en/, accessed on 1 December 2020).

Of the 20 NTDs recognized by WHO, 12 are caused by parasites (parasite-caused NTDs): Chagas disease (American trypanosomiasis), Dracunculiasis (Guinea-worm disease), Echinococcosis, Foodborne trematode infections, Human African trypanosomiasis (sleeping sickness), Leishmaniasis, Lymphatic filariasis (Elephantiasis), Onchocerciasis (river blindness), Scabies and other ectoparasites, Schistosomiasis (Bilharzia), Soil-transmitted helminthiases, and Taeniasis and Cysticercosis (https://www.who.int/neglected_diseases/diseases/en/, accessed on 1 December 2020). It is likely that all of the world's population living below the World Bank poverty line of US$1.90 per day are infected with one or more of these NTDs, corresponding to, at least, 10% of the global population [2]. Based on data provided by the 2017 Global Burden of Disease Study (GBD), Kyu et al. [3] calculated that over 17 million disability adjusted life years (DALYs) are caused by NTDs. This represents 4.7% of the total DALYs by any communicable, maternal, neonatal or nutritional disease [3]. Among the parasite-caused

NTDs, the most prominent morbidities came from lymphatic filariasis, foodborne trematodiases, and schistosomiasis. On the other hand, the deadliest diseases according to the Global Health Estimates (GHE, 2016) by the WHO are schistosomiasis, cysticercosis, and echinococcosis, each causing over 20,000 deaths a year (https://www.who.int/healthinfo/global_burden_disease/en/, accessed on 25 November 2020). Global attention tends to focus on killer diseases, although NTDs disable and disfigure more than they kill [4]. A summary of the morbidity and mortality data for parasite-caused NTDs is shown in Figure 1. While all those numbers offer context to the current situation in tropical regions, it is important to emphasize the fluctuations that epidemiological data suffer. This is especially critical in tropical regions where data collection remains a very demanding task, and vast underestimations of both incidence and mortality have been reported in previous editions of the GBD [5].

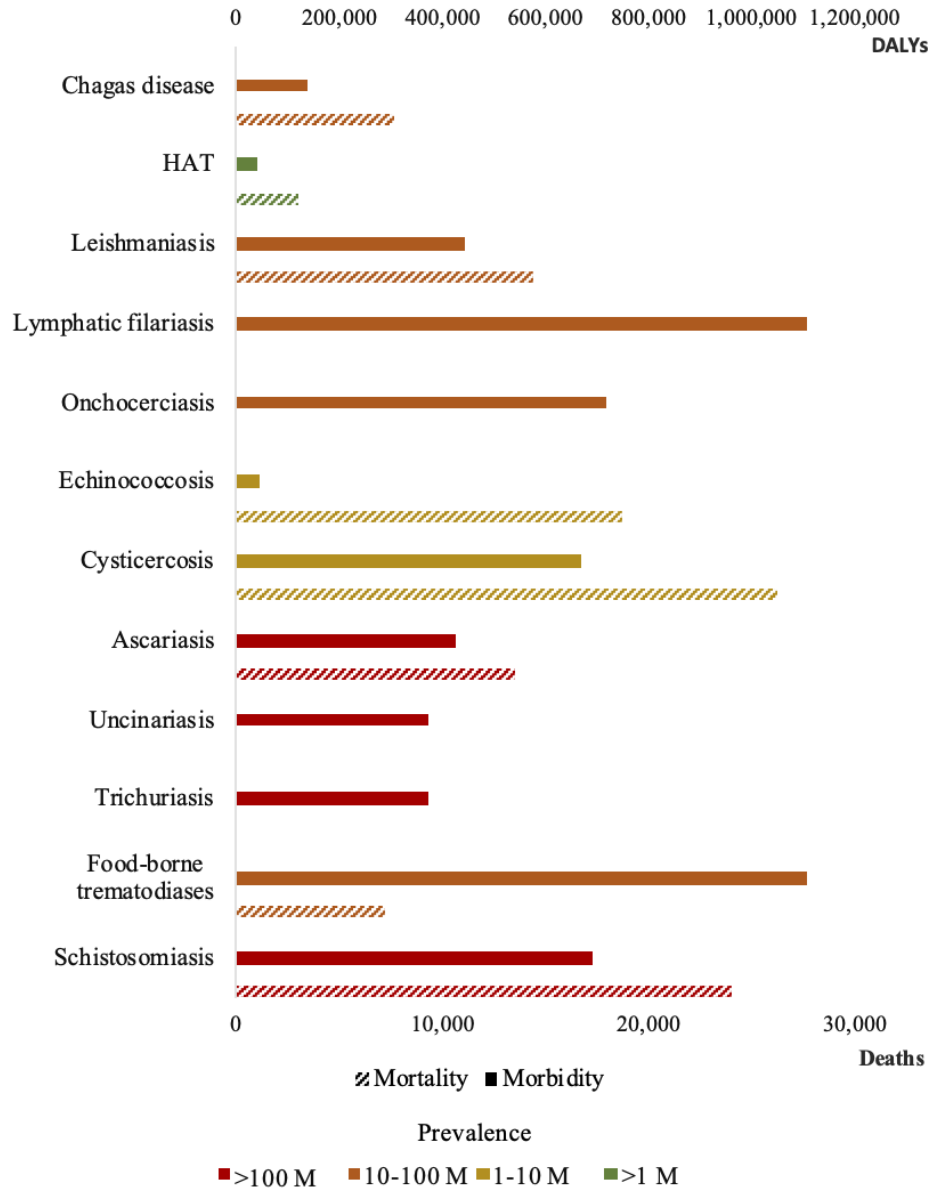

Figure 1. Estimated burden of parasite-caused Neglected Tropical Diseases (NTDs). Solid bars represent disability adjusted life years (DALYs) data, obtained from Kyu et al. [3]. Cross-lined bars represent mortality data, obtained from Global Health Estimates (GHE) 2016 [6]. Color of the bars represents prevalence values (referred as M, million people), obtained from Hotez et al. [7].

In the new WHO road map for NTDs (2021–2030) a particular focus is directed to diagnosis monitoring and evaluation, access and logistics, and advocacy and founding to attain

the sustainable development goals. The engagement of companies and foundations has been key to support advances in schistosomiasis, soil-transmitted helminthiases, or HAT diagnosis. However, the overall investment in diagnosis development has been very low, representing only 5% of research and development investment for NTDs, which as a hole, has decreased 10% in the last 10 years [8]. Although significant improvements have been made regarding chemotherapy, the same has not happened to the diagnostic tools to guide it. Classical NTDs diagnosis is hampered by the lack of a gold-standard and, in general, the lack of sensitivity and specificity. Accurate diagnostic tests are commercially available but are mostly laboratory-based, thus, not widely accessible in low-income countries [9]. PCR-based methods are expensive and infrastructure-demanding, therefore, not ideal for point-of-care (POC) tests. In this context, methods of isothermal nucleic acid amplification technology (INAAT) have emerged as a promising alternative. They can be performed with simple equipment, combining the sensitivity and specificity of molecular methods with a reduced response-time and cost. These methods have been recently reviewed by Zhao et al. [10]. Among them, LAMP (loop-mediated isothermal amplification) has become the preferred method, due to its sensitivity, specificity, rapidity, low cost and resistance to inhibitors [11,12] and represents 60% of all INAAT publications [13]. There are numerous well-established applications of LAMP technology in the diagnostic of bacterial, viral, fungal, and parasitic diseases in humans, animals, and plants [14,15]. Compared to PCR-based techniques, the simplicity of LAMP makes it suitable for field-testing in developing countries [16,17] and an ideal candidate to develop POC molecular diagnostic tools. In recent years, a great variety of approaches of the LAMP technology in a field-friendly display have been released, such as, lateral flow dipstick and lab-on-chip layouts [18], microfluidic-based methods [19], in combination with metallic nanoparticles [20], or coupled with smart phone-based technology [21].

2. Loop-Mediated Isothermal Amplification

LAMP method was first introduced by Notomi et al. in 2000 [11] and was patented by Eiken Chemical Co., Ltd. (http://www.eiken.com.cn/, accessed on 1 December 2020). LAMP is based on auto-cycling strand displacement DNA synthesis performed under isothermal conditions (60–65 °C for 45–60 min) in the presence of a *Bst* polymerase [11]. In silico designed *Bst* mutants have been developed to improve processivity, fidelity, stability, and tolerance to amplification inhibitors, thus increasing robustness of the LAMP technique [22]. The LAMP reaction requires four primers (two inner and two outer primers), which specifically recognize six distinct sequences in target DNA, thus ensuing high specificity for amplification. The inner primers are called forward inner primer (FIP) and backward inner primer (BIP), and each contains two sequences (usually linked by a poly-T linker) corresponding to the sense and antisense sequences of the target DNA. The outer primers are called forward outer primer (F3) and backward outer primer (B3) (see Figure 2a). The amplification process can be divided into two phases. At the first phase, FIP hybridizes to the target DNA and *Bst* polymerase stars complementary strand synthesis. The F3 starts strand displacement of the elongate FIP primer, releasing single stranded DNA (ssDNA). That ssDNA is used as template for the backward primers. The BIP hybridizes and starts strand synthesis at the ssDNA and then is displaced by the B3 primer. Now, as the 3′ and 5′ ends are complementary to sequences further inwards, stem-loops DNA structures are formed and subsequently used as targets to start an exponential amplification second phase (see Figure 2b). In the second phase, self-priming and the elongation of 3′ end induces displacement of the 5′ end and subsequently, the hairpin comes off and the newly synthesized strand folded. Further self-priming repetitions generate many amplicons with cauliflower-like structures. In addition, FIP and BIP primers now hybridize to the loop structures formed and initialize strand synthesis and subsequent displacement. This method operates on the fundamental principle of the production of a large quantity of DNA amplification products with a mutually complementary sequence and an alternating, repeated structure [11,23]. Nagamine et al. [24] introduced loop primers (LF, loop-forward; LB, loop-backward), thus shortening the reaction time by approximately 30 min. Consequently, a six-

primer design can be used in LAMP reactions (two inner, two outer and two loop primers), targeting in up to eight different sequences, compared to only two in typically PCR-based methods. For ease of explanation see animations at: http://loopamp.eiken.co.jp/e/lamp/anim.html; www.neb.com, accessed on 1 December 2020.

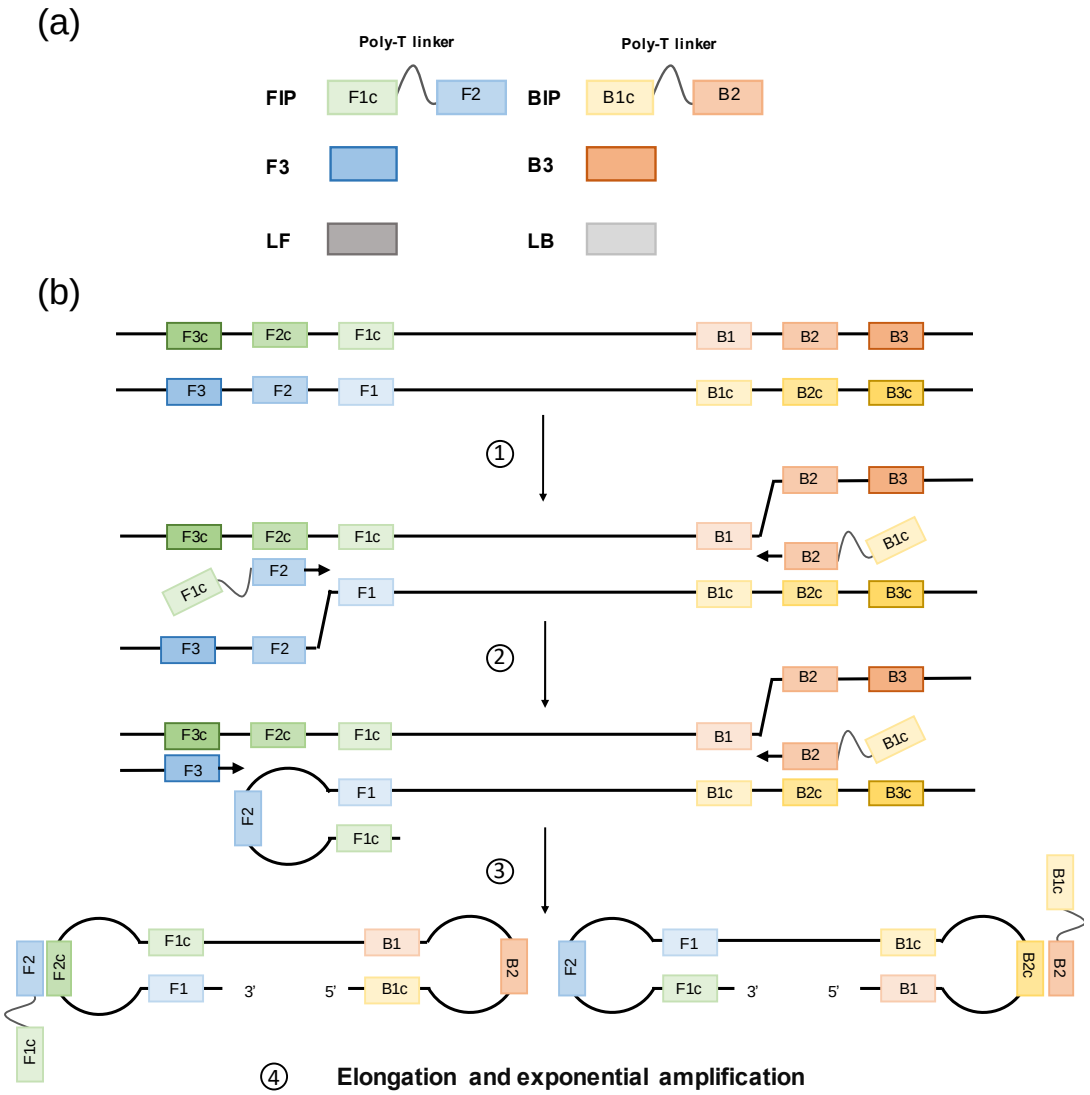

Figure 2. Loop-mediated isothermal amplification (LAMP): primer design and mechanism. (**a**) A typical set of LAMP primers is represented. LAMP reaction requires four primers, two inner primers (forward inner primer (FIP) and backward inner primer (BIP)) and two outer primers (F3 and B3). FIP and BIP each contains two sequences (usually linked by a poly-T linker) corresponding to the sense and antisense sequences of the target DNA. Additional loop primers (loop-forward (LF) and loop-backward (LB)), can be included, shortening the reaction time up to 30 minutes. (**b**) LAMP amplification process can be divided into two phases. At the first phase: 1. FIP hybridizes to the target DNA and *Bst* polymerase starts complementary strand synthesis. 2. The outer primer F3 starts strand displacement of the elongate FIP primer, releasing single stranded DNA (ssDNA). That ssDNA is used as template for the backward primers. The inner primer BIP hybridizes and starts strand synthesis at the ssDNA and then is displaced by the B3 primer. 3. Now, as the 3′ and 5′ ends are complementary to sequences further inwards, stem-loops DNA structures are formed and subsequently used as targets to start an exponential amplification second phase. 4. In the second phase, self-priming and the elongation of 3′ end induces displacement of the 5′ end and subsequently, the hairpin comes off and the newly synthesized strand folded. Further self-priming repetitions generate many amplicons with cauliflower-like structures. In addition, FIP and BIP primers now hybridize to the loop structures formed and initialize strand synthesis and subsequent displacement.

3. LAMP Development in Parasite-Caused NTDs

The potential of LAMP as a possible POC diagnostic test for NTDs was clear from the publication of the technique in the year 2000 and, in 2003, the first LAMP assay for human African trypanosomes DNA detection was published [25], representing the start of a new diagnostic approach for NTDs. Since then, many researchers have reported LAMP assays for parasite-caused-NTDs as an alternative molecular tool to PCR-based methods. However, to date, only a few of the methods developed have been tested in real field conditions. A timeline of first LAMP assays described for each parasite-caused NTD is show in Figure 3.

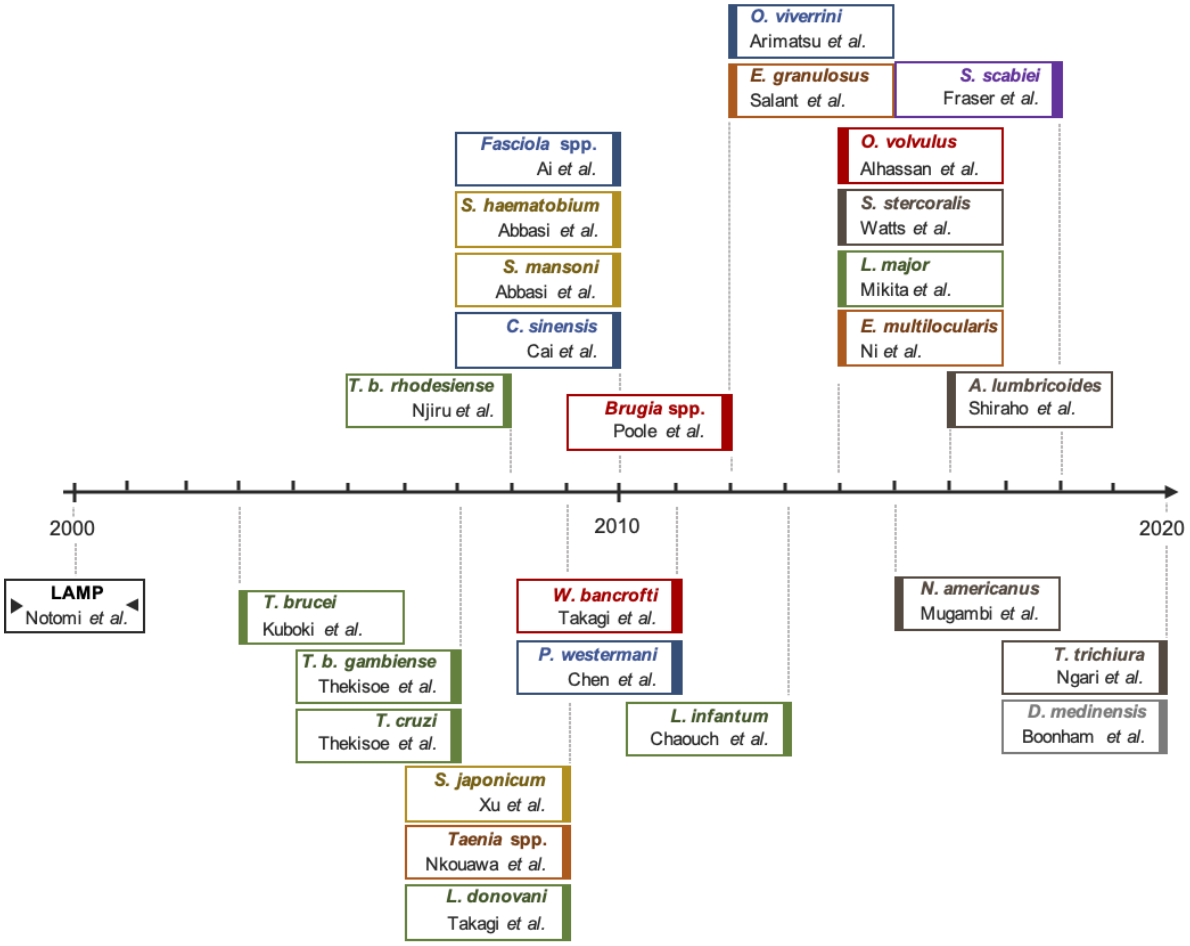

Figure 3. Timeline of first LAMP tests development for parasite-caused NTDs. Color code; orange, Cestodiases: *Taenia* spp., *Echinococcus granulosus* (*E. granulosus*) *Echinococcus multilocularis* (*E. multilocularis*); blue, Food-borne trematodiases: *Fasciola* spp., *Clonorchis sinensis* (*C. sinensis*) *Paragonimus westermani* (*P. westermani*), *Opistorchis viverrini* (*O. viverrini*); brown, Soil-transmitted helminthiasis: *Strongyloides stercoralis* (*S. stercoralis*), *Necator americanus* (*N. americanus*), *Ascaris lumbricoides* (*A. lumbricoides*), *Trichuris trichiura* (*T. trichiura*); green, Protozoa: *Trypanosoma brucei* (*T. brucei*), *Trypanosoma brucei gambiense* (*T. b. gambiense*), *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*), *Trypanosoma cruzi* (*T. cruzi*), *Leishmania infantum* (*L. infantum*) and *Leishmania major* (*L. major*); grey, Dracunculiasis: *Dracunculus medinensis* (*D. medinensis*); red, Lymphatic filariasis: *Brugia* spp., *Wuchereria bancrofti* (*W. bancrofti*) and *Onchocerca volvulus* (*O. volvulus*); violet, Scabies: *Sarcoptes scabei* (*S. scabei*); yellow, Schistosomiasis: *Schistosoma mansoni* (*S. mansoni*), *Schistosoma haematobium* (*S. haematobium*) and *S. japonicum* (*S. japonicum*). References in the figure: Notomi et al. [11], Kuboki et al. [25], Thekisoe et al. [26], Njiru et al. [27], Xu et al. [28], Nkouawa et al. [29], Ai et al. [30], Abbasi et al. [31], Cai et al. [32], Takagi et al. [33], Takagi et al. [34], Chen et al. [35], Arimatsu et al. [36], Salant et al. [37], Poole et al. [38], Chaouch et al. [39], Alhassan et al. [40], Watts et al. [41], Mikita et al. [42], Ni et al. [43], Mugambi et al. [44], Shiraho et al. [45], Fraser et al. [46], Ngari et al. [47], Boonham et al. [48].

Over the years, LAMP assays development and evaluation have followed similar steps for most parasite-caused NTDs: (1) target selection, primer design, set-up, and optimization with purified parasite genomic DNA (gDNA) and synthetic DNA; (2) feasibility of application in different specimens; (3) clinical application using patients' samples; and (4) improvements towards a true POC test. Other relevant studies needed for test development in particular parasite-caused NTDs are the assessment of the efficacy testing intermediate hosts or vectors as xenomonitoring tool, and evaluation in experimental animal models. Those complementary studies allow the assessment of important variables like, inclusivity (recognition of different strains or genotypes of the same species) and exclusivity (discrimination between species) values, the evaluation of treatment success, early-stage of infection diagnosis, infection dynamics, or species-specific identification.

3.1. Genomic Target Selection and LAMP Optimization

Similar to other molecular-based diagnostic methods, selection of genomic targets focusses on highly specific and highly repeated sequences to obtain both high specificity and sensitivity. Some targets are commonly used for nearly all species, particularly nuclear ribosomal sequences, including the internal transcribed spacers 1 and 2 (ITS1 and ITS2), the intergenic spacer (IGS) and the 18S and 28S ribosomal DNA (18S rRNA and 28S rRNA); and mitochondrial sequences, such as NADH dehydrogenase subunits 1 and 5 (nad1 and nad5) or cytochrome c oxidase subunit 1 (cox1). The principal exception is concerning Leishmania species where the main target used is the highly repeated kinetoplast DNA (kDNA). Other frequently targeted sequences are satellite sequences, retrotransposons and genes coding for structural proteins or enzymes (Figure 4). LAMP primer design is more complex than for PCR. There are various systems for LAMP primer design available but the most popular is Primer Explorer, an online free software (https://primerexplorer.jp/e/).

A large number of LAMP studies working with parasite gDNA have consistently shown that LAMP reaches at least similar sensitivity values to those obtained with PCR-based methods. Remarkably, some studies have shown up to 100 to 1000 times more sensitivity for LAMP than PCR, as in the detection of *Paragonimus westermani* using the ITS2 DNA region [35] and *Clonorchis sinensis* targeting the cathepsin B3 gene [32], that reach a limit of detection as low as 10 ag/µL. Nevertheless, most LAMP assays reach sensitivities ranging from 1 to 100 fg/µL.

3.2. LAMP in Molecular Xenomonitoring

A number of studies have shown the usefulness of LAMP as a disease and transmission surveillance tool, especially valuable in low-prevalence areas, where other conventional techniques often lack sensitivity and accuracy. To date, LAMP has been successfully used for the detection of parasites in their insect vectors, including, triatomines (Chagas disease), tsetse flies (HAT), sandflies (leishmaniasis), mosquitoes (LF), black flies (onchocerciasis) and also snail intermediate hosts for schistosomiasis Particularly valuable results have been reported in transmission assessment surveys (TAS) for detection of *Wuchereria bancrofti* DNA in Anopheles and Culex mosquitoes collected in regions of Guinea [49] and Nigeria [50]. In those areas, the transmission of LF-causing parasites is suggested to be unsustainable due to the decline, or absence, of circulating filarial antigen (CFA) in the population. However, *W. bancrofti* DNA was detected by LAMP in mosquitoes, which had tested negative by microscopy, PCR, or both [49,50]. These results lead to the recommendation that filarial infection prevalence in the human and mosquito populations should be re-assessed periodically. Further valuable are a number of studies of the LAMP method for detecting schistosomes-infected intermediate host snails. In experimental infections of snails, LAMP could detect parasite DNA during the prepatent phase of infection (as soon as one day after miracidial exposure) in both individual snails and pooled samples [51]. LAMP has also been evaluated for the detection of *Schistosoma* species in large-scale screening of pooled field-collected snails for analyzing the transmission of schistosomiasis, especially in low-transmission areas. The results of these studies agree

that LAMP is a rapid, sensitive, and a cost-effective tool to screen large numbers of snail samples compared with other PCR-based methods. The usefulness of LAMP to identify foci of transmission in order to build risk maps of schistosomiasis is also apparent in these publications and could be a contributing factor in control campaigns [52–56].

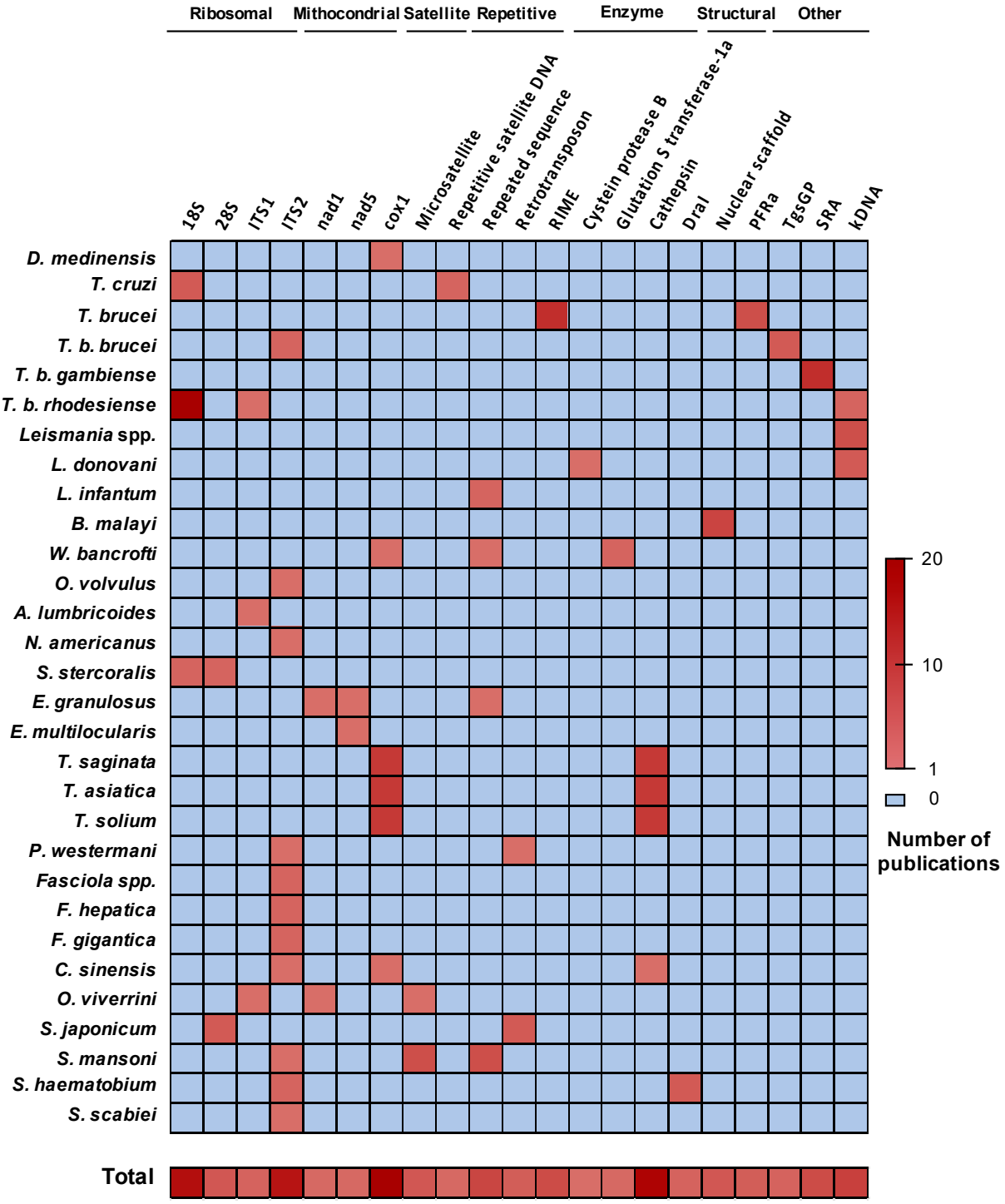

Figure 4. Heat map of the target sequences used for LAMP in parasite-caused NTDs. Sequence abbreviations, from left to right: Microsat (Microsatellite), Rep sat (Repetitive Satellite), Rep Seq (Repetitive sequence), Retrotrans (Retrotransposon), RIME (Repetitive insertion mobile element), CpB (Cystein protease B), GST1a (Glutation-S-Transferase 1a), Nucl scaffold (Nuclear scaffold); PFRa (Paraflagellar Rod) TgsGP (*T. b. gambiense* specific gene,) SRA (serum-resistance associated gene) kDNA (kinetoplast DNA). Data obtained from all the publications used in this review.

3.3. LAMP in Experimental Infections

Within the main limitations of both classical microscopy and serology diagnosis are the inability to detect acute infections and the irregular performance through the course of the parasite infections. In vivo studies in animal models have repeatedly shown the value of LAMP as an early-detection diagnostic tool in comparison to PCR-based methods. Regarding HAT, LAMP could detect *T. b. gambiense* in mice blood samples as soon as two days post-infection (dpi) and throughout the course of the infection, whereas PCR became positive at day 6 dpi and yielded an irregular infection detection [25]. In *T. b. gambiense*-infected monkeys monitoring during 180 dpi, LAMP and PCR were compared using serum, cerebrospinal fluid (CSF), saliva, and urine samples that were collected on a weekly basis, with a significantly higher efficiency of LAMP versus PCR in serum (100% at 7 dpi), saliva (100%; 21–77 dpi), and urine (80%; 28–91 dpi) [57]. In echinococcosis, when monitoring experimentally infected dogs, LAMP performed in faeces became positive at 22 dpi (vs. 26 dpi using PCR, 25 dpi using copro-ELISA, and 69 dpi using microscopy) for *E. granulosus* [58], and at 12 dpi (vs. 17 dpi using PCR and 44 dpi using microscopy) for *E. multilocularis* [43]. These results are very promising for echinococcosis, for which early detection and treatment can prevent hydatid cysts development. The increased sensitivity achieved using LAMP in comparison to PCR was also reported in the analysis of blood samples of dogs experimentally infected with *P. westermani* metacercariae, allowing three weeks earlier detection by LAMP than PCR (2 weeks p.i. vs. 5 weeks p.i.) [59]. In experimental schistosome-infection animal models, LAMP has been evaluated to detect *S. mansoni* DNA in serum, plasma [60], urine [61], and in stool samples [62,63] from infected mice, as well as DNA of *S. japonicum* in stool, serum [28,64], and blood samples [65] from infected rabbits. In all those studies, schistosome-derived DNA was detected in the acute phase of the infection, before the microscopic detection of parasite eggs in faeces [28,60,62] and even before other diagnostic methods, both immunological [62,64] and molecular [65]. This proves the high sensitivity of LAMP as a tool for monitoring active infections in many different biological specimens and a potential method for early diagnosis of human schistosomiasis.

3.4. LAMP in Clinical Studies

To date, most human clinical studies testing LAMP in parasite-caused NTDs are very limited and only a handful present a relatively large sample size. A selection of the most representative based on their sample size, results, and novelty is summarized in Table 1. It is imperative to carry on large-scale studies to further validate the LAMP technique. Nevertheless, a few studies are worth highlight. An example is the specific detection of *T. b. rhodesiense* using DNA eluted from FTA (Flinders Technology Associates) cards spotted with blood from HAT patients collected in Tanzania [66]. Clinical samples were tested by LAMP targeting RIME [27] and SRA [67] regions (see Figure 4) and by a *T. b. gambiense*-specific PCR. The high level of concordance (98.4%) and agreement (kappa value, 0.85) obtained between RIME-LAMP and SRA-LAMP demonstrated the possibility that either test could be used to reliably diagnose *T. b. rhodesiense*-HAT, needing five times less reaction time than PCR [66]. Another study for HAT diagnosis using RIME-LAMP was conducted in Uganda on blood samples from *T. b. gambiense* HAT patients [68]. RIME-LAMP was compared with other isothermal amplification test, the nucleic acid sequence-based amplification (NASBA) combined with oligo-chromatography (OC) [69]. NASBA-OC showed a significantly higher sensitivity than RIME-LAMP for *T. b. gambiense*, but LAMP needed less equipment and time compared to NASBA-OC.

Several studies using different *Leishmania*-LAMP approaches have widely evaluated the clinical utility of different human samples and different DNA extraction methods for cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and Post-kala-azar dermal leishmaniasis (PKDL) diagnosis in different endemic areas [70–75]. All the *Leishmania*-LAMP assays developed to date have been recently exhaustively reviewed and discussed by Nzelu et al. [76] and Silva et al. [77]. In general, bone marrow, and specially whole

blood samples, achieve the higher sensitivity and specificity values, satisfying WHO standard rates [78].

With regard to soil-transmitted helminthiasis, the largest clinical study has been performed to detect *Necator americanus* in human stool samples comparing Kato-Katz (KK) with LAMP [44]. Comparison of both techniques showed an overall 97% clinical sensitivity for the LAMP assay.

In relation to taeniasis, a LAMP to detect *Taenia solium* was carried out in blood samples from patients suffering from neurocysticercosis (NCC) and results were compared in different clinical situations [79]. Conventional LAMP was adapted into a real-time assay showing higher sensitivity in patients with extra-parenchymal brain cysts (86.7%) than those with intra-parenchymal brain cysts (71.8%), all with high specificity (90.2%). The number of cysts did not significantly affect sensitivity. In addition, a higher sensitivity compared to enzyme-linked immunoelectrotransfer blot (EITB) when performed in patients with single cysts and patients with calcified cysts was recorded, thus showing a high value of the technique in any clinical situation.

Among foodborne trematodiases, LAMP-based assays have been used principally in clinical surveys of clonorchiasis and opistorchiasis. Rahman et al. [80] developed a LAMP for *Clonorchis sinensis* detection which was tested on human stool samples with confirmed infection both by two KK smears and one real-time PCR. The sensitivity and specificity of the LAMP relative to the combined result of KK and real-time PCR resulted 97.1% and 100%, respectively. The only two false-negative results obtained by LAMP had only 12 eggs per gram of faeces (EPG) by KK. This low number of eggs, combined with their heterogeneous distribution in stool, might have resulted in a lack of eggs in the sample portion used for DNA extraction. This slightly lower sensitivity would be tolerable as low-burden infections would rarely result in cholangiocarcinoma if untreated. In exchange, LAMP assay saves a minimum of 2 h to diagnosis and greatly reduce infrastructure required [80]. Additionally, it would also allow clinicians to reduce double-checks needed for an accurate diagnosis, thereby improving control programs and treatment. Concerning opistorchiasis, an *Opisthorchis viverrini*-LAMP was tested using stool samples microscopically selected from schoolchildren in Khon Kaen Province, Thailand. A diagnostic sensitivity of 100% with a proved analytical sensitivity of 1 pg/μL was obtained with LAMP. However, a poor clinical specificity of 61.5% was obtained, suggesting missed eggs by microscopy in light infections or cross-reactions with other organisms. This remains unclear as specificity was not evaluated in the development of this LAMP [36]. Those results showed a clear limitation in the gold standard diagnostic tool (light microscopy) used for comparison as, the differences in sensitivity between techniques lead to a very low specificity value (61.5%), that may not be an accurate representation of the diagnostic value of LAMP in the case of *O. viverrini* diagnosis.

Finally, several studies have evaluated the clinical application of LAMP in diagnosis of human schistosomiasis. LAMP has been applied to detect *Schistosoma haematobium* in field conditions using both purified DNA and heat-treated urine samples in comparison with microscopy in human urine samples collected in an endemic area in Cubal, Angola [81]. The overall prevalence detected by LAMP was significantly higher than microscopy when testing both purified DNA (73.8% vs. 50.6%) and crude urine samples (63.4% vs. 50.6%). The reproducibility of LAMP tests in a well-equipped laboratory decreased especially in crude urine samples, probably because of the inappropriate samples storage over time [81]. Another study evaluated a LAMP to detect *S. haematobium* DNA in urine samples collected from suspected patients for urogenital schistosomiasis attending outpatient clinic in Imbaba Cairo, Egypt. LAMP resulted in a 100% sensitivity and 63.16% specificity when compared with conventional urine filtration followed by microscopy for egg detection [82]. Regarding *S. mansoni* DNA detection in clinical stool samples, a first survey using LAMP was conducted in a low-transmission area in Umbuzeiro, Brazil [55]. Using KK as the reference test, LAMP resulted in an overall sensitivity of 92.86% and 80.11% specificity.

Table 1. Summary of relevant studies of LAMP in parasite-caused NTDs.

Disease	Application [1]				Specimen [2,3]	Clinical Studies			Key Points
	VE	AM	HS	PT		n [4]	Sensitivity	Specificity	
Dracunculiasis	✓	✓	✓	✗	N/A [48]	N/A	N/A	N/A	Test applied in adult worms recovered from humans, not in human specimens.
Chagas	✓	✓	✓	✗	Blood [83–85]	27 [83] 33 [84] 46 [85]	100% 73.9% 93%	100% 100% 100%	Accurate diagnosis in one test, regardless the clinical situation of the patient.
HAT	✓	✓	✓	✗	Blood [25,66,68,86] Buffy coat [87] CSF [87] Bone marrow [87] Sera [57] Saliva [57] Urine [57]	128 [66] 355 [86] 181 [68]	95.3–93.8% 87.3–93% 76.9%	N/T 92.8–96.4% 100%	Non-invasive samples such us saliva and urine useful substitutes of highly invasive CSF or bone marrow. Highly sensitive technique, fitting for the last stages of HAT control and elimination.
Leishmaniasis									
VL	✓	✓	✓	✗		186 [73]	97.6–100%	99.1%	
					Blood [70–74]	55 [72]	96.4%	98.5%	
					Buffy coat [71]	30 [70]	83%	100%	
					Saliva [75]	50 [71]	92.3%	100%	
					Bone marrow [72]	267 [74]	98.3%	96.6%	
CL	✓	✓	✓	✗	Skin [70–72]	43 [70]	98%	100%	
						105 [71]	95%	86%	
PKDL	✓	✓	✓	✗		62 [72]	96.2%	98.5%	
Lymphatic filariasis	✓	✗	✓	✗	Blood [34]	N/A	N/A	N/A	Valuable for molecular xenomonitoring in low-prevalence areas and epidemiological control post-MDA [5].
Onchocerciasis	✓	✗	✓	✗	Skin [88,89]	70 [88] 146 [89]	65.7% 88.2%	N/T 99.2%	
Trichuriasis	✗	✓	✓	✗	Stool [47,90] Urine [90]	137 [47]	77%	88%	Urine might be a viable alternative to stool in epidemiological studies, but further evidence is needed. *Ancylostoma duodenale* does not have a specific LAMP designed yet.
Ascariasis	✗	✗	✓	✗	Stool [45]	40 [45]	96.3%	61.5%	
Uncinariasis	✗	✗	✓	✗	Stool [44]	106 [44]	97%	100%	
Strongyloidiasis	✗	✓	✓	✗	Stool [41,91,92] Serum [91] Broncho alveolar [91] Urine [92,93]	28 [41] 396 [91]	96.4% 77.4%	N/T 100%	

Table 1. *Cont.*

Disease	Application [1]				Specimen [2,3]	Clinical Studies			Key Points
	VE	AM	HS	PT		n [4]	Sensitivity	Specificity	
Echinococcosis	✗	✓	✓	✗	Stool [37,43,58] Hydatid cysts [94]	N/A	N/A	N/A	Good enough performance to avoid resource-demanding imaging techniques. Promising results in early infection detection, key in these diseases prognosis.
Taeniasis	✗	✗	✓	✗	Stool [95,96] Blood [79]	43 [95] 100 [79]	86% 74%	100% 90.2%	
Paragonimiasis	✓	✓	✓	✗	Blood [59] Sputum [35] Pleural fluid [35]	N/A	N/A	N/A	Larger studies with human clinical samples are required. Highly variable analytical sensitivity and specificity results.
Fascioliasis	✗	✓	✓	✗	Stool [97]	N/A	N/A	N/A	
Clonorchiasis	✓	✗	✓	✗	Stool [80]	120 [80]	97.1%	100%	
Opistorchiasis	✓	✗	✓	✗	Stool [36]	50 [36]	100%	61.5%	
Schistosomiasis						50 [28]	96.7%	100%	Consistently shows similar or better performance than the other available diagnostic tools. Sufficient evidence in large clinical studies to start its implementation in public health of endemic and non-endemic regions
S. japonicum	✓	✓	✓	✓	Plasma [60] Serum [28,60,64] Urine [61,81,98,99]	110 [64] 94 [98] 172 [81]	95.5% 100% 86.2%	100% 86.7% N/T	
S. haematobium	✓	✓	✓	✗	Stool [28,55,62,63] Blood [65]	162 [55] 86 [99]	92.9% 100%	80.1 100%	
S. mansoni	✓	✓	✓	✗		383 [63]	97%	100%	
Scabies	✗	✓	✗	✗	Skin [46]	N/A	N/A	N/A	-

[1] VE: vectors; AM: animal models; HS: human studies; PT: post-treatment studies. ✓ indicates that there are studies performed in this category; ✗ indicates there are no studies performed in this category. [2] In this category the parasite and the intermediate host are excluded. [3] Detection methods used were: SYBR Green I [28,35–37,44,45,47,55,57,60–64,66,68,72,74,80,81,83,87,90,92,93,97–99], electrophoresis [25,28,34,35,37,41,44,45,47,55,58,61–63,65,75,79–81,83,87,90,92–99], real-time detection [36,46,48,57,59,60,79,84,88,89,91,94], turbidity [34,57,71,80,88,96], calcein [84,85], hydroxynaphtol blue [36,88,94], fluorescence detection reagent (Eiken Chemical Co., Ltd.) [70,71,73,86], malachite green [75], neutral red [88], SYTO-82 [41], and lateral-flow dipstick [59]. [4] n: sample size. [5] MDA: massive drug administration. N/A: not applicable; N/T: not tested.

3.5. LAMP in Post-Therapy Monitoring

To date, very few studies have evaluated LAMP for monitoring the effectiveness of chemotherapy in parasite-caused NTDs. Stands out the work performed in *Schistosoma japonicum* infections in experimentally infected rabbits [28,64,65] and one in patients [64]. In the study conducted by Xu et al. [28], detection of *S. japonicum* DNA in rabbit sera infected with a high dose of 500 parasite cercariae became negative at 12-week post-infection after praziquantel administration. The results obtained by Wang et al. [65] in detection of *S. japonicum* DNA in experimentally infected rabbits with 200 cercariae following artesunate or praziquantel treatment demonstrated the higher sensitivity of LAMP compared to PCR for evaluation. *S. japonicum* DNA in rabbit sera remained detectable by PCR up 12 and 8 week post-treatment with artesunate and praziquantel, respectively, whereas DNA remained detectable by LAMP up 20 weeks in 50% and 66% of rabbit sera treated with artesunate and praziquantel, respectively. Finally, Xu et al. [64] evaluated the utility of LAMP assay for detection of light infections in experimentally infected rabbits and evaluation of chemotherapy efficacy both in animals and in patients. In this study, rabbits were infected using low infection doses (30 cercariae) and subsequently treated with praziquantel. LAMP could detect *S. japonicum* DNA in sera from infected rabbits as soon as the 3rd dpi and became negative at 10-week post-therapy, thus indicating the utility of LAMP in early diagnosis of light infection schistosomiasis, and in monitoring the effectiveness of treatment. In this same study, LAMP was also evaluated as a tool to assess the response to treatment in 47 patients' sera infected with *S. japonicum* after treatment with praziquantel at 3, 6, and 9 months post-treatment. The parasite DNA in serum was not detected in 31.9%, 61.7%, and 83% at 3, 6 and 9 months post-therapy. IHA and ELISA only reached at nine months a conversion rate of 31.9% and 25.5%, respectively.

These studies indicate that the LAMP technique has potential for monitoring the effectiveness of schistosomiasis treatment. Nevertheless, further studies are needed with other parasite-caused NTDs to determine the usefulness of LAMP in assessing the efficacy of treatment and as a diagnostic tool after preventive chemotherapy campaigns.

4. LAMP as Point-of-Care Test

All parasite-caused NTDs, except dracunculiasis, have at least one available treatment and, access to those drugs, has significantly improved in recent years. However, accurate patient identification is still a major limitation in NTDs management and control, and dramatically contributes to the sustained burden they present worldwide [100]. Likewise, overtreatment or mistreatment, also consequence of a poor diagnostic capacity [101], lead to drug wasting and disease resurgence, respectively. Moreover, over-sustained treatment or under-dosage, which could be avoided using accurate diagnostic tools, could lead to drug resistance. Still, this is not due the lack of new diagnostic tools, rather the inability of those methods to reach low-resource settings. Particularly, molecular tools have not replaced classical methods, although consistently showing better results at the laboratory, being often more sensitive so needed as later stages of control. The challenge of affordable and simple molecular diagnostics development is not new, in 2002 was identified as the most important challenge of biotechnology contributing to improve developing countries health [102]. Since then, numerous techniques have been on the spotlight in parasite infections detection: real-time PCR (2002); LAMP (2003); multiplex ligation-dependent probe amplification (MLPA) (2005); high-resolution melt curve analysis (HRM) (2009); or digital PCR (dPCR) (2016). Despite of all this, none are actually routinely used in field settings [103]. In 2006, the acronym ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable) was proposed by the WHO Sexually Transmitted Diseases Diagnostics Initiative (SDI) as a set of criteria that any diagnostic method must achieve to be considered as a POC test in low resource settings [104]. This term has been recently updated (so-called REASSURED), including: Real-time connectivity and Ease of specimen collection and Environmental friendliness [105] (Figure 5).

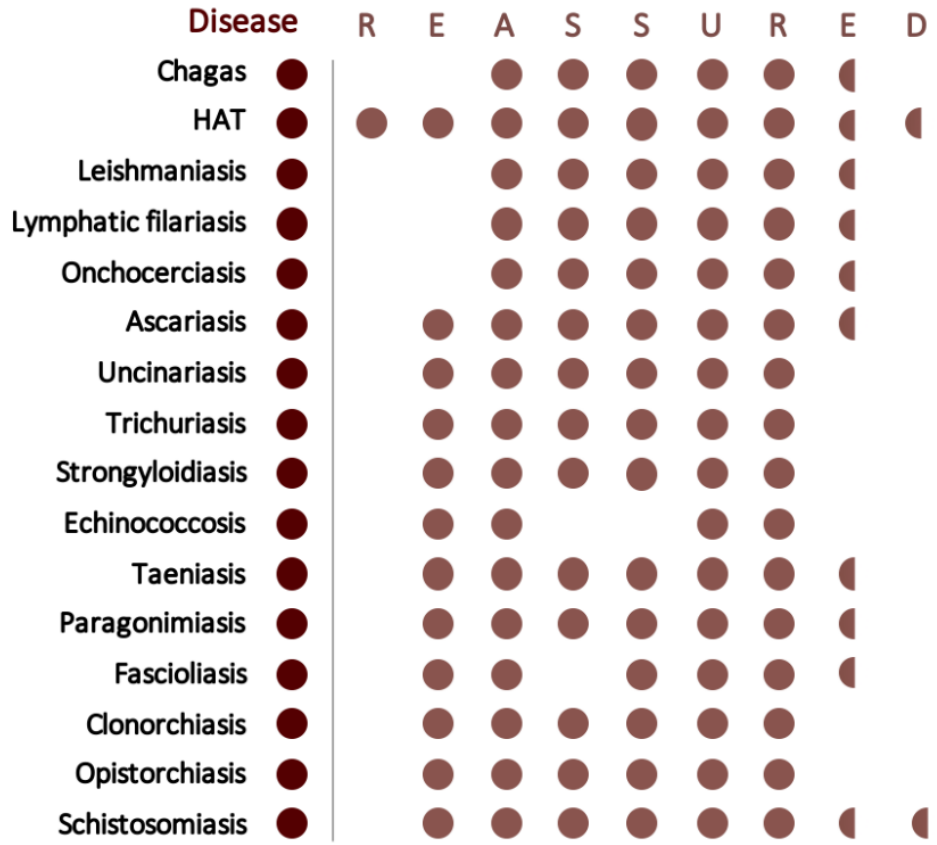

Figure 5. REASSURED criteria fulfillment of designed LAMP methods for the different parasite-caused NTDs. Red dots indicate that a criterion is fulfilled for the corresponding disease. Absence of dot means that criterion is not yet accomplished. Half-dots signify that steps have been taken to achieve those features; however, they are not yet met. An assay is considered sensitive with 75% or more clinical sensitivity. An assay is considered specific if no cross-reaction take place with other human infecting parasites. Dracunculiasis and scabies are excluded of this figure due to lack of sufficient information.

Unfortunately, frequently in tests development, achieving one of these features means trading-off another. When focusing on NAAT, concurrent accuracy, accessibility and affordability are almost never met. Higher accuracy usually leads to a lower accessibility and reduced affordability and vice versa. Thus, NAAT frequently present high accuracy, maybe valid for National Health Care Systems, but poor accessibility and affordability for communitarian and primary health care level [105]. Although LAMP has now become 20 years old [11], recent technological advances, might turn it into an ideal solution to those limitations, notwithstanding the necessary improvements still to be made for its deployment in resource-constrained settings.

4.1. Real-Time Connectivity

Smartphone technology accessibility is increasing exponentially worldwide, with over 60% adoption in 2017, and 70% expected in 2020. Even in Sub-Saharan Africa, where it was close to 40% in 2017, and 60% is expected in 2020 [106]. Smartphone-based diagnostic tests may provide useful applications for NTDs diagnostics in remote areas and greatly facilitate epidemiological surveys. A handheld digital microfluidic device for LAMP (so-called LampPort) with a fully Bluetooth control with a tablet has been recently presented as a proof of concept using *Trypanosoma brucei* as a model [107]. The on-chip detection sensitivity reached 40 DNA copies and endpoint naked-eye visualization was tested adding SYBR Green I. The system greatly reduced post-amplification contaminations but did not completely remove them, so further integration of sample preparation

on-chip is needed [107]. Other smartphone-based LAMP systems have been also evaluated for other tropical diseases, including Zika, Chikungunya, and Dengue viruses [108] and malaria [109]. All these recent developments show a promising future, nonetheless, they should be carefully validated in clinical settings and compared to current diagnostic standards before implementation.

4.2. Ease of Specimen Collection

Two simultaneous requirements need to be met, a non-invasive specimen collection and little-to-non processing of the specimen pre-diagnosis. LAMP has proved to be as sensitive as real-time PCR without any prior nucleic acid purification in a great variety of body fluids (i.e., plasma, blood, urine, saliva, or semen) [110]. This feature has allowed to substitute invasive-specimen collection, such serum for *T. b. gambiense* detection, for non-invasive-specimen collection, such as urine or saliva, without compromising sensitivity of the test [57]. It has also allowed to use alternative specimens, such as urine samples, for molecular diagnosis of intestinal parasites, instead of stool samples, which are harder to handle and store [72,96]. Urine samples have already been successfully evaluated in LAMP assays for schistosomiasis [80,96,97] and strongyloidiasis [93] human diagnosis. Not only the specimen collection has improved, but also the sample processing has been reduced. This is of critical importance since nucleic acid purification is regarded as the primary bottleneck preventing adoption of NAAT outside the well-equipped laboratory [111]. Several approaches combining a simple DNA processing with LAMP have been applied for several parasites-caused NTDs without any need for costly laboratory instrumentation and skilled personnel. One example is the rapid-heat LAMPellet method (RHE-LAMP) for *Schistosoma haematobium* detection in clinical urine samples using only a 15 min 95 °C heat lysis step [98]. For *Leishmania infantum* detection, a simple boil-spin protocol with a rapid centrifugation and incubation at 90 °C has been used to process heparinized blood samples [112]. More sophisticated strategies have focused on incorporating the nucleic acid extraction step into a microfluidic chip, reducing handling and potential contaminations, as is the case for *Schistosoma mansoni* detection [60]. In all, the combination of rapid nucleic acid extraction protocols and LAMP assays have been presented for the successful detection of *T. brucei* [66,113], *Leishmania* spp. [42,75,112], *Onchocerca volvulus* [40], *Taenia* spp. [114], *S. mansoni* [51,55,99,115], and *S. haematobium* [81,98].

4.3. Affordable

No benchmarks are settled on what is considered an affordable diagnostic test; however, $0.5–1 has been accepted for HIV and malaria while up to $10 for tuberculosis [105]. For parasites-caused NTDs, being vastly diseases of the poor, research should target the cheaper end of the spectrum. LAMP technology markedly reduces costs of molecular diagnostic compared to PCR-based methods. Equipment is reduced from an expensive thermocycler to a simple heating block, water-bath, or non-instrument nucleic acid amplification (NINA) heating devices [116]. As stated above, in LAMP assays, nucleic acid extraction can be by-passed thus reducing an important additional cost. Moreover, results detection, can be naked-eye visualized with Mg^{2+} dependent dyes, such as malachite green [117] or hydroxynaphtol blue [118], much cheaper than DNA-binding dyes or molecular probes. Estimates have been made for lymphatic filariasis diagnosis, whose LAMP assay for *W. bancrofti* detection, costs approximately $0.82 (PCR ≈ $2.20) [34]. For schistosomiasis diagnosis, the differences in price are even more significant. While LAMP only costs $0.71–2 per sample, PCR goes up to $6.4–7.7 and even, classical diagnostic techniques, such as, ELISA ($1.5) or KK ($2.00–2.67) are more expensive [119]. However, a bias can be attributed to this estimation as DNA purification is not considered when calculating LAMP pricing.

4.4. User-Friendliness

Two complementary solutions should be mentioned here: colorimetric detection of LAMP results and ready-to-use reaction formats. The colorimetric evaluation allows for untrained personnel to easily interpret the results. Regarding the latter, novel stabilized master mixes for LAMP reactions avoid cold chain maintenance and allow untrained personnel to easily perform the diagnosis. Currently, these "LoopAmp kits" are scarce, but their improvement is an ongoing task in the case of kinetoplastid parasites, including *T. cruzi* [84], *T. brucei* [86], and *Leishmania* spp. [73,112]. In a recent work, our group [120] presented a novel protocol for long-term preservation of LAMP master mixes for *S. mansoni* detection through a simple 30 min one-step protocol based on the use of threhalose as cryoprotectant to produce functional ready-to-use reaction mixes. Another dry-LAMP approaches for schistosomiasis based on a different cryoprotectants (i.e., sucrose) have also been reported [54].

4.5. Rapid and Robust

Since LAMP tests can be performed isothermally for 45–60 min using a wide variety of biological specimens, greater robustness and shorter run times are achieved compared to conventional PCR. The robustness of LAMP reactions has also been enhanced with the improvements in speed, sensitivity, inhibitors tolerance, or stability to enable room temperature set-up of the *Bst* DNA polymerases family (http://www.neb-online.de/wp-content/uploads/2015/04/NEB_isothermal_amp.pdf, accessed on 10 January 2021). Moreover, these novel engineered polymerases are also suitable for ready-to-use formats that enables long-term storage at ambient temperature [121]. Additionally, real-time LAMP assays are significantly faster than PCR, showing results as soon as 16 min for *T. cruzi* [83] or 18 min for *Leishmania* spp. [112] in comparison with 2–4 h employed by conventional PCR.

4.6. Equipment Free

LAMP is not completely equipment-free, but it greatly reduces it. If DNA extraction is avoided, centrifuges can be circumvented all together. Moreover, the use of dry-LAMP protocols for ready-to-use tests allow to maintain all reagents at room temperature until the reaction is performed, thus greatly reducing additional equipment needed in field settings. The two most common strategies to avoid any equipment is the use of microfluidic chips and lateral flow dipstick (LFD). Among the first group, the work of Wan et al. [122] in *T. brucei* for HAT diagnosis stands out. They developed a chip based on low-Tm molecular beacons DNA probes that allows a 10x reduction in reagent consumption, with a LAMP reaction time of 40 min and a sensitivity of 10 copies. Unfortunately, clinical studies for most of these methodologies are still lacking. A LFD, classically used in serological tests, can be combined with LAMP technology. Once again, an example is the combination of RIME-LAMP for the detection of *T. brucei* with a LFD. The LFD-RIME-LAMP is based on specific labeling F1c and B1c primers with a fluorescein isothiocyanate (FITC) and a biotinalyted molecular probe. Using LFD-RIME-LAMP in clinical samples, *T. brucei* was detected in both bone marrow and CSF [123]. LAMP has also been combined with a LFD for the detection of *P. westermani* DNA [59]. Other approaches have been tested under field settings as equipment-free solutions for LAMP assays, including non-instrumented nucleic acid amplification (NINA) devices for the detection of filarial parasites DNA [124] or the use of a simple kettle for *Taenia* spp. DNA detection [114].

4.7. Deliverable to End-Users

Despite all the recent advances above-mentioned, no significant changes in current diagnostic protocols for parasites-caused NTDs have included LAMP yet. The technique has been available since 2000 [11] and has not yet broken the barriers of true POC testing. In fact, neither have any of the other NAAT. It is worth highlighting that in the Report of the first meeting of the WHO Diagnostic Technical Advisory Group for Neglected Tropical Diseases, in 2019, LAMP was a recommended current diagnostic technique for HAT and schistosomi-

asis, and the need for POC nucleic acid amplification diagnostic methods was acknowledge for echinococcosis, foodborne trematodiases, and taeniasis/cysticercosis [125]. This might have an effect in the near future in the deployment of LAMP and other molecular assays to the field.

Currently, LAMP presents a number of limitations that need to be acknowledge: non-applicable for cloning, primer design is subject to more constrains than other NAAT, high risk of carry-over contamination, and multiplexing approaches for multiple pathogen detection are highly complex and poorly still developed [126]. Furthermore, the lack of an internal control to rule out extraction failures and evaluate the presence or absence of inhibitors in the sample is a crucial limitation. This is a must in RT-qPCR commercial kits; however, most LAMP kits, whether home-made or commercial, do not include them. The reason behind it is probably the primer design complexity and the lack of a standardized technology to multiplexing LAMP assays.

There are other limitations that affect the deployment of new diagnostic assays that are not directly related with the technique itself. As discussed above, the limited investing in diagnostic development, only 5% of the NTDs research founding, that has also decreased overall (10% over the last 10 years) [8], significantly hamper any kind diagnostic improvement. Additionally, in the absence of massive infrastructure or health care facilities development, NTDs control campaings require integrated approaches, that are often complex and chaotic, and thus attract less funding and political actor investment than a new vaccine or a theoretically perfect diagnosis. Local programs should be designed and performed by locals and, while program managers often based their strategies in the WHO or other international entities, there is sometimes need for tailor-made solutions. In the case of diagnostics, cost-effectiveness of a particular assay can only be addressed at a local level [127].

Specifically addressing LAMP, the multiplexing drawback is very concerning, since co-infections are frequent in endemic regions and often times obscure clinical diagnosis. On the bright side, some multiplex-LAMP (mLAMP) approaches are beginning to appear for detection of parasite-caused NTDs. An example is combining mLAMP with dot enzyme-linked immunosorbent assay (dot-ELISA) for discrimination of the three human *Taenia* species by labelling species-specific FIP primers with fluorescein isothiocyanate (FITC), digoxigenin (DIG), and tetramethylrhodamine (TAMRA), and BIP primers labelled with biotin [128]. Another interesting multiplexed approach is a two-stage isothermal amplification method in a microfluidic format that consist of a dubbed rapid amplification (RAMP) first stage follow by a second-stage LAMP assay. This assay has been designed in a 16-plex, 2-stage RAMP assay to concurrent detection in 40 min up to 16 different targets of DNA and RNA from different pathogens, including helminths such as *S. mansoni*, *S. hematobium*, *S. japonicum*, *Brugia malayi*, and *Strongyloides stercoralis*. This multiplexed assay could provide healthcare personnel in endemic areas with a molecular tool to detect multiple pathogens in a single sample without a need to send the sample to a reference laboratory [115].

Thus, there is a clear need to share knowledge within the diagnostics field in the developing world to facilitate the development and deployment of the latest molecular tools, that can be extremely valuable for improving global health [101].

5. Conclusions

Parasite-caused NTDs are the most predominant and still representing a major Public Health concern in many developing countries, with the highest rates of disease burden, particularly lymphatic filariasis and foodborne trematodiases. The need for novel fast, affordable, specific, sensitive, robust, and easy to use diagnostic tools is only increasing to support the efforts towards control and elimination. Theoretically, INAAT fulfills all these needs, but to date only LAMP has been used in all parasite-caused NTDs. For NTDs caused by protozoa, LAMP has been particularly promoted in recent years. "Loopamp amplification prototype kits" in a ready-to-use format are available for Chagas disease,

leishmaniasis, and HAT. In this regard, an optimistic future is upon us. In general, studies performed on clinical evaluation of LAMP for parasite-caused NTDs due to helminths show highly variable sensitivity and specificity, according to the parasite species and type of samples analyzed. Particularly, for intestinal helminths infections diagnosis and urogenital schistosomiasis, is very difficult to determine the sensitivity and specificity of LAMP assays due to the lack of a true "gold-standard" against which to compare, considering the low sensitivity of stool-based microscopic methods or urine filtration methods, routinely used in field surveys. Taking into account the potential effectiveness of the LAMP assays in helminths-derived DNA detection in urine, as well as its easier handling, processing and storage in low-resource settings, the use of patients' urine samples would be a good alternative approach for helminths molecular detection. Until now, all of the studies about LAMP for parasite-caused NTD diagnosis agree that the clinical application of LAMP technology should only be considered as a pilot test. We expect that the new supportive technology (such as LFD, microchips, lab-on-chips, portable fluorometers, or smartphone apps) will help to meet the proposed REASSURED criteria, allowing LAMP to reach those who need it most. Additionally, sustained and targeted funding and political support are needed to validate and implement the technique. Overall, the current merits of LAMP technology outweigh its disadvantages.

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1.4.

Loop-mediated isothermal amplification in schistosomiasis

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RESUMEN

En la actualidad, la esquistosomosis es la NTD con mayor prevalencia, y sobre la que más ha avanzado la tecnología LAMP para su diagnóstico. Nuestro grupo de investigación lleva años trabajando en el desarrollo de ensayos LAMP para la detección de diferentes especies de esquistosomas. Por tanto, es un punto de partida idóneo para tratar de adaptar el método LAMP al diagnóstico de esta enfermedad "a pie de paciente". Así, en esta sección se incluye el artículo de revisión "*Loop-mediated isothermal amplification in schistosomiasis*", que detalla en profundidad los últimos avances realizados.

La técnica Kato-Katz sigue siendo el diagnóstico de referencia para la esquistosomosis intestinal. Sin embargo, las técnicas moleculares disponibles, y particularmente la tecnología LAMP, presentan numerosas ventajas. En primer lugar, permiten alcanzar una sensibilidad muy superior a la de la técnica de referencia. Esto se debe, en parte, a una selección de secuencias diana adecuada que se centra en las regiones mitocondriales y nucleares del genoma altamente repetidas. Así, se obtiene un proceso de amplificación exponencial de alto rendimiento. Además, la tecnología LAMP ha sido utilizada con éxito para la monitorización de la infección de hospedadores intermediaros, mostrándose capaz de detectar un único caracol infectado entre mil no infectados. Por tanto, podría ser una técnica valiosa no solo desde el punto de vista diagnóstico, sino también eco-epidemiológico. Además, la técnica de Kato-Katz requiere de muestras de heces, habitualmente difíciles de obtener, almacenar y transportar apropiadamente. Se ha demostrado que el uso de otras muestras más sencillas de obtener, como la orina, puede ofrecer resultados sensibles mediante LAMP tanto en modelos animales como en muestras humanas, no solo para *Schistosoma haematobium* sino también para *S. mansoni.*

Aunque los estudios clínicos disponibles son aún limitados, se han evaluado métodos LAMP para detectar la infección humana por las tres principales especies causantes de la esquistosomosis: *S. mansoni, S. haematobium* y *S. japonicum*. De manera consistente, la tecnología LAMP ha demostrado una elevada sensibilidad y especificidad, superior en algunos casos a la de la PCR y a la de ensayos serológicos mediante ELISA (del inglés, *Enzyme linked immunoabsorbent assay*) e IHA (del inglés, *Indirect haemagglutination assay*). Además, a diferencia del resto de NTDs causadas por parásitos, la tecnología LAMP se ha utilizado para evaluar la eficacia del tratamiento con praziquantel, tanto en modelos animales como en humanos. Estudios en *S.*

japonicum demostraron el potencial de la técnica para monitorizar el éxito del tratamiento, tanto en fases agudas como crónicas de la enfermedad. Sin embargo, la diferencia entre la detección de ADN residual en muestras biológicas y la presencia y viabilidad de entidades biológicas completas como huevos o gusanos sigue siendo objeto de debate.

En este contexto, la esquistosomosis se presenta como el modelo ideal para aplicar la tecnología LAMP como diagnóstico a pie de paciente. Además, existen ejemplos en la literatura que demuestran la posibilidad de combinar su utilización con métodos de extracción rápida de ácidos nucleicos. Nuestro grupo ha demostrado que el simple calentamiento de muestras clínicas de orina a 95ºC durante 15 minutos es suficiente para realizar un diagnóstico mediante LAMP sensible de la infección por *S. haematobium*. También ha confirmado ser más asequible económicamente que el Kato-Katz, el ELISA o la PCR. Nos queda afrontar retos reales como la limitada capacidad que tenemos en este momento de detectar simultáneamente varios patógenos, esencial en zonas endémicas donde las coinfecciones son la norma. La aparición de especies híbridas que pudieran limitar la sensibilidad y especificidad de la técnica es también un reto que debemos afrontar.

Ya sea como técnica única o como complemento a otras técnicas de diagnóstico a pie de paciente, con el análisis realizado en esta revisión podemos concluir que la técnica LAMP sería una herramienta de gran valor para incorporarla en el diagnóstico de la esquistosomosis. Para poder hacerlo de manera fiable, todavía son necesarios estudios a gran escala que confirmen los resultados obtenidos en ensayos preliminares. También se requiere de una mejora en la tecnología suplementaria como son: la estabilización de reactivos, el uso de dispositivos portátiles, la conectividad a tiempo real con los laboratorios de referencia, la facilidad de manejo o la conservación y el transporte de las muestras.

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Review

Loop-Mediated Isothermal Amplification in Schistosomiasis

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Abstract: Human schistosomiasis is one of the most important parasitic diseases, causing around 250 million cases (mostly in Africa) and 280,000–500,000 deaths every year. Due to the limited resources and the far-removed nature of many endemic areas, the implementation of new, sensitive and specific diagnostic tools has had little success. This is particularly true for PCR-based molecular methods that require expensive equipment and trained personnel to be executed. Loop-mediated isothermal amplification (LAMP) along with other isothermal techniques appeared in the early 21st century as an alternative to those methods, overcoming some of the aforementioned limitations and achieving a more inexpensive diagnostic. However, to this date, neither LAMP nor any other isothermal technique have signified a meaningful change in the way schistosomiasis diagnosis is routinely performed. Here, we present the recent developments in LAMP-based schistosomiasis diagnosis. We expose the main advantages and disadvantages of LAMP technology over PCR and other classical diagnostic methods focusing in various research approaches on intermediate hosts, animal models and patients. We also examine its potential clinical application in post-therapy monitoring, as well as its usefulness as a point-of-care test.

Keywords: schistosomiasis; LAMP; diagnosis; point-of-care; neglected tropical diseases; molecular diagnostics

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1. Introduction

Human schistosomiasis is the most important helminthic Neglected Tropical Disease (NTD), causing significant morbidity and mortality [1]. It is a chronic waterborne parasitic disease caused by several flatworm (blood flukes) trematodes of the genus *Schistosoma*. It is transmitted in 78 countries with over 250 million estimated cases (mostly in Africa), around 280,000–500,000 deaths every year, and a DALYs index of 3.3 million per year [2]. The three most widespread and clinically impactful *Schistosoma* species affecting humans are *Schistosoma haematobium* (Africa and the Middle East), *S. mansoni* (Africa, South America, the Middle East and the Caribbean), and *S. japonicum* (Indonesia, China, Southeast Asia and the Philippines). Moreover, there are four additional *Schistosoma* species able to infect humans, which present a lower prevalence: *S. mekongi*, *S. guineensis*, *S. intercalatum* and *S. malayensis* [3]. Additionally, the hybridization between *Schistosoma* species is an increasing concern [4]. Schistosomiasis is acquired by contact with freshwater contaminated with cercariae penetrating the skin that are disseminated by specific intermediate-host snails [1,3]. Transmission typically occurs in tropical and subtropical regions (80–90% of cases in sub-Saharan Africa). Nevertheless, recent outbreaks caused by schistosome hybrids in the south of Europe have proved the reemergence potential of the disease in temperate regions [5,6]. Clinically, three stages are typically distinguished: cercarial dermatitis, acute and chronic schistosomiasis. *Cercarial dermatitis* is due to skin penetration of cercariae, which cause a maculopapular pruritic reaction that lasts for 24–48 h. This is

notably more common among migrants or travelers than residents in endemic areas [7]. *Acute schistosomiasis* (Katayama fever) usually occurs 2–4 weeks after infection, mostly in naive individuals, with rapid fever, fatigue, myalgia, malaise and eosinophilia, a consequence of a hypersensitivity reaction to the migrating schistosomulum. Rarely observed in people in schistosomes-endemic areas [8]. Finally, *chronic schistosomiasis* (months or years after infection) results from the granulomatous reaction around eggs trapped in the tissues [1]. "Classic" manifestations are species-dependent, being mainly intestinal and hepatic symptoms (*S. mansoni*, *S. japonicum*, *S. mekongi and S. intercalatum*) except in *S. haematobium* infections, which cause urogenital symptoms [1,2,9]. Additionally, *S. haematobium* chronic infection has been strongly correlated with bladder squamous cell carcinoma [10]. Ectopic lesions can also occur, and organs affected by ectopic granulomas include the central nervous system, genital organs, skin and eyes [11].

Schistosomiasis diagnosis requires different methods in acute and chronic infections. An active *Schistosoma* infection is definitively diagnosed by microscopic examination of excreted eggs in stool (*S. mansoni, S. japonicum, S. intercalum, S. guineensis* and *S. mekongi*) by Kato-Katz thick smear (KK), or in urine (*S. haematobium*) by filtration or sedimentation techniques. Acute infections, particularly those of low intensity, are frequently missed by microscopy [1,3]. A large number of serological approaches detecting antigens or antibodies have been widely evaluated in endemic areas, for imported or travel-related schistosomiasis, for epidemiological studies and control programs. However, significant differences in sensitivity and specificity exist, aggravated by a lack of standardization [12,13]. Numerous PCR-based assays have also been employed both in the diagnosis of animal and human schistosomiasis [14], being especially valuable in simultaneous detection and identification of *Schistosoma* species [15]. Despite being highly sensitive and accurate, the complex PCR-based methods for schistosomiasis are more difficult to adapt to field studies. In this context, isothermal nucleic acid amplification tests (iNAAT), particularly LAMP technology, much more suited for limited-resource settings, have represented a very promising alternative. Here, we present an overview highlighting the most relevant research performed with LAMP technology for schistosomiasis diagnosis. We discussed its application in different fields, from epidemiological surveys to clinical practice, including studies in intermediate hosts, in animal models and in humans, as well as its potential as a tool to assess schistosomiasis treatment success. We also discuss its role in the development of true point-of-care diagnostics for schistosomiasis, exposing both its advantages and disadvantages and pointing out the steps that are still needed to move forward.

2. Loop-Mediated Isothermal Amplification (LAMP)

LAMP technique was first introduced by Notomi et al. in 2000 [16] and, to date, it is the most widely used iNAAT among alternatives to PCR-based technologies. Briefly, LAMP assay is a one-step amplification reaction that amplifies a target DNA or RNA under isothermal conditions (60–65 °C) employing a DNA polymerase (*Bst* polymerase) with strand-displacement activity, along with two inner primers (FIP, BIP; forward and backward inner primers) and two outer primers (F3, B3; forward and backward outer primers) which specifically recognize six separate regions within the target. Shortly after LAMP description, Nagamine et al. [17] reported a major improvement by adding extra LAMP loop primers (LF, LB; loop-forward and loop-backward primers), thus shortening the reaction time by around 30 min. Hence, a six-primer design can be used in LAMP reactions, targeting in up to eight different regions, compared to only two in typically PCR-based methodology. Another important feature of LAMP is the numerous approaches towards result detection, including gel electrophoresis with characteristic amplicon ladder-like pattern [16], naked-eye monitoring of turbidity caused by the precipitation of magnesium pyrophosphate [18,19] as well as end-point detection by the addition of colorimetric dyes, either post-amplification [20] or pre-amplification [21–24]. The latest advances in end-point detection and in real-time monitoring of LAMP have been recently reviewed by Zhang et al. [25]. Compared to other molecular techniques, the simplicity of LAMP makes

it ideal for field-testing in developing countries [18,21]. In recent years, a great variety of approaches have been used to further ensure that LAMP becomes a field-friendly tool, including lateral flow dipsticks and lab-on-chip layouts [26], microfluidic-based methods [27], combination with metallic nanoparticles [28], control through smart phone-based technology [29] and within a red blood cell [30].

3. LAMP and Schistosomiasis

A number of LAMP assays have been designed for the species-specific detection of the three main species causing human schistosomiasis (*S. haematobium*, *S. japonicum* and *S. mansoni*) and have been applied to schistosomiasis diagnosis, to detect schistosomes-infected snails, and to evaluate efficacy of chemotherapy, both in animal models and human patients. Recently, a LAMP for species-specific detection of the most important veterinary species (*S. bovis*) and a LAMP for simultaneous detection of different *Schistosoma* species have been also reported [31]. The selected molecular targets mostly used for LAMP designing for the detection of *Schistosoma* species are shown in Figure 1, and different assays features are summarized in Table 1.

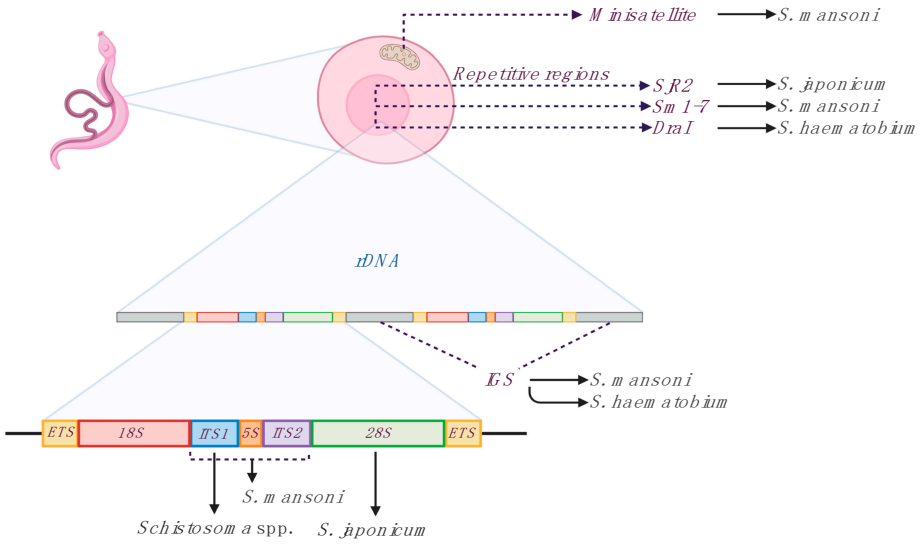

Figure 1. Main targets used for loop-mediated isothermal amplification (LAMP) designing for detection of *Schistosoma* species. Three groups of sequences are the most used for primer sets designing: mithocondrial sequences, repetitive nuclear sequences and ribosomal nuclear sequences (rDNA). Minisatellite; mithocondrial minisatellite sequence. Repetitive regions; SjR2, *S. japonicum* non-long terminal repeat retrotransposon; Sm1–7, *S. mansoni* 121 bp tandemly arranged repeated sequence; DraI, *S. haematobium* 121 bp tandemly arranged repeated sequence. Ribosomal nuclear sequences (rDNA); IGS, intergenic spacer sequences; ITS1 and ITS2, internal transcribed spacers; 5S, small subunit of ribosomal RNA; 18S, 28S, major subunits of ribosomal RNA; ETS, external transcribed spacers. Sm1–7 and DraI targets are here represented as species-specific, although they are considered more group-specific [32]. Figure created with BioRender software (https://biorender.com/).

3.1. LAMP in Schistosomes Infected Snails

To evaluate the efficiency of LAMP detecting schistosomes-infected snails, several experimental infections of snails of the genus *Oncomelania* (*S. japonicum*) [33], *Bulinus* (*S. haematobium*) [34] and *Biomphalaria* (*S. mansoni*) [34,35] have been carried out. In all cases, LAMP could detect schistosomes DNA during the early prepatent phase of infection (as soon as one day after exposure to one miracidium), ergo, before cercarial release, in both individual snails and pooled samples (up to 1 infected snail within 1000 non-infected snails [36]. LAMP has also been evaluated for the detection of *S. japonicum*,

S. haematobium and *S. mansoni* in large-scale screening of pooled field-collected snails to evaluate schistosomiasis transmission, especially in low-prevalence areas [36–40]. In general, the studies performed in the intermediate host reveal that LAMP can be used as a rapid, sensitive, and inexpensive tool to screen large numbers of intermediate hosts compared to other molecular methods. Moreover, the usefulness of the tool to indentify transmission foci and build infection-risk maps has been shown for both *S. mansoni* [39] and *S. japonicum* [33,36]. Thus, the application of the technique could support schistosomiasis control campaigns.

3.2. LAMP in Schistosomes Experimental Infections

Animal models have profoundly contributed to the design and evaluation of LAMP assays for schistosomiasis, particularly for *S. mansoni* and *S. japonicum*. They have allowed the testing of the performance of the assays in different specimens as well as the sensitivity of the diagnosis along the course of the infection. In the case of *S. mansoni*, LAMP has been used to detect cell-free DNA (cfDNA) in infected mice in serum, plasma [41], stool samples [42,43] and urine [44]. For *S. japonicum*, cfDNA has been detected in stool, serum [45,46] and blood samples [47], obtained from infected rabbits. For both species, cfDNA from the parasite was detected in the acute phase of the infection, before Schistosoma eggs were detected in faeces [41,42,45] and even before immunological [42,46] or molecular [47] methods. Therefore, LAMP has shown a high potential as an early-detection diagnostic tool, as well as a sensitive diagnostic method for active infections.

Table 1. LAMP assays for *Schistosoma* species detection and their clinical application.

Assay Features					Clinical Application				
Species [1]	**Target** [2]	**Detection** [3]	**Sensitivity** [4]	**Specimen** [5]	***n*** [6]	**Sensitivity (LAMP+/Ref+)**	**Specificity (LAMP−/Ref−)**	**Ref. Diag** [7]	**Ref.**
S spp.	*ITS-1*	SGI/Gel	0.1 pg *Sh* 1 pg *Sm/Si* 10 pg *Sb*	gDNA	N/A	N/A	N/A	N/A	Fernández-Soto et al. [31]
Sh	*DraI*	SGI/Gel	0.1 fg	gDNA, snails	N/A	N/A	N/A	N/A	Abbasi et al. [34]
	DraI	SGI/Gel	N/D	Snails	N/A	N/A	N/A	N/A	Hamburguer et al. [37]
	IGS	SGI/Gel	1 fg	gDNA, Urine	94	100% (18/18)	86.7% (68/76)	Micro	Gandasegui et al. [48]
	DraI	SGI/Gel	N/D	Urine	86	100% (72/72)	100% (14/14)	PCR	Lodh et al. [49]
	IGS	T/SGI/Gel	N/D	Urine	172	86.2% (75/87)	N/D	Micro	Gandasegui et al. [50]
Sm	*Sm1–7*	SGI/Gel	0.1 fg	gDNA, Snails	N/A	N/A	N/A	N/A	Abbasi et al. [34]
	Sm1–7	SGI/Gel	N/D	Snails	N/A	N/A	N/A	N/A	Hamburguer et al. [37]
	Mito	SGI/Gel	1 fg	gDNA, mice stool	N/A	N/A	N/A	N/A	Fernández-Soto et al. [42]
	Sm1–7	RT-EG	0.5 fg	gDNA, mice blood, serum	N/A	N/A	N/A	N/A	Song et al. [41]
	28S-18S	SGI/Gel	0.1 fg	gDNA, snails	N/A	N/A	N/A	N/A	Gandasegui et al. [35]
	Sm1–7	SGI/Gel	N/D	Urine	86	100% (81/81)	100% (5/5)	KK, PCR	Lodh et al. [49]
	ITS	SGI/Gel	70 fg	gDNA, snails	N/A	N/A	N/A	N/A	Caldeira et al. [38]
	Mito	SGI/Gel	N/D	Snails, stool	162	92.86% (12/13)	80.11% (112/149)	KK	Gandasegui et al. [39]
	Sm1–7	SGI/Gel	32 fg	gDNA, stool	383	97% (166/171)	100% (207/207)	KK	Mwangi et al. [43]
	Sm1–7	SGI/Gel		Urine	111	100% (97/111)	100% (14/14)	KK, PCR	Price et al. [51]
	Mito	RT-EG/SGI/Gel	0.01 fg/µL	Urine	28	71.4% (5/7)	71.4% (15/21)	KK	Fernández-Soto et al. [44]
	Mito	RT-EG/SGI/Gel	N/D	gDNA, hepatic, skin, appendix	N/A	N/A	N/A	N/A	García-Bernalt Diego et al. [52]

Table 1. *Cont.*

Assay Features					Clinical Application				
Species [1]	**Target** [2]	**Detection** [3]	**Sensitivity** [4]	**Specimen** [5]	***n*** [6]	**Sensitivity (LAMP+/Ref+)**	**Specificity (LAMP−/Ref−)**	**Ref. Diag** [7]	**Ref.**
Sj	*SjR2*	SGI/Gel	0.08 fg	gDNA, serum	50	96.7% (29/30)	100% (20/20)	Micro, PCR	Xu et al. [45]
	28S	T/Gel	100 fg	gDNA, snails	N/A	N/A	N/A	N/A	Kumagi et al. [33]
	SjR2	Gel	0.1 fg	gDNA, rabbits blood	N/A	N/A	N/A	N/A	Wang et al. [47]
	28S	Calcein	100 fg	gDNA, snails	N/A	N/A	N/A	N/A	Tong et al. [36]
	SjR2	SG I	N/D	Human, rabbits serum	170	95.5% (105/110)	100% (60/60)	KK	Xu et al. [46]
	28S	Calcein	N/D	Snails	N/A	N/A	N/A	N/A	Qin et al. [40]

[1] Species: *S* spp. (*Schistosoma* spp.) *Sh* (*S. haematobium*) *Sm* (*S. mansoni*) *Sj* (*S. japonicum*), [2] Mito: mitochondrial minisatellite. For all other abreviations see Figure 1, [3] SG I: SYBR Green I; Gel: Electrophoresis; T: Turbidity; RT-EG: Real-time EvaGreen fluorescence detection, [4] *Si: S. intercalatum; Sb: S. bovis,* [5] gDNA: parasite genomic DNA, [6] *n*: sample size, number of patients (when applicable, [7] Ref. diag: Technique used as diagnostic reference. Micro: Microscopy; KK: Kato-Katz technique, N/A: Non-applicable; N/D: Non-disclosed.

3.3. LAMP in Human Schistosomiasis Diagnosis

A number of studies have been conducted to evaluate the clinical application of LAMP in the diagnosis of human schistosomiasis. Different human samples have been tested and performance has been compared with various diagnostic methods. Two studies conducted by Xu et al. [45,46] have applied LAMP to serum samples from people infected with *S. japonicum* (determined by KK), living in endemic areas in Hunan Province, China. In a first study, to evaluate LAMP, 50 serum samples, including 30 *S. japonicum*-positive and 20 *S. japonicum*-negative controls from healthy individuals, were analysed and a 96.7% sensitivity compared to 60% by PCR was obtained [45]. Later, in a second study, 110 patient serum samples *S. japonicum*-positive were also analysed by LAMP and compared to ELISA and indirect hemagglutination assay (IHA) techniques. The sensitivity and specificity of LAMP resulted in 95.5% and 100%, respectively, whereas sensitivity and specificity of ELISA and IHA was 84.6% and 85.7%, and 91.8% and 88.1%, respectively [46]. These differences must be considered in the light of the different objectives of the diagnostic techniques in relation to the different clinical states of the disease. While serological techniques may indicate a past infection (or indirectly an active infection), molecular diagnostics target active infections (or at least residual DNA), thus being much more sensitive in acute states. In both studies, serum samples from people living in non-endemic areas of *S. japonicum* infections were used to determinate the high specificity obtained with LAMP. However, despite being people from non-endemic schistosomiasis areas, antibody detecting methods (ELISA and IHA) showed cross reactivity and lower specificity than LAMP assay [45,46]. On the other hand, 10/60 (16.7%) residents in endemic areas of schistosomiasis recognized as "healthy" people (*S. japonicum*-negative by KK, ELISA, and IHA) were diagnosed as LAMP-positive, suggesting that classical methods may lack sensitivity for low-intensity infections diagnosis [46]. Those results could also imply an imperfect specificity, although this reasoning is weakened by the lack of cross-reactivity in non-endemic subjects. LAMP has been also evaluated in the clinical determination of *S. mansoni* in stool [39] and urine samples [44,49,51], as well as *S. haematobium* in urine samples [48–50,53]. Regarding *S. mansoni* DNA detection in clinical stool samples, a first survey using the so-called SmMIT-LAMP was conducted in the low-transmission area of Umbuzeiro, Brazil [39]. Considering the parasitological findings by KK as reference, the SmMIT-LAMP resulted in an overall sensitivity of 92.86% and 80.11% specificity with a negative predicted value (NPV) of 99.33% but a scarce 26% positive predicted value (PPV). The percentage of false negative registered in this study can be partly explained with the greater sensitivity of SmMIT-LAMP over the classical KK technique, especially in those patients with a low egg-count in areas of low *S. mansoni* transmission [39]. In large-scale field trials, collecting stool samples to diagnose intestinal schistosomiasis can

be extremely laborious and urine samples have been proposed as a good alternative as a source of cfDNA, due to their better handling and storage. In this sense, several studies have demonstrated the detection of *S. mansoni* cfDNA in human urine samples by different LAMP assays, including the one based on the 121 bp Sm1–7 repeated sequence in filtered urine samples field-collected in Ghana [49] and Zambia [51] and other using the SmMIT-LAMP in long-term frozen patients' urine samples [52]. Consequently, the combination of the high efficiency of LAMP with urine samples could be suitable to use not only for well-equipped laboratories, but also for poor-resource laboratories in *S. mansoni*-endemic areas. Additionally, SmMIT-LAMP was also successfully used on a skin biopsy as a real-time LAMP assay to confirm ectopic cutaneous schistosomiasis caused by *S. mansoni*, a proof of concept of the usefulness of LAMP in acute schistosomiasis diagnosis [54].

The microscopic detection of excreted ova in urine samples remains the 'gold standard' diagnostic method for *S. haematobium* infection despite its low sensitivity, high day-to-day variability and inefficacy in the acute stage of the disease. With the aim to solve this, a LAMP assay specifically designed for the detection of *S. haematobium* in patients' urine samples was first developed by Gandasegui et al. in 2015. Compared to microscopy (69.2%), LAMP resulted in a higher sensitivity (86.7%); moreover, the simple heating of urinary pellets for DNA purification (the Rapid-Heat LAMPellet method) was effective to detect *S. haematobium* through LAMP in several urine samples with confirmed infection [48]. Later, this LAMP procedure was applied under field conditions using both purified DNA and heat-treated urine samples in comparison with microscopy in 172 human urine samples collected in a schistosomiasis-endemic area in Cubal, Angola [50]. The overall prevalence detected by LAMP was significantly higher than microscopy when testing purified DNA (73.8% vs. 50.6%), even when testing crude urine samples (63.4% vs. 50.6%). Nevertheless, the reproducibility of LAMP tests in a well-equipped laboratory only reached 72.1% and 49.5% of coincidences in DNA or crude urine, respectively. Test performance, especially in crude urine samples (usually containing many inhibitors which may interfere in DNA amplification), was probably affected by inappropriate sample storage (suffering from numerous freezing and thawing cycles) resulting in deterioration of DNA over time [50]. In a study conducted by Lodh et al. [49] in a schistosomiasis-endemic area of Ghana, LAMP was also used to detect *S. haematobium* in urine samples using two different DNA extraction methods, standard extraction kit and field usable LAMP-PURE kit. Urine samples were collected from 86 individuals with no previous parasitological examination for *S. haematobium* and were evaluated by LAMP and PCR. LAMP amplification for both extractions showed similar sensitivity (72/86; 84%) when compared with PCR (70/86; 81%) showing that LAMP for detecting *S. haematobium*-specific DNA is an effective diagnostic tool, equal to PCR amplification [49]. Another study evaluated a LAMP method based on ribosomal IGS DNA to detect *S. haematobium* in 69 urine samples collected from suspected patients for urogenital schistosomiasis attending outpatient clinic in Imbaba Cairo, Egypt. LAMP resulted in a 100% sensitivity and 63.16% specificity when compared with conventional urine filtration followed by microscopical egg detection [53].

3.4. LAMP to Evaluate Treatment Success

The evaluation of schistosomiasis treatment success with diagnostic tools has been traditionally very limited, due to the low sensitivity of parasitological methods, particularly in light infections, and the limited value of antibody-based immunological methods. On the other hand, serum-PCR methods have shown their usefulness to evaluate treatment success on a long-term basis (1-year after initial treatment), not in early treatment monitoring [55]. In this sense, LAMP assays have been used to evaluate the efficacy of chemotherapy in schistosomiasis. However, studies are still limited to *S. japonicum* infections, performed both in experimentally infected animals [45–47] and in patients [46]. In a first study, Xu et al. [45] studied praziquantel treatment in rabbits experimentally infected with a high dose of *S. japonicum* (500 cercariae). They showed that LAMP become negative after 20 weeks post-infection (12-week post-treatment), two weeks longer than with PCR. Subse-

quently, Wang et al. [47] tested the value of LAMP to evaluate treatment success in lighter *S. japonicum* experimentally infected (200 cercariae) rabbits. In this case, both artesunate and praziquantel treatment were evaluated. Again, LAMP showed a higher sensitivity than PCR, detecting *S. japonicum* DNA in rabbit sera up to 20 weeks post-treatment in 50% and 66% of the cases, treated with artesunate and praziquantel, respectively. PCR was able to detect *S. japonicum* DNA only up to 12 and 8 weeks post-treatment with artesunate and praziquantel, respectively [47]. Finally, in an extension of their previous work, Xu et al. [46] also evaluated LAMP effectiveness for the detection of light infections in experimentally infected rabbits pre- and post-treatment and also in chemotherapy efficacy in human patients. On the one hand, rabbits were infected with 30 *S. japonicum* cercariae and subsequently treated with praziquantel. LAMP could detect *S. japonicum* DNA in sera from infected rabbits at the third day post-infection and became negative at 10 weeks post-therapy, showing the usefulness of LAMP in early diagnosis of light infections and also in treatment success evaluation. ELISA and IHA techniques were used to compare and assess LAMP results. As expected, they were not useful for early diagnosis (ELISA and IHA gave positive results at 5 and 4-weeks post-infection, respectively) and, anti-schistosome antibodies were detected by both techniques during 23 weeks after treatment [46]. On the other hand, treatment efficacy was evaluated in 47 patients' sera infected with *S. japonicum*. The parasite DNA in serum was not detected in 31.9%, 61.7% and 83% of patients at 3 months, 6 months and 9 months post-therapy, respectively. LAMP negative conversion rates were higher than those of IHA and ELISA, that reached only 31.9% and 25.5%, respectively, at 9 months post-treatment (Figure 2). These results seem to indicate that LAMP technique has potential for monitoring the effectiveness of schistosomiasis treatment in a long-term approach, not only in early treatment monitoring. However, further studies are needed to determine the usefulness of LAMP in assessing the efficacy of treatment. In addition, the role of free DNA in biological samples, as well as our capacity to link DNA detection levels to biological entities (i.e., eggs or worms) is still challenging for many parasites [56] and needs to be clearly addressed to give real meaning to the results obtained. Specifically, for *Schistosoma* spp. infections, three different hypotheses could explain the mentioned results. Firstly, the subcurative doses of praziquantel regularly used, that would generate persistence infection in some patients. Secondly, DNA derived from degenerating eggs trapped in tissues could be slowly being released, thus, yielding false-positive results regarding active infections. Finally, single-sex *Schistosoma* infections have been described. Those infections could be producing positive results without the excretion of eggs [55].

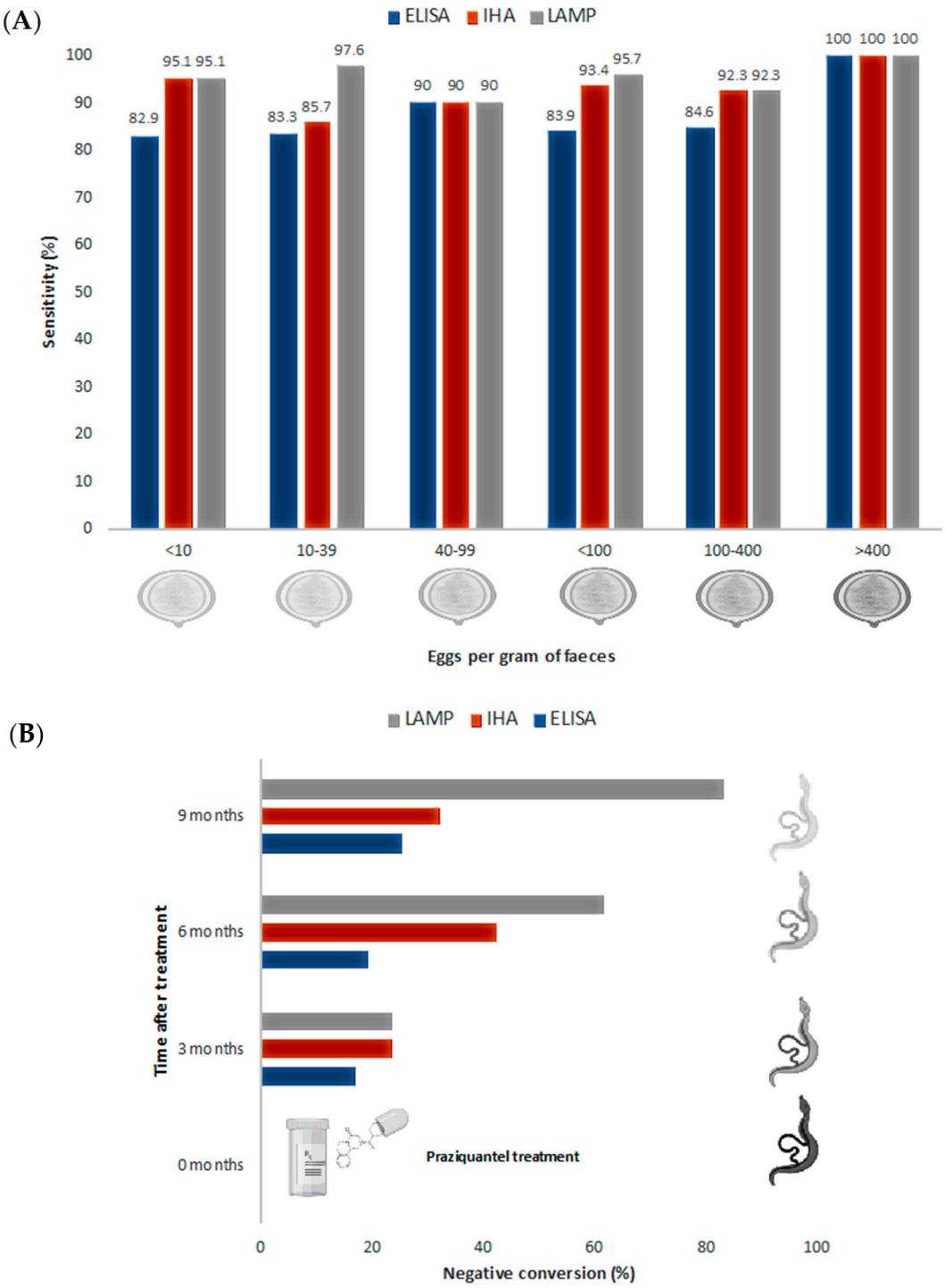

Figure 2. Sensitivity and post-treatment evaluation comparison for *S. japonicum* experimental infection by ELISA, IHA and LAMP. (**A**) ELISA, IHA and LAMP sensitivity comparison, related to the number of eggs per gram of faeces. (**B**) Evaluation of negative conversion rates after praziquantel treatment by ELISA, IHA and LAMP. Data obtained from Xu et al. [46].

3.5. Towards a True Point-of-Care Schistosomiasis Diagnostic?

Accurate patient identification is still a major limitation in NTDs management and control, dramatically contributing to the sustained burden that many of these diseases still present [57]. Molecular tools, highly sensitive and precise methodologies, have not yet replaced microscopy or serology in schistosomiasis diagnosis despite repeatedly showing better results at the laboratory. This is partly caused by the difficulty of deploying molecular

methods to the field. Such methodologies should fulfill the rules established by the acronym ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable) a set of criteria that must be achieved for any diagnostic method to be considered a point-of-care (POC) test [58]. The term has been recently revisited and modernized to REASSURED by Land et al. [59], including: Real-time connectivity and Ease of specimen collection and Environmental friendliness. In this direction, several studies show promising results contributing to develop accurate and specific schistosomiasis POC diagnosis.

To date, nucleic acid purification is considered the most important challenge preventing molecular diagnostics adoption from reaching the field [60]. In this sense, *S. haematobium* detection in clinical urine samples with LAMP has been accomplished with a simplified DNA extraction consisting on a 15-min 95 °C lysis step (so-called rapid-heat LAMPellet method) [34,49]. The ability of LAMP to amplify DNA without prior extraction has been proved in a wide variety of body fluids (i.e., plasma, blood, urine, saliva or semen) [61]. This has allowed the use of alternative specimens, such as urine samples, for the molecular diagnosis of intestinal schistosomiasis [44,49,51]. As handling and storage of large numbers of stool samples for field surveys is very demanding, urine has been proposed as more suitable specimen for large-scale field studies [44,62]. Nevertheless, further evidence is needed to prove the usefulness of urine for intestinal schistosomiasis diagnosis. As nucleic acid purification can be avoided and detection of LAMP results can be done with economical dyes [22,23], a considerable reduction in the final cost is achieved. LAMP costs are estimated to be 0.71–2$ per sample while for PCR it is 6.4–7.7$, ELISA is 1.5$, and KK is 2.00–2.67$ [63]. However, a bias can be attributed to those estimations as DNA purification is not included and, to date, rapid extraction methods reproducibility has not been proved in schistosomiasis large-scale surveys.

Another interesting approach to develop POC diagnostic for schistosomiasis is the use of ready-to-use formats, stabilized reaction mixes for LAMP that permit avoiding cold chain maintenance and facilitate the performance of the diagnosis by untrained personnel. Our group has recently presented a novel protocol for long-term preservation of LAMP master mixes for *S. mansoni* detection. We developed a simple one-step protocol based on threhalose as cryoprotectan to produce functional ready-to-use reaction mixes in at least 3 weeks or over five months when storing at room temperature or at 4 °C, respectively [52]. Other dry-LAMP approaches for schistosomiasis based on distinct cryoprotectans (i.e., sucrose) have been reported too [37], thus potentially allowing them to work at room temperature and reducing equipment needed in field settings. Despite all recent advances, no significant changes in current diagnostic protocols for schistosomiasis have included LAMP method. This technology has been available since 2000 [16] but it is not yet a true POC test, nor are any other PCR-based techniques.

It should also be noted that LAMP presents a several important disadvantages: it is non-applicable for cloning, it has a highly constrained primer design, the risk of carry-over contamination is elevated, and multiplexing approaches are still scarce and mechanistically, very complex [64]. In addition, partial hybridization of one or more LAMP primers to fragmented genomic host DNA or with phylogenetically related non-target microorganisms abundant in non-sterile biological materials may yield occasionally random amplification by the *Bst* polymerase used for LAMP. The difficulty to develop multiplex approaches is the most concerning out of the limitations described in the case of schistosomiasis, since co-infections are very frequent in endemic regions, adding one more layer of complexity to an already difficult diagnosis. However, some multiplex-LAMP (mLAMP) approaches are beginning to appear for detection of a number of parasites causing infection diseases and a two-stage isothermal amplification method has been applied to schistosomiasis. Briefly, a microfluidic chip has been designed to perform a dubbed rapid amplification (RAMP) first-stage follow by a second-stage LAMP assay. This assay has been designed in a 16-plex, 2-stage RAMP assay to simultaneously detect up to 16 different targets of DNA

and RNA from different parasites in just 40 min, including *S. mansoni*, *S. hematobium* and *S. japonicum* [65].

Finally, although LAMP presents sensitivity and specificity features to be a standalone diagnostic technique, the combination with other useful diagnostic tests should also be considered. This is especially true for the widely used detection of circulating cathodic antigen (CCA) and circulating anodic antigen (CAA). Both have already been developed adapted to lateral flow dipsticks, specifically CAA for *S. japonicum* and CCA for *S. mansoni*. Still, CCA antigen presents limitations detecting *S. haematobium*, thus is only considered effective in regions where only *S. mansoni* infections occur [14]. Thus, LAMP would be a great complementary diagnostic tool in those regions where infections by both *S. mansoni* and *S. haematobium* occur.

On the basis of the REASSURED criteria [59] defined above, LAMP already fulfills most of the requirements. It is affordable, sensitive, specific, rapid and robust by defi-nition, and efforts have been made to develop supportive technology to make it more user friendly. Nevertheless, some others still need improvement. The ease of specimen collec-tion is still hampered by the lack of validation of alternative specimens (i.e., urine for *S. mansoni* diagnostic) in large scale-studies, as well as the true capability of rapid and equipment-free DNA purification strategies. Additionally, it is very unlikely that LAMP becomes a completely equipment free technology, but it can be reduced and simplified to the fullest (through lateral flow dipsticks, microchips and other lab-on-chip displays) and, critically, made more affordable. This, combined with the extension of smartphone tech-nology in Sub-Saharan Africa, and the development of smartphone diagnostic strategies [66], could finally bridge the gap of real-time connectivity and data management in far remote areas. However, real world application of this technology has been coming for years now, and it has not yet been delivered to those who needed most. Here, the extensive research made and the steps needed ahead have been highlighted. Still, the sustained invariability on the diagnostic tools used in tropical diseases to date begs the question: Is it the Research or is it the Health Systems?

4. Conclusions

Schistosomiasis still represents a very pressing health problem in many countries of the world. There is a critical need for new specific and sensitive diagnostic tools that are inexpensive and easy to transport and use. Although being one of the NTDs for which more work has been done, clinical applications of LAMP for schistosomiasis diagnosis are still limited and only applied in research scenarios, not routinely in the clinic. Larger, in-depth studies are imperative to move forward in the real-world application of this method. We believe that with the help of new supportive technology (i.e., including lateral flow disks, microchips, lab-on-chips, smartphone apps) LAMP might be able to finally reach endemic areas. LAMP results obtained for schistosomiasis have proved to be comparable to or better than those of the common diagnostic methods and it could be a great candidate to finally get molecular testing to the field as a true POC test.

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1.5

The future of point-of-care nucleic acid amplification diagnostics after COVID-19: time to walk the walk

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RESUMEN

El estallido de la pandemia de COVID-19 ha generado una revolución profunda en el campo del diagnóstico en general, y en el del diagnóstico molecular en particular. Por ello, es interesante finalizar la introducción de este trabajo con un repaso al diagnóstico molecular a pie de paciente desarrollado para SARS-CoV-2 y discutir el potencial impacto que estos avances podrían tener en la detección de otras infecciones. Todo ello está recogido en esta sección con el artículo de revisión "*The future of point-of-care nucleic acid amplification diagnostics after COVID-19: time to walk the walk*".

Desde el inicio de la pandemia se han diagnosticado más de 610 millones de casos, con más de 6,5 millones de muertes confirmadas. Por desgracia, se estima que la cifra real podría estar más cercana a los 20 millones de fallecidos. La crisis generada por SARS-CoV-2 ha obligado a la comunidad científica a desarrollar herramientas para el control y el manejo de las enfermedades a un ritmo nunca visto. Ese control se basa en gran medida en el uso de diagnósticos rápidos y precisos que permitan realizar pruebas a gran escala.

El diagnóstico de referencia para las infecciones víricas es la RT-qPCR, que proporciona resultados consistentes y fiables, pero está limitada por sus requisitos técnicos y de infraestructura. Sin embargo, gracias a diversas mejoras como la automatización de los procesos de extracción de ácidos nucleicos de las muestras, la estabilización de reactivos y la miniaturización y abaratamiento de los dispositivos necesarios para realizar la reacción, se ha conseguido, en cierta medida, llevar esta técnica a condiciones a pie de paciente al menos en países desarrollados. Algunos de los ensayos de RT-qPCR para SARS-CoV-2, son los primeros ejemplos de test basados en esta tecnología que se han aprobado para dicho uso.

Sin embargo, la aplicabilidad de la RT-qPCR en condiciones de recursos limitados está más restringida que las técnicas isotérmicas de amplificación de ácidos nucleicos. Por primera vez, las grandes organizaciones internacionales de la salud, como la FDA o la OMS, han aprobado ensayos basados en técnicas isotérmicas, principalmente LAMP y RPA, para su uso como test diagnóstico único para una enfermedad infecciosa. De hecho, con la normalización del automuestreo y el desarrollo de plataformas integradas de tipo *sample-to-answer*, diversos ensayos LAMP han sido autorizados para realizarse en casa por los propios pacientes, sin supervisión

de personal cualificado. Esto representa un salto inmenso en la aplicación en el "mundo real" de esta tecnología, impensable antes de la pandemia.

También la detección de ácidos nucleicos basados en sistemas CRISPR ha sido enormemente impulsada durante la crisis, generando las primeras plataformas de diagnóstico comerciales basadas en esta tecnología. Esto abre un mundo de posibilidades en términos de detección simultanea de patógenos, de monitorización de mutaciones de interés o incluso de resistencias a antibióticos mediante métodos sencillos y de fácil accesibilidad.

Esta revisión nos ha demostrado que el campo del diagnóstico molecular se mueve en la dirección de ensayos asequibles, dispositivos portátiles y facilidad de uso que nosotros también proponemos en este trabajo. Ahora queda preguntarse si el impulso proporcionado por la situación excepcional vivida durante la pandemia tendrá un efecto duradero, no solo en esta enfermedad, sino en tantas otras que necesitan de una mejora en el diagnóstico.

CAPÍTULO II

Hipótesis y objetivos

2.1. Hipótesis

Aunque en la actualidad existen diversos métodos de diagnóstico basados en la amplificación isotérmica de ácidos nucleicos, no se aplican de manera rutinaria en la práctica clínica, especialmente en zonas de escasos recursos. La tecnología LAMP podría ser una buena alternativa a métodos moleculares complejos como la PCR, presentando ventajas de rapidez, simplicidad y bajo coste. Para poder conseguir un diagnóstico útil en la práctica clínica basado en la tecnología LAMP, es necesaria su adaptación y combinación con otras tecnologías que permitan almacenar y transportar los reactivos de reacción sin necesidad de mantener una cadena de frío. Además, es preciso disponer de dispositivos pequeños, portátiles, autónomos y de funcionamiento a tiempo real que lleven a cabo las reacciones de amplificación. Para una mayor facilidad de uso e interpretación, la monitorización de las reacciones debería efectuarse a través de dispositivos inteligentes mediante aplicaciones móviles para el registro, gestión y análisis de los resultados obtenidos.

2.2. Objetivos

El **objetivo general** de esta Tesis Doctoral es adaptar la tecnología LAMP para su validación como método molecular en el diagnóstico de las enfermedades infecciosas.

Los **objetivos específicos** son:

I. Optimizar un método sencillo y rápido de estabilización de los reactivos de la reacción LAMP que permita su almacenamiento a temperatura ambiente en un formato listo para el uso.

II. Desarrollar un dispositivo portátil para realizar reacciones de amplificación isotérmica de ácidos nucleicos con monitorización a tiempo real mediante colorimetría, controlado por una aplicación móvil que haga posible el registro, gestión y análisis sencillo de los resultados.

III. Diseñar ensayos RT-LAMP para la detección de ARN del virus SARS-CoV-2 y definir su aplicabilidad en diferentes muestras clínicas.

IV. Validar la capacidad diagnóstica del dispositivo portátil con diferentes ensayos LAMP comparando su funcionamiento con técnicas moleculares de referencia y otros dispositivos comerciales.

CAPÍTULO III

Artículos de investigación y propiedad intelectual

3.1

Progress in loop-mediated isothermal amplification assay for detection of Schistosoma mansoni DNA: towards a ready-to-use test

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RESUMEN

El primer reto que nos propusimos en este trabajo fue desarrollar una metodología que permitiera estabilizar y almacenar a temperatura ambiente reactivos necesarios para realizar el LAMP en un formato "listo para su uso". De esta manera, los reactivos podrían transportarse y utilizarse en zonas en las que el mantenimiento de la cadena de frío es un problema importante. Además, el diagnóstico lo podría realizar personal con poca experiencia al no requerir de la preparación previa de mezclas de reacción. Se evitan también posibles contaminaciones al disponer ya de las mezclas preparadas. En trabajos anteriores, la mayoría de los métodos de estabilización de reactivos estaban basados en técnicas de liofilización, que eliminan el agua de las mezclas de reacción mediante un proceso de congelación rápida y una posterior sublimación en presencia de distintos crioprotectores. Aunque la liofilización es un proceso fiable, nos planteaba *a priori* varios problemas: (*i*) el equipamiento necesario para realizarlo es muy complejo y costoso; (*ii*) cuando se trabaja con volúmenes muy pequeños (entre 25 µL y 15 µL en nuestro caso) el proceso de liofilización puede ser difícil de optimizar y (*iii*) el protocolo de liofilización es largo y tedioso, requiriendo numerosas fases, algunas de varias horas, a diferentes temperaturas y presiones para conseguir las condiciones de congelación y posterior sublimación óptimas.

Con estas limitaciones en mente, decidimos optar por un método más sencillo: la estabilización por desecación. En este caso, no hay un proceso de congelación y las mezclas de reacción solo se someten a vacío, permitiendo retirar el agua en presencia de un crioprotector (p.ej. trehalosa). Esto evita procesos de activación enzimática, oxidación y degradación que podrían afectar a la funcionalidad de las mezclas almacenadas a lo largo del tiempo. Se ensayaron diversas variaciones de este protocolo, con o sin centrifugación, estabilizando parte o todos los reactivos y se obtuvo un protocolo simple de un único paso de media hora a temperatura ambiente, en el que todos los reactivos se unen en un único *pellet*. Por tanto, desarrollamos y aplicamos una metodología mucho más sencilla que la mayoría de las descritas previamente.

En un trabajo anterior a esta Tesis Doctoral, nuestro grupo de investigación desarrolló un método basado en la tecnología LAMP para detectar ADN de *Schistosoma mansoni* (SmMIT-LAMP) utilizando un modelo murino de infección experimental. El SmMIT-LAMP permitió el diagnóstico precoz de la esquistosomosis

en muestras de heces de los ratones infectados. Posteriormente, se utilizó con éxito en la detección de ADN de *S. mansoni* en muestras de heces de individuos en zona endémica de Brasil. De manera más reciente, se ha demostrado su utilidad para la detección de *S. mansoni* en muestras de orina. Teniendo en cuenta que el SmMIT-LAMP ya se había valorado con éxito "en fresco" (sin proceso de desecación) en distintas situaciones y tipos de muestras, decidimos utilizarlo como modelo de ensayo LAMP para aplicar la estabilización de los reactivos necesarios para su uso.

De este primer artículo de investigación se derivan varias conclusiones: (*i*) es posible estabilizar reactivos LAMP en un formato listo para el uso mediante un protocolo de desecación sencillo y rápido; (*ii*) se puede estabilizar el ADN control junto con los reactivos de reacción y obtener amplificación; (*iii*) en muestras de biopsias de distintos tejidos los resultados obtenidos mediante SmMIT-LAMP en fresco se corresponden con los que se obtienen con los reactivos estabilizados; (*iv*) este protocolo es aplicable tanto para reacciones convencionales como para reacciones a tiempo real; (*v*) el protocolo de estabilización desarrollado permite almacenar los reactivos estabilizados sin pérdida de funcionalidad durante, al menos, 5 meses a -4 ºC y 3 semanas a 25 ºC.

Sin embargo, no podemos obviar las limitaciones del protocolo desarrollado. En primer lugar, la cinética de la reacción empeora claramente tras la estabilización. Esto se puede atribuir, en parte, al tiempo de rehidratación previo que requieren los componentes antes de comenzar la reacción, y también a que la funcionalidad de la enzima disminuye tras la desecación. En segundo lugar, la vida útil de las mezclas estabilizadas y almacenadas a temperatura ambiente se limitó a las a tres semanas. Aunque obtuvimos cierta limitación en la vida útil de las mezclas, la estabilización permitiría preparar lotes de reactivos en un centro de referencia y hacer campañas cortas y planificadas de diagnóstico a pie de paciente en zonas endémicas. La causa de la pérdida de funcionalidad podría ser la cristalización de alguna de las sales presentes en el tampón de reacción, que favorecería la ruptura de la protección ofrecida por la trehalosa, comenzando así distintos procesos de oxidación y degradación de los distintos componentes de la reacción.

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Progress in loop-mediated isothermal amplification assay for detection of *Schistosoma mansoni* DNA: towards a ready-to-use test

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Schistosomiasis is one of the most prevalent Neglected Tropical Disease, affecting approximately 250 million people worldwide. *Schistosoma mansoni* is the most important species causing human intestinal schistosomiasis. Despite significant efforts in recent decades, the global disease burden of schistosomiasis remains extremely high. This could partly be attributed to the absence of accurate diagnostic tools, primarily in endemic areas. Loop-mediated isothermal amplification (LAMP) is increasingly used in molecular diagnostics as a field-friendly alternative to many other complex molecular methods and it has been proposed as an ideal candidate for revolutionizing point-of-care molecular diagnostics. In a previous work, a LAMP-based method to detect *S. mansoni* DNA (SmMIT-LAMP) was developed by our research group for early diagnosis of active schistosomiasis in an experimental infection murine model. The SmMIT-LAMP has been further successfully evaluated in both human stool and snail samples and, recently, in human urine samples. In this study, we developed an important improvement for SmMIT-LAMP molecular assay, transforming it into a cold maintenance dry format suitable for potentially manufacturing as kit for ready-to-use for schistosomiasis diagnosis. This procedure could be applied to create dry LAMP kits for a laboratory setting and for diagnostic applications for other neglected tropical diseases.

The World Health Organization (WHO) identifies Neglected Tropical Diseases (NTD) as a diverse group of communicable chronic, debilitating and often stigmatizing infectious diseases affecting more than one thousand million people in tropical and subtropical regions along 149 countries, especially in populations living in extreme poverty and inadequate sanitation[1]. One of these NTD is schistosomiasis, a parasitic disease caused by trematode worms (blood flukes) of the genus *Schistosoma*. There are two major forms of human schistosomiasis: urogenital schistosomiasis, caused by *Schistosoma haematobium*, and intestinal schistosomiasis, caused by any of the organisms *S. mansoni*, *S. intercalatum*, *S. guineensis*, *S. japonicum*, and *S. mekongi*. It is estimated that 779 million people live within high-risk-of-infection areas and 250 million are infected, more than 80% of them living in sub-Saharan Africa[2]. The disease accounts for an estimated 1.9 million disability-adjusted life years (DALYs) annually[3]. In recent years, autochthonous outbreaks of urogenital schistosomiasis in the south of Europe have been reported[4]. All these figures emphasize the importance and the need for control and elimination of schistosomiasis. Despite significant efforts in recent decades, the global disease burden of schistosomiasis remains extremely high since a regular treatment with praziquantel, provision of water, sanitation and hygiene, new complimentary drugs, local recommendations for snail control, surveillance and management of hotspots, and long-term, well-structured control programmes are still needed in endemic areas. Besides, more accurate diagnostic tools for detecting schistosome infections are also required to help in the overall control of schistomiasis.

The traditional Kato-Katz fecal microscopic examination for counting schistosome eggs and immunology-based analyses detecting schistosome-derived circulating anodic (CAA) and cathodic (CCA) antigens mainly lack sensitivity in low-intensity infections and posttreatment conditions[5,6]. Antibody detection also

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lacks specificity, causing a high level of cross-reactivity[6]. Several PCR-based molecular approaches –conventional PCR (cPCR), real-time quantitative PCR (qPCR), droplet digital PCR (ddPCR)- have been proven effective in detection of schistosome-derived DNA with more sensitivity than parasitological and serological methods, especially in chronic infections[7] and in low transmission areas[8]. However, diagnostic assays based on PCR are not widely used in resource-limited settings because they are time-consuming, complex, and require skilled personnel and expensive equipment[9]. Hence, the development of novel diagnostics methods for NTD complying with features, such us low cost, rapidity, usable with different kinds of samples, simple operation and interpretation, and detection capability with high sensitivity and specificity are vital to address the present limitations in use of PCR-based tests in low-income countries.

In recent years, a number of nucleic acid isothermal amplification techniques trying to fulfill these requirements have been developed[10]. Among the most promising is the loop-mediated isothermal amplification (LAMP) assay[11]. LAMP was initially reported in 2000 as a single tube method for rapid nucleic acids amplification under isothermal conditions with high specificity and efficiency[12], allowing the direct visual discrimination of positive results[13]. Since then, LAMP technology has been successfully used for detection of many infectious agents in both animals and plants[14]. Nowadays, of the 20 NTD recognized by WHO (http://www.who.int/neglected_diseases/diseases/en/), LAMP reactions for 16 of them have been reported, including schistosomiasis[11]. The cheapness and user-friendliness of LAMP method, together with the increasing development of miniaturized instrument-free LAMP systems as lab-on-chip, makes it a cost-effective and simple tool that provides an alternative to PCR assays for low-cost chip-based point of care (POC) diagnostic applications in low-resource settings[15–18]. Even so, currently micro-LAMP systems for POC diagnostics still have limitations and do not completely satisfy WHO criteria of equipment-free/electricity-free operation[17]. One of the major obstacles for the application of LAMP in NTD-endemic countries has been the difficulty in maintaining the cold-chain to preserve reagents. Thus, the challenge is to develop a LAMP kit in a ready-to-use format with dried reagents useful for quick and simple application in field conditions. LAMP kits for Tuberculosis and Malaria are quite developed and already commercialized (available in Human Diagnostic Worldwide; https://www.human.de/products/molecular-dx/). Regarding NTD, the only LAMP prototype kits in dried form developed are for Chagas disease, Human African Trypanosomiasis[19], and for multiplexing Dengue and Chikungunya viral infections[20].

The two most common procedures used to stabilize pre-mixed LAMP reagents in a dried kit format are drying[21] and lyophilization[22,23]. Lyophilization protocols allow all LAMP reagents to be initially included[20,22,24,25], while in drying protocols at least a two-step dry-up is required, keeping the remaining components on ice for final addition to the mix at the time to run LAMP reaction[21,26]. In both cases, experimentally processes are laborious and require sophisticated equipment.

In general, stabilization protocols of reagents by drying are based on the use of molecules like trehalose, due to its ability to resist extreme dehydration and temperature conditions[27]. Besides, since it is produced naturally by a number of microorganisms, it is easy and cheap to obtain[28]. The stabilizing capacity of trehalose is a result of its physical properties: (*i*) interacts directly with the compound and protect it during the drying process and, (*ii*) limits the mobility of the compound increasing their state of hydration[29]. It has been proven that trehalose is useful for the preservation of reagents (including primers, dNTPs and enzymes) for PCR[30–32] and LAMP[21]. Besides this application, it has been demonstrated to improve the analytical performance of other isothermal nucleic acid amplification methods, such as the exponential amplification reaction (EXPAR)[33].

A LAMP method for detection of *S. mansoni* DNA (so called, SmMIT-LAMP) was previously established by our group for early diagnosis of active schistosomiasis in experimental infection in a murine model[34]. Recently, the SmMIT-LAMP was successfully evaluated in both human stool and snail samples from a low-transmission schistosomiasis-endemic area in Brazil[35] and, even more recently, also in human urine samples[36].

Here, in order to improve the established LAMP technique for *S. mansoni* DNA detection, we report the development of a desiccation procedure to stabilize the SmMIT-LAMP reagents in a single tube for conventional or real-time potential ready-to-use application in diagnosis of schistosomiasis *mansoni*.

Material and Methods

DNA extraction for molecular analysis. *Schistosoma mansoni DNA*. Genomic *S. mansoni* DNA was extracted from frozen adult worms available in our laboratory using NucleoSpin Tissue kit (MACHEREY-NAGEL, Germany) following manufacturers' instructions. The concentration was measured using a NanoDrop (ND-1000; THERMO FISHER SCIENTIFIC, USA) and diluted to a final concentration of 0.5 ng/μL to be used as positive control (2 μL; 1 ng) in all LAMP trials.

DNA from patients' tissue samples. DNA from 3 patients' tissue samples with microscopy-confirmed infection with *S. mansoni* was tested by LAMP: cutaneous and hepatic biopsies (kindly provided by the University Hospital Vall d'Hebron, Barcelona, Spain) and an appendix biopsy (kindly provided by the Department of Parasitology, National Centre for Microbiology, Institute of Health Carlos III, Majadahonda, Madrid, Spain). DNA was isolated from tissue samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following manufacturers' instructions and then stored at −20 °C until use in LAMP reactions. The tissue samples were collected from adult patients attending at Hospitals as part of public health diagnostic activities. They signed an informed consent form.

Conventional SmMIT-LAMP. Fresh conventional LAMP reactions to amplify *S. mansoni* DNA were performed using the set of four primers and conditions previously described by Fernández -Soto *et al.*[34]. In brief, in a volume of 25 μL were mixed: betaine (1 M) (SIGMA, USA); Isothermal Amplification Buffer 1X, supplementary $MgSO_4$ (6 mM) and 8U of *Bst* polymerase 2.0 WarmStart (NEW ENGLAND BIOLABS, UK); dNTPs (1,4 mM each); FIP/BIP (40 pmol/μL each) and F3/B3 (5 pmol/μL each) (BIORON). The cLAMP reactions were carried out in a Dry-Bath (No. DB-006) at 65 °C for 1 h followed up by 10 min at 80 °C to stop amplification.

Amplification products were visualized with naked eye by adding 2 μL of SYBR Green I 1000× (INVITROGEN) in each post-amplified tube. LAMP products were also monitored on 1.5% agarose gels when required.

Real-time SmMIT-LAMP. Each 25 μL liquid freshly prepared real-time LAMP reaction to amplify *S. mansoni* DNA contained the same reagents as those for conventional SmMIT-LAMP together with 0.40 μL of EvaGreen 20X in water (BIOTIUM) to monitor the fluorescence over time. In some trials, betaine was removed from reaction mixes. The real-time SmMIT-LAMP reactions were performed in 8-tube Genie Strips on a portable Genie III device (OPTIGENE Ltd., Horsham, UK) at 65 °C for 60 min followed up by 10 min at 80 °C. Amplicons were confirmed on 1.5% agarose gels when required.

Stabilization procedures of LAMP reagents for conventional and real-time tests. To carry out the stabilization of the conventional SmMIT-LAMP mixtures we based our trials on a previous protocol described by Hayasida *et al.*[21] that uses a drying procedure with trehalose. Briefly, this two-step protocol consisted in: first, primers and dye ("colori-fluorometric indicator", CFI: a mix of hydroxyl-naphtol blue and GelGreen dissolved in distilled water) were air dried in the presence of 0,56 μL of trehalose (2 M) and 0,14 μL of 50% glycerol for 30 min under a flow of clean air in the top of a 0.2 mL microtube. Secondly, *Bst* polymerase and dNTPs were dried in the presence of additional 1.5 μL of trehalose for 15 min. Finally, reaction mixes were further dried over 24 h under a vacuum in a container with P_2O_5 and silica gel, and then stored in an aluminium bag with zeolite molecular sieves.

Based on the above, we tried to simplify this drying protocol by subjecting the SmMIT-LAMP reagents to vacuum process using: (1) centrifugation (so called, concentration) or (2) without centrifugation (so called, desiccation) following two or single dry-up steps as shown in Supplementary Fig. S1 (S1) and stated as follows.

Drying by concentration (S1.1). Open 1.5 mL tubes containing the SmMIT-LAMP reagents were subjected to vacuum in a Concentrator Plus (EPPENDORF, Germany) while centrifuged (V-AQ mode) at 1400 rpm at room temperature (RT). This action was carried out using the following drying procedures:

a. **Two-step dry-up** (**S1.1a**). First, a mix containing 1.8 μL of primers, 0.56 μL of trehalose 2M (SIGMA, USA) and 0.14 μL of 50% glycerol was placed in the bottom of the tubes and concentrated for 30 min thus obtaining a dry pellet. Next, a mix containing 3.5 μL dNTPs, 1 μL *Bst* polymerase and 1.5 μL of trehalose (2M) was incorporated on top of pellet to be concentrated for additional 15 min. For the LAMP reaction, reconstitution of pellets was performed in 2.5 μL of buffer, 1.5 μL of $MgSO_4$ and 19 μL of water (and in 2 μL of DNA template, if applicable) mixed up to a resulting final volume of 25 μL.
b. **One-step dry-up** (**S1.1b**). A mix containing 1.8 μL of primers, 3.5 μL of dNTPs, 1 μL of *Bst* polymerase, 2.06 μL of trehalose and 0.14 μL of 50% glycerol was placed in the bottom of the tubes and concentrated for 30 min. Rehydration of the formed pellets was performed as indicated in the previous section (a).
c. **All in one-step dry-up** (**S1.1c**). All necessary SmMIT-LAMP reagents were placed in the bottom of the tubes in the presence of 2 μL of trehalose (no glycerol) and then concentrated for a single step of 30 min. For later rehydration, only water up to a final volume of 25 μL was added (and 2 μL of DNA template, if applicable).

Drying by desiccation (S1.2). This procedure was performed in all in one-step dry-up. Open 1.5 mL tubes containing all necessary SmMIT-LAMP reagents placed in the bottom in the presence of 2 μL of trehalose (no glycerol) were exposed to vacuum in a Concentrator Plus (EPPENDORF, Germany) without centrifugation (D-AQ mode) at RT for 30 min. For subsequently reaction, only water up to a final volume of 25 μL was added (and 2 μL of DNA template, if applicable).

To carry out the stabilized real-time SmMIT-LAMP tests, drying was performed in all in one-step desiccation procedure (that is, without centrifuge) in open 8-tube Genie Strips (OPTIGENE, UK) with or without the pre-addition of EvaGreen 20X (0.40 μL) in water to the LAMP master mixes (Supplementary Fig. S2). In some trials, DNA from *S. mansoni* was mixed with the other reagents from the beginning of the desiccation procedure to be used as ready-to-use positive control in tests. For later real-time reactions, the pellets were reconstituted in different volumes depending on the reagents included in the pre-desiccation process: (*i*) 25 μL water when including all LAMP components (counting with *S. mansoni* DNA); (*ii*) 23 μL water and 2 μL DNA template of *S. mansoni*; and (*iii*) 22.6 μL water, 2 μL DNA template of *S. mansoni* and 0.40 μL EvaGreen. After reconstituting the dried pellet by gently pipetting on the bottom of the tubes, the reaction was carried out in a portable Genie III device. In some assays, only 4/8-tube strips were prepared with dried reagents, thus keeping 4 tubes free to use with fresh liquid LAMP mixes to compare results running the same amplification real-time assay.

All SmMIT-LAMP and real-time SmMIT-LAMP dried reactions were carried out in a heat block and in a Genie III device, respectively, at 65 °C for 120 min followed up by 10 min at 80 °C to stop amplification. Amplification products were visualized with naked eye by adding 2 μL of SYBR Green I 1000× (INVITROGEN) in each post-amplified tube, by fluorescence monitoring and, by electrophoresis when required.

Storage stability of dry-reagent LAMP mix. To estimate the stability and functionality over time, desiccated SmMIT-LAMP mixtures were stored at RT and 4 °C in cardboard storage boxes with some Silica Gel desiccant pouches inside to protect against moisture until use. After rehydration of the pellets, the LAMP reactions were performed on a heat block at 1 week, 3 weeks, 1 month, 3 months and 5 months.

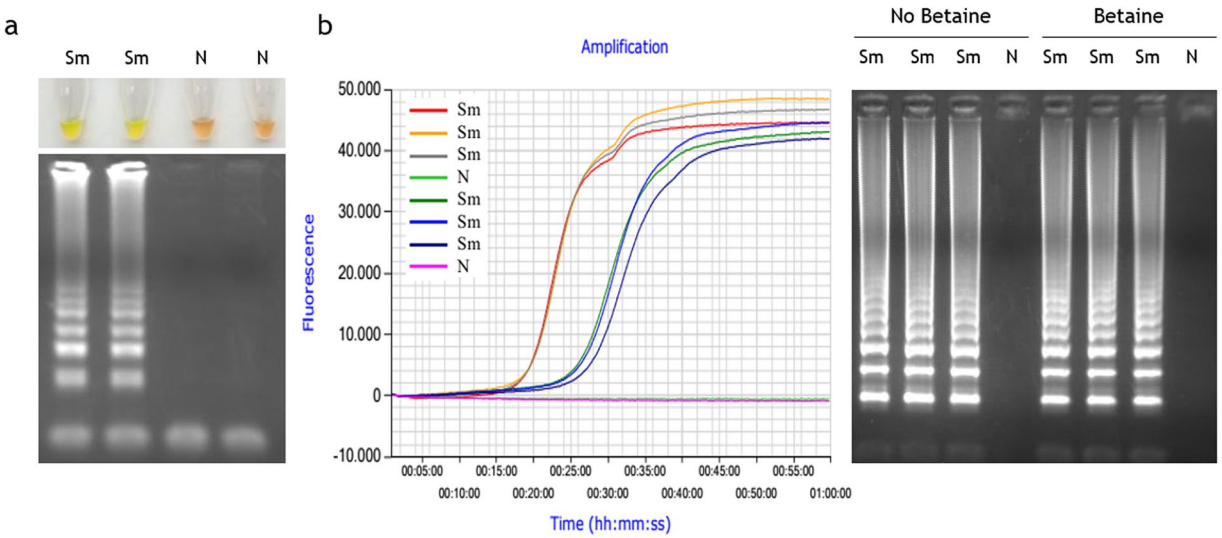

Figure 1. Comparison between conventional and real-time SmMIT-LAMP assays. (**a**) Conventional SmMIT-LAMP. Visual assessment based on addition of SYBR Green I after amplification (top) and electrophoresis on agarose gel (bottom). (**b**) Real-time SmMIT-LAMP. Time-course of EvaGreen 20X fluorescence signal over time (left) and electrophoresis results of free-betaine and 1 M betaine SmMIT-LAMP reactions (right). Sm, positive control (*S. mansoni* DNA; 1 ng); N, negative control (ultrapure water, no DNA). All electrophoresis gel images were obtained with an exposure between 700 ms and 1.2 s in a Gel documentation system, UVItec, UK. Real-time graph is directly captured from Genie Explorer Software (V2.0.5.5) result report.

Results and Discussion

Comparison of conventional and real-time SmMIT-LAMP assays. SmMIT-LAMP result comparison between conventional and real-time conditions is shown in Fig. 1. Endpoint results at 65–70 min (counting 60 min for reaction plus 5–10 min of inactivation) were clearly observed with naked eye by adding the fluorescent dye SYBR Green I post-amplification. The positive LAMP reactions turned to green; otherwise, they remained orange. Correlation of positive colorimetric results with the typical ladder of multiple bands on agarose gels was clear (Fig. 1a). Real-time SmMIT-LAMP was carried out on a portable device using the same reaction conditions but testing including or not betaine in the master mix. Real-time reactions worked properly in both cases and a strong correlation between the signal of the fluorescent EvaGreen dye and electrophoresis was obtained (Fig. 1b). However, removing betaine from the mixture resulted on a 10 min reduction in the amplification time while showing identical intensity of electrophoresis bands. These findings were consistent with recent observations described by Ma *et al.*[37] that associate an increased efficiency of the real-time LAMP with betaine-free conditions. Several mechanisms of action have been proposed for betaine, as increasing DNA accessibility[38], preventing secondary structure formation in GC-rich region[39] or pH dependent ion exchange[40]. Nevertheless, other studies consider that betaine has no effect at certain concentrations on the effectiveness of the LAMP assay[41,42]. In our study, 1 M betaine had no negative effect on real-time SmMIT-LAMP amplification, but betaine-free assay was faster, so all reactions from then on were performed without betaine.

Stabilized conventional LAMP assay. Both concentration and desiccation procedures yielded stable and well-adhered pellets at the bottom of the tubes. Subsequent reconstitution of the pellets by gently pipetting was performed and conventional LAMP reactions were run for 2 h. The incubation time was increased up to 2 h because we obtained better amplification results in preliminary tests working with stabilized mixes. Figure 2 shows the comparison of the results of conventional SmMIT-LAMP (Fig. 2a) and dry SmMIT-LAMP prepared with all different tested stabilization protocols: concentration, in two and one (Fig. 2b) or all-in-one (Fig. 2c) dry-up steps, and desiccation (Fig. 2d). Drying by concentration resulted in a successful amplification when the reaction buffer and supplementary $MgSO_4$ were not included in the stabilized master mix, regardless the use of a two-step or a one-step drying protocol. The visible color change results and those on electrophoresis showing bands coincided (Fig. 2b). By contrast, the concentration procedure in all-in-one step (including the reaction buffer from the beginning of the drying procedure) failed and no amplification was obtained (Fig. 2c). A probable explanation for this could be related to the instability of the master mix, since during the drying process the *Bst* polymerase is exposed to a high salt concentration when it is mixed with the reaction buffer, which causes the enzyme to become destabilized. This hypothesis has been recently reported for Phi29 polymerase used in rolling circle amplification (RCA) technique, and the separation of buffer and other salts into a different drying step has also been proposed for increasing significantly stability of master mixes storage over time[43,44]. In line with those findings, in our study, the two-step concentration protocol lead to functional master mixes but one-step concentration (including reaction buffer) did not. In addition, the centrifugation process probably favoured the formation of salt crystals precipitates that ended up inhibiting the subsequent amplification. Notwithstanding the evidence, a two-step concentration protocol would increase the time and complexity of the stabilization

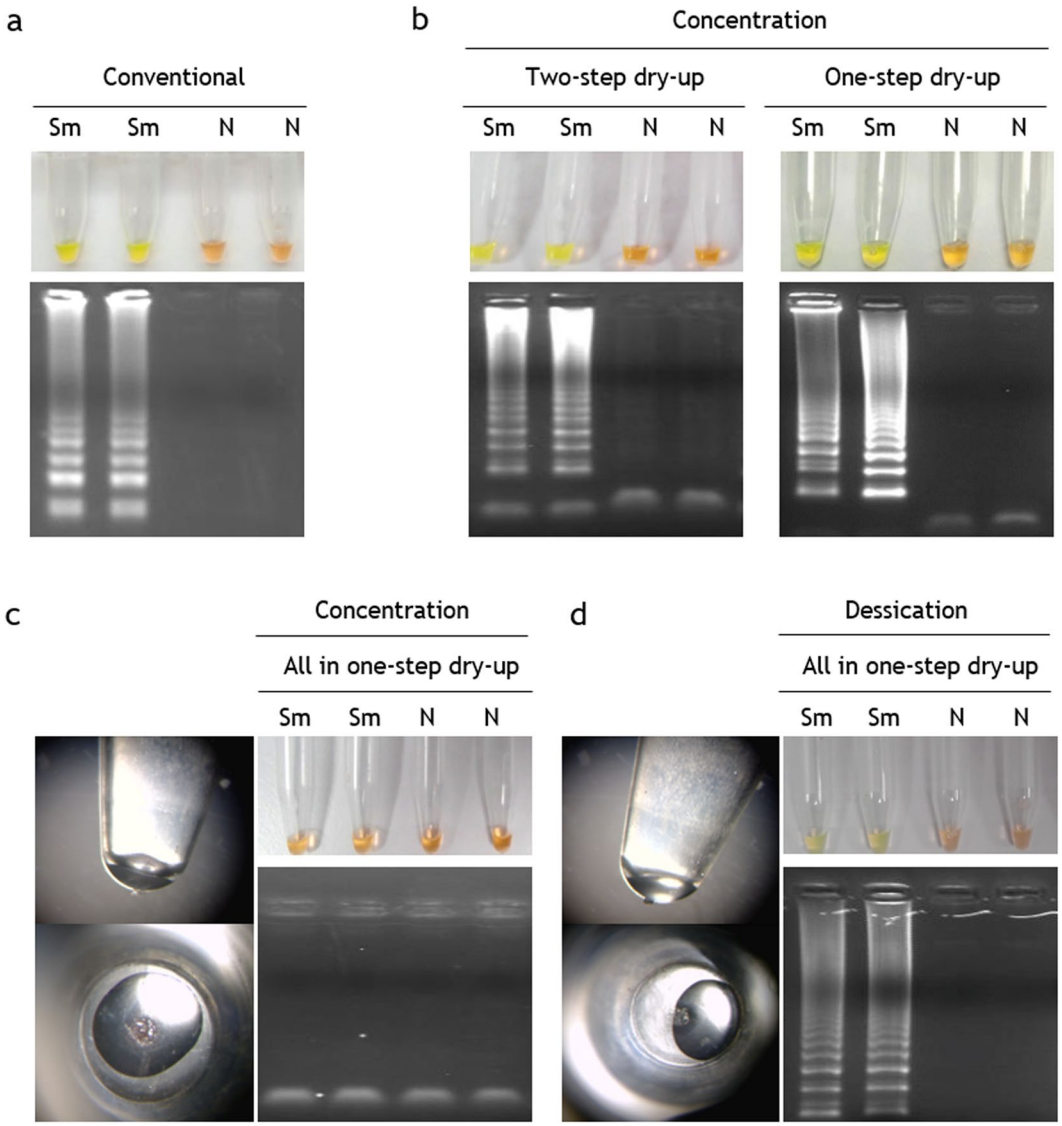

Figure 2. Operation of conventional SmMIT-LAMP and dry SmMIT-LAMP. (**a**) Results of conventional SmMIT-LAMP by color change and electrophoresis. (**b**) Results of SmMIT-LAMP after drying reagents by concentration following two-(left) and one-(right) step dry-up. (**c**) Results of SmMIT-LAMP after drying reagents by concentration following all in one-step dry-up. The pellet at the bottom of the tube obtained by 30 min concentration at RT is shown on the left. (**d**) Results of SmMIT-LAMP after drying reagents by desiccation following all in one-step dry-up. The pellet at the bottom of the tube obtained by 30 min concentration at RT is shown on the left. Sm, positive control (*S. mansoni* DNA; 1 ng); N, negative control (ultrapure water, no DNA). Electrophoresis gel images shown are cropped from different gels. All were obtained with an exposure between 700 ms and 1.2 s in a Gel documentation system, UVItec, UK.

procedure in comparison to the one-step concentration protocol. Thus, in order to avoid possible precipitation by centrifuging and attempt the stabilization in an easier one-step protocol, we tried to stabilize all necessary SmMIT-LAMP reagents in one-step dry up desiccation procedure without centrifugation. Proceeding in this way, the subsequent reconstitution of reagents worked well and amplification results were observed both by colour change and in agarose gels electrophoresis (Fig. 2d). By avoiding centrifugation procedure (and therefore, special rotor adapters in concentrator), the desiccation protocol allowed us to stabilize the SmMIT-LAMP reagents not only in individual tubes, but also in 8-tube strips to perform the reaction in real-time using the portable Genie III device. Furthermore, by simple desiccation, multi-well plates could be prepared with stabilized master mixes allowing a greater number of samples to be analysed using either thermal cyclers in well-equipped laboratories or simple ovens or stoves when performing in low-resources settings.

Stabilized real-time SmMIT-LAMP assay. The evaluation of desiccation procedure used in real-time SmMIT-LAMP is shown in Fig. 3. The 8-tube strips, containing four dried and four fresh liquid mixtures (operating controls), were incubated under isothermal conditions a 65 °C in a portable Genie III device. When reaction

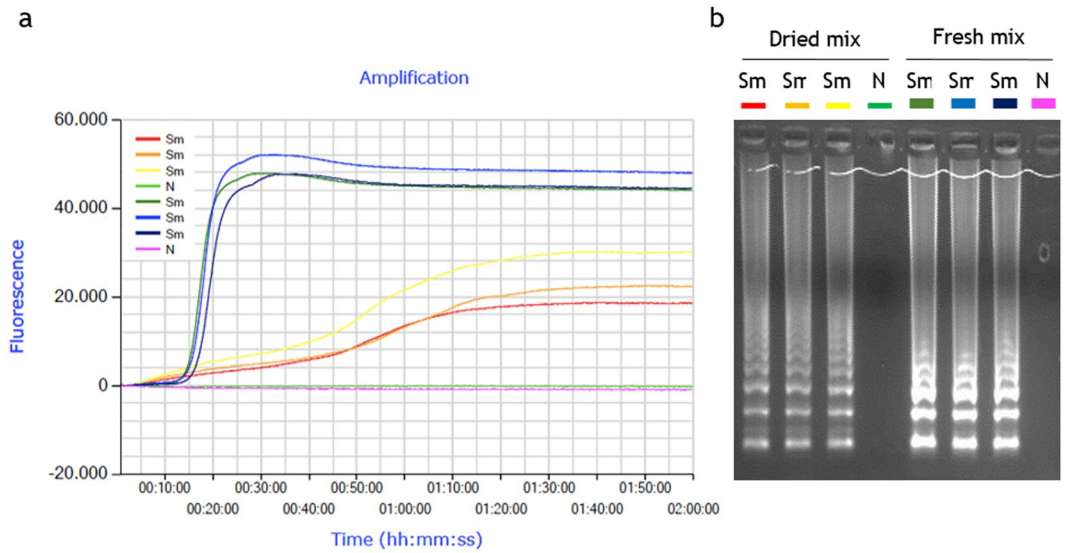

Figure 3. Operation of desiccation procedure to use in real-time SmMIT-LAMP. The assay was ran at 65 °C for a 2 h program in a portable Genie III device. (**a**) LAMP amplification with fluorescence detection with EvaGreen dye. (**b**) Agarose gel electrophoresis showing amplification results for both dried and fresh mixes. Sm, positive control (*S. mansoni* DNA; 1 ng); N, negative control (ultrapure water, no DNA). Real-time graph is directly captured from Genie Explorer Software (V2.0.5.5) result report.

was running for 1 h (standard incubation time), only liquid mixtures yielded amplification results. A subsequent increase in reaction time (establishing a 2 h program) allowed us to obtained amplification also in dried mixtures, but later in time (aprox. 65–70 min *vs*. 20 min) and with a lower amplification level (30.000 *vs*. 50.000) than fresh liquid mixtures (Fig. 3a). Despite this, time-course of fluorescence during the reaction for dried mixtures was visibly detected and good quality amplification products appeared as typical ladder pattern on gel electrophoresis (Fig. 3b). A possible explanation for delay in DNA amplification after drying the reaction mixtures could be the role of trehalose in retarding conformational dynamics in dehydrated protein systems[45], because in dried trehalose systems the guest molecules are homogeneously integrated into a hydrogen-bond network of water-trehalose which strongly restricts their mobility[46]. A probable restriction in mobility of *Bst* polymerase in a trehalose matrix at low water content could result into lower enzymatic activity and, therefore, in lower efficiency in DNA amplification.

Stabilization of *S. mansoni* DNA for ready-to-use as positive control. The desiccation procedure for real-time SmMIT-LAMP assays was also evaluated in 8-tube strips with mixes containing pre-incorporated *S. mansoni* genomic DNA (1 ng) as positive control ready-to-use for further diagnostic purposes (Fig. 4). Besides, EvaGreen® dye was included or not in desiccation protocol. Next, dry-reagent LAMP mixes were reconstituted by adding just water (to those mixes containing *S. mansoni* DNA and dye) or water and fresh EvaGreen (to those mixes containing *S. mansoni* DNA but not dye). After 2 h of incubation, positive results were clearly observed by electrophoresis for both types of mixes, although with differences in fluorescence readings. The two types of dried mixes (with or without dried DNA-binding dye) yielded the same results in gel electrophoresis (Fig. 4b), but atypical small fluorescence readings appeared at the initial stages of the reactions when EvaGreen was previously dried (Fig. 4a). This could suggest a possible pre-amplification interaction between dye and DNA of *S. mansoni* during the desiccation process that ended up in a modification of the fluorescence signal during the reaction. The absence of information on this event in the literature does not allow us to compare our results, but we speculate with the possibility that this effect might be caused by an increase in the concentration of reagents after drying. The desiccation process would favor the interaction between dye and DNA, shifting the reaction equilibrium towards the bound conformation of EvaGreen and dsDNA, resulting in a small fluorescence signal[47]. This small fluorescence peak would disappear when the reagents become rehydrated and the reaction develops.

Evaluation of the stabilized real-time SmMIT-LAMP assay on patients' tissue samples. DNAs from three patients' tissue samples (skin, hepatic and appendix) with parasitological confirmed *S. mansoni* infection were used as "true samples" to test the potential applicability of the dry-reagent SmMIT-LAMP as ready-to-use test for schistosomiasis diagnosis (Fig. 5). To verify and compare results, a conventional real-time SmMIT-LAMP was also assessed (Fig. 5a). It is known that real-time SmMIT-LAMP works well with clinical samples, since we recently used it on patient's skin biopsies to confirm the diagnosis of ectopic cutaneous schistosomiasis[48]. Both fresh and desiccated SmMIT-LAMP mixtures yielded amplification signals for *S. mansoni*-positive control and tissue samples. Nevertheless, a delay in the appearance of positive results and a decrease in the fluorescence signals were observed when using desiccated mixtures (Fig. 5b). As already discussed, this result would be in accordance with the potential restriction in mobility of *Bst* polymerase in a trehalose matrix at low water content, resulting in lower efficiency in DNA amplification.

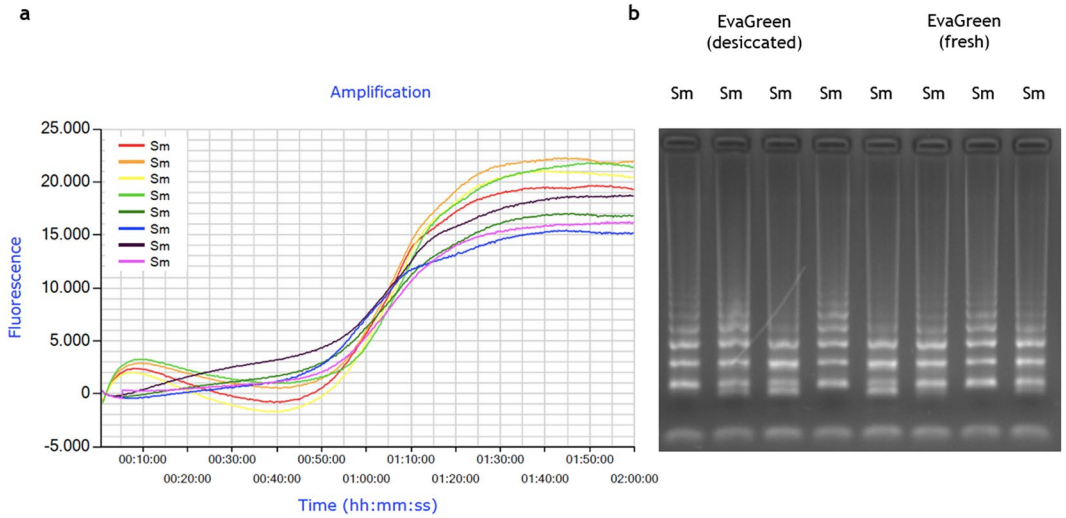

Figure 4. Results for real-time SmMIT-LAMP amplification using desiccated *Schistosoma mansoni* DNA as positive control ready-to-use. The assay was ran at 65 °C for a 2 h program in a portable Genie III device. (**a**) LAMP amplification with fluorescence detection with desiccated and fresh EvaGreen dye. (**b**) Agarose gel electrophoresis. Sm, positive control (*S. mansoni* DNA; 1 ng); Real-time graph was directly captured from Genie Explorer Software (V2.0.5.5) result report.

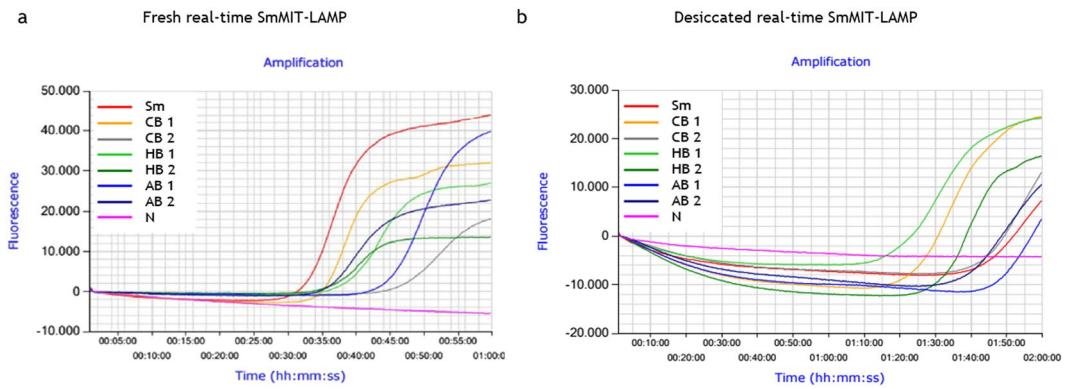

Figure 5. Amplification curves for *S. mansoni* in patients' tissue samples using fresh and desiccated real-time SmMIT-LAMP mixtures. Outputs from the LAMP run using (**a**) fresh master mixes and (**b**) desiccated master mixes. Tissue samples were analyzed in duplicate (1 and 2); CB1, CB2: cutaneous biopsy; HB1, HB2: hepatic biopsy; AB1, AB2: appendix biopsy. Sm, positive control (*S. mansoni* DNA; 1 ng); N, negative control (ultrapure water, no DNA). Real-time graphs are directly captured from Genie Explorer Software (V2.0.5.5) result report. Sample names in the legend were manually added to the image.

Stability performance over time of desiccated SmMIT-LAMP for conventional assays. The performance of the dry-reagent LAMP mix was assessed periodically for conventional assays by rehydration at one week, 3 weeks, one month, 3 months and 5 months (Fig. 6). Storage of dry-reagent LAMP mix at RT was found to be functional for 3 weeks (Fig. 6a). Remarkably, the dry-reagent LAMP mix proved to be stable up to 5 months at 4 °C (Fig. 6b).

A number of studies have attempted to address the problem of stabilizing molecular reagents for LAMP to be stored outside of the cold-chain, aiming to be applied for diagnostic purposes in field settings. Thus, different pre-mixed LAMP assays have been developed using lyophilization[22,23,49], drying[21,26,50] or combining different procedures, such as previous deglycerolization of *Bst* polymerase, followed by lyophilization of LAMP reagents[51]. Most studies highlight the proper operation of the dry-LAMP developed, but there are a number of differences and inaccuracies regarding the stability and functionality of the dry-reagent LAMP mix over time. Thus, in a study detecting *Leptospira* DNA, lyophilized reagents are stable for nearly 3 months when stored at 4 °C and 1 month when stored at 25 °C[49]. In another study, a dehydrated reagent mixture obtained by lyophilisation for detection of Zaire Ebola RNA Virus remained stable at RT for nearly 60 days with little loss of activity[22]. In the work of Howson *et al.*[23], a lyophilized RT-LAMP for specific detection of foot-and-mouth virus was developed; in this case, although lyophilisation of reagents had no impact on the performance of the assay when compared

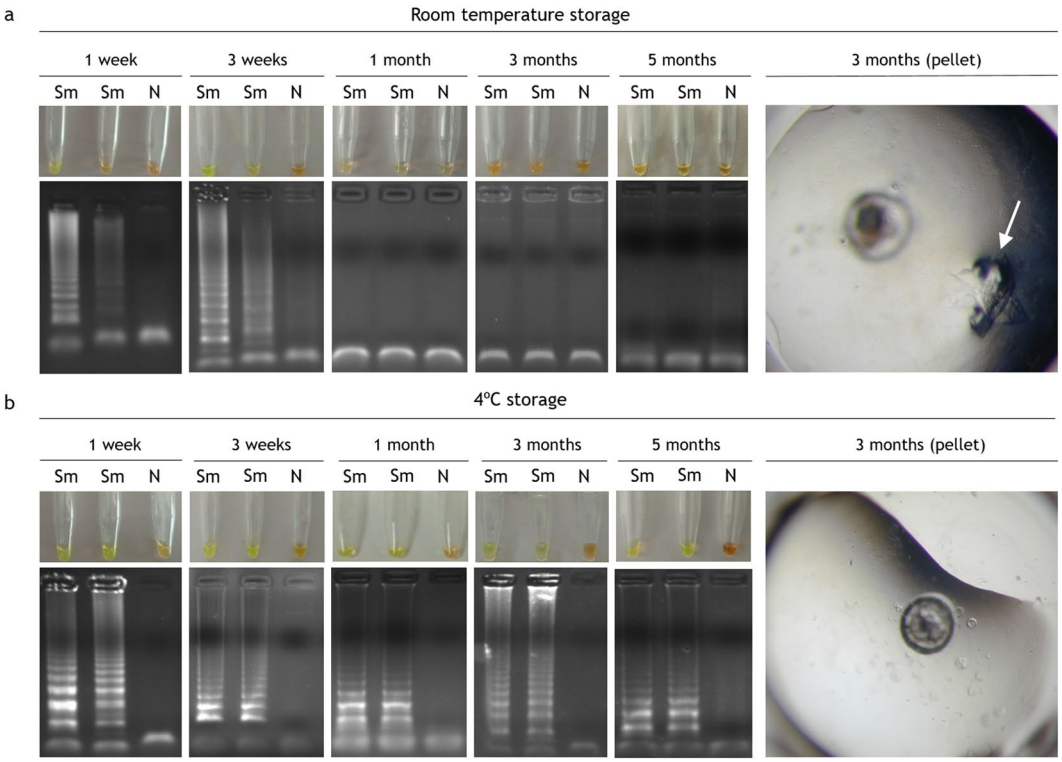

Figure 6. LAMP results of stabilized mixes over time at room temperature and 4 °C storage. (**a**) Room temperature storage. Visual inspection of reaction tubes positive (green) and negative (orange) and gel electrophoresis of DNA products. On the right, a pellet with a precipitate of crystals (white arrow) after 3 months storage at RT. (**b**) 4 °C storage. Visual inspection of reaction tubes positive (green) and negative (orange) and gel electrophoresis of DNA products. On the right, a pellet without any precipitate of crystals after 3 months storage at 4 °C. All electrophoresis gel images were obtained with an exposure between 700 ms and 1.2 s in a Gel documentation system, UVItec, UK. All gel images were captured within a day of the performance of the corresponding LAMP reaction.

to the equivalent "wet" reagents, no information about functionality over time is presented or discussed. On the other hand, several other studies on drying and stabilization of LAMP reagents using trehalose as cryoprotectant to prolong shelf-life at ambient temperature, have been published for detection of *Trypanosoma brucei rhodesiense*[21], both *Plasmodium falciparum* and non-*P. falciparum*[26] and *Trypanosoma evansi*[50]. In the study of Hayashida *et al.*[21], the sensitivity of the dry RIME-LAMP -for detection of the repetitive insertion mobile element (RIME) target in *T. b. rhodesiense*) did not change after a 7-month storage at RT. However, with the same methodology, later used for the stabilization of the LAMP reagents for detection of *Plasmodium* spp.[26] or *T. evansi*[50], no information regarding functionality over time is disclosed. Finally, in the study of Engku Nur Syafirah *et al.*[51], the dry-reagent LAMP mix developed for detection of toxigenic *Vibrio cholerae*, was found to be stable for 1 month at 4°C, 25°C and 37 °C; in addition, using the Q_{10} method, the shelf-life for this dry-LAMP was estimated at least 90.75 days at 25 °C.

In our work, the dry-reagent LAMP mix for *S. mansoni* detection resulted noticeably less stable over time at RT than the dry LAMP for *T. b. rhodesiense* detection developed by Hayashida *et al.*[21] (3 weeks *vs.* 7 months, respectively). Probably, our attempt to dry the reagents in a single step was detrimental to the stability over time because of the accumulation of trehalose crystals in the dry pellets (Fig. 6a; white arrow). As discussed in previous reports[43,44], under RT storage conditions, the presence of the reaction buffer in the stabilized reagent mix may favor crystallization of trehalose disrupting the oxidation barrier formed by the disaccharide and hindering the three-dimensional structure of the polymerase, thus resulting in a decrease or total loss of functionality. Despite this limitation, maintaining the functionality for at least 3 weeks at RT would allow us to prepare and distribute a set of dried SmMIT-LAMP master mixes to be used within a few weeks in field surveys of schistosomiasis in resource-limited areas. Further studies using other stabilizers are needed to achieve a better thermal stability of the LAMP reagents at ambient temperature. In this way, a new simple method that uses pullulan or mixture of pullulan and trehalose to achieve the long-term stabilization of LAMP master mixes has been recently reported[44].

By contrast, no crystallization was observed when storing the dry-reagent LAMP mix at 4 °C (Fig. 6b), thus likely maintaining the functionality of the mixture until at least 5 months of storage. To the best of our knowledge, this is the longest period of storage of stabilized LAMP reagents at 4 °C while maintaining functionality. Although our stabilized SmMIT-LAMP assay require a cold storage, its ready-to-use features have improved the previous conventional LAMP, reducing the possibilities of cross contamination during multiple pipetting steps in master mix preparation and repeated freezing and thawing of reagents prior use.

Point-of-care testing (POCT) is defined as laboratory diagnostic testing performed at or near the site where clinical care is delivered, allowing a rapid outcome and, potentially, the application of an early treatment[52]. The ideal POCT should be user-friendly and as simple as possible so that, even those without technical or clinical knowledge, would be able to use it and understand its response[53]. The ASSURED (Affordable, Sensitive, Specific, User-friendly, Robust and Rapid, Equipment-free and Deliverable) criteria established by the WHO, is a general benchmark to work towards developing POCT diagnosis[54] and it needs to be revisited for LAMP application in endemic areas. LAMP technology is a field-friendly alternative to many other complex molecular methods[55] and it has been proposed as an ideal candidate for revolutionizing point-of-care molecular diagnostics. In addition, novel LAMP-based platforms have been developed for pathogen detection and significant improvements have been made also in monitoring LAMP results, both end-point and real-time, thus bringing closer the LAMP technology to a realistic point-of-care format for resource-poor endemic areas[56]. The simplification, both in the number of steps and the infrastructure needed, would be a great advance in manufacturing and lowering the cost of preparation.

In summary, we present here the development and application of a novel and simple desiccation procedure for drying LAMP reagents using trehalose that can be also adapted for conventional and real-time amplification assays. The one-step dry-up protocol is simpler and faster than those previously reported and allows to maintain the functionality for at least 3 weeks at RT and up to 5 months at 4 °C. Our work demonstrates an important improvement for SmMIT-LAMP molecular assay, transformed into a cold maintenance dry format suitable for manufacturing as kit for ready-to-use for *S. mansoni* DNA detection. This procedure could be applied to create ready-to-use molecular dry LAMP kits for a laboratory setting and for POC diagnostic applications for several other NTD. Optimization and improvement of long-term stability at ambient temperature for a real application as a POCT in field conditions is still needed and currently ongoing.

Data Availability

No additional data (other than stated in the manuscript) was produced or used for the preparation of the manuscript. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

J.G.B., P.F.S., A.M.A. conceived and designed the experiments, J.G.B., P.F.S., B.C.V., S.A.C., B.F.S., A.G.S., B.V.S., J.L.A., conducted the experiments, P.F.S., A.M.A., funding acquisition, J.G.B. and P.F.S. prepared figures; J.G.B., P.F.S., B.C.V., B.F.S., A.M.A., analyzed the results, J.G.B., P.F.S., A.M.A., wrote and reviewed the manuscript.

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Progress in loop-mediated isothermal amplification assay for detection of *Schistosoma mansoni* DNA: towards a ready-to-use test.

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Supplementary Information

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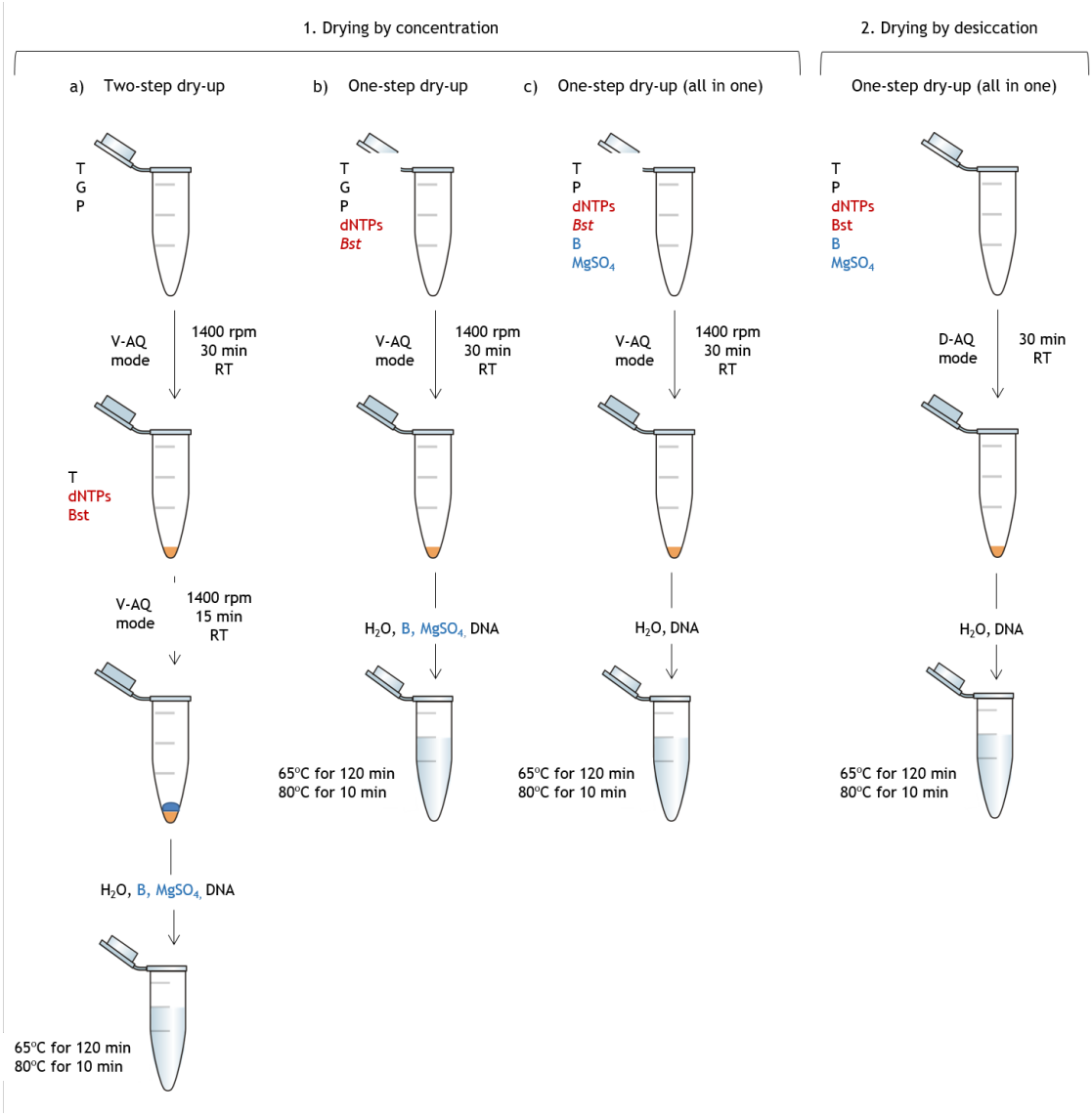
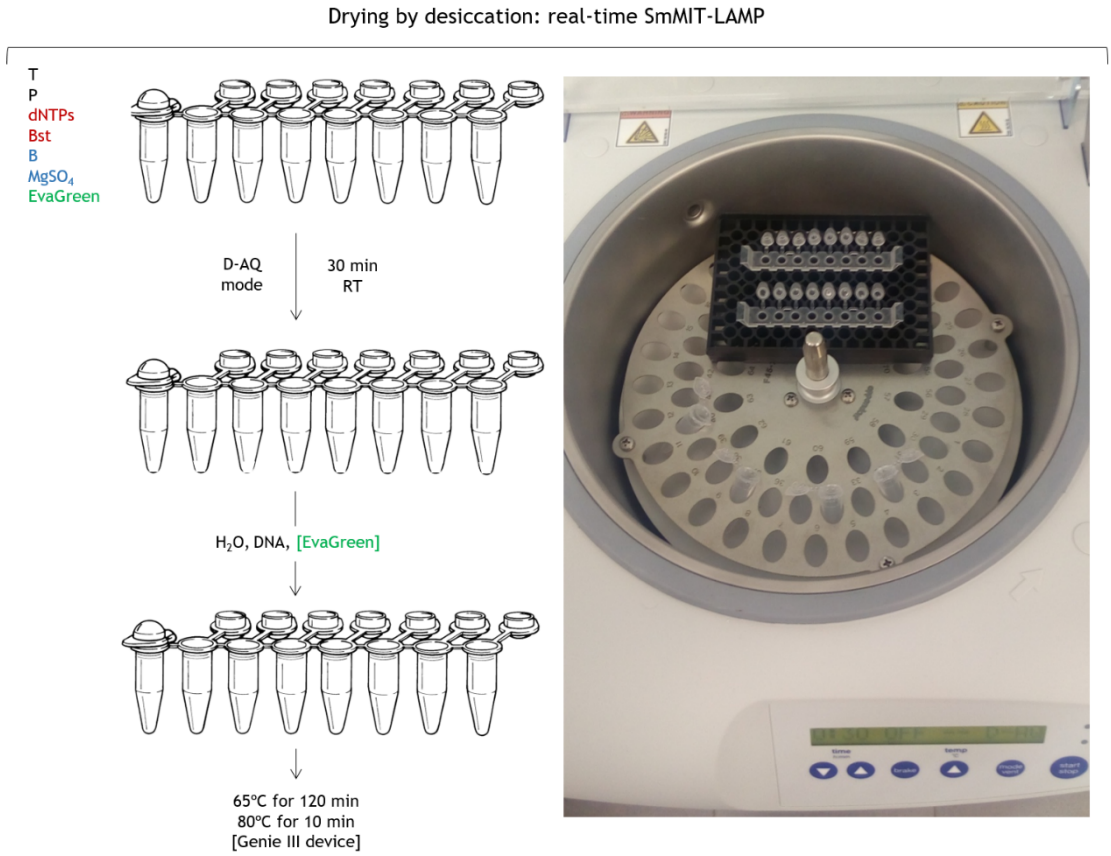
Supplementary Figure 1



Supplementary Figure 1 (S1). Stabilization procedures of SmMIT-LAMP reagents for conventional assays. 1. Drying by concentration. (1a) Concentration protocol following two dry-up steps. (1b) Concentration protocol following one dry-up step. (1c) Concentration protocol following one dry-up step (all in one). 2. Drying by desiccation using one dry-up step (all in one). T, trehalose; G, glycerol; P, primers; dNTPs, deoxynucleotides; Bst, *Bst* polymerase 2.0 WarmStart; B, isothermal buffer; $MgSO_4$, magnesioum sulphate; DNA, deoxyribonucleic acid; V-AQ mode, with centrifugation; D-AQ, without centrifugation; RT, room temperature; rpm, revolutions per minute.

Supplementary Figure 2



Supplementary Figure 2 (S2). Stabilization procedures of SmMIT-LAMP reagents for real-time assays. Drying was performed in all in one-step desiccation procedure in open 8-tube strips with or without the pre-addition of EvaGreen dye. T, trehalose; P, primers; dNTPs, deoxynucleotides; Bst, *Bst* polymerase 2.0 WarmStart; B, isothermal buffer; $MgSO_4$, magnesium sulphate; EvaGreen (included in pre-desiccation), D-AQ, without centrifugation; RT, room temperature; rpm, revolutions per minute; DNA, deoxyribonucleic acid; [EvaGreen], added in rehydration step.

3.2

Dispositivo de diagnóstico de enfermedades tropicales desatendidas

ES 1 259 239 U

Inventores

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Oficina Española de Patentes y Marcas

RESUMEN

Dentro de los objetivos de este trabajo se incluyen el desarrollo y la evaluación de un dispositivo portátil que permita realizar reacciones de amplificación isotérmica a tiempo real. Se podrían haber tomado numerosos enfoques, desde un dispositivo portátil capaz de leer señales fluorescentes, un diseño de tipo microfluídico o de *lab-on-a-chip* o incluso mediante la combinación de la técnica LAMP con dispositivos electroquímicos o tiras inmunocromatográficas. Sin embargo, siguiendo con el principio fundamental que ha regido esta Tesis Doctoral, buscamos una alternativa lo más sencilla y asequible posible, que nos permitiera utilizar material de laboratorio de uso habitual y que fuera un método fácilmente interpretable por alguien con poca experiencia, como por ejemplo un método colorimétrico.

Por ello, en colaboración con investigadores del grupo de investigación en Bioinformática, Sistemas Informáticos Inteligentes y Tecnología Educativa (BISITE) de la Universidad de Salamanca, desarrollamos el SMART-LAMP, un dispositivo portátil capaz de realizar reacciones de amplificación isotérmica y leer los resultados a tiempo real, basándose en un simple cambio colorimétrico de las muestras. Esta invención se protegió mediante un Modelo de Utilidad, bajo el título "*Dispositivo de diagnóstico de enfermedades tropicales desatendidas*", concedido por la Oficina Española de Patentes y Marcas a 5 de abril de 2021, confiriendo una protección de 10 años al dispositivo desde la fecha de solicitud (14 de diciembre de 2020).

La capacidad de lectura a tiempo real de los resultados que tiene el SMART-LAMP, se basa, a diferencia de la gran mayoría de dispositivos comerciales de RT-qPCR o amplificación isotérmica, en una lectura de cambios colorimétricos mediante unos sensores RGB, mucho más asequibles que los sensores para una lectura mediante fluorescencia. Además, la utilización de estos sensores permite flexibilidad en cuanto a los colorantes que se pueden utilizar para la detección de la amplificación. Cualquiera puede ser válido mientras se mantenga en el espectro visible y la lectura de resultados no está restringida a canales específicos de emisión y absorción como ocurre en los dispositivos basados en fluorescencia.

El dispositivo tiene un diseño muy sencillo, con un módulo de incubación que acepta ocho muestras, en tubos estándar de polipropileno de 0,2 mL. Un módulo de lectura, que comprende los lectores RGB y unas iluminaciones tipo LED cenitales a las muestras para homogeneizar las condiciones de lectura de color; y un módulo de gestión, que está conectado al módulo de incubación y que procesa toda la

información obtenida por el dispositivo. Esta información incluye tanto los resultados obtenidos de la reacción, como el posicionamiento GPS del dispositivo y la información del paciente. Estos datos pueden transmitirse a un dispositivo móvil (p.ej. *smartphones* o *tablets*) o a un ordenador de manera fácil mediante una conexión Bluetooth. Por tanto, el dispositivo resulta independiente de la disponibilidad de conexión a internet que haya en el lugar de trabajo. Por último, tiene un módulo de visualización que permite controlar si el dispositivo está o no activo, la temperatura a la que se encuentra y el tiempo transcurrido y restante de reacción.

Está alimentado de tres baterías de Ion-Litio extraíbles, intercambiables y recargables (en el propio dispositivo o con un cargador externo), pudiendo ser independiente de la corriente eléctrica durante largos periodos de tiempo. El dispositivo se controla de manera muy sencilla a través de una aplicación móvil, que permite modificar las condiciones de reacción, generar una base de datos con información relevante de los pacientes analizados, seguir a tiempo real la evolución de los valores RGB de cada muestra y visualizar y almacenar todos los resultados.

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TÍTULO
DE
MODELO DE UTILIDAD

Cumplidos los requisitos previstos en la vigente Ley 24/2015, de 24 de julio, de Patentes, se expide el presente TÍTULO, acreditativo de la concesión del Modelo de Utilidad.

Se otorga al titular un derecho de exclusiva en todo el territorio nacional, bajo las condiciones y con las limitaciones en la Ley de Patentes. La duración del modelo de utilidad será de **diez años** contados a partir de la fecha de presentación de la solicitud (14/12/2020).

El modelo de utilidad se concede sin perjuicio de tercero y sin garantía del Estado en cuanto a la validez y a la utilidad del objeto sobre el que recae.

Para mantener en vigor el modelo de utilidad concedido, deberán abonarse las tasas anuales establecidas, a partir de la tercera anualidad. Asimismo, deberá explotarse el objeto de la invención, bien por su titular o por medio de persona autorizada de acuerdo con el sistema de licencias previsto legalmente, dentro del plazo de cuatro años a partir de la fecha de presentación de la solicitud del modelo de utilidad, o de tres años desde la publicación de la concesión en el Boletín Oficial de la Propiedad Industrial, aplicándose el plazo que expire más tarde.

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(54) Título: **DISPOSITIVO DE DIAGNÓSTICO DE ENFERMEDADES TROPICALES DESATENDIDAS**

ES 1 259 239 U

DESCRIPCIÓN

DISPOSITIVO DE DIAGNÓSTICO DE ENFERMEDADES TROPICALES DESATENDIDAS

OBJETO DE LA INVENCIÓN

El objeto de la invención es un dispositivo de diagnóstico de enfermedades tropicales desatendidas con capacidad de medir una reacción de amplificación isotérmica de ácidos nucleicos tipo LAMP, a tiempo real, haciendo uso de la detección colorimétrica en la lectura de resultados.

ANTECEDENTES DE LA INVENCIÓN

Las enfermedades tropicales desatendidas (NTD, *Neglected Tropical Diseases*) son un grupo diverso de enfermedades infecciosas crónicas, debilitantes y, a menudo, estigmatizantes, que afectan a más de mil millones de personas en ciento cuarenta y nueve países de zonas tropicales y subtropicales, con especial incidencia en poblaciones que viven en condiciones de extrema pobreza. Actualmente, incluso se han llegado a notificar casos autóctonos en el sur de Europa.

Uno de los problemas más importantes que se plantean en las NTD es su diagnóstico, debido a la falta de sensibilidad de los métodos parasitológicos clásicos, la falta de especificidad y reacciones cruzadas de los métodos serológicos, la falta de estandarización y coste elevado de los métodos moleculares, así como la inespecificidad de sus manifestaciones clínicas, principalmente en la fase aguda de la enfermedad.

Teniendo en cuenta estas limitaciones, es necesario el desarrollo y aplicación de nuevos métodos que reúnan las características ideales para el diagnóstico como alta sensibilidad y especificidad, facilidad de uso e interpretación, utilización de diferentes tipos de muestras, rapidez, bajo coste

y potencialmente aplicables en zonas endémicas de la enfermedad con escasos recursos económicos.

Para resolver estos problemas, se puede utilizar la técnica de amplificación isotérmica de ácidos nucleicos (LAMP, *Loop-mediated isothermal amplification*). Esta técnica puede llevarse a cabo en diversos dispositivos portátiles y se ha utilizado en el diagnóstico de distintas enfermedades infecciosas. Todos estos dispositivos se basan en la detección de la amplificación de ADN por una señal fluorescente.

Los fluoróforos necesarios en la mezcla de reacción para obtener la señal fluorescente y los requerimientos técnicos para su lectura suponen un importante encarecimiento, limitando su uso en zonas endémicas.

DESCRIPCIÓN DE LA INVENCIÓN

La presente invención logra resolver los problemas planteados anteriormente con un dispositivo pequeño, portátil, de gran autonomía, capaz de medir la amplificación isotérmica de ácidos nucleicos a tiempo real por un cambio colorimétrico, detectado *in situ* con unos lectores RGB.

Todo ello en un dispositivo controlado y cuyos resultados pueden visualizarse y analizarse a tiempo real a través de una aplicación de teléfono móvil.

Esta invención soluciona además las desventajas derivadas del número limitado de canales de los dispositivos de fluorescencia, que son dos únicamente en los dispositivos portátiles actuales, y permite el uso del dispositivo con un conjunto mucho más variado de indicadores, a elección del usuario.

Concretamente, el dispositivo de diagnóstico de enfermedades tropicales desatendidas tiene la capacidad de medir una reacción isotérmica de amplificación de ácidos nucleicos tipo LAMP, a tiempo real, por cambio colorimétrico, de al menos una muestra, produciéndose una amplificación de ADN (secuencia diana), lo que permite la detección altamente sensible de patógenos.

La reacción LAMP es isotérmica y utiliza una enzima polimerasa de ADN cuya propiedad es la del desplazamiento de la cadena de ADN junto con su propiedad habitual de polimerización.

Además, la técnica LAMP amplifica el ADN del patógeno permitiendo una visualización directa de la reacción, por la liberación durante esta, de pirofosfatos que causan turbidez que, al unirse a unos iones de magnesio o manganeso presentes en la reacción, generan un precipitado que provoca un aumento de la turbidez de la mezcla de la reacción.

Al llevarse a cabo la reacción, se genera una señal química de reconocimiento altamente sensible, por lo que se permite la discriminación visual de los resultados.

El dispositivo comprende, en primer lugar, un módulo de incubación de muestras destinado a recibir las muestras junto con una mezcla de reacción, a una temperatura controlada y durante un tiempo concreto, en un ambiente seco y seguro. El tiempo de procesado de las muestras es configurable minuto a minuto.

El módulo de incubación de las muestras comprende unos indicadores de evolución de la reacción, como pueden ser unos LEDs o indicadores acústicos que informan acerca de la temperatura y tiempo de la reacción.

Además, este módulo comprende unos elementos de seguridad con la capacidad de detectar y alertar cuando se alcanzan temperaturas muy elevadas en el dispositivo que podrían ocasionar quemaduras u otro tipo de lesiones.

En segundo lugar, el dispositivo comprende un módulo de lectura del cambio colorimétrico de la reacción, que comprende unos lectores RGB (*Red Green Blue*) de la reacción, destinados a posicionarse sobre las muestras cuando se cierra el dispositivo. La distinción de los valores RGB por parte de los lectores consiste en la obtención de unos pulsos eléctricos diferentes que son proporcionales a la intensidad del color detectado.

Además, el módulo de lectura comprende una retroiluminación de la reacción, que queda posicionada sobre los lectores RGB y las muestras, iluminando ambas desde arriba cuando se cierra el dispositivo, facilitando la lectura con independencia de las condiciones del ambiente, y permitiendo el uso del dispositivo en zonas endémicas sujetas a condiciones ambientales variables.

La lectura de estos colores se hace por tanto de manera automática con los lectores RGB, pero el dispositivo comprende también unas ventanas de validación visual de la muestra y la reacción, que permiten la obtención de resultados a tiempo real.

En tercer lugar, el dispositivo comprende un módulo de gestión de información, conectado al módulo de incubación, con capacidad de almacenar los valores RGB, extraer resultados, así como almacenar datos relacionados con la muestra y transmitirlos a otros dispositivos externos.

Los datos recopilados por el módulo de gestión de información pueden ser tanto los relativos a los resultados obtenidos de la lectura del cambio colorimétrico de la reacción, como la posición GPS, información del paciente,

los datos del responsable de la toma de las muestras, información del dispositivo, u otra información relevante.

El módulo de gestión de la información tiene la capacidad de transmitir estos datos recopilados a través de GDM/LTE, una tarjeta SIM, Wifi, Bluetooth o utilizando una conexión VPN. Además, los datos pueden ser almacenados en una tarjeta SD interna o cualquier otro sistema de almacenamiento alternativo.

Finalmente, el dispositivo comprende una batería recargable y reemplazable. Se puede monitorizar el estado de carga de dicha batería, y permite la recarga desde vehículos, red eléctrica convencional u otros. La batería es intercambiable, no teniendo que esperar a su recarga para seguir utilizando el dispositivo.

Adicionalmente, el dispositivo puede comprender un módulo de visualización de datos, como una pantalla LCD alfanumérica, que permite al usuario visualizar y validar los resultados, realizar operaciones de envío de información, etc.

DESCRIPCIÓN DE LOS DIBUJOS

Para complementar la descripción que se está realizando y con objeto de ayudar a una mejor comprensión de las características de la invención, de acuerdo con un ejemplo preferente de realización práctica de la misma, se acompaña como parte integrante de dicha descripción, un juego de dibujos en donde con carácter ilustrativo y no limitativo, se ha representado lo siguiente:

Figura 1.- Muestra una vista superior del dispositivo de diagnóstico de enfermedades tropicales desatendidas.

Figura 2.- Muestra una vista superior del dispositivo en la que se pueden observar las muestras posicionadas en el módulo de incubación.

Figura 3.- Muestra una vista frontal del dispositivo en el que se observa la retroiluminación del módulo de lectura.

Figura 4.- Muestra una vista inferior evidenciando la batería (5).

Figura 5.- Muestra una vista lateral evidenciado los indicadores luminosos (10) de la carga de la batería.

Figura 6.- Muestra una vista posterior evidenciando el ventilador (11), la toma de corriente (12) y el puerto USB (13).

REALIZACIÓN PREFERENTE DE LA INVENCIÓN

A la vista de las figuras descritas anteriormente, se puede observar un ejemplo de realización no limitativo del dispositivo (1) de diagnóstico de enfermedades tropicales desatendidas con capacidad de medir una reacción de amplificación isotérmica de ácidos nucleicos tipo LAMP a tiempo real por un cambio colorimétrico, en al menos una muestra (9).

El dispositivo (1) comprende, en primer lugar, un módulo de incubación (2) de las muestras (9) a temperatura controlada mientras se produce la reacción y el consiguiente cambio colorimétrico, destinado a recoger las muestras (9) junto con una mezcla de reacción.

El módulo de incubación (2) tiene la capacidad de incubar las muestras (9) a una temperatura comprendida entre 35ºC y 90ºC durante un periodo de tiempo de 5-120 minutos, ajustando la temperatura a intervalos de 1ºC, con precisión de ±0.5ºC y manteniendo estos valores constantes en un ambiente

seco y seguro, durante un tiempo de procesado configurable minuto a minuto, dependiendo de la muestra (9) analizada.

El módulo de incubación (2) comprende unos indicadores (6) visuales o acústicos que informan acerca de la temperatura y tiempo de la reacción.

Para la generación de calor el módulo comprende unas células de Peltier y para la estabilización de la temperatura un algoritmo de control de temperatura PID (Proporcional Integral y Derivativo) que mantiene la precisión de ±0.5ºC.

El módulo de incubación (2) comprende unos elementos de seguridad con la capacidad de detectar y alertar cuando se alcanzan temperaturas muy elevadas en el dispositivo (1) que podrían ocasionar quemaduras u otro tipo de lesiones. Además, los materiales que constituyen el dispositivo (1) son aislantes térmicamente e ignífugos en los puntos en los que se alcanzan temperaturas elevadas y que son susceptibles de provocar incendios en caso de fallo.

En segundo lugar, el dispositivo comprende un módulo de lectura (3) del cambio colorimétrico de la reacción, en el que unos lectores RGB tipo HDJD-S822 determinan los valores RGB de dicho cambio colorimétrico. Este módulo comprende también una retroiluminación (8) de la reacción que facilita el procesado de los valores RGB con independencia de las condiciones ambiente, facilitando el uso del dispositivo (1) en zonas endémicas sujetas a condiciones ambientales variables.

La distinción de los valores RGB por parte de los lectores RGB consisten en la obtención de unos pulsos eléctricos diferentes que son proporcionales a la intensidad del color detectado.

La lectura de estos colores se hace por tanto de manera automática utilizando los lectores RGB, pero también se permite la validación visual, permitiendo la obtención de resultados a tiempo real mientras tiene lugar la reacción de cada muestra (9) en el módulo de incubación. Para ello, el dispositivo (1) comprende ventanas de validación visual, desde los que se puede observar directamente la muestra (9) y la reacción.

En tercer lugar, el dispositivo comprende un módulo de gestión de información (4), conectado al módulo de lectura (3), con capacidad de almacenar los valores RGB registrados por los lectores RGB, extraer resultados, así como almacenar datos relacionados con las muestras (9) y transmitirlos a otros dispositivos externos.

Los datos recopilados en el módulo de gestión de información (4) son tanto los relativos a los resultados obtenidos de la lectura de las muestras (9), como la posición GPS, información del paciente, los datos del responsable de la toma de las muestras (9), información del dispositivo, u otra información relevante.

El propio módulo de gestión de información (4) tiene la capacidad de trasmitir los datos recopilados a través de GDM/LTE, una tarjeta SIM, Wifi, Bluetooth o utilizando una conexión VPN. Además, tienen la capacidad de almacenar los datos en una tarjeta SD interna o cualquier otro sistema de almacenamiento alternativo.

Los datos almacenados están cifrados, salvaguardando así la identidad de los usuarios y permitiendo certificar tanto la procedencia como la corrección de los mismos, cumpliendo las leyes internacionales de protección de datos.

Finalmente, el dispositivo (1) comprende una batería (5) de polímero de iones de Litio (LiPo). El dispositivo (1) tiene la capacidad de monitorizar el estado de carga de dicha batería, que se puede recargar desde vehículos,

red eléctrica convencional u otros. La batería es intercambiable, no teniendo que esperar a su recarga para seguir utilizando el dispositivo (1).

Adicionalmente, el dispositivo (1) comprende un módulo de visualización de datos (7), que es una pantalla LCD alfanumérica de dieciséis caracteres y dos líneas, conectada al módulo de gestión de información (4), destinada a que el usuario pueda visualizar y validar los resultados, realizar operaciones de envío de información, etc.

Además, el dispositivo puede comprender indicadores luminosos (10) de la carga de la batería, un ventilador (11), toma de corriente (12) y puerto USB (13).

Los datos obtenidos proporcionan una información que se puede visualizar y analizar, y que permite asimismo predecir y analizar la evolución de las distintas enfermedades de forma descentralizada. Esta información se almacena en una nube, permitiendo el acceso en tiempo real y de forma distribuida.

REIVINDICACIONES

1.- Dispositivo (1) de diagnóstico de enfermedades tropicales desatendidas con capacidad de medir una reacción de amplificación isotérmica de amplificación de ácidos nucleicos tipo LAMP en al menos una muestra (9), a tiempo real, por cambio colorimétrico, caracterizado porque comprende:

- un módulo de incubación (2) de las muestras (9) a una temperatura controlada, mientras se produce la reacción, destinado a recibir las muestras (9) junto con una mezcla de reacción,
- un módulo de lectura (3) de cambio colorimétrico, que comprende unos lectores RGB del cambio colorimétrico de la reacción, destinados a posicionarse sobre las muestras (9), y una retroiluminación (8) de la reacción posicionada sobre los lectores RGB y la muestra (9),
- un módulo de gestión de información (4) conectado al módulo de lectura (3), con capacidad de almacenar los valores RGB, extraer resultados, almacenar datos relacionados con la muestra (9) y transmitirlos a otros dispositivos externos,
- una batería (5) recargable y reemplazable.

2.- El dispositivo (1) de la reivindicación 1, en el que el módulo de incubación (2) tiene la capacidad de incubar las muestras (9) en un rango de temperaturas comprendido entre 35ºC y 90ºC.

3.- El dispositivo (1) de la reivindicación 1, en el que el módulo de incubación (2) tiene la capacidad de incubar las muestras (9) durante un periodo de tiempo comprendido entre 5 y 120 minutos.

4.- El dispositivo (1) de la reivindicación 1, en el que el módulo de incubación (2) comprende adicionalmente unos indicadores (6) luminosos o acústicos de la temperatura y tiempo de la incubación de las muestras (9).

5.- El dispositivo (1) de la reivindicación 1, en el que el módulo de incubación (2) comprende un control PID (Proporcional Integral y Derivativo) de la temperatura.

6.- El dispositivo (1) de la reivindicación 1, que comprende adicionalmente unas alarmas conectadas al módulo de incubación (2) que se activan en el caso de que la temperatura de la reacción aumente de manera descontrolado o excesiva.

7.- El dispositivo (1) de la reivindicación 1, en el que los lectores RGB del módulo de lectura (3) son de tipo HDJD-S822.

8.- El dispositivo (1) de la reivindicación 1, en el que los datos almacenados en el módulo de gestión de la información (4) son seleccionados entre la posición GPS, información del paciente, el responsable de toma de las muestras (9) y la información del dispositivo (1).

9.- El dispositivo (1) de la reivindicación 1, en el que el módulo de gestión de información (4) tiene la capacidad de transmitir los datos a un dispositivo externo haciendo uso de un sistema seleccionado entre tarjeta SIM, WiFi, Bluetooth y conexión VPN.

10.- El dispositivo (1) de la reivindicación 1, en el que la batería (5) es de polímero de iones de litio (LiPo).

11.- El dispositivo (1) de la reivindicación 1, que comprende adicionalmente un módulo de visualización (7) de datos conectado al módulo de gestión de la información (4), que permite la visualización y envío de los datos a dispositivos externos.

12.- El dispositivo (1) de la reivindicación 1, que comprende adicionalmente indicadores luminosos (10) de la carga de la batería, un ventilador (11), toma de corriente (12) y puerto USB (13).

5

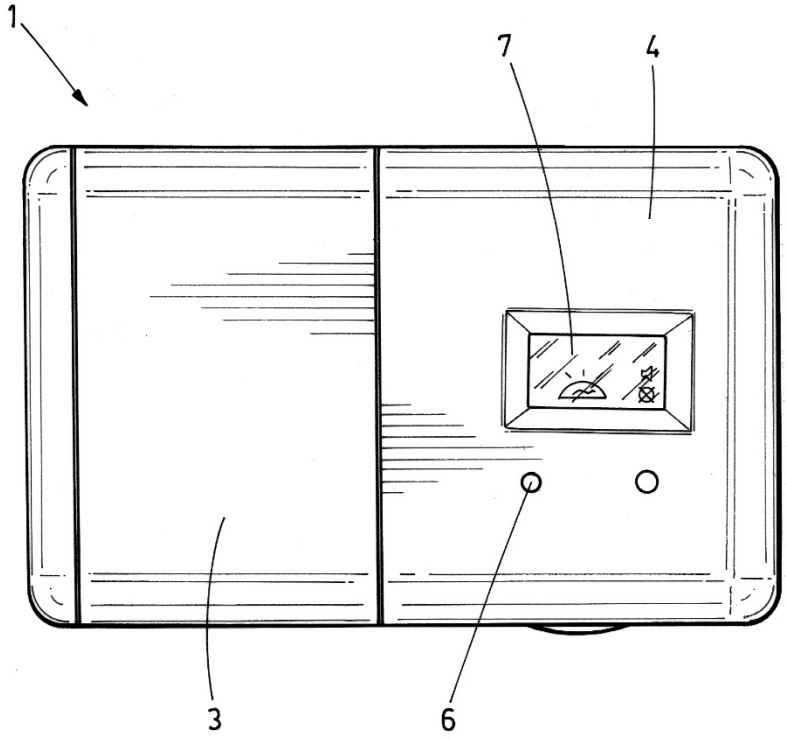

FIG.1

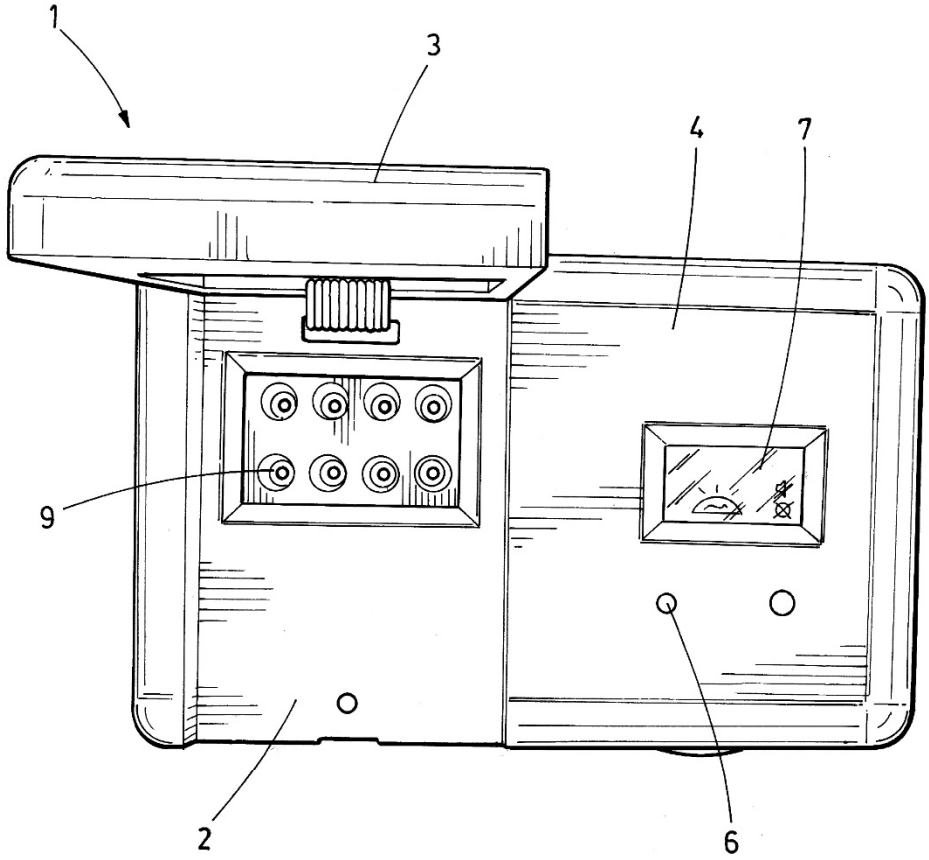

FIG.2

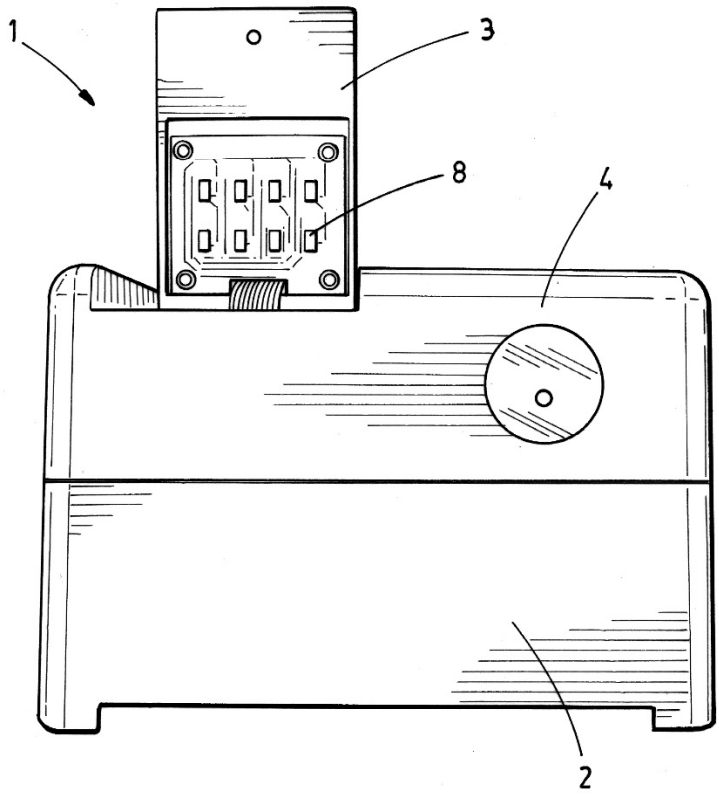

FIG.3

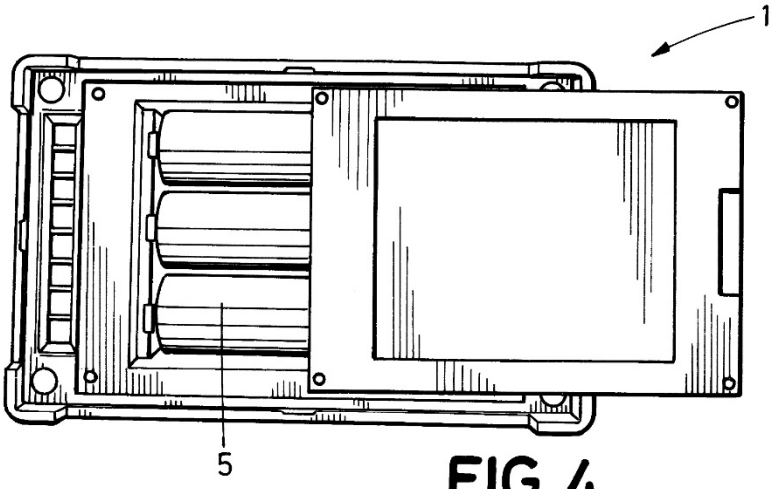

FIG. 4

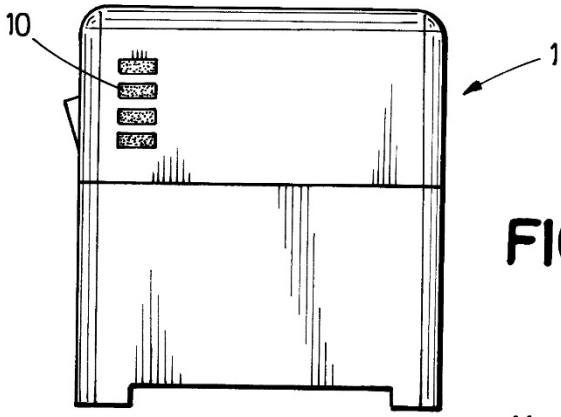

FIG.5

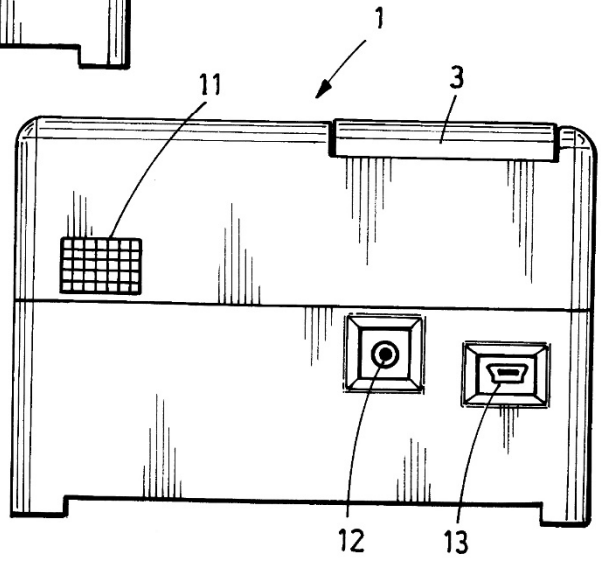

FIG.6

3.3

A Simple, Affordable, Rapid, Stabilized, Colorimetric, Versatile RT-LAMP assay to detect SARS-CoV-2

Juan García-Bernalt Diego, Pedro Fernández-Soto, Marta Domínguez-Gil, Moncef Belhassen-García, Juan Luis Muñoz Bellido, Antonio Muro

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RESUMEN

La emergencia de la pandemia de COVID-19 comprometió la actividad de muchos laboratorios para intentar dar una respuesta rápida y eficaz a la enfermedad. La necesidad de realizar cribados masivos en instalaciones poco preparadas para llevar a cabo pruebas de laboratorio (pabellones, aeropuertos, parkings, …) y que ofrecieran un resultado rápido, nos hizo considerar la técnica LAMP como una alternativa interesante a los métodos disponibles en los primeros compases de la crisis. Además, nos permitió modificar y evaluar nuestro protocolo de estabilización de los reactivos, con la intención de mejorar la estabilidad en el tiempo y valorar la puesta a punto del método para un nuevo patógeno. También, comprobamos que el proceso de retrotranscripción previo a la amplificación LAMP necesario para la detección de SARS-CoV-2, no se viese afectado por la estabilización de los componentes de la reacción.

En un enfoque integral, se seleccionaron ocho dianas diferentes, incluyendo regiones clave del genoma del virus (*ORF1ab*, *ORF1b* y genes *S*, *E*, *M* y *N*). Obtuvimos los mejores resultados con los ensayos dirigidos a las regiones *ORF1ab*, *E* y *N*. Se comprobó que estos ensayos eran sensibles, especialmente los dirigidos a la secuencia *ORF1ab* y *N*. También resultaron específicos tanto *in silico* como *in vitro*. Además, los resultados de los ensayos dirigidos a la región *ORF1ab* y al gen *N* (N15-RT-LAMP) demostraron una sensibilidad similar a la de la RT-qPCR. Comprobamos que la correlación entre RT-qPCR y RT-LAMP se mantenía independientemente de que el ensayo se realizara a tiempo real o colorimétricamente.

Con los resultados obtenidos en el primer artículo de investigación, decidimos modificar el protocolo de estabilización. El objetivo era separar las sales probablemente que generaban la cristalización de la enzima, componente más sensible que el resto a los procesos de degradación y oxidación. Para ello, manteniendo el protocolo de desecación de media hora a temperatura ambiente, dividimos la mezcla de reactivos en dos volúmenes en el mismo tubo para ser secados a la vez. El tampón de reacción, el $MgSO_4$ y el colorante se secaban en la tapa del tubo en presencia de trehalosa (2M) y el resto de los componentes, también en presencia de trehalosa (2M), se secaban en el fondo del mismo tubo. De esta manera, obteníamos dos *pellets* bien formados y adheridos, cuyos componentes solo entrarían en contacto en el momento de rehidratar las mezclas, simplemente volteando el tubo de reacción.

Este sencillo cambio demostró una mejora muy importante en los resultados con reactivos estabilizados. Se mejoró la cinética y la estabilidad a temperatura ambiente, incrementándose a más de dos meses.

En conclusión, en este trabajo desarrollamos un método mejorado de estabilización de los componentes de una reacción tipo LAMP, observando que era efectivo para la detección de secuencias de otros patógenos. Comprobamos que la adición de los componentes necesarios para la retrotranscripción antes de la amplificación LAMP no tenía efectos significativos y demostramos que los resultados obtenidos con este protocolo eran comparables a los obtenidos por RT-qPCR como técnica de referencia.

No podemos obviar las limitaciones que presenta este estudio, como el pequeño tamaño muestral o la falta de sensibilidad de algunos ensayos desarrollados para detectar el virus en muestras con cargas virales muy bajas.

diagnostics

MDPI

Article

A Simple, Affordable, Rapid, Stabilized, Colorimetric, Versatile RT-LAMP Assay to Detect SARS-CoV-2

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Abstract: The SARS-CoV-2 pandemic has forced all countries worldwide to rapidly develop and implement widespread testing to control and manage the Coronavirus Disease 2019 (COVID-19). reverse-transcription (RT)-qPCR is the gold standard molecular diagnostic method for COVID-19, mostly in automated testing platforms. These systems are accurate and effective, but also costly, time-consuming, high-technological, infrastructure-dependent, and currently suffer from commercial reagent supply shortages. The reverse-transcription loop-mediated isothermal amplification (RT-LAMP) can be used as an alternative testing method. Here, we present a novel versatile (real-time and colorimetric) RT-LAMP for the simple (one-step), affordable (~1.7 €/sample), and rapid detection of SARS-CoV-2 targeting both *ORF1ab* and *N* genes of the novel virus genome. We demonstrate the assay on RT-qPCR-positive clinical samples, obtaining most positive results under 25 min. In addition, a novel 30-min one-step drying protocol has been developed to stabilize the RT-LAMP reaction mixtures, allowing them to be stored at room temperature functionally for up to two months, as predicted by the Q_{10}. This Dry-RT-LAMP methodology is suitable for potentially ready-to-use COVID-19 diagnosis. After further testing and validation, it could be easily applied both in developed and in low-income countries yielding rapid and reliable results.

Keywords: SARS-CoV-2; RT-LAMP; molecular diagnostics; dry-RT-LAMP; point-of-care

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1. Introduction

Coronavirus disease 19 (COVID-19) is an infection caused by the novel coronavirus SARS-CoV-2, which emerged in China in December of 2019, becoming the seventh member of the *Coronaviridae* family to infect humans [1]. Although it is less severe than other previously described coronaviruses infecting humans, such as SARS or MERS coronaviruses, it has a significantly higher transmission capacity. This elevated transmission compelled the World Health Organization (WHO) to declare a global health emergency on 31 January 2020 and, subsequently, a pandemic situation on 11 March 2020 [2]. Until the date of writing this manuscript, the COVID-19 pandemic had already affected over 114 million people worldwide and caused over 2.5 million deaths (https://coronavirus.jhu.edu/map.html; accessed on 2 March 2021).

For COVID-19, a mean incubation period of 6.4 days (ranging from 2.1 to 11.1 days) is estimated [3]. Clinical manifestations can range from mild flu-like symptoms to severe or critical, and patients can present an either symptomatic or asymptomatic infection. The

later seem to account for 40 to 45% of cases [4]. The most prevalent symptoms are fever, cough (either productive or not) and myalgia or fatigue, but other signs such as headache, hemoptysis and diarrhea may appear [5,6]. In severe presentations, COVID-19 is associated with pneumonia and acute respiratory distress syndrome (ARDS), for which elderly and chronic disease patients are particularly susceptible [7]. SARS-CoV-2 can also affect the gastrointestinal, nervous, or cardiovascular systems [6]. Timely diagnosis in the early infection stages is hindered by the aforementioned incubation period together with the asymptomatic course or unspecific manifestations of the illness in a high proportion of patients [5]. Early diagnosis allows a prompt intervention, reducing the risk of developing more serious complications. Moreover, one of the main challenges to contain the spread of COVID-19 is the identification of asymptomatic cases.

In that sense, nucleic acid detection-based tests are reliable and accurate approaches for viral infection detection. Specifically, reverse-transcription (RT)-qPCR is the main molecular method used for the detection of all kinds of coronaviruses, including SARS-CoV-2 [8,9]. Currently, COVID-19 RT-qPCR-based tests target mainly the ORF1ab region of SARS-CoV-2 genome, combined with genes coding for the E and N proteins. However, protocols for commercial RT-qPCR kits uses different reagents and different combination of the aforementioned genes depending on the country [10]. Moreover, the devices to perform RT-qPCR result in a wide range of costs and processing times, as well as variations in tests accuracy [11]. Moreover, RT-qPCR technology is not easily adaptable for point-of-care diagnosis in low-resource settings due to the need for temperature cycling. An increasingly recognized alternative is loop-mediated isothermal amplification (LAMP) technology, a highly efficient, specific, and rapid technology to amplify DNA at a constant temperature, using two or three primer sets and a *Bst* polymerase with high strand displacement activity [12]. Many prominent advantages of LAMP over PCR-based technologies in terms of sensitivity, specificity, rapidity, robustness, and cost have been extensively informed [13]. Additionally, different approaches have been developed to allow LAMP reagents storage, in a single tube at room temperature over extended periods, to be used for point-of-care testing [14]. Reverse-transcription LAMP (RT-LAMP) combines LAMP to amplify DNA from an RNA target in one-step reaction by directly adding a dedicated reverse transciptase[10] or a DNA polymerase with reverse transcriptase activity to the reaction mixture. RT-LAMP shares the versatility and all the benefits of LAMP technology and has already been developed for the detection of numerous RNA viruses including virus influenza, Zika, Ebola, and MERS [15]. The prominent prospect of RT-LAMP in the context of COVID-19 diagnosis has been recently discussed by Augustine et al. [16].

Thus, since the outbreak of COVID-19, in parallel with the emergence of new in-house and commercial RT-qPCR assays to detect SARS-CoV-2 RNA, numerous RT-LAMP assays have been rapidly developed mainly targeting ORF1ab [17–24] and gene N [17,19,22,25–30] sequences, which are the regions recommended for RT-qPCR by the Centre of Disease Control and Prevention (CDC, Atlanta, USA) [31]. Although less explored, some assays have also targeted Gen S [17,19], Gen E [30] and Gen M sequences [32]. A comparison of different RT-LAMP for SARS-CoV-2 detection, including master mixes, primer sets, targeting genes, readout monitoring, and analytical sensitivity has been recently summarized and examined by Thompson & Lei [33]. Those studies expose a wide variety of methodologies, sensitivities, and results. Furthermore, a commercial technology based on RT-LAMP, ID NOW COVID-19 (ABBOTT LABORATORIES, Chicago, USA) has been granted the Emergency Use Authorization (EUA) by the FDA [9]. RT-LAMP in combination with other molecular techniques such as CRISPR-Cas12 [30] or recombinase polymerase assay (RPA) [24], has also been optimized for SARS-CoV-2 detection. Additionally, various strategies to avoid RNA extraction and purification, one of the main bottlenecks molecular testing is facing now, have been presented, mainly for nasopharyngeal swabs [24] and saliva [34] analyses.

In this paper, with the aim of contributing to an effective COVID-19 diagnosis, we present a novel, specific, sensitive, rapid, and versatile RT-LAMP assay for SARS-CoV-2

RNA detection. We demonstrate our RT-LAMP assay proper operation on clinical samples by using a portable real-time device and in conventional colorimetric trials easily visualized with the naked eye. Furthermore, we developed a new simple one-step desiccation procedure to stabilize the RT-LAMP reagents in a single tube for potentially ready-to-use COVID-19 diagnosis. The Dry-RT-LAMP methodology does not require complex instrumentation and it is much faster to perform (30 min) than other available alternatives such as lyophilization. The Dry-RT-LAMP format could be very useful for easy testing in situations of high diagnostic demand and in low-resources settings thus contributing to rapid diagnosis for COVID-19.

2. Materials and Methods

2.1. Clinical Specimens

Nasopharyngeal swabs specimens were collected in Sample Preservation Solution (MOLE BIOSCIENCE, SUNGO Europe B.V., Amsterdam, The Netherlands) as part of the routine testing of patients for COVID-19 at the University Hospital of Salamanca, Salamanca, Spain. Collected samples were delivered to the Laboratory of Microbiology, and then processed in a biosafety level 2 cabin until inactivation by mixing with a lysis buffer.

2.2. RNA Isolation and RT-qPCR Amplification

Samples were processed either by performing RNA isolation (NUCLISENS EASYMAG, BIOMÉRIEUX, France) and RT and amplification (VIASURE SARS-CoV-2 Real-Time PCR Detection Kit, CERTEST BIOTECH, Spain) separately, or in an integrated way in an automated platform (COBAS 6800, ROCHE, Switzerland) following manufactures' instructions. RT-qPCR for the detection of SARS-CoV-2 RNA was performed using commercial kits and reagents targeting ORF1ab and gen N (VIASURE SARS-CoV-2) or ORF1ab and gen E (COBAS SARS-CoV-2 Test) following manufacturers' instructions. Aliquots of purified RNA samples were stored at −80 °C until further analysis. For RNA samples analyzed, SARS-CoV-2 Cycle threshold (Ct) values for ORF1ab, E, or N amplified targets were used as reference for the RT-LAMP assays.

2.3. Viral RNA-Positive Control and Patients' RNA Samples Selected

An RNA isolate amplified by VIASURE SARS-CoV-2 Real-Time PCR Detection Kit from a positive COVID-19 patient with a Ct = 25 min for ORF1ab target and a Ct = 29 min for gen N was selected as a well-established RNA-positive control to set up the COVID-LAMP assays (hereafter, C+). The Ct values allowed us to determinate a concentration between 10^5 and 10^4 copies per RT-qPCR reaction (cpr) (5 µL of sample), attending to the VIASURE SARS-CoV-2 Real-Time PCR Detection Kit's handbook (https://www.certest.es/wp-content/uploads/2020/03/IU-NCO212enes0420-rev.01.pdf, accessed on 10 November 2020). Therefore, a concentration of 2×10^4 cpr (1 µL of C+) was estimated for analytical sensitivity calculations.

To further test COVID-LAMP effectiveness, 20 RNA isolates from COVID-19 patients were selected and distributed into four groups according to Ct values obtained from RT-qPCR: group 1 (n = 6), RT-qPCR-positive for both ORF1ab (Ct $\leq$ 30 min) and E/N genes; group 2 (n = 6), RT-qPCR-positive for both ORF1ab (Ct > 30 min) and E/N genes; group 3 (n = 2), RT-qPCR-positive for ORF1ab and RT-qPCR-negative for gen E; group 4 (n = 6), RT-qPCR-negative for ORF1ab and RT-qPCR-positive for N gene. Groups of samples, Ct values for amplified targets, and commercial kits used in RT-qPCR are indicated in Table 2.

2.4. RT-LAMP Primer Design

Primer sets used for LAMP were based on the SARS-CoV-2 complete genome sequence from the NCBI nucleotide database (GenBank: MN908947.3) [35] to target specific regions in ORF1ab, ORF1b, S, E, M, and N genes. The primer set used to target the conserved sequence of ORF1ab was previously described by El-Tholoth et al. [24]. Primer sets targeting ORF1b and S genes were original designs, based upon recently reported sequences used for RT-

qPCR detection of SARS-CoV-2 by Wu et al. [35], with the appropriate modifications to fit RT-LAMP constrains. Primer sets targeting E, M, and N genes were also original designs. All primer sets were designed using the open access Primer Explorer V5 software tool (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan) at the website: https://primerexplorer.jp/e/; accessed on 1 May 2020. Once the best parameters have been considered, single primer sets were selected for ORF1ab, ORF1b, E, and M genes; for S (S447, S555) and N (N5, N15) genes two primer sets targeting different regions within each gene were selected. Each primer set included two outer primers (F3 and B3), two inner primers (FIP and BIP) and, for ORF1ab, S447, N5, and N15 primer sets, two additional loop primers (LF and LB) were designed and selected. All the primers were synthesized (synthesis scale, 0.025 µmol; purification, desalt; solution, water) by EUROFINS GENOMICS (Ebersber, Germany). The localization of LAMP targets on the genome of SARS-CoV-2 is represented in Figure 1. Sequences of the oligonucleotide primer sets finally selected are listed in Table 1.

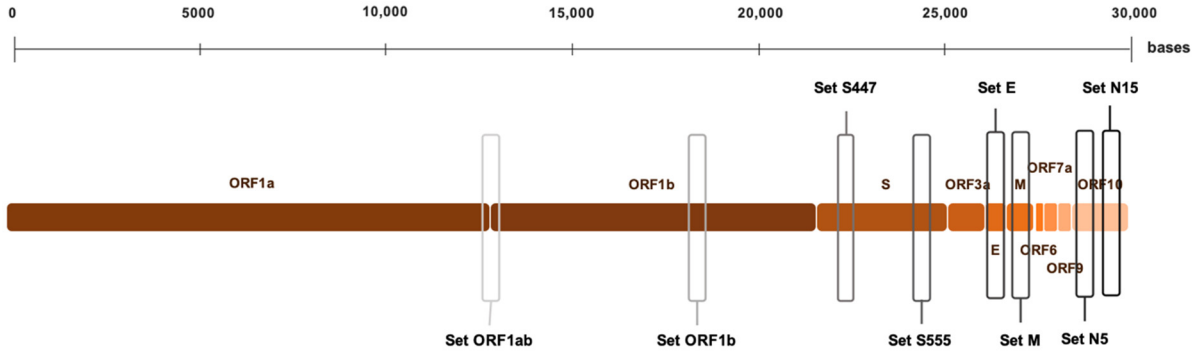

Figure 1. Schematic representation of COVID-LAMP target localization within SARS-CoV-2 genome. Genbank sequence accession number: MN908947.3 [35].

Table 1. Primer sets used in this study for the detection of SARS-CoV-2 through reverse-transcription loop-mediated isothermal amplification (RT-LAMP).

Set	Primer [1]	Sequence 5′→3′	Length (nt)	Gene Position
ORF1ab [2]	F3	TGCTTCAGTCAGCTGATG	18	13,434–13,636
	B3	TTAAATTGTCATCTTCGTCCTT	22	
	FIP	CAGTACTAGTGCCTGTGCCGCACAATCGTTTTT AAACGGGT	41	
	BIP	TCGTATACAGGGCTTTTGACATCTATCTTGGAAG CGACAACAA	43	
	LF	CTGCACTTACACCGCAA	17	
	LB	GTAGCTGGTTTTGCTAAATTCC	22	
ORF1b [3]	F3	CACAGACTTTGTGAATGAGTT	21	15,654–15,896
	B3	GTCAGTCTCAGTCCAACAT	19	
	FIP	CTATTGAAACACACAACAGCATCGCATATTTGC GTAAACATTTCTCA	47	
	BIP	TATGCATCTCAAGGTCTAGTGGCTATGCTTCAGA CATAAAAACATTG	47	
S447 [3]	F3	GTTTCTGCCTTTCCAACAA	19	22,985–23,415
	B3	AACAGGGACTTCTGTGCA	18	
	FIP	TCAAGAATCTCAAGTGTCTGTGGTGGCAGAGA CATTGCTGA	41	
	BIP	ACCATGTTCTTTTGGTGGTGTCAACATCCTGATA AAGAACAGC	43	
	LF	TCACGGACAGCATCAGTAGTG	21	
	LB	CAGGAACAAATACTTCTAACCAGGT	25	

Table 1. *Cont.*

Set	Primer [1]	Sequence 5′→3′	Length (nt)	Gene Position
S555 [3]	F3	CTATGCAAATGGCTTATAGGTT	22	24,182–24,736
	B3	AGTTGTTTAACAAGCGTGTT	20	
	FIP	GCACTATTAAATTGGTTGGCAATCATAATGGTAT TGGAGTTACACAGA	48	
	BIP	ATTGGCAAAATTCAAGACTCACTTTTGTGCATTT TGGTTGACC	43	
E [4]	F3	TCATTCGTTTCGGAAGAGA	19	26,245–26,472
	B3	AGGAACTCTAGAAGAATTCAGAT	23	
	FIP	TGTAACTAGCAAGAATACCACGAAACAGGTACG TTAATAGTTAATAGCG	49	
	BIP	GCTTCGATTGTGTGCGTACTCGAGAGTAAACGT AAAAAGAAGG	43	
M [4]	F3	GTTTCCTATTCCTTACATGGATT	23	26,597–26,801
	B3	AGCCACATCAAGCCTACA	18	
	FIP	CCATAACAGCCAGAGGAAAATTAACCTTCTACAA TTTGCCTATGCC	46	
	BIP	AACTTTAGCTTGTTTTGTGCTTGCACAAGCCATT GCGATAGC	42	
N5 [4]	F3	CCAGAATGGAGAACGCAGTG	20	28,355–28,570
	B3	CCGTCACCACCACGAATT	18	
	FIP	AGCGGTGAACCAAGACGCAGGGCGCGATCAAAA CAACG	38	
	BIP	AATTCCCTCGAGGACAAGGCGAGCTCTTCGGTAG TAGCCAA	41	
	LF	ATTATTGGGTAAACCTTGGGGC	22	
	LB	ATTAACACCAATAGCAGTCCAGATG	25	
N15 [4]	F3	AGATCACATTGGCACCCG	18	28,703–28,915
	B3	CCATTGCCAGCCATTCTAGC	20	
	FIP	TGCTCCCTTCTGCGTAGAAGCCAATGCTGCAATCGTGCTAC	41	
	BIP	GGCGGCAGTCAAGCCTCTTCCCTACTGCTGCCTGGAGTT	39	
	LF	GCAATGTTGTTCCTTGAGGAAGTT	24	
	LB	CCTCATCACGTAGTCGCAACAG	22	

F3, forward primer; B3, backward primer; BIP, backward inner primer; FIP, forward inner primer; LB, loop backward; LF, loop forward; [1] Primer concentrations remain unchanged for all sets: 1.6 µM FIP/BIP, 0.2 µM F3/B3, 0.4 µM LF/LB; [2] Primer set previously described by El-Tholoh et al. [24]; [3] Primer sets (original design) based upon sequences used for RT-qPCR detection of SARS-CoV-2 by Wu et al. [35]; [4] Primer sets (original design).

2.5. RT-LAMP Reaction

For the one-step RT-LAMP reaction, two reaction mixtures containing different polymerases were evaluated. On one hand, RT-LAMP assay was performed using *Bst* 3.0 DNA Polymerase (*Bst* 3.0) (NEW ENGLAND BIOLABS Ltd., Ipswich, USA) for both isothermal amplification performance and reverse-transcription. On the other hand, RT-LAMP assays were performed in the presence of two enzymes: *Bst* 2.0 WarmStart DNA Polymerase (*Bst* 2.0 WS) and WarmStart *RTx* Reverse Transcriptase (*RTx* WS) (NEW ENGLAND BIOLABS Ltd., Ipswich, USA). Briefly, RT-LAMP reaction mixtures (15 µL) contained 1.6 µM FIP/BIP primers, 0.2 µM F3/B3 primers, 0.4 µM LF/LB primers (if applicable), 1.4 mM of each dNTP, (BIORON GmBH, Römerberg, Germany) 0.13 M of D-(+)-Trehalose dihydrate (Sigma-Aldrich, USA) (from now on, trehalose), 6mM $MgSO_4$, and 1× Isothermal Amplification Buffer II (20 mM Tris-HCl (pH 8.8), 150 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20) for *Bst* 3.0 DNA polymerase (0.32 U/µL) or 1× Amplification Buffer (20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20) for *Bst* 2.0 WS (0.32 U/µL) and *RTx* WS (0.3 µL), with 1 µL of template RNA (C+, for positive control; ultrapure water for negative control). To establish the optimal reaction conditions, the one-step RT-LAMP assay was real-time evaluated at different temperatures and reaction

times in 8-tube Genie Strips on a portable Genie III device (OPTIGENE Ltd., Horsham, UK). In this case, 0.24 µL of EvaGreen 20× in water (BIOTIUM, San Francisco, USA) was added to the reaction mix before the reaction started. For *Bst* 3.0 reactions, temperatures ranging 62 °C to 72 °C (in 2 °C increments) and reaction times of 45, 60, and 80 min were tested. For *Bst* 2.0 WS and *RTx* WS reactions, different temperatures (63 °C, 64 °C, 65 °C) and reaction times (45, 60, 80 min) were also tested. All reactions were performed in duplicates.

In addition, conventional colorimetric one-step RT-LAMP reactions were also performed with *Bst* 2.0 WS and *RTx* WS in a heating block. For this, attending to samples availability limitations, eight RNA isolates representing the four groups of COVID-19 patients' samples used in the study were selected. Results were visually inspected by the naked eye based on the color change observed (green for a positive result and orange for a negative result) with 1 µL of SYBR Green I 1000× (INVITROGEN, USA) added post-amplification to each tube. To avoid potential cross-contamination with amplified products, the tubes were briefly centrifuged and carefully opened in a laminar flow hood before adding the dye.

2.6. Sensitivity and Specificity

To assess the analytical sensitivity of the primer sets in the detection of SARS-CoV-2, serial dilutions (10-fold) of the C+ in nuclease-free water (diluted from 1× to 10^{-4}) were prepared and used to determine the limit of detection of the RT-LAMP assays.

To confirm the specificity of the evaluated primers a BLASTN local search and alignment analysis was carried out in GenBank online databases (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 1 September 2020) against currently available nucleotide sequences for other human respiratory viruses and other human-infecting viruses. Comparison included: Influenza A virus (taxid:11320), Influenza B virus (taxid:11520), Human parainfluenza virus 1 strain Washington/1964 (taxid:188538), Human parainfluenza virus 2 (strain Toshiba) (taxid:11214), Human parainfluenza virus 4a (taxid:11224), Human adenovirus 4a (taxid:35263), Human adenovirus 7 (taxid:10519), Enterovirus A (taxid:138948), Enterovirus B (taxid:138949), Enterovirus C (taxid:138950), Metapneumovirus (taxid:162387), Respiratory Syncytial Virus (taxid:12331), Zika virus (taxid:64320), Dengue virus (taxid:12637), Chikungunya virus (taxid:37124), and Middle East respiratory syndrome-related coronavirus (MERS-CoV) (taxid:1335626).

Additionally, the RT-LAMP primer sets were cross-tested for specificity against a panel of 13 RNA isolates of related coronaviruses obtained from patients infected with Coronavirus NL63, Coronavirus OC43, Bocavirus, Rinovirus, Metapneumovirus, Respiratory Syncytial Virus A, Respiratory Syncytial Virus B, Enterovirus, Parainfluenzae 1, Influenza H1N1, Influenza A H3, Influenza A H1, and Influenza B. These RNA isolates were provided by the laboratory of the National Influenza Centre of Valladolid (University Clinical Hospital of Valladolid, Valladolid, Castilla y León, Spain). This laboratory is part of a network of 126 laboratories around the world linked to the WHO responsible for the characterization and diagnosis of circulating influenza viruses.

2.7. Stabilization for Long-Term Room-Temperature Storage: Dry-RT-LAMP

We optimized the RT-LAMP reaction protocol for potential ready-to-use COVID-19 RT-LAMP test. For long-term room temperature storage, the master mixes containing the two enzymes and primer sets ORF1ab, N5, or N15 were stabilized by a vacuum process without centrifugation (so called, desiccation) in a Concentrator Plus (EPPENDORF, Hamburg, Germany) at RT for 30 min, following a single dry-up step as previously described elsewhere [14], with some modifications. In brief, RT-LAMP master mixes were dried in open 8-tube Genie Strips (OPTIGENE, Horsham, UK) separately in two partial mixes: one containing primers, dNTPs, and polymerases placed in the bottom of the tube in the presence of 1.8 µL of trehalose 2M; other containing Isothermal Buffer 10×, $MgSO_4$, and 0.24 µL EvaGreen 10× in the tube cap in the presence of 2.25 µL of trehalose 2M. The desiccation procedure yielded two stable and well-adhered pellets in both cap and bottom

of the tubes. To estimate the stability and functionality over time, the desiccated 8-tube strips were stored at 25 °C, 37 °C, and 45 °C for up to 28 days in paperboard storage boxes with some Silica Gel desiccant pouches inside to protect against moisture until use. After rehydration with ultrapure water (for negative controls) or ultrapure water containing RNA (for C+ or RNA samples), the real-time RT-LAMP assays with Genie III were performed at 63 °C for up to 120 min at 0, 1, 7, 14, 21, and 28 days post-desiccation.

2.8. Estimation of the Shelf-Life of the Dry-RT-LAMP Mixes

To estimate the shelf-life of the dry-reagent RT-LAMP mixes the accelerated ageing technique (also known as Q_{10} method) described by Clark (1991) was employed [36]. The shelf-life can be determined by either a real-time or an accelerated ageing test where Arrhenius Law is applied in a simulated environment. The method was conducted by exposing the dry-reagent RT-LAMP mixes to different temperatures (25 °C, 37 °C, 45 °C) for up to 28 days and assessing the functionality of the dried RT-LAMP reagents periodically, at 0, 1, 7, 14, 21, and 28 days post-desiccation. Data obtained were used to calculate the assay stability at RT (25 °C) with the following formulas:

$$AF = Q_{10}^{[0.1\times(Te-Ta)]}$$

$$AG = t_e \times AF$$

$$Estimated\ Shelf-life = AG + t_e$$

where AF is the acceleration factor used to correlate the shelf-life of the product at a lower temperature than the one used to perform the experiment; Q_{10} factor measures the temperature sensitivity of an enzymatic reaction rate due to an increase by 10 °C; T_e represents the elevated temperature; AG is the accelerated age; t_e is the length of time storage at elevated temperature, and T_a is the ambient temperature (RT; 25 °C). Since for most biological reactions Q_{10} ~ 2 or 3 [37], we established a conservative value for Q_{10} = 1.9 to perform all the calculations. The evaluation of the estimated shelf-life was performed for dried master mixes mentioned above for up to 28 days using C+ as template. Additionally, dried N15-RT-LAMP master mixes for up to one week were also tested using RNA isolates as templates.

3. Results

3.1. RT-LAMP Primer Sets Screening and Selection

The first screening of each primer set designed was performed using the *Bst* 3.0 DNA polymerase in real-time conditions to amplify C+ template at different temperatures and reaction times described in the 'Methods' section. Frequently, non-specific amplification was obtained, and results were rather irregular and not reproducible. Therefore, further use in RT-LAMP amplification of SARS-CoV-2 RNA was discarded.

The second screening of each primer set was also performed in real-time conditions using the combination of the *Bst* 2.0 WS and RT × WS. After testing different RT-LAMP conditions, a reaction time of 60 min at 63 °C plus 5–10 min of inactivation was considered the most appropriate for performance comparison among primer sets. Subsequently, the accuracy and efficiency of each primer set was evaluated through RT-LAMP using C+ as template in duplicate. Results obtained are shown in Figure 2. Four of the eight primer sets were selected for further evaluation based on the shorter time to positivity (Tp)—thus meaning the fastest amplification—reproducibility and the absence of non-specific amplifications: set ORF1ab (Tp = 20.5 min), set E (Tp = 43.5 min), set N5 (Tp = 20 min), and set N15 (Tp = 15 min). The remaining primer sets were discarded for further assessment because of relatively early non-specific amplifications (set ORF1b), failed amplification (set S447), poor amplification and reproducibility (set M), or long Tp value (set S555).

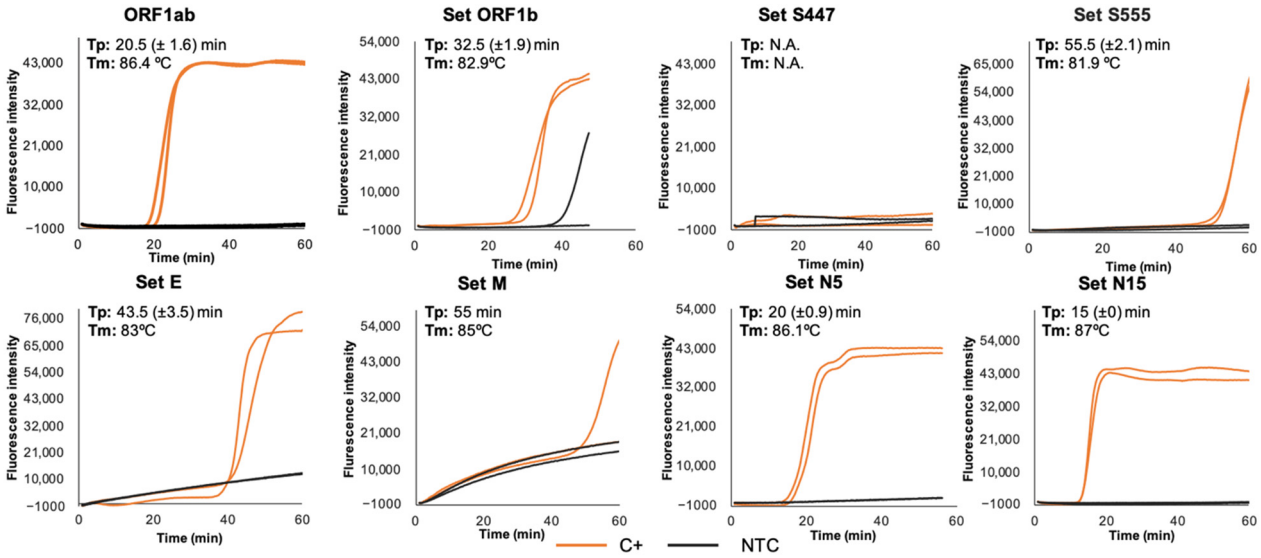

Figure 2. Real-time RT-LAMP assays performed using the eight primer sets evaluated for the detection of SARS-CoV-2. EvaGreen 20× fluorescence signal over time for primer sets ORF1ab, ORF1b, S447, S555, E, M, N5, and N15 is shown. Orange lines (C+, positive control); black lines (NTC, non-template control). Time to positivity with standard error (Tp (±SE); min) and melting temperatures (Tm; °C) for each primer set are indicated. All reactions were performed in duplicates.

3.2. Sensitivity and Specificity of RT-LAMP

Regarding to the sensitivity of the RT-LAMP assays, 10-fold serial dilutions of C+ was amplified by real-time RT-LAMP to determine the lower limit of detection. Analytical sensitivities for selected primer sets ORF1ab, E, N5, and N15 are shown in Figure 3. The results indicated that RT-LAMP assays using sets ORF1ab, N5, and N15 were 10 times more sensitive than RT-LAMP assay using set E (1:100 vs. 1:10 dilution, respectively). According to this, an approximate limit of detection of 2×10^2 cpr for RT-LAMP using sets ORF1ab, N5, and N15, and 2×10^3 cpr for RT-LAMP using set E was established.

No significant similarity between targets selected for SARS-CoV-2 detection and other sequences reported for possible human-infecting viruses was in silico detected, when searching in databases. Furthermore, no RNA isolates from patients infected with related coronaviruses was amplified when using the selected primer sets that resulted in the most efficient in amplifying SARS-CoV-2 RNA (set ORF1ab, set E, set N5, set N15), thus indicating the high specificity of the established RT-LAMP assays (Figure 4).

3.3. Clinical Samples Testing

Based on the analysis with RT-qPCR, 20 RNA isolates from COVID-19 patients were analyzed by RT-LAMP with the most efficient primer sets in this study: set ORF1ab, set E, set N5, and set N15. The comparison of the Ct values obtained by RT-qPCR and Tp values of RT-LAMP assays is shown in Table 2. When testing the six RT-qPCR-positive samples of group 1 (ORF1ab+; Ct $\leq$ 30/E+ or N+) the RT-LAMP assays using primer sets ORF1ab, N5, and N15, each detected 6/6 (100% sensitivity) with shorter Tp values for all samples than Ct values obtained by RT-qPCR. It needs to be highlighted that Tp for RT-LAMP includes both retro-transcription and amplification processes in a one-step reaction while Ct of RT-PCR accounts only for the amplification time but not retro-transcription time. The RT-LAMP using primer set E detected 5/6 samples (83.3% sensitivity), with a long Tp = 72 min for sample 2, the only one tested by COBAS RT-qPCR for gene E (Ct = 26) in this group. The Ct values for the four remaining positive samples for gene E resulted equal or higher than those of the VIASURE RT-qPCR for N gene.

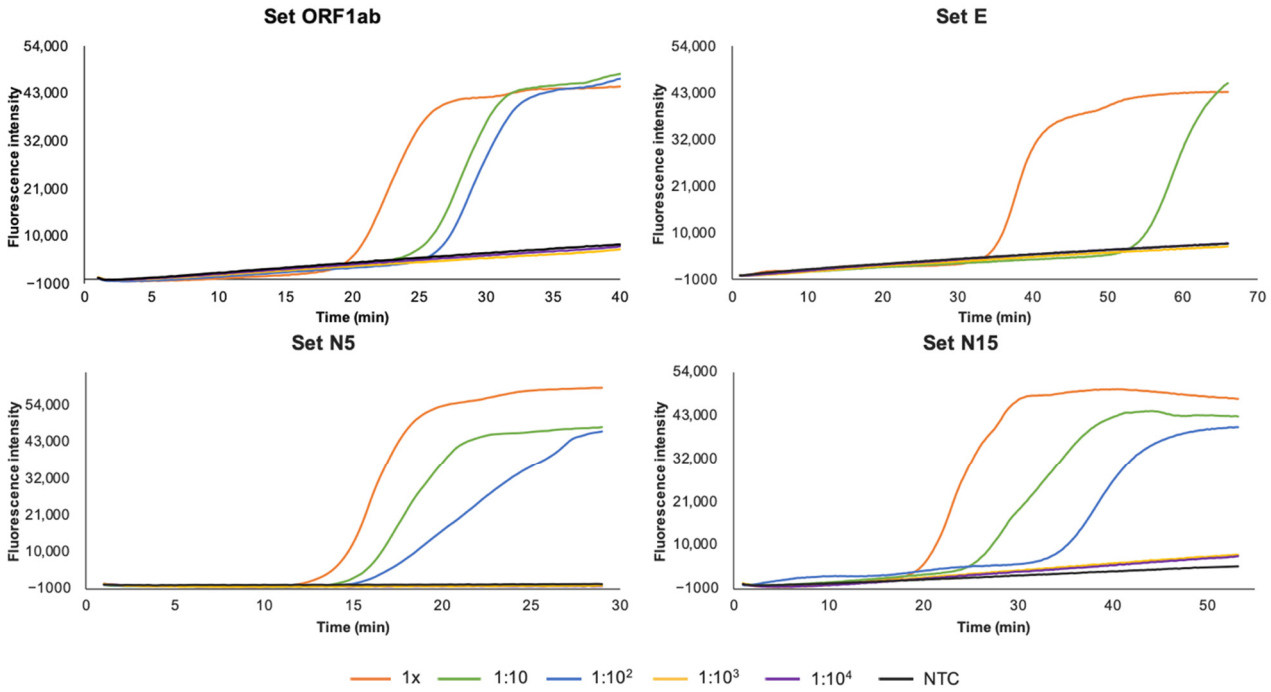

Figure 3. Sensitivity assessment of the RT-LAMP assays for SARS-CoV-2 RNA detection using primer sets ORF1ab, E, N5, and N15. The 10-fold dilutions ($1\times$-$1{:}10^4$) of positive control (C+) are represented by different color lines; non-template control (NTC) is represented by black lines.

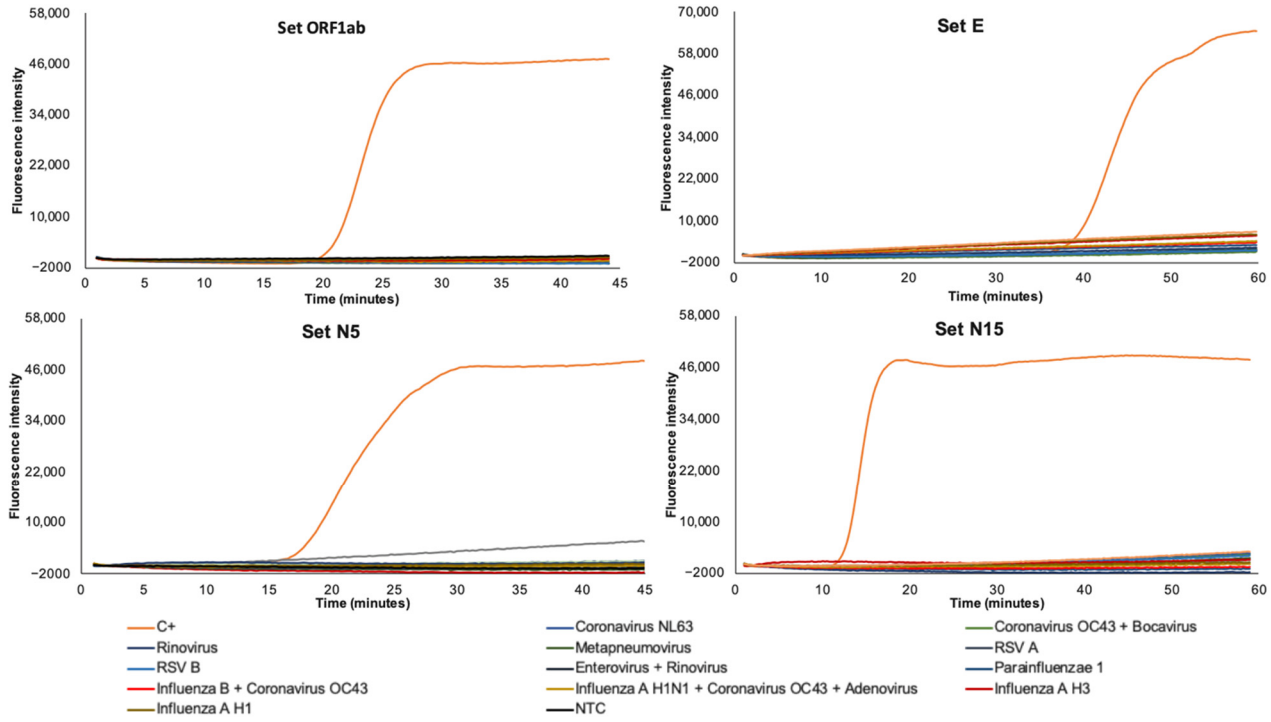

Figure 4. Specificity assessment of the RT-LAMP assays for SARS-CoV-2 RNA detection using primer sets ORF1ab, E, N5, and N15. A panel of 13 purified RNA isolates of related viruses obtained from infected patients are included: Coronavirus NL63, Coronavirus OC43, Bocavirus, Rinovirus, Metapneumovirus, Respiratory Syncytial Virus A, Respiratory Syncytial Virus B, Enterovirus, Parainfluenzae 1, Influenza H1N1, Influenza A H3, Influenza A H1, and Influenza B. One sample contained RNA from two viruses (Influenza B + Coronavirus OC43); other sample contained RNA from three viruses (Influenza A H1 + Coronavirus OC43 + Adenovirus).

Table 2. Comparison of the results of cycle threshold (Ct) values obtained by RT-qPCR and time to positivity (Tp) values obtained by RT-LAMP assays using primer sets ORF1ab, N5, N15, and E in testing 20 RNA isolates from COVID-19 patients. Groups of samples, commercial Real-Time PCR Detection Kits targeting ORF1ab and E/N used in RT-qPCR tests, and primer sets evaluated for RT-LAMP assays are indicated. Ct and Tp values are indicated in minutes.

			RT-qPCR Ct Values		Real-Time RT-LAMP Tp Values			
Groups	**No. Sample**	**Commercial Kit** [1]	**ORF1ab**	**E/N**	**ORF1ab**	**N5**	**N15**	**E**
Group 1 ORF1ab+ (Ct < 30) E/N+	1	VIASURE	22	27	15	14	9	27
	2	COBAS	25	26	18	16	13	72
	3	VIASURE	25	29	15	15	19	43
	4	VIASURE	26	25	17	16	11	28
	5	VIASURE	27	31	17	18	13	-
	6	VIASURE	27	30	19	18	13	35
Group 2 ORF1ab+ (Ct > 30) E/N+	7	VIASURE	32	31	20	22	13	60
	8	COBAS	32	35	35	23	49	-
	9	VIASURE	33	39	23	20	39	-
	10	COBAS	33	36	31	-	60	-
	11	COBAS	34	36	-	-	45	-
	12	VIASURE	39	36	-	-	36	-
Group 3 ORF1ab+ E−	13	COBAS	36	-	39	-	29	-
	14	COBAS	33	-	30	18	47	-
Group 4 ORF1ab- N+	15	VIASURE	-	25	34	-	46	-
	16	VIASURE	-	38	28	-	31	-
	17	VIASURE	-	38	-	-	-	-
	18	VIASURE	-	40	43	-	60	-
	19	VIASURE	-	41	-	-	58	-
	20	VIASURE	-	41	-	-	30	-

[1] A different commercial kit for RNA extraction was used to perform RT-qPCR using the commercial Real-Time PCR Detection Kits. NUCLISENS EASYMAG, BIOMÉRIEUX, France, for VIASURE SARS-CoV-2 Real-Time PCR Detection Kit, CERTEST BIOTECH, Spain; an integrated system in an automated platform for COBAS 6800, ROCHE, Switzerland, following manufactures' instructions.

When testing the six RT-qPCR-positive samples of group 2 (ORF1ab+; Ct > 30/E+ or N+) the RT-LAMP using primer set ORF1ab detected 4/6 samples (66.6%) with shorter (nos. 7, 9, and 10) or similar (no. 8) Tp values than the Ct values obtained by RT-qPCR for both the ORF1ab and the E/N targets. The RT-LAMP using primer set N5 detected 3/6 samples (50%) with much shorter Tp values (nos. 7, 8, 9) than the Ct values obtained for RT-qPCR. The RT-LAMP using primer set N15 amplified 6/6 (100%) samples with relatively long Tp values for samples 8 (Tp = 49), 10 (Tp = 60 min) and 11 (Tp = 45 min) in comparison to the Ct values obtained by RT-qPCR. For samples nos. 9 and 12, the Tp values were similar than RT-qPCR results (Ct = 39 and Ct = 36, respectively). For no. 7, a very short Tp = 13 min was obtained. The RT-LAMP using primer set E only amplified 1/6 samples (16.6%) (sample no. 7), with a Tp = 60 min, a value much longer than the one obtained by RT-qPCR.

The samples nos. 13 and 14 of the group 3 (ORF1ab+; Ct > 30/E−) were amplified by RT-LAMP using the primer set ORF1ab with very similar Tp values than RT-qPCR Ct values. The primer set N5 amplified the sample no. 14 with a very short Tp = 18 min in comparison to Ct = 33 obtained for OFR1ab by RT-qPCR. The primer set N15 amplified the two samples, but not the primer set E.

When testing the six samples included in the group 4 (ORF1ab-/N+), the primer set ORF1ab amplified 3 samples (nos. 15, 16, 18). The primer set N15 detected 5/6 samples (86.3%) that resulted N+ by VIASURE RT-qPCR, nevertheless, the primer set N5 did not amplify any sample. The primer set E, either. In all, considering the few positive results

obtained with the primer set E (6/12 confirmed positives (both PCR targets positives); 50%) this RT-LAMP assay was discarded for further testing.

To evaluate the conventional colorimetric RT-LAMP assay we selected 8 RNA isolates representing the four groups of samples used in the study: samples nos. 4, 5, 6 (ORF1ab+; Ct $\leq$ 30/E+ or N+); samples nos. 7, 12 (ORF1ab+; Ct > 30/E+ or N+); sample no. 14 (ORF1ab+/E+), and samples nos. 15, 16 (ORF1ab-/N+). The samples were tested using the primer sets ORF1ab, N5, and N15. The performance of each RT-LAMP assay is shown in Figure 5. Green fluorescence was clearly observed in the successful RT-LAMP reactions, while it remained original orange in the negative reactions. For the selected samples, the color change matched 100% with the results obtained in real-time RT-LAMP assays.

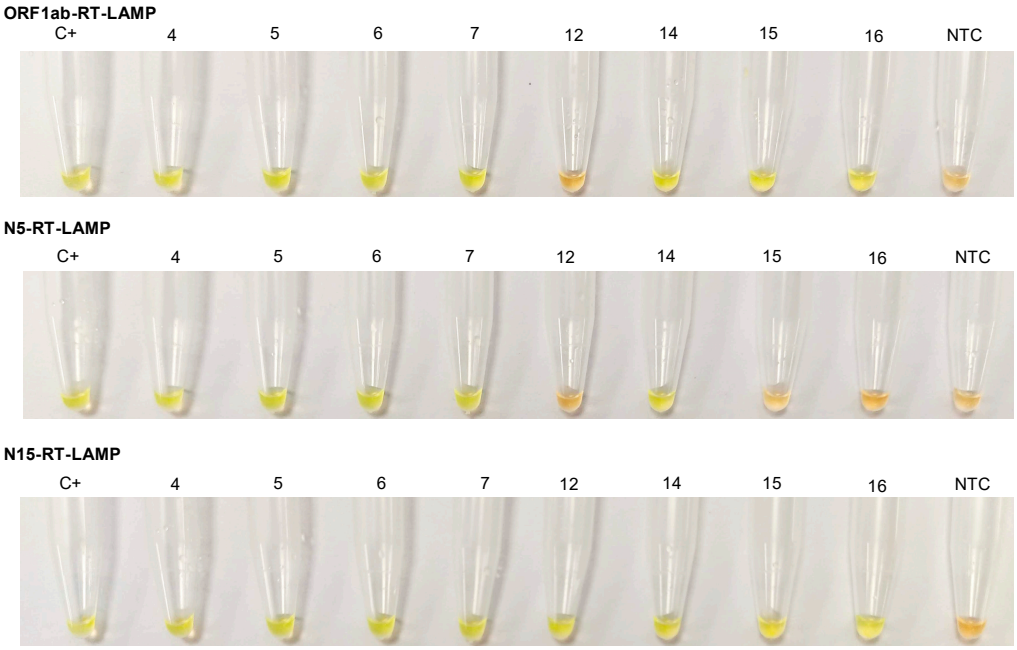

Figure 5. Conventional colorimetric RT-LAMP assays using the primer sets ORF1ab, N5, and N15. Eight RNA isolates from COVID-19 patients (samples nos. 4, 5, 6, 7, 12, 14, 15, and 16) were analyzed by colorimetric RT-LAMP using SYBR Green I fluorescent dye. Green (positive samples), orange (negative samples). C+, RNA-positive control; NTC, non-template control.

3.4. Stability and Functionality Over Time of Dry-RT-LAMP Mixes

As the primer sets ORF1ab, N5, and N15 offered the best results in the amplification of the COVID-19 patients' RNA isolates they were selected for further stabilization assays. The results obtained in Dry-RT-LAMP tests for each primer set are shown in Figure 6. In general, reconstitution of dry reagents worked well, and amplification was obtained for the three primer sets used at RT (25 °C), but a delay in amplification during the reaction for dried mixtures was observed in comparison to fresh mixes. Thus, just after desiccation of reagents (at day 0) an increase in Tp values was noticed for primer sets ORF1ab (Tp = 20.5 min to Tp = 32 min) and N5 (Tp = 19 min to Tp = 29 min). Significantly, no variation of Tp value was registered for primer set N15 after desiccation (Tp = 15 min). Despite the increase in Tp values over time, storage of dry-reagent RT-LAMP assays at RT was found to be functional for 14, 21, and 28 days when using primer sets ORF1ab, N5, and N15, respectively. Remarkably, the Dry-N15-RT-LAMP assay proved to be stable up to 28 days with a very reasonable Tp = 80 min. According to values of the stability times obtained at 37 °C (21 days) and 45 °C (14 days), the Q_{10} method predicted up to 66 days and 64 days of shelf-life at room temperature, respectively.

Dry-N15-RT-LAMP assay was also tested at 0, 1 and 7-days post-desiccation with the same samples used in colorimetric RT-LAMP assays (nos. 4, 5, 6, 7, 12, 14, 15, and 16). Results obtained are shown in Figure 7. As for C+ amplification trials, an increase in

the Tp values was observed in comparison to fresh reactions. The only sample with no amplification at any post-desiccation time was the no. 12, which presented the highest Ct values in RT-qPCR for both ORF1b (Ct = 39) and N (Ct = 36). The sample no. 14 (with a high Ct = 33 for ORF1ab and E− by RT-qPCR) was amplified with long Tp values at 0 (Tp = 81 min), 1 (Tp = 85 min) and 7 (Tp = 75 min) days post-desiccation; interestingly, a long Tp = 47 min was also obtained with N15-RT-LAMP fresh mixture. The samples nos. 15 and 16 (with no amplification of ORF1ab and N+ by RT-qPCR) amplified with high Tp values at 0 and 1day post-desiccation, respectively. These samples also presented a relatively long Tp values in fresh N15-RT-LAMP. The samples nos. 4, 5, 6 (group 1; Ct $\leq$ 30) and 7 (group 2; Ct > 30) were all amplified at 0, 1, and 7-days post-desiccation with very reasonable Tp values in comparison to fresh mixtures.

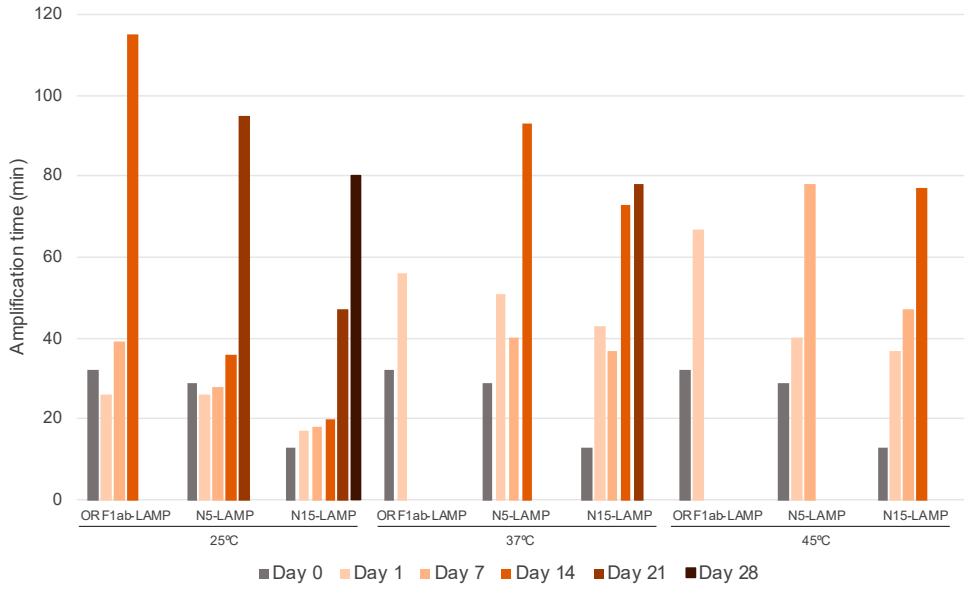

Figure 6. Amplification time of Dry-RT-LAMP assays as a function of storage time and temperature. Amplification times of C+ in RT-LAMP assays performed with dry reagents including primer sets ORF1ab, N5, and N15 tested at 0, 1, 7, 14, 21, and 28-days post-desiccation is shown. The different storage temperature (25 °C, 37 °C and 45 °C) is also indicated.

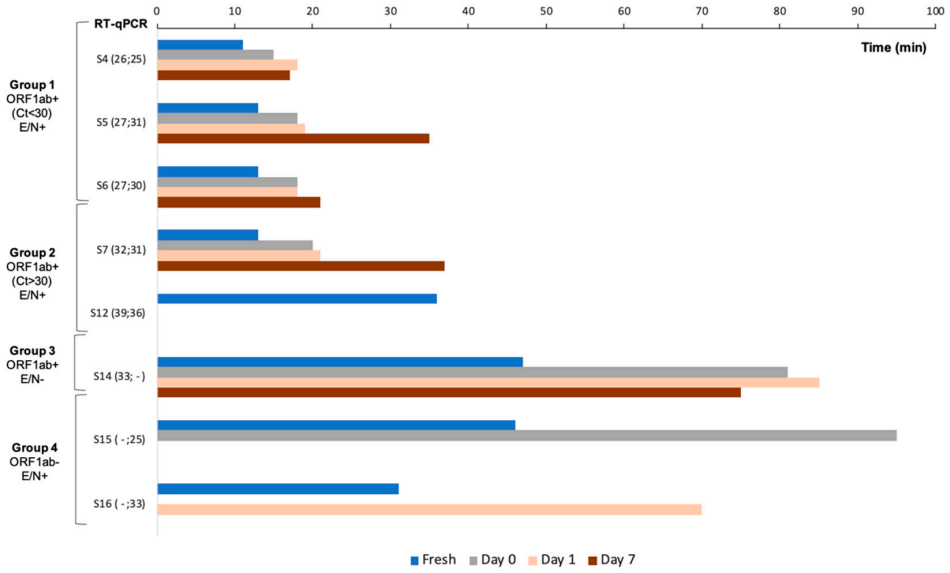

Figure 7. Dry-RT-LAMP assessment using primers set N15 in RNA isolates from COVID-19 patients. Amplification times of samples (S) nos. 4, 5, 6, 7, 12, 14, 15, and 16, performed with dry reagents including primer set N15 at 0, 1, and 7-days post-desiccation are shown. Blue bars (fresh) indicate amplification times obtained in N15-RT-LAMP assays using non-dried reactions (fresh liquid mixes) as reference for comparison. All those reactions were singles.

4. Discussion

In this work, five regions from SARS-CoV-2 viral genome were studied, including the open reading frames ORF1a and ORF1b, and genes S, E, and N. For this, eight sequence-specific RT-LAMP primer sets were designed and screened for the detection of the novel coronavirus. Our RT-LAMP reaction was optimized using a well-established RNA-positive control from a COVID-19 patient and primer sets targeting ORF1ab, gene E, and gene N were finally selected. For most RT-LAMP assays detecting SARS-CoV-2, the genes ORF1ab and N have been used as the principal targets for amplification, to ensure specific and sensitive detection [33]. In fact, in our trials, we obtained the best performance in detecting SARS-CoV-2 RNA targeting these two genes, not only in amplification times, but also in analytical sensitivity. First, we established the proper operation of the primer sets designed, specificity, and sensitivity in the amplification of the selected SARS-CoV-2 RNA target sequences. In set-up trials, the RT-LAMP reactions using *Bst* 3.0 DNA polymerase did not work well and, frequently, non-specific amplification was observed. It has been previously described several differences in efficiency of *Bst* 3.0 compared to *Bst* 2.0 WS depending on time and temperature for amplification [38]. As *Bst* 3.0 has an optimal temperature range for amplification between 68–70 °C, it is possible that when testing different temperatures in the optimization of RT-LAMP, a suboptimal annealing of our primer sets occurred, thus probably increasing the irregular and non-specific amplification. In addition, it has been recently described a significantly increased tendency of *Bst* 3.0 to yield false-positive results in comparison to *Bst* 2.0. These unspecific products were characterized by a higher Tm than specific products which also occurred in our *Bst* 3.0 reactions. These false positives have been associated with the interaction of multiple primers and the template switching and terminal transferase activities of the polymerase, combined with a lack of $3' \rightarrow 5'$ exonuclease activity [39]. In this sense, although some previously reported RT-LAMP tests for diagnosis of COVID-19 appear to have worked well using *Bst* 3.0 [40,41], different primers sequences targeting gene N were used and then annealing could be probably more effective or be less constrained by primer interaction. Nevertheless, most studies use a combination of a *Bst* 2.0 WS DNA polymerase and *RTx* WS in one-step reaction to amplify gene ORF1ab or gene N [33]. In our trials, optimization of RT-LAMP was finally carried out using the combination of the two enzymes and the best performance resulted for the amplification of regions ORF1ab, E, and N (using both primer sets N5 and N15). The shortest Tp values for RT-LAMP assays were obtained targeting gene N, particularly with primer set N15 (Tp = 15 min). On the other hand, ORF1b was prone to false-positive results so it was removed. Some background signal was detected in the case of E and M primer sets; however; it was not due to DNA amplification, but rather related to the real-time device measurements.

Regarding specificity, RT-LAMP showed to be highly specific for SARS-CoV-2 since no cross-reaction resulted in silico comparisons, although validation with real samples is needed. RNA isolates from other human respiratory viruses were evaluated did not show cross-reaction either. As may occur with any molecular-based test, possible mutations can arise in viral sequences and may affect primer annealing, thus causing a failure in further amplifications. To date, of the mutations reported for SARS-CoV-2 [42], none of them match in the sequences targeted by the primer sets used in RT-LAMP assays. In addition, it seems that mutations (most of them single-nucleotide alterations between viruses from different people) makes SARS-CoV-2 change much more slowly as it spreads [43].

RT-LAMP reactions targeting ORF1ab and N (using sets N5 and N15) demonstrated a limit of detection of 200 copies of SARS-CoV-2 RNA/reaction. The sensitivity of amplification based on gene E detection was proved to be lower (2000 copies/reaction) than ORF1ab or N genes. This might be caused by the slower kinetics of the RT-LAMP reaction targeting gen E, which lacks in loop primers, and therefore does not allow the detection of low concentrations of viral RNA within a reasonable time. Moreover, most of the RT-LAMP assays developed to date do target genes ORF1ab or N [33], and do not gene E, thus suggesting poorer results when using this target. Other published studies have reported sensitivities

as low as 2 copies/reaction for both ORF1ab and N genes [28] or 3 copies/reaction for gene ORF1ab [20]. However, those results for analytical sensitivity in detecting SARS-CoV-2 RNA were found using synthesized RNA fragments of genes N and ORF1ab obtained from in vitro transcription instead real RNA isolates from COVID-19 patients. Our results are in line with those larger clinical studies in which RT-LAMP assays present analytical sensitivities around hundreds of copies of SARS-CoV-2 genomic RNA [17,25,44].

A great variability in viral load in COVID-19 patients has been reported, ranging from 641 copies/mL to 1.34×10^{11} copies/mL (with a median of 7.99×10^4 in throat samples and 7.52×10^5 in sputum samples) and 1.69×10^5 copies/mL in a nasal swab sample [45]. Other studies testing SARS-CoV-2 positive patients estimated a viral load ranging from 1 copy/μL to 10^8 copies/μL, with most samples ranging from 10^4–10^8 copies/μL [46] or median viral load of 1440 copies/μL in nasopharyngeal swab samples [47]. In addition, a study performed by Yu et al. [48] showed that the viral loads in the early and progressive stages were significantly higher (over 46,000 copies) than in the recovery stage of the disease (over 1200 copies). Despite these variations in viral load of COVID-19 patients, our RT-LAMP assay resulted sensitive enough for detection of SARS-CoV-2 RNA in clinical samples, as it seems to indicate the comparison of Ct values with those Tp values obtained with RT-qPCR. Thus, for samples with a Ct $\leq$ 30 obtained by RT-qPCR (group 1), we found an excellent sensitivity and specificity values for viral RNA by RT-LAMP assays using primer sets ORF1ab, N5, and N15. The Tp values for the three RT-LAMP assays resulted much shorter than those obtained with RT-qPCR. This fact is even more significant if we take into account that to calculate the Tp of the RT-LAMP assay, both the time dedicated to retro-transcription and amplification are considered, while the Ct of the RT-qPCR does not include the time dedicated to retro-transcription. In general, for clinical samples with Ct > 30 (or with only one RT-qPCR-amplified target; suggestive positives), RT-LAMP assays were initially less sensitive, and amplification was not obtained in all samples. However, the RT-LAMP with the primers set N15, although with high threshold time values, tested positive in all but one sample, probably suggesting a sensitivity significantly lower than 200 copies/reaction for N15-RT-LAMP.

In this work, we have developed three highly efficient RT-LAMP assays for the detection of SARS-CoV-2 RNA. Considering those samples with a RT-qPCR-positive result for two different targets (nos. 1–12; confirmed positives), value of sensitivities resulted in 75% (9/12) for N5-RT-LAMP, 83.3% (10/12) for ORF1ab-RT-LAMP, and 100% (12/12) for N15-RT-LAMP. Furthermore, if only samples nos. 1–10 are considered, with a RT-qPCR Ct $\leq$ 33 for ORF1ab (equivalent to approximately 10–100 copies), sensitivities of ORF1ab-RT-LAMP and N5-RT-LAMP increase to 100% and 90%, respectively. It should be also noted that Tp values for N5-RT-LAMP were considerably lower than the Ct values for RT-qPCR targeting gene N (nos. 1, 3, 4, 5, 6, 7, and 9), and all positive result could be detected with excellent reaction times under 25 min. All these results suggest that any of the three RT-LAMP assays would be able to detect COVID-19 patients in all disease stages (early, progressive and recovery) according to the currently known data on viral load of SARS-CoV-2 in clinical samples [45–48]. The significant correlation between RT-qPCR and RT-LAMP threshold times obtained—particularly in theoretically high viral load samples (Ct $\leq$ 30)—together with the absolute agreement between real-time and conventional colorimetric RT-LAMP assays, increase the confidence in our results. Nevertheless, the increased variability in Tp values that N15-RT-LAMP presented with theoretically low viral load samples (Ct > 30) cannot be disregarded. In this respect, it is important to note that a lack of correlation between speed and sensitivity in isothermal amplification reactions has been previously reported [49] and reactions with higher efficiency can have substantially longer times to be positive, thus contradicting the intuition derived from qPCR reactions. We are aware of the limitations of our study in terms of the sample size and we acknowledge that further studies to examine the reproducibility of N15-RT-LAMP in testing larger sets of clinical samples, both positive and negative, are needed. We also acknowledge the limitations in sensitivity of sets N5 and ORF1ab for the detection of

positive samples with low viral load, with RT-qPCR Ct over 33. Those limitations should also be investigated in larger sets of clinical samples.

To develop a RT-LAMP as simple as possible to carry out in any condition for SARS-CoV-2 detection, we tried to keep all necessary components in a non-reactive state using tubes containing dry master mixes coated on the inner walls and caps. In a previous work, we successfully developed a simple desiccation procedure for drying LAMP reagents adapted for conventional and real-time amplifications assays [14]. Now, for COVID-19 RT-LAMP test, that protocol has been modified to achieve better thermal stability of dehydrated RT-LAMP mixes at ambient temperature along time. A Dry-RT-LAMP format can overcomes the requirement of cold storage facilities and temperature-controlled shipping [50,51], allows the omission of adding reagents individually, making the process easier and faster, and avoids possible cross-contamination during multiple pipetting steps in master mix preparation. The new 30 min one-step dry-up protocol was applied for RT-LAMP mixes containing primer sets ORF1ab, N5, and N15, resulting in functional amplifications of the C+ after storage at RT (25 °C) for up 14, 21, and 28 days, respectively. At this moment, we are not aware of the underlying cause of differences in stability when using different primer sets, but it could be possible than the higher efficiency showed by RT-LAMP with the primers set N15 allowed to amplify viral RNA after longer periods of storage. On the other hand, the longer dry components are storage at RT, the longer the reaction incubation time to achieve amplification is needed. An increase in reaction time, as well as a reduction in the amplification level in comparison to fresh liquid mixtures was already described by our group in operation of desiccation LAMP procedure [14]. In any case, very reasonable amplification times of 80 min (for set N15), 95 min (for set N5) and 115 min (for set ORF1ab) were observed before functionality loss after storage for 28, 21, or 14 days, respectively. Additionally, the Q_{10} method predicts a shelf-life for Dry-RT-LAMP using primer set N15 of over 64–66 days at 25 °C. Subsequently, the Dry-N15-RT-LAMP format was selected to test those eight samples used in conventional colorimetric RT-LAMP assay at 0, 1, and 7 days post-desiccation. In analysis, an expected increase in the Tp values was observed in comparison to results obtained in N15-RT-LAMP fresh liquid reactions. Thus, those samples with long Tp values when testing in fresh using primer set N15 (presumably with a very low viral load or marginal positives: nos. 12, 14, 15, and 16) did not work very well at post-desiccation times, resulting in no amplification or in amplification with Tp values much longer. By contrast, those samples with short Tp values in fresh testing (presumably with medium/high viral load: nos. 4, 5, 6 and 7), despite increase slightly in reaction time, were consistently detected over post-desiccation time.

In summary, we have developed a novel, rapid, specific and sensitive RT-LAMP test for SARS-CoV-2 RNA detection in clinical samples by targeting gene N with a specific-sequence primer set N15. Our RT-LAMP assay can be simply performed both as a single-tube isothermal colorimetric method without any expensive equipment requirement and in a real-time platform. The results can be detected as soon as 9 min after the reaction starts and obtain close to 100% sensitivity within 60 min. Moreover, the procedure is easily adaptable to a dry format that could be stored and delivered at room temperature. At this moment, maintaining the functionality for at least 2 months at RT, would allow us to prepare and distribute a set of dried RT-LAMP master mixes to be used within a few weeks in settings where detection of SARS-CoV-2 is required at the point of collection, such as schools, nursing homes, or rural medical centers. This feature, which can be achieved by a simple and fast process in comparison to other available options, mainly lyophilization, could represent a great contribution to fast molecular SARS-CoV-2 diagnostic tools. Additionally, the affordability of the test is apparent, as the price per reaction in this study was 1.76 € for fresh mixes when using SYBR Green I and 1.69 € when using Eva Green 20x. The desiccation process only added 0.02 € per reaction. However, it is important to highlight that RNA purification would add over 3.50 € per sample depending on the commercial extraction kit used. Thus, the price per reaction is significantly cheaper than a standard RT-qPCR test which is approximately 7–10 € without considering previous RNA

purification. Notwithstanding its limitations, the possibility of avoid the RNA extraction, or the combination of this RT-LAMP with some rapid RNA purification methods already described [22,34,52], could allow easy testing in situations of rapid diagnostic demand and in low-resource settings and areas of difficult access, where the limited testing capacity is one of the main challenges in the COVID-19 response [53], reducing considerably its price too. More work on this with the aim to improve and achieve a point-of-care (POC) molecular diagnosis of COVID-19 will be performed in the future.

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Institutional Review Board Statement: The study protocol was approved by the Clinical Research Ethics Committee of Investigation with Drugs of the University Hospital of Salamanca, Spain (CEIMC 2020.06. 530) on 19 October 2020. The procedures described here were carried out in accordance with the ethical standards described in the Revised Declaration of Helsinki in 2013. All patient data were anonymized and unidentified.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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3.4

Detection of SARS-CoV-2 RNA in urine by RT-LAMP: a very rare finding

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RESUMEN

La utilización de orina como muestra diagnóstica presenta diversas ventajas: (*i*) facilidad de obtención, (*ii*) mayor aceptación por parte del paciente, (*iii*) posibilidad de trabajar con grandes volúmenes de muestra y (*iv*) relativa facilidad de extracción y purificación de ácidos nucleicos. Aunque *a priori* no tenía por qué ser la muestra más apropiada para el diagnóstico de SARS-CoV-2, decidimos estudiar si era posible amplificar ARN del virus en orina. Además, nos planteamos si la detección de ARN tenía alguna correlación con la sintomatología y con comorbilidad de los pacientes estudiados.

Estructuramos el estudio con tres grupos de pacientes (n = 300): grupo 1 de pacientes con RT-qPCR positiva en muestras nasofaríngeas (n = 100), grupo 2 de pacientes con RT-qPCR negativa en muestras nasofaríngeas y sintomatología compatible (n = 100) y grupo 3 de pacientes con RT-qPCR positiva en muestras nasofaríngeas, pero ya recuperados de la enfermedad (n=100). Se obtuvieron muestras de orina de estos pacientes paralelamente a la toma de la muestra nasofaríngea para la RT-qPCR, que se usó como técnica de referencia (grupos 1 y 2) o en el momento de recibir el alta hospitalaria (grupo 3). Todas las muestras se evaluaron mediante el método N15-RT-LAMP (colorimétrico y a tiempo real), que resultó el más sensible de los desarrollados en el trabajo anterior.

Durante el análisis en el grupo de pacientes con RT-qPCR nasofaríngea positiva (grupo 1) solo se encontró una tasa de positividad del 4 % mediante N15-RT-LAMP colorimétrica y un 2 % mediante RT-qPCR y RT-LAMP a tiempo real. Esto nos permitió concluir que: (*i*) la muestra de orina no es apropiada para la detección molecular de SARS-CoV-2. La detección de pacientes positivos, por baja que sea, solo se produce en aquellos individuos con infección activa, pero no en individuos recuperados, lo que parece indicar que la eliminación de las partículas virales no es habitual a través de la vía renal; (*ii*) no hay correlación evidente entre la positividad en orina con síntomas clínicos o comorbilidades, a diferencia de lo que afirman otros estudios; (*iii*) a concentraciones muy bajas de ARN del virus, la técnica de RT-LAMP colorimétrica tiene más sensibilidad que la de RT-LAMP a tiempo real. Este hecho es atribuible a la adición pre-amplificación del colorante intercalante de ADN EvaGreen, que ha demostrado cierto carácter inhibitorio en otros estudios, incluso a bajas concentraciones. Así, la utilización de colorantes que no se unan directamente al

ADN parece una mejor alternativa para monitorizar reacciones a tiempo real, estrategia que se va a seguir en el siguiente artículo de investigación.

Aunque el estudio presentado tiene sus limitaciones, como la falta de datos de carga viral en las RT-qPCR nasofaríngeas o el bajo número de positivos obtenidos, es, a fecha de su publicación, el trabajo más extenso sobre la detección de SARS-CoV-2 en muestras clínicas de orina.

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Detection of SARS-CoV-2 RNA in Urine by RT-LAMP: A Very Rare Finding

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Abstract: Detection of SARS-CoV-2 is routinely performed in naso/oropharyngeal swabs samples from patients via RT-qPCR. The RT-LAMP technology has also been used for viral RNA detection in respiratory specimens with both high sensitivity and specificity. Recently, we developed a novel RT-LAMP test for SARS-CoV-2 RNA detection in nasopharyngeal swab specimens (named, N15-RT-LAMP) that can be performed as a single-tube colorimetric method, in a real-time platform, and as dry-LAMP. To date, there has been very little success in detecting SARS-CoV-2 RNA in urine by RT-qPCR, and the information regarding urine viral excretion is still scarce and not comprehensive. Here, we tested our N15-RT-LAMP on the urine of 300 patients admitted to the Hospital of Salamanca, Spain with clinical suspicion of COVID-19, who had a nasopharyngeal swab RT-qPCR-positive (n = 100), negative (n = 100), and positive with disease recovery (n = 100) result. The positive group was also tested by RT-qPCR for comparison to N15-RT-LAMP. Only a 4% positivity rate was found in the positive group via colorimetric N15-RT-LAMP and 2% via RT-qPCR. Our results are consistent with those obtained in other studies that the presence of SARS-CoV-2 RNA in urine is a very rare finding. The absence of SARS-CoV-2 RNA in urine in the recovered patients might suggest that the urinary route is very rarely used for viral particle clearance.

Keywords: SARS-CoV-2; urine; COVID-19; RT-LAMP; RT-qPCR; molecular diagnostics

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1. Introduction

The infection caused by SARS-CoV-2 (COVID-19) affects mainly the respiratory system, and the typical symptoms at onset are fever, dry cough, fatigue, and dyspnea. Most patients present a good prognosis, while a few develop severe complications such as acute respiratory distress syndrome (ARDS) which can lead to death [1,2]. SARS-CoV-2 infects human cells using the angiotensin-converting enzyme 2 (ACE2) receptor [3–5]. Due to the expression across a wide variety of human tissues of ACE2, COVID-19 infection can lead to renal, hematological, skin, neurological, cardiovascular, gastrointestinal, liver, endocrine, and ophthalmological involvement, with different degrees of severity in patients [6,7]. The potential systemic dissemination of SARS-CoV-2 suggests that detection of viral particles or viral RNA might be possible in several biologic fluids depending on the patients' disease presentation.

Nucleic acid amplification tests (NAATs) are considered the most appropriate methods for screening suspected SARS-CoV-2 cases, with the reverse transcription-polymerase chain

reaction (RT-qPCR) the gold standard test to confirm infection [8,9]. Respiratory samples are considered the most efficient specimens for the isolation and detection of SARS-CoV-2 RNA, particularly upper respiratory tract samples (nasopharyngeal or oropharyngeal swabs) in the early stages of infection, and lower respiratory tract samples in the later stages of infection (mainly sputum, if produced) [8]. Other clinical specimens such us saliva, stool, urine, serum, blood, tears [10–12] and seminal fluid [13] have been tested for the presence of SARS-CoV-2 with varying degrees of efficiency.

Regarding urine, despite being an easy sample to obtain, less intrusive and with less potential risk of infection for health workers, to date, there has been very little success in detecting viral RNA by RT-qPCR [11]. In fact, the evidence regarding the presence, viability, and infectivity of SARS-CoV-2 in urine is sparse. Additionally, the few methodologically sound studies using RT-qPCR for detection included a small number of specimens and found a very low rate of positivity [14]. On the other hand, there are still conflicting opinions as to whether the presence of the virus in urine can be an indicator of severity or worse prognosis of the disease [15–19].

A recent study has shown that the spike protein of SARS-CoV-2, particularly subunit S1, can be detected in the urine of patients with a nasopharyngeal RT-qPCR-positive result using an antigen-capture assay. However, sensitivity of this methodology is highly reduced compared to nasopharyngeal RT-qPCR (only 25% of SARS-CoV-2 infected individuals are detected) [20].

In addition, the RT-qPCR methodology is not easily adaptable for point-of-care diagnosis in situations requiring rapid diagnosis. To solve this, an increasingly recognized alternative is loop-mediated isothermal amplification (LAMP) technology. Since the LAMP technique was first described [21], it has been used to detect a target nucleic acid (DNA or RNA) mainly for molecular diagnostics purposes without sophisticated equipment. LAMP can amplify DNA from an RNA sample (RT-LAMP) in a one-step reaction by the addition of a reverse transcriptase to the LAMP reaction or a DNA-polymerase with retrotranscriptase activity [22]. In addition, the LAMP assay can operate at different pH and temperature ranges and with crude samples, which is advantageous for working with multiple clinical samples [23]. In this regard, it has been used for the detection of SARS-CoV-2 RNA, with sensitivities well over 90% when using purified RNA samples [24] and reaching 85% when using unprocessed pharyngeal samples [25]. Furthermore, a color RT-LAMP based diagnostic assay has been recently approved for emergency use by the U.S. Food and Drug Administration (FDA) for COVID-19 diagnostics [26].

Recently, we developed an RT-LAMP test for SARS-CoV-2 RNA detection in clinical nasopharyngeal swabs specimens by targeting gene N with a specific-sequence primer set N15 (N15-RT-LAMP) that can be performed as a single-tube colorimetric method, in a real-time platform, and as dry-LAMP. This assay proved to be specific for SARS-CoV-2 and showed a limit of detection of 200 copies per reaction (cpr) [27]. The RT-LAMP methodology has already been tested successfully in the detection of SARS-CoV-2 RNA in urine but only using artificially spiked samples with various concentrations of SARS-CoV-2 RNA [28]. However, to the best of our knowledge, there are no clinical studies evaluating the usefulness of RT-LAMP as a SARS-CoV-2 detection molecular tool in COVID-19 patients' urine samples. Thus, the objective of the present study was to evaluate the usefulness of RT-LAMP for the detection of SARS-CoV-2 RNA in urine samples. To do so, we tested our N15-RT-LAMP method on urine of 300 patients (including COVID-19 positive, negative and recovered patients) admitted with COVID-19 clinical suspicion at the Hospital of Salamanca, Spain. We also compared N15-RT-LAMP results in urine samples with those obtained by RT-qPCR in nasopharyngeal swabs. Additionally, for the first time, urine samples from a recovered group of patients were evaluated by RT-LAMP for the presence of SARS-CoV-2 RNA.

2. Materials and Methods

2.1. Nasopharyngeal Specimen Collection, RNA Isolation and RT-qPCR

Nasopharyngeal swab specimens were collected from admitted patients of the University Hospital of Salamanca, Salamanca, Spain, with compatible COVID-19 symptoms. Collected samples were first preserved in sample preservation solution (MOLE BIOSCIENCE, SUNGO Europe B.V., Amsterdam, The Netherlands), delivered to the Laboratory of Microbiology, and then processed in a biosafety level 2 cabin until inactivation by mixing with a lysis buffer. Nasopharyngeal swabs were processed in an integrated platform for both RNA isolation and RT-qPCR (COBAS 6800, ROCHE, Basel, Switzerland), targeting ORF1ab and E gene of SARS-CoV-2, following the manufacturer's instructions.

2.2. Urine Specimen Collection, RNA Isolation and RT-qPCR Analysis

Along with the nasopharyngeal samples, urine samples for routine analysis were also collected from inpatients. An aliquot of these samples was reserved for COVID-19 analysis. Based on the results obtained from the nasopharyngeal swabs RT-qPCR tests, a total of 300 patient urine samples were selected and grouped as follows (Figure 1): Group 1: 100 urine samples from patients with a RT-qPCR positive result and symptoms of COVID-19 (PP, positive patients); Group 2: 100 urine samples from patients with a RT-qPCR negative result, but with compatible symptoms of disease (NP, negative patients); and Group 3: 100 urine samples from symptomatic patients with RT-qPCR positive on admission, but recovered and discharged from hospital (RP, recovered patients). PP urine samples were obtained within 24 h of a positive result by RT-qPCR in nasopharyngeal swabs; RP urine samples were obtained 7 days after hospital discharge, with a negative RT-qPCR result in nasopharyngeal swabs. Patients selected in the study presented the typical symptoms described for COVID-19 including cough, fatigue, sputum production, sore throat, headache and shortness of breath, among the most frequent [1].

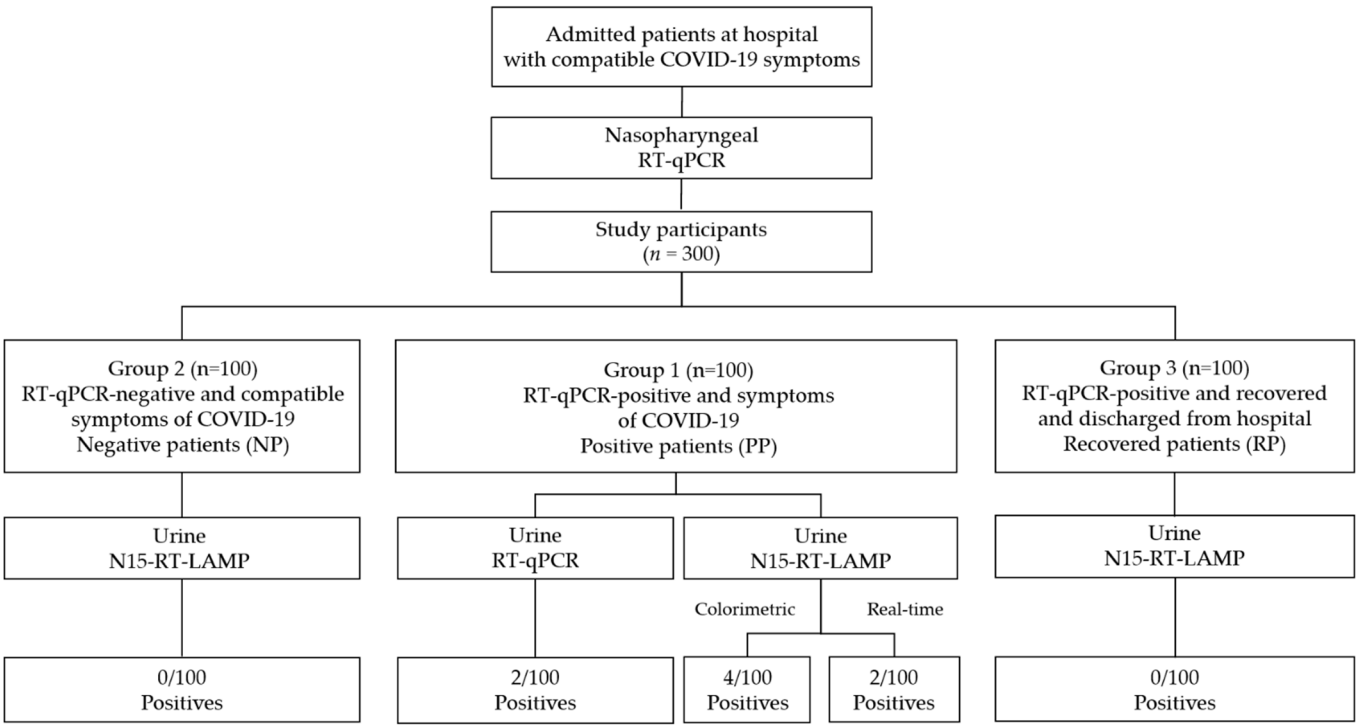

Figure 1. Flowchart describing the process of patient group selection and urine sample analysis strategy. RT-qPCR, reverse transcription-polymerase chain reaction; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

Urine samples were individually collected in conventional sterile urine containers and delivered to the e-INTRO group's laboratory for further processing in a biosafety level 2 cabin until inactivation by mixing with the lysis buffer. Subsequently, 1 mL from each urine sample was concentrated to a final volume of 200 µL that was used as the input for RNA extraction using the NZY Viral RNA Isolation Kit (NZYTECH, Lisbon, Portugal) following the manufacturer's instructions. Afterwards, the 100 urine samples from the PP group were analyzed by RT-qPCR using the SARS-CoV-2 One-Step RT-PCR Kit, CE-IVD (NZYTECH, Lisbon, Portugal) following the recommended conditions, and viral copy numbers were calculated according to the manufacturer's instructions.

2.3. RT-LAMP Analysis

All 300 RNA isolates from urine samples were analyzed by a one-step conventional colorimetric RT-LAMP targeting a region of 212 base pairs (bp) of the N gene of SARS-CoV-2, recently described by our group and referred as N15-RT-LAMP [27]. In brief, colorimetric N15-RT-LAMP assays were performed in the presence of two enzymes: *Bst* 2.0 WarmStart DNA polymerase (*Bst* 2.0 WS) and WarmStart RTx reverse transcriptase (RTx WS) (NEW ENGLAND BIOLABS Ltd., Ipswich, MA, USA) in a volume of 15 µL containing 1.6 µM FIP/BIP primers, 0.2 µM F3/B3 primers, 0.4 µM LF/LB primers, 1.4 mM of each dNTP (BIORON GmBH, Römerberg, Germany), 0.13 M of D-(+)-trehalose dihydrate (Sigma-Aldrich, St. Louis, MO, USA) (hereinafter trehalose, for short), 6 mM $MgSO_4$, 1× 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), *Bst* 2.0 WS (0.32 U/µL) and *RTx* WS (0.3 µL), with 2 µL of template RNA. All RT-LAMP reactions were performed at 63 °C for 45 min in a heating block, followed by 10 min at 80 °C for enzyme inactivation. Results were evaluated by the naked eye using 1 µL of SYBR Green I 1000× (INVITROGEN, Carlsbad, CA, USA) added post-amplification to each reaction tube. For positive results, the dye turns to an intense green/yellow, whilst negative reactions remain orange. To avoid potential post-amplification contamination, each tube was briefly centrifuged and carefully opened in a laminar flow hood to add the intercalating dye.

In addition, real-time N15-RT-LAMP assays were performed in 8-tube Genie Strips on a portable Genie III device (OPTIGENE Ltd., Horsham, UK) using the same reaction mixes as previously described but adding 0.24 µL of EvaGreen 20× in water (BIOTIUM, San Francisco, CA, USA) before the reaction started. In all N15-RT-LAMP assays, positive (2 µL of RNA purified from a nasopharyngeal swab from a patient with a RT-qPCR-positive result for SARS-CoV-2; Ct = 25 for ORF1ab) and negative (2 µL of ultrapure water) controls were included. All positive results were confirmed in triplicates.

3. Results

As stated above, the 300 patients included in this study were divided into three groups of 100 individuals each according to the nasopharyngeal swabs RT-qPCR testing: PP, NP, and RP. The gender ratio in the study was 51.67% female and 48.33% male, with a higher proportion of women in NP group (57% vs. 43%) than in PP group (39% vs. 61%). Overall, the mean age was 66.47 years (standard deviation (SD) = ±16.78), with a minimum age of 20 and a maximum of 99, which was lower in RP group (64.90 ± 13.60) than in PP group (68.14 ± 17.80).

First, the 100 urine RNA isolates from the PP group were analyzed by RT-qPCR in our laboratory (see Figure 1). Only 2/100 (2%) were found positive (nos. PP80 and PP36), with cycle threshold (Ct) values resulting in 23.65 and 36.76, respectively. Then, all the 300 urine RNA isolates were tested by our conventional colorimetric N15-RT-LAMP and 4/100 (4%) samples were positive in PP group (nos. PP21, PP36, PP58, and PP80), including the 2 positive samples previously obtained by RT-qPCR analysis (see Figures 1 and 2a). No positives were detected in the NP group (0/100) or in the RP group (0/100).

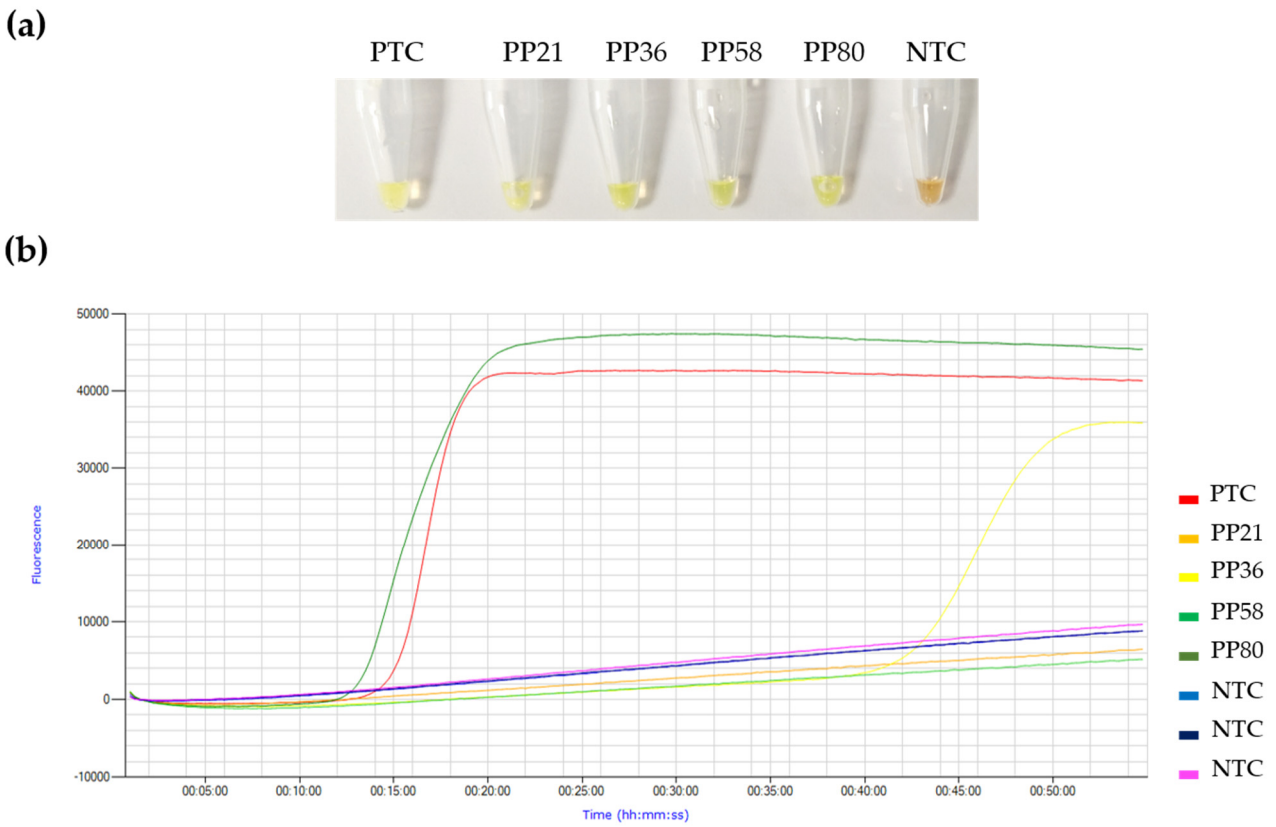

Figure 2. Detection of SARS-CoV-2 RNA in urine samples by colorimetric and real-time N15-RT-LAMP. (**a**) Colorimetric N15-RT-LAMP: results detected with SYBR Green I. Green/yellow, positive results; orange, negative results. (**b**) Real-time N15-RT-LAMP: results detected by fluorescence in a Genie III device. PTC, positive template control (2 µL of RNA purified from a nasopharyngeal swab from a patient with a RT-qPCR-positive result for SARS-CoV-2; Ct = 25 for ORF1ab); PP21, PP36, PP58, and PP80, patient urine samples; NTC, negative template control (2 µL of ultrapure water instead RNA).

The 4 urine RNA positive samples were also tested by real-time N15-RT-LAMP for a better comparison with RT-qPCR results (Figure 2b). Only the 2 RT-qPCR positives (nos. PP80 and PP36) were also positive by real-time N15-RT-LAMP, with a time to positivity (Tp) values of 14.5 min and 45 min, corresponding to Ct values by RT-qPCR of 23.65 and 36.76, respectively.

Relevant available clinical data from the four positive patients are presented in Table 1. After an analysis of the symptoms, clinical presentation, and medical history of the four positive participants, no relevant common clinical pattern was found.

Table 1. Most relevant clinical data of the four patients who tested positive for SARS-CoV-2 in a urine sample by N15-RT-LAMP.

	PP21	**PP36**	**PP58**	**PP80**
Demografic data				
Age (years)	63	73	33	92
Sex	Male	Female	Male	Male
Immunosuppression	No	Yes	No	Yes
Immunosupression cause	N/A	Diabetes mellitus	N/A	Renal insufficiency

Table 1. *Cont.*

	PP21	PP36	PP58	PP80
Previous clinical data				
Non-COVID-19 Disease	Dyslipemia, Renal insufficiency I, Anxiety	Diabetes mellitus, Arterial hypertension, Cognitive impairment, Dyslipemia	Asthma, Dyslipemia	Renal insufficiency III, Ischemic heart disease, Depression
Previous medical treatment	Atorvastatine, Diazepam	Losartan, Rosuvastatine, Amantadine	Pravastatine/fenofibrate, Salbutanol	Acetylsalicylic acid, Bisopropol, Furosemide, Omeprazole
COVID-19 clinical data				
Fever	Yes	Yes	Yes	No
Dyspnea	Yes	Yes	Yes	Yes
Cough	Yes	Yes	Yes	No
Ageusia/Anosmia	No	Yes	No	No
Myalgia	Yes	No	No	No
Asthenia	No	Yes	No	No
Diarrhea	No	No	No	No
Microbiological diagnosis	Yes	Yes	Yes	Yes
RT-qPCR	Positive	Positive	Positive	Positive
Days since symptom onset	14	3	10	1
Diagnosis	Bilateral pneumonia	Bilateral pneumonia	Bilateral pneumonia	Bilateral pneumonia
Specific treatment for COVID-19				
Steroids	Yes	Yes	No	Yes
Tocilizumab	Yes	Yes	No	No
Remdesevir	No	No	No	No
Heparin	Yes	Yes	No	No
Others		Hydroxychloroquine	Amoxicillin	Lopinavir/Ritonavir-Hydroxychloroquine-Cefthriaxone
Evolution of COVID-19				
Stay (days)	10	5	0	3
ICU	No	No	No	No
Dead	No	No	No	Yes
Other diagnosis during COVID-19 stay	Lupus discoid	No	No	No

4. Discussion

At present, information on SARS-CoV-2 is already extensive, but urine viral excretion tests regarding both the presence and virulence of the virus are still very scarce. Although the WHO has recommended in its guidelines to consider urine testing for all symptomatic patients and contact persons [8], the presence of SARS-CoV-2 in urine samples is still poorly investigated in the current literature and a few methodologically sound studies have reported only a very low rate of positivity [14]. It should be noted that, despite the rarity of the event, it can occur. The results from our study reinforce this point.

Remarkably, out of a group of 100 patients admitted to hospital with COVID-19 symptoms and a nasopharyngeal swab RT-qPCR-positive result, only two (2/100; 2%) tested positive for the presence of SARS-CoV-2 RNA by RT-qPCR when urine samples were analyzed. In these two positive samples, a great variation in viral load was detected, with one sample having a viral copy number of around 500,000 copies/mL, and the other just

50 copies/mL. Notwithstanding the difference in viral load, no differences in the severity of COVID-19 were observed in these patients.

A study performed by Yu et al. [29] using droplet digital PCR (ddPCR) for an accurate quantification of viral load, showed great variation in viral load among COVID-19 patients. In that study, the viral loads in the early and progressive stages of COVID-19 were significantly higher (over 46,000 copies) than in the recovery stage of the disease (over 1200 copies) when analyzing different types of samples, including nasal swabs, throat swabs, sputum, blood and urine. However, no positive results were found in blood or urine. To date, only two studies have provided the viral copy number of SARS-CoV-2 detected by RT-qPCR in urine. In a study by Peng et al. [30], a concentration of 322 copies/mL was found in the urine of a patient with COVID-19 symptoms. In another study by Frithiof et al. [16], a mean concentration of 1200 copies/mL (range 300–2800) was found in six critically ill COVID-19 patients. The paucity of previous studies makes comparison with our results difficult. Nevertheless, the data seem to indicate that the number of viral copies in the urine of patients with COVID-19 symptoms may be highly variable, as was also the case in our two RT-qPCR-positive patients.

In the overall analysis, our conventional colorimetric N15-RT-LAMP detected four positive urine samples from PP group, including the two samples positive by RT-qPCR. Although slight, this higher positivity rate (4% vs. 2% by RT-qPCR) suggests a higher sensitivity of our LAMP method over RT-qPCR for analysis of RNA isolates from urine. Unexpectedly, this higher positivity rate was not found when N15-RT-LAMP was performed under real-time conditions, as identical results were obtained on the same two RT-qPCR-positive samples. The fact that two urine samples were positive by conventional colorimetric N15-RT-LAMP, but not in real-time settings, could be due to the presence of pre-amplified EvaGreen fluorescent dye in the reaction mixes for real-time monitoring. It has been shown in LAMP assays that EvaGreen can produce a partial inhibition of the reaction by reducing both the reaction rate and final amplification levels [31]. This does not occur in conventional colorimetric LAMP assay since the SYBR Green I dye is added when the amplification is already finished. This would be an advantage for analysis since only a heating block would be needed to carry out the reaction. Another possible explanation for the amplification of these two samples by conventional, but not real-time, N15-RT-LAMP, could have been non-specific amplification or post-amplification contamination by opening the tubes and adding SYBR Green I at the end of the reaction. However, we sincerely believe that the specificity demonstrated by our N15-RT-LAMP in its previous development and set up [27], the gentle handling of the tubes in a laminar flow hood, and the triplicate confirmation of positive results, rule out those possibilities.

The current gold standard for diagnosing COVID-19 is based on molecular tests of RT-qPCR to detect the RNA of the virus in respiratory samples such as nasopharyngeal swabs or bronchial aspirate using different protocols and target sequences available in the WHO database [8,32]. However, it is important to note that RT-qPCR is not fail-safe and can also give false negatives if viral load is low or if the correct time-window of viral replication is missed. In this sense, the COVID-19 incubation period is estimated to be 5 days, but false negative results may occur within the first week of infection [33]. In addition, possible sources of RT-qPCR false results can include laboratory errors at three different stages including, pre-analytical, analytical and post-analytical phases [34]. Furthermore, the commercially available diagnostics kits in RT-qPCR have different features, mainly connected to the viral target tested and the limit of detection. Significantly, the higher the limit of detection, the more risk of false negatives [35].

A recent systematic review with meta-analysis conducted by Böger et al. [36]. compared the clinical performance of RT-qPCR tests for SARS-CoV-2 detection using different samples (including oral saliva, deep-throat saliva, posterior oropharyngeal saliva, sputum, urine, feces, and tears) against standard specimens (nasopharyngeal and oropharyngeal swabs). As a result, oral saliva proved to be the most promising sample for the detection of SARS-CoV-2, not only because of the high diagnostic accuracy obtained (above 90%), but

also because it allows self-collection (decreasing the risk of exposure of health-care workers to infections) and reduces the waiting time for sample collection (favoring epidemiological measures). Unfortunately, for urine, the authors did not find enough studies to calculate estimates and, yet again, no data are provided in meta-analysis making comparison with our results impossible.

To date, the only systematic review of the literature to investigate the presence of SARS-CoV-2 specifically in human urine is the one conducted by Bröniman et al. [14]. The study concluded that: (i) this finding is still poorly investigated (0.6%; 34/5674 articles); (ii) only 7 studies reported positive results with a very low rate of positivity and, (iii) 90% of the patients with multiple urine analysis displayed a positive RT-qPCR only at one single point in time. On the other hand, some studies have indicated that, in addition to being a rare occurrence, urinary viral secretion was not associated with acute kidney injury or severity of COVID-19 disease [15,16]. However, several other studies have found that those subjects with SARS-CoV-2 in the urine at admission to hospital and its persistence during hospitalization had more severe COVID-19 [18,19] and a worse clinical course [19]. Thus, there are different studies with conflicting results regarding the presence of SARS-CoV-2 RNA in urine and the progression of COVID-19. However, all these studies are based on very limited sample sizes and agree in recognizing the need for more large-scale studies to better assess this hypothesis and possible future implications. In our study, we found a low positivity rate in urine samples (4%) in the PP group. Among the four positive patients, the one with the highest viral load (PP80; see Table 1) had the worst prognosis (death). Nonetheless, stage III renal insufficiency and the advanced age of the patient could explain both the presence of RNA in urine and the disease prognosis. Apart from these observations, no common clinical features were found among all four positives.

We acknowledge the limitations of our study. The study lacks viral load data from patients' nasopharyngeal samples, which makes it difficult to contextualize urine-positive samples within the positive group of patients. In addition, plasma samples could have been informative for these positive results. Although several studies have included plasma for the detection of SARS-CoV-2 by nucleic acid testing with variable results (mainly in the early stages of the disease, raising doubts about its diagnostic value) it could be of added value in determining or predicting the severity of COVID-19 [37]. Finally, due to the very limited positive results, our study lacks strong statistical analysis.

Overall, compared to previous studies on the molecular detection of SARS-CoV-2 RNA in human urine, our work using N15-RT-LAMP technology provides a larger number of urine samples analyzed from three groups of patients with COVID-19, including symptomatic positives, symptom-compatible negatives and, for the first time, patients recovered from the disease. Our results are consistent with those obtained in other studies in finding that the presence of SARS-CoV-2 RNA in urine is highly unlikely. Additionally, the absence of SARS-CoV-2 RNA in urine in our recovered patients could indicate that the urinary route is very rarely used for the clearance of viral particles.

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Institutional Review Board Statement: All participants enrolled in the study voluntarily and informed consent was obtained from all subjects. The study protocol was approved by the Clinical Research Ethics Committee of Investigation with Drugs of the University Hospital of Salamanca, Spain (CEIMC 2020.06.530) on 19 October 2020. The procedures described here were carried out in accordance with the ethical standards described in the Revised Declaration of Helsinki in 2013. All patient data were anonymized and unidentified.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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3.5

SMART-LAMP: A smartphone-operated handheld device for real-time colorimetric point-of-care diagnosis of infectious diseases via loop-mediated isothermal amplification

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RESUMEN

La utilidad del dispositivo SMART-LAMP fue finalmente valorada en un último trabajo de investigación publicado bajo el título "*SMART-LAMP: A smartphone-operated handheld device for real-time colorimetric point-of-care diagnosis of infectious diseases via loop-mediated isothermal amplification*". Para hacer una evaluación lo más exhaustiva posible podemos dividir este trabajo en cuatro bloques: (*i*) evaluación de los valores básicos de lectura colorimétrica y perfiles de temperatura y comparación entre tres prototipos; (*ii*) optimización de cambios colorimétricos y condiciones de estabilización; (*iii*) valoración de la capacidad predictiva y sensibilidad del SMART-LAMP para cuatro ensayos LAMP y; (*iv*) aplicación del dispositivo en muestras clínicas y comparación con técnicas de referencia y otros dispositivos comerciales.

Con respecto a la lectura colorimétrica del SMART-LAMP, comparamos las lecturas de los tres prototipos en cada uno de sus pocillos para 8 diluciones diferentes del colorante verde malaquita. Este es un colorante de uso rutinario en biología molecular y de fácil adquisición. Comprobamos que para colores similares a los que ofrece una amplificación típica LAMP, no había diferencias significativas de lectura entre prototipos. En términos del perfil térmico, los prototipos demostraron ser capaces de mantener la temperatura seleccionada de manera muy estable y alcanzar las temperaturas de reacción (65 ºC) o inhibición (80 ºC; a término de la reacción) en unos pocos minutos. Los tiempos necesarios para pasar de 25 ºC a 65 ºC y de 65 ºC a 80 ºC aumentaban a medida que el porcentaje de las baterías en los dispositivos se reducía. Eran, en cualquier caso, superiores a los de dispositivos comerciales utilizados como referencia, el Genie III (Optigene, Reino Unido) o el termociclador PCRmax ECO 48 (Illumina, Estados Unidos) utilizados como referencia. Esto podría tener un efecto en la especificidad de los ensayos en el dispositivo SMART-LAMP, aunque la prueba piloto realizada en muestras de pacientes de COVID-19 demostrara posteriormente que no es así.

En segundo lugar, demostramos que se puede obtener un cambio colorimétrico evidente entre resultados positivos y negativos utilizando 0.008 % p/v del colorante verde malaquita. Curiosamente, la estabilización del colorante junto con el resto de los reactivos potenciaba de manera clara la diferencia colorimétrica entre los resultados positivos y negativos. Hasta la fecha, no hemos podido dilucidar la causa de este fenómeno. En cualquier caso, el cambio colorimétrico resultó evidente

incluso utilizando reactivos estabilizados y almacenados durante más de un mes a temperatura ambiente. Además, comprobamos que en las reacciones LAMP que incluían *loop primers* (SARS-CoV-2 y *Schistosoma haematobium*) la cinética de reacción no empeoraba tras la estabilización, mientras que en las que no los incluían (*S. mansoni* y *Strongyloides* spp.) el deterioro era mucho menor que al utilizar el protocolo de estabilización desarrollado en el primer artículo de investigación.

En tercer lugar, demostramos que el dispositivo SMART-LAMP era capaz de diferenciar entre resultados positivos y negativos con gran precisión. Para ello, la mejor medida era la reducción en el componente rojo del modelo RGB entre el minuto 10 de reacción y el final de esta. Estableciendo como umbral de positividad una reducción de 25 unidades en el componente rojo, obteníamos valores predictivos positivos y negativos por encima del 85 % para todos nuestros ensayos. Estos valores predictivos no se veían afectados por periodos largos de almacenamiento de los reactivos listos para el uso a temperatura ambiente (>30 días). Poder establecer un único umbral de positividad para todos los ensayos nos llevó a pensar que la puesta a punto y optimización de ensayos para la detección de otros patógenos en el SMART-LAMP debería ser relativamente sencilla. Demostramos también que la sensibilidad de nuestros ensayos se mantenía en el SMART-LAMP, en comparación con una amplificación a tiempo real medida por fluorescencia en el dispositivo comercial Genie III.

Por último, realizamos un estudio piloto utilizando muestras de 80 pacientes con sospecha de COVID-19 (60 positivos y 20 negativos). Obtuvimos una sensibilidad y especificidad comparables a la de la técnica de referencia RT-qPCR, así como a la obtenida mediante RT-LAMP a tiempo real medida por fluorescencia, tanto con reactivos sin estabilizar como estabilizados. Si bien, la correlación entre los umbrales de ciclo (Ct) de la RT-qPCR y el tiempo de positividad (Tp) del RT-LAMP, se vio reducida cuando utilizamos el SMART-LAMP en comparación con el dispositivo comercial Genie III.

En conclusión, demostramos que el uso combinado del dispositivo SMART-LAMP con mezclas de LAMP estabilizadas, almacenadas a largos periodos de tiempo a temperatura ambiente, proporciona una mejora hacia un verdadero diagnóstico a pie de paciente de las enfermedades infecciosas en entornos con una infraestructura limitada. Atendiendo a los resultados obtenidos, consideramos que nuestra propuesta podría adaptarse fácilmente al diagnóstico de otras enfermedades infecciosas.

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SMART-LAMP: A Smartphone-Operated Handheld Device for Real-Time Colorimetric Point-of-Care Diagnosis of Infectious Diseases via Loop-Mediated Isothermal Amplification

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Abstract: Nucleic acid amplification diagnostics offer outstanding features of sensitivity and specificity. However, they still lack speed and robustness, require extensive infrastructure, and are neither affordable nor user-friendly. Thus, they have not been extensively applied in point-of-care diagnostics, particularly in low-resource settings. In this work, we have combined the loop-mediated isothermal amplification (LAMP) technology with a handheld portable device (SMART-LAMP) developed to perform real-time isothermal nucleic acid amplification reactions, based on simple colorimetric measurements, all of which are Bluetooth-controlled by a dedicated smartphone app. We have validated its diagnostic utility regarding different infectious diseases, including Schistosomiasis, Strongyloidiasis, and COVID-19, and analyzed clinical samples from suspected COVID-19 patients. Finally, we have proved that the combination of long-term stabilized LAMP master mixes, stored and transported at room temperature with our developed SMART-LAMP device, provides an improvement towards true point-of-care diagnosis of infectious diseases in settings with limited infrastructure. Our proposal could be easily adapted to the diagnosis of other infectious diseases.

Keywords: loop-mediated isothermal amplification; point-of-care diagnostics; infectious diseases SARS-CoV-2; mHealth; Neglected Tropical Diseases

1. Introduction

The lack of affordable and simple molecular diagnostic tools for infectious diseases represents a long standing bottleneck in the health improvement of developing countries [1]. This problem has been tackled through the development of point-of-care tests (POCTs), defined as the rapid detection of analytes near the patient to enable better diagnosis, monitoring, and management of diseases [2]. Nowadays, some forms of POCTs have been reported for many Neglected Tropical Diseases (NTDs), including schistosomiasis (rapid tests for cathodic and anodic circulating antigens), as well as strongyloidiasis and other soil-transmitted helminthiasis (microscopy tool Kankanet) [3]. Furthermore,

since the advent of the COVID-19 pandemic, the importance of these POCTs has also been highlighted in developed countries. As of April, 2022, close to five billion molecular tests have been performed for the detection of SARS-CoV-2 [4] and, in an effort to overcome that massive technical challenge, over 400 different rapid tests have already been developed [5].

Ideally, POCT tools should be cheap, portable, simple, fast and capable of quantification [6]. The benchmark to assess those features is the ASSURED criterion (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable) defined in 2004 by the World Health Organization Special Program for Research and Training in Tropical Disease (WHO/TDR) [7]. This criterion has been recently updated to REASSURED, including Real-time connectivity and Ease of specimen collection [8].

A comparison of the main POCTs for the diagnosis of infectious diseases, namely nucleic acid amplification tests (NAATs), antigen detection and antibody detection, reveals that NAATs present the highest sensitivity and specificity [9]. However, they exhibit poor levels of rapidness and robustness, they usually require equipment, and are not affordable nor user-friendly [8]. Alternatively, several isothermal amplification techniques have been developed. Amongst them, LAMP technology is the most widely used alternative. The LAMP method is a one-step amplification reaction based on auto-cycling strand displacement DNA synthesis performed under isothermal conditions (60–65 °C for 45–60 min) in the presence of a *Bst* polymerase using four primers (two inner and two outer primers). The LAMP method specifically recognizes six distinct sequences in target DNA, thus ensuring high specificity, efficiency and rapidity for amplification [10]. Additionally, reverse-transcription LAMP (RT-LAMP) amplifies DNA from an RNA target in a one-step reaction by directly adding a dedicated reverse transcriptase (RTase) or a DNA polymerase with RTase activity to the reaction mixture sharing all benefits of LAMP technology [11]. LAMP, together with other isothermal amplification techniques, overcomes some of the shortcomings classic NAATs present, particularly speed and price limitations. Nevertheless, other limitations, such as user-friendliness, equipment or deliverability, need supportive technology to be fulfilled [12].

A relatively new approach to tackle those limitations is the combination of POCT with mobile health (mHealth), defined by the WHO Global Observatory as "medical and public health practice supported by mobile devices, such as mobile phones, patient monitoring devices, personal digital assistants (PDAs), and other wireless devices" [13]. Combined with diagnostic devices, mHealth can improve the diagnostic and control strategies of infectious diseases [14]. Additionally, the worldwide increase in smartphone adoption, which had already reached 67% of the global population by 2020 and is expected to reach 70% by 2025 [15], could democratize access to mHealth tools.

Various examples of the combination of mHealth devices and LAMP assays have already been presented. Most of these approaches combine an integrated platform to perform the LAMP reaction, usually in the form of a microfluidic cassette, and a smartphone as a readout tool to evaluate fluorescence generated upon amplification [16–20]. Accurate quantification has been achieved through mathematical processing of the signal detected by smartphone cameras [21]. A few examples make use of microfluidic designs combined with colorimetric detection via the RGB (red, green and blue) color sensors of the smartphone camera. This colorimetric detection of amplification is based on Mg^{2+} dependent indicator hydroxynaphthol blue [22,23], thus considerably simplifying the result analysis and cheapening the overall costs of the test. Accurate quantification of genomic DNA has also been achieved, although no patient samples have been examined [22]. Notwithstanding this, other approaches, such as redox indicators [24], lateral-flow assays [25] or centrifugal displays [26], have also been combined with LAMP. Microfluidic designs present the main advantage of the integration of the various steps of a multistep diagnostic assay, including sample handling, processing as well as signal amplification and detection [27]. Although they show a promising future, they also pose numerous questions regarding the following: automation (most assays require extensive user intervention for sample

preparation prior to amplification, as well as for reagent addition to the diagnostic platform); the need to improve sensitivity, selectivity and stability of sensing moduli; scalability and clinical validation [28].

Attending to some of those limitations, in this work we present the SMART-LAMP, a handheld portable device to perform real-time isothermal amplification reactions, based on simple colorimetric measurements in standard 0.2 mL Eppendorf tubes, controlled via Bluetooth by a dedicated SMART-LAMP app on a smartphone. We show its applicability as a POCT tool using stabilized reaction mixes that can be stored and transported at room temperature (RT) for extended periods of time. We tested the applicability of SMART-LAMP for the detection of different infectious agents: *Schistosoma mansoni, S. haematobium, Strongyloides* spp. and SARS-CoV-2. Additionally, we tested the diagnostic utility of SMART-LAMP on patients suspected of having COVID-19.

2. Materials and Methods

2.1. Human Samples

The study protocol was approved by the Clinical Research Ethics Committee of Investigation with Drugs of the University Hospital of Salamanca, Spain (CEIMC 2020.06.530) on 19 October 2020. The procedures described here were carried out in accordance with the ethical standards described in the Revised Declaration of Helsinki in 2013. All patient data were anonymized and unidentified.

2.2. DNA and RNA Purification

For amplification trials, genomic DNA (gDNA) from *Schistosoma mansoni, S. haematobium* and *Strongyloides venezuelensis,* as well as SARS-CoV-2 RNA samples from positive COVID-19 patients, were used. DNA from *S. mansoni* and *S. haematobium* was obtained from freeze-stored adult worms. DNA from *S. venezuelensis* was purified from freeze-stored infective third-stage larvae (iL3). DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, GmH & Co., Düren, Germany) following the manufacturers' instructions. Purified DNA was measured in triplicate using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and then diluted in ultrapure water to a final concentration of 5 ng/μL.

SARS-CoV-2 RNA samples were obtained from nasopharyngeal swabs from suspected COVID-19 cases collected and stored in Sample Preservation Solution (MOLE BIOSCIENCE, SUNGO Europe B.V., Amsterdam, The Netherlands) as part of the routine testing of patients for COVID-19 at the University Hospital of Salamanca (Salamanca, Spain). Swabs were transported to the e-INTRO group laboratory and processed in a biosafety level 2 cabin until inactivation by mixing with the lysis buffer. Afterwards, RNA was isolated using NZY Viral RNA Isolation Kit (NZYTECH, Lisbon, Portugal), following manufacturers' instructions.

2.3. Fresh-LAMP and Fresh-RT-LAMP Assays

For LAMP assays, we used the set of primers and reaction conditions previously described by our group for DNA detection of S. mansoni [29], S. haematobium [30], Strongyloides spp. [31] and SARS-CoV-2 RNA detection [32]. In brief, fresh-LAMP reaction mixtures (15 μL) contained 1.6 M FIP/BIP primers, 0.2 μM F3/B3 primers, 0.4 μM LF/LB primers (if applicable), 1.4 mM of each dNTP, (BIORON GmBH, Römerberg, Germany), 6 mM $MgSO_4$, and 1× Isothermal Buffer (20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20) for Bst 2.0 Warm Start (WS) (0.32 U/μL) (NEW ENGLAND BIOLABS Ltd., Ipswich, MA, USA). Reactions were performed at 65 °C for up to 1 h. Fresh-RT-LAMP reactions for SARS-CoV-2 contained the same reagents and concentrations as fresh-LAMP, with an additional WS RTx Reverse Transcriptase enzyme (0.3 μL) (NEW ENGLAND BIOLABS Ltd., Ipswich, MA, USA). Reactions were performed at 63 °C for up to 40 min.

Amplification detection was performed via real-time fluorescence monitoring or real-time RGB color monitoring. Real-time fluorescence-based reactions were performed either in the portable Genie III instrument (OPTIGENE, Horsham, UK) or the PCRmax ECO 48 real time PCR system (PCRmax, Stone, UK) using 0.24 μL of EvaGreen (EG) 20× dye (BIOTIUM, Inc, Fremont, CA, USA). For real-time colorimetric-based reactions, performed in the SMART-LAMP prototypes, 0.008% *w/v* of malachite green (MG) (Sigma-Aldrich, San Luis, CA, USA) was included in the master mixes. In all tests, 2 μL of nucleic acid template (DNA or RNA) for the positive control (PTC), or ultrapure water for the negative control (NTC), were added to the reaction mixture.

2.4. Dry-LAMP: Stabilization of LAMP Reaction Components for Ready-to-Use Tests

Dry-LAMP (for DNA detection) and dry-RT-LAMP (for RNA detection) reaction mixes were obtained by desiccation of fresh-LAMP mixes in the presence of 2M trehalose (Sigma-Aldrich, San Luis, CA, USA) using a protocol previously described by our group [33] and later improved [32]. Briefly, fresh master mixes were dried separately in two partial mixes. One was placed in the bottom of the tubes containing the primer mix, dNTPs, *Bst* 2.0 WS and *RTx* WS (if applicable) with 1.8 μL of 2M trehalose. The other was pipetted in the tube caps, containing isothermal buffer, additional $MgSO_4$, and the dye (MG or EG) in the presence of 2.25 μL of 2M trehalose. Then, the stabilization consisted of a 30 min single-step desiccation protocol in a Concentrator Plus (Eppendorf, Hamburg, Germany) at RT. This procedure did not require lyophilization, greatly reducing the technical requirements.

Subsequently, desiccated reaction mixes were stored protected from the light at RT until use. Upon rehydration, tubes were placed upside down for 2 min to accomplish full reconstitution of the master mix before the reaction started.

Reactions were performed under the same conditions as the fresh-LAMP reaction. Amplification was also detected by the same strategies. To carry out the dry-LAMP assays, reaction times for *S. mansoni* and *Strongyloides* spp. were increased to 90 min, while reaction times for dry-RT-LAMP SARS-CoV-2 were increased to 50 min. For *S. haematobium* amplification, the reaction time remained 60 min long in both fresh- and dry-LAMP formats.

2.5. SMART-LAMP: Principal Modules and Characteristics

The SMART-LAMP device comprises a 3D-printed handheld platform manufactured with Fused Deposition Modelling (FDM) technology in polylactide (PLA) material for the simultaneous incubation and real-time data acquisition of eight samples. It has a modular design which comprises: (i) a sample incubation module, (ii) a sample reading module and, (iii) a transmission module.

The *sample incubation module* maintains the sample at 60–70 °C for a period of up to 120 min. Intelligent algorithms were designed to allow this module to modify both the temperature (ranging from 40 °C to 80 °C) and the processing time (ranging from 5 to 120 min). It also includes LED and acoustic indicators that provide information about the device status (such as "not ready", "ready to use", "connected" or "in-use"), temperature and the elapsed and remaining reaction times. To stabilize the temperature a Proportional Integral and Derivative temperature control algorithm (PID) was applied [34] maintaining the temperature with a ±0.9 °C accuracy. In brief, the PID was implemented through the PID_v1 library of Brett Beauregard [35] with values of Kp = 4, Ki = 1 and Kd = 1, adjusted via trial and error. Due to the thermal damping caused by the thermo-block and its isolation, the PID was deactivated when the temperature reached the target. Temperature maintenance was achieved manually to relieve the microcontroller unit (MCU) from computational load, as results were the same as the ones obtained via PID control. Temperature is calculated with the Steinhart-Hart equation. Three measurements were realized to obtain A, B and C coefficients of the equation. The system continuously monitors the

temperature to ensure safety and prevent fires, whereby audible alarms are triggered via software in the event of excessive or uncontrolled temperature rises.

The *sample reading module* consists of photoresistors and fiber optics elements. A TCS3472 color sensor was used as detector, which provides a digital return of red, green, blue (RGB), and clear light sensing values. The high sensitivity, wide dynamic range, and infrared filter (IR Block filter) make the TCS3472 an ideal color sensor solution for use under varying lighting conditions and through attenuating materials. Additionally, overhead white LED lights were located over the samples providing the right light for effective reading.

The *transmission module* includes four systems necessary for the reception and protection of the sample results and other relevant information: data reception, data protection, transmission and storage. Regarding data reception, along with the results of the sample, the system incorporates other complementary information related to the context in which the sample is taken, such as GPS position, information about the patient, healthcare professional responsible for taking sample, measurement equipment information, incidents, etc. The information is stored in compliance with the HL7 specifications [36] Data protection includes all the elements needed to encrypt the data, safeguard the identity of the users, and certify both the origin and the correction thereof, so that all international data protection laws are complied with. In terms of transmission, the virtual organization facilitates communication through Bluetooth Low Energy (BLE) to send data to a connected device, following the standards of the Edge Computing architecture. Where storage is concerned, the module stores the data collected by means of an internal Process Context Block (PCB) memory to facilitate delayed retransmission, in case there is no signal coverage [37].

In addition to the three fundamental modules, SMART-LAMP is designed to be used in areas with and without an internet or electrical connection through two main features: (i) the generation and recharge of the battery. The device uses MC73831T and three Li-ion batteries (NCR186508), chosen to power the entire system. They render well in terms of discharge capacity and in the number of possible recharges [38] and (ii) SMART-LAMP Interaction Interface, which allows the device to visualize and validate the results, send information, backups, etc. The interface is designed both at the level of the data measurement system, and at the level of the analysis and information storage system. The generated information can be visualized on personal computers as well as on tablets or smartphones, and is adaptable to different screen formats. The visualization is also displayed on the SMART-LAMP device on an alphanumeric LCD screen with 16 characters and 2 lines. The interface may analyze the information considering geographical, local and/or personal parameters. In addition, a mobile application has been created to display the results in real time through a friendly interface on the iOS (Apple Inc., Los Altos, California, USA) operating system, where measurement data can be accessed. The application also allows interaction with the SMART-LAMP device, starting or forcing the stop of a measurement, as well as registering a user, linking to a nearby device and monitoring the battery level of the device. Although the smartphone app is not yet publicly available, it will be ready for download in the near future via the website https://smartlamp.es/en (accessed on 7 June 2022).

Single-line circuit sketches of SMART-LAMP components are shown in Figure S1 and printed circuit boards in Figure S2.

2.6. SMART-Lamp Assessment

2.6.1. Color Readout and Temperature Profile

For color readout assessment, 2-fold serial dilutions of MG dye in water were measured with the three SMART-LAMP prototypes, ranging from 0.1% *w*/*v* to 7.81 × 10^{-4}% *w*/*v*. Each dilution was measured in triplicate in each well of each prototype.

The temperature profile was assessed by measuring the ramping times of the device between RT and reaction temperature (25 °C to 65 °C), between reaction temperature and inhibition temperature (65 °C to 80 °C), and back to reaction temperature (80 °C to 65 °C). To evaluate the influence of the batteries in the ramping times, measurements with 100%, 75%, 50%, 25%, and under 25% of battery charge were taken. Temperature stability was assessed for 1 h at 65 °C, with measurements taken every 5 min.

2.6.2. Positive and Negative Predictive Values

Four positive controls (PTCs) and four negative controls (NTCs) from each fresh-LAMP and dry-LAMP assay were used to establish the best measurement to discriminate positive and negative results. RGB values were monitored at real-time with the SMART-LAMP. Absolute values of each component at the end of the reaction (t_{final}), as well as relative differences between color components at t_{final} and reaction times 0 min (t_0), 5 min (t_5), 10 min (t_{10}), and 15 min (t_{15}) were considered.

Once the most appropriate measurement was established, 40 controls (20 PTCs and 20 NTCs) were tested for each dry-LAMP to establish the positivity threshold value and to calculate positive predictive value (PPV) and negative predictive value (NPV) for the SMART-LAMP. To account for any possible effect of long-term room-temperature storage of dried mixes, 20 controls (10 PTCs and 10 NTCs) were tested after >30-days of room-temperature storage.

2.6.3. Analytical Sensitivity

To assess the sensitivity of dry-LAMP mixes, 10-fold serial dilutions, starting from 5 ng/μL of each template, were prepared and tested, both with EG in the Genie III device and with MG as dyes in the SMART-LAMP prototypes. For each LAMP assay, dilutions were prepared down to the limit of detection described in their original descriptions: 10 fg in the case of *S. mansoni* and *S. haematobium* [29,30], and 10 pg for *Strongyloides* spp. [31]. For LAMP amplification of SARS-CoV-2, RNA from a patient sample with a RT-qPCR Ct value of 25, for the ORF1ab region, was used as a PTC; similarly, 10-fold serial dilutions, down to the 1:100 limit of detection originally described[30], were prepared to assess sensitivity.

2.7. Proof of Concept: COVID-19 Patients Sample Analysis

Eighty RNA samples from patients with symptoms compatible with COVID-19 disease (20 negative and 60 positive results via RT-qPCR) were used as a proof of concept of the applicability of the SMART-LAMP for diagnostic purposes. For comparison, the 80 samples were first analyzed by RT-qPCR in a PCRmax ECO 48 real time PCR system using the SARS-CoV-2 One-Step RT-PCR Kit–IVD (Nzytech, Lisbon, Portugal), following manufacturers' instructions. The 80 RNA samples were also tested by fresh-RT-qLAMP and dry-RT-qLAMP in the Genie III instrument and dry-RT-qLAMP in the SMART-LAMP prototypes. Correlation of positive results between the RT-qPCR kit and the three different LAMP protocols were measured and evaluated via Pearson's correlation.

2.8. Statistical Analysis

Statistical analysis was performed in R (version 3.6.3). Packages ggplot2, ggbeeswarm, ggpubr, grid, EnvStats and dplyr were used to analyze and visualize the data. Comparisons among the three SMART-LAMP prototypes were performed via one-way analysis of variance (ANOVA). Comparisons between positive and negative values were evaluated via the Mann-Whitney U test. Correlations assays were performed via Pearson's correlation.

3. Results and Discussion

3.1. Device Design and Construction

The SMART-LAMP was digitally designed with SolidWorks, which yielded a render of the prototype that can be observed in Figure 1a. The prototype cases were 3D-printed and manually assembled with all the necessary electronic components. The SMART-LAMP prototypes presented dimensions of 12.5 cm × 7.8 cm × 8.8 cm and 850 g of weight (including three NCR186508 power batteries).

This design proved to be robust enough to withstand temperatures above 80 °C without any sign of deterioration in the three different constructed prototypes. Battery life was found to be 8–9 h at reaction temperature (65 °C) which, for a typical 1 h reaction, would allow for 7 to 8 reactions before battery replenishing. The sample incubation module, encompassing a heating block, was designed to simultaneously fit eight universal 0.2 mL Eppendorf tubes (see Figures 1b,c). The heating of the sample block was achieved by two polyamide heaters of 4.8 W at 12 V. The block was thermically isolated with a rock wool covering. In addition, to protect the device case, batteries and all the SMART-LAMP components, a fan was activated to extract hot air from the interior of the devices.

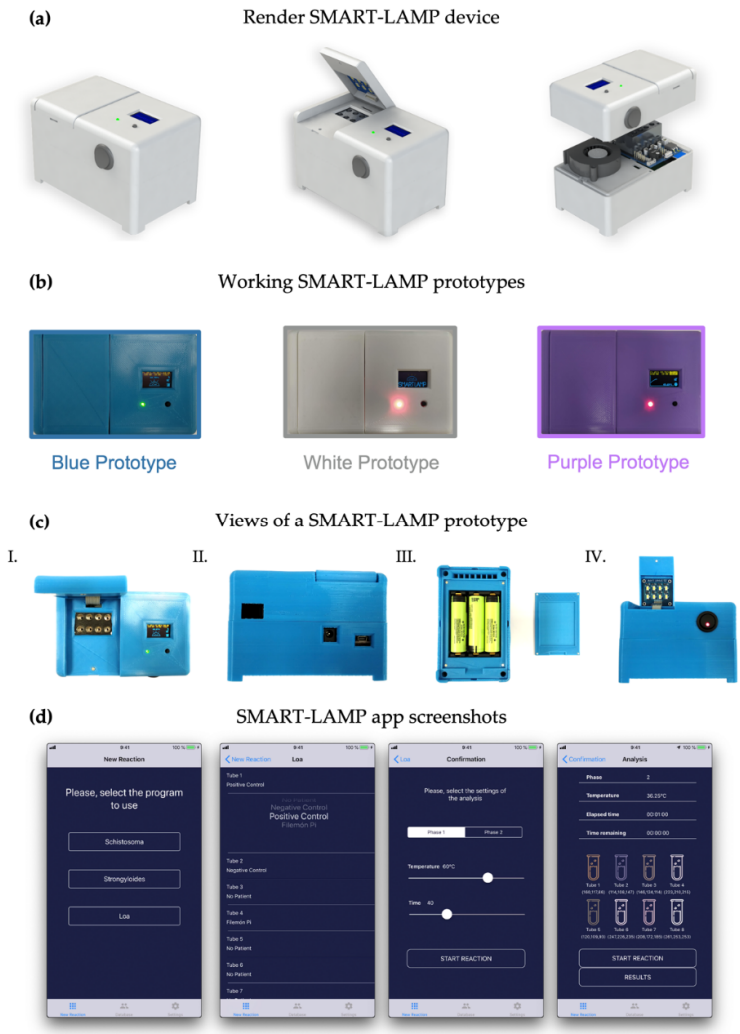

Figure 1. Design and principal features of the SMART-LAMP and SMART-LAMP app: (**a**) Render of the SMART-LAMP prototype with SolidWorks. From left to right: closed SMART-LAMP; SMART-LAMP with the lid of the incubation module opened; and internal structure of the SMART-LAMP prototype; (**b**) Top view of the three working SMART-LAMP prototypes used; (**c**) Different

views of the one SMART-LAMP device: I. Top view of the SMART-LAMP device with the incubation module lid opened; II. Posterior view with all ports and device connections exposed, from left to right: fan opening, power supply port and USB port for computer connection; III. Inferior view with the exposed MC73831T and three Li-ion batteries that power the SMART-LAMP device. To the right, the lid used to cover the batteries which is magnetically attached to the device for easy removal and reinstalment; IV. Frontal view of the SMART-LAMP with the lid opened, exposing the overhead with LED used to illuminate samples while RGB measures are taken; (**d**) SMART-LAMP app screenshots. From left to right: reaction program selection, sample identification, duration and temperature setup through sliding buttons, interface of the SMART-LAMP app while a reaction is being carried out, showing the temperature of the device, the time elapsed and the RGB measurements of each well.

The production cost of the device has been estimated to be around €300. A list of all necessary materials for building the device can be found in Table S1.

All three prototypes efficiently connected via Bluetooth to both an iPad Air and an iPhone 11. The SMART-LAMP iOS app allowed for an easy selection of reaction conditions and a continuous monitoring of RGB values of each well. At the end of the reaction, the researcher could export all data in csv format, as well as revise the color profile of each tube within the app (Figures 1d and S3). The time of reaction and GPS location could also be extracted. A patient database could be created within the app to store clinically relevant data from the patients.

Other demonstrations of devices performing isothermal amplification can be found in the literature. However, most of them are based on intricate chip or microfluidic designs and use fluorescence as a measurement of amplification [16–20].

Despite lacking a microfluidic design, the SMART-LAMP is still compact and even smaller than other devices already described for LAMP [39]. The simple structure of SMART-LAMP, operated like a regular thermo-block, avoids complex schemes that often hamper the deployment of microfluidic diagnostic tests [40].

3.2. Color Readout and Temperature Profile Assessment of the SMART-LAMP

Two-fold serial dilutions of malachite green (MG) dye in water were measured with the three SMART-LAMP devices, ranging from 0.1% *w*/*v* to 7.81×10^{-4}% *w*/*v*. As expected, higher concentrations of the dye resulted in lower RGB values and lower concentrations in higher RGB values. Variability among measurements in each device increased as the dye concentration decreased. However, differences among devices were significant at high MG concentrations, while they were not at low concentrations (Figure 2a). The low concentrations resulted in colors that were the most similar to the ones obtained in real LAMP reactions. No statistically significant differences were found among the devices for those dilutions; thus, the following experimentation used the three devices indistinctively. Moreover, absolute RGB values were not considered a good indicator of DNA amplification, due to the high variability detected in color readout.

Focusing on the temperature profile, SMART-LAMP was able to rise from RT (25 °C) to reaction temperature (65 °C) in 4–8 min depending on the battery percentage of the working prototype. To inhibit the enzymatic activity at the end of the LAMP reaction, the SMART-LAMP was able to rise from reaction temperature (65 °C) to inhibition temperature (80 °C) in 2–5 min depending on the battery percentage. Although the inhibition temperature was not necessary in real-time amplification reactions, it may nevertheless be of interest when subsequent down-stream analysis is required with the amplified products (e.g., electrophoresis, band purification), or for end-point colorimetric visualization of the results. The cooldown of the device from inhibition temperature to reaction temperature was maintained around 3 min, regardless of the battery percentage (Figure 2c). Once the reaction temperature was achieved, it was maintained constant throughout the reaction. In all three prototypes, the temperature never cooled under the predetermined value, and never over-heated by more than 0.9 °C (Figure 2b).

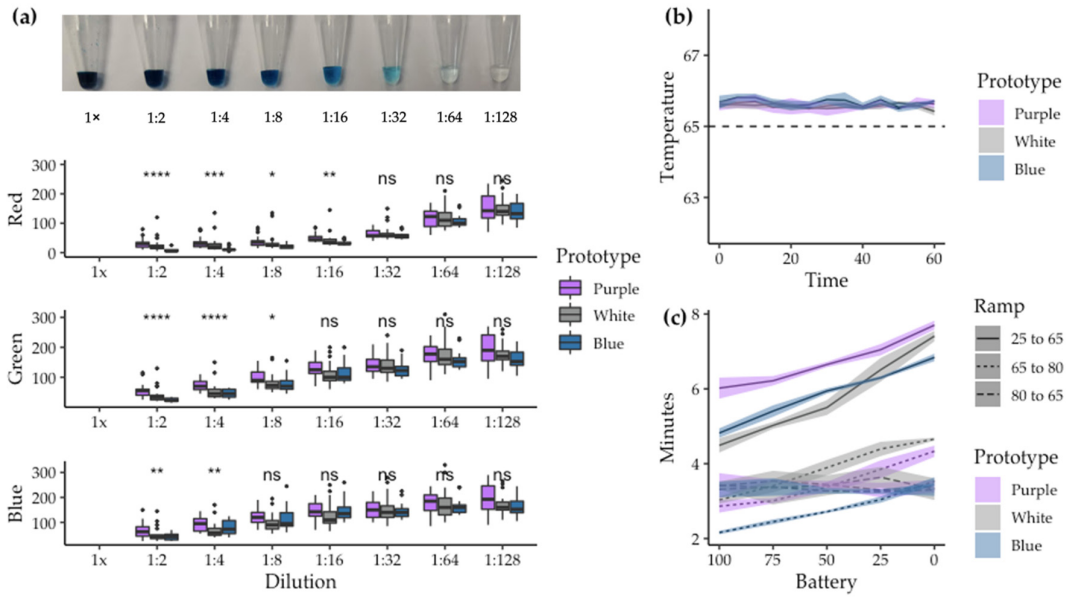

Figure 2. Basic measurements of color readout and temperature profile of the SMART-LAMP devices: (**a**) Color readout of the SMART-LAMP with MG dilutions in water. In the top picture, 15 μL of MG dilutions in water in 0.2 mL Eppendorf tubes are presented, ranging from a 0.1% *w/v* dilution (1×) down to 7.81 × 10^{-4}% *w/v* (1:128), in a 2-fold serial dilution fashion. Boxplots representing RGB values for all eight dilutions are presented (from top to bottom: Red component, Green component and Blue component), measured in triplets in each well of each prototype. ANOVA-test results comparing the three prototypes are presented. (n/s: non-significant, *: *p*-value ≤ 0.05, **: *p*-value ≤ 0.01, ***: *p*-value ≤ 0.001, ****: *p*-value ≤ 0.0001). (**b**) Temperature profile for one hour reaction at 65 °C in each prototype. Measured in triplets. (**c**) Ramping times to achieve reaction temperature (65 °C) from room temperature (25 °C), from reaction temperature to inhibition temperature (80 °C) and from inhibition temperature back to reaction temperature. Measured for different battery percentages. Each measurement was taken in triplicates.

3.3. Positive and Negative Predictive Values of SMART-LAMP

To assess the predictive positive capacity of the SMART-LAMP devices, the difference between the final value (t_{final}) of RGB components and the values of RGB components at 0 (t_0), 5 min, 10 min (t_{10}) and 15 min (t_{15}) after the reaction started were evaluated for both fresh-LAMP and dry-LAMP reactions. For dry-LAMP reactions, stable desiccation of all reaction mixtures was achieved and well attached pellets were formed, both in the cap and at the bottom of the tubes (Figure S4). The pellets remained well-preserved for more than 30 days at RT.

Four positive controls (PTC) and four negative controls (NTC) of each LAMP assay were used (*Schistosoma mansoni*, *S. haematobium*, *Strongyloides* spp. and SARS-CoV-2). For each time-point, differences between PTC and NTC were more significant for dry-LAMP than fresh-LAMP assays. For fresh-LAMP assays, the only measurement that resulted in significant differences between positives and negatives (evaluated by the Mann-Whitney U test) was obtained analyzing the red and green components. Specifically, the reduction in the red component between t_{15} and t_{final} presented the most significant difference between positive and negative. For dry-LAMP reactions, the best predictor of amplification was also the reduction in the red component of RGB between times t_{10} or t_{15} and t_{final} (Figure 3). Greater significance was found in dry-LAMP reactions than in fresh-LAMP reactions which correlated with the increased colorimetric variation that could be observed by the naked eye (Figure S4). Given that the fastest amplifications we have detected with dried mixes were close to 15 min, the reduction of the red component of RGB between t_{10} and t_{final} was selected as the predictor of positivity.

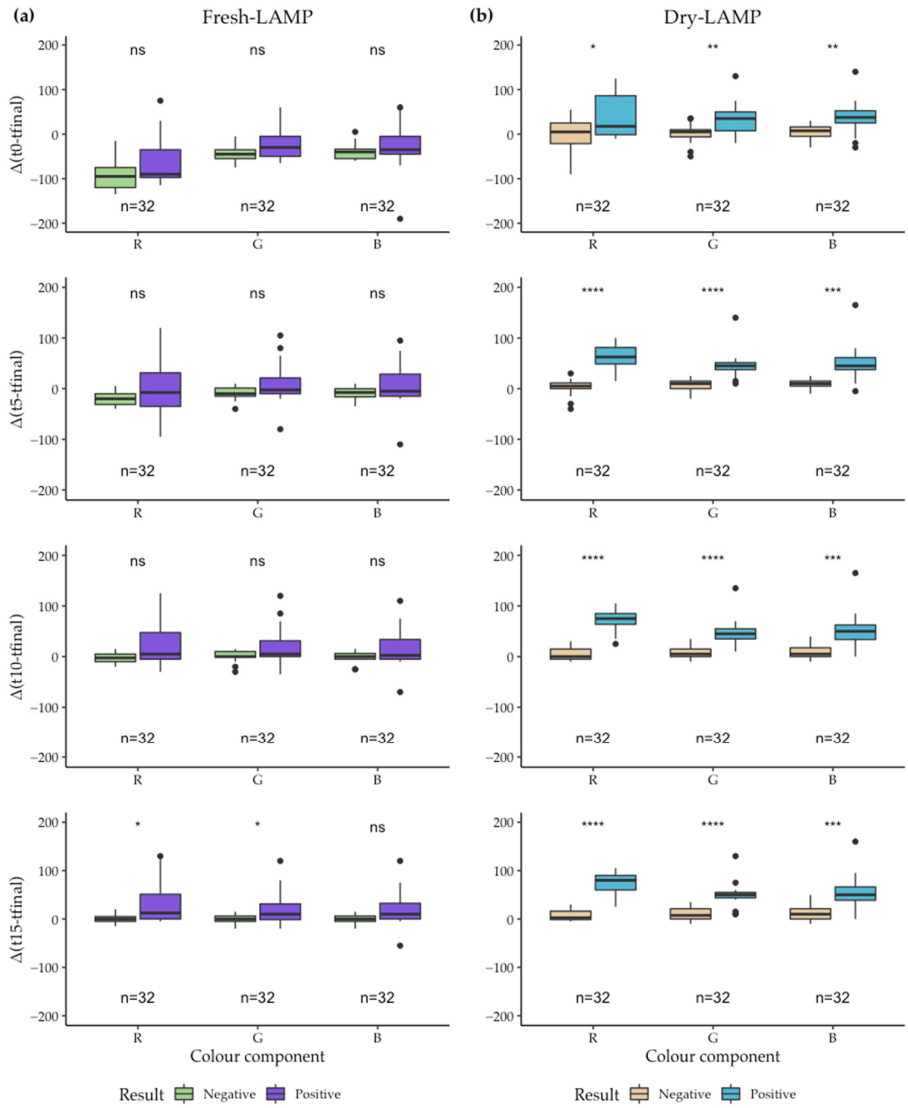

Figure 3. Differences between the final RGB values and the RGB values at 0, 5, 10 and 15 min for fresh-LAMP and dry-LAMP reactions measured by SMART-LAMP devices: (**a**) For fresh-LAMP and (**b**) dry-LAMP a total of 32 LAMP reactions ($n = 32$) were performed: 8 LAMP reactions (including 4 PTC and $ NTC) for *Schistosoma mansoni*, *S. haematobium*, *Strongyloides* spp. and SARS-CoV-2. Mann-Whitney U test: n/s: non-significant, *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, ****: p-value ≤ 0.0001.

Next, 20 PTCs and 20 NTCs from each dry-LAMP assay were used to establish the positivity threshold value. To do so, 40 dry-LAMP mixes of each LAMP assay were stored at RT and rehydrated within a 7-day period. In all dry-LAMP assays a decrease in the red component of RGB of 25 units between the value at t_{10} (10 min after the start of the reaction) and t_{final} (at the end of the reaction) maximized the positive and negative predictive capacity of the SMART-LAMP (Figure 4). Thus, any difference equal or superior to 25 was considered positive, any difference under 25 was considered negative. Then, to evaluate the effect of long-term storage in color measurements, 20 dry-LAMP mixes were stored for over 30 days at RT, followed by the analysis of 10 PTCs and 10 NTCs in the SMART-LAMP. The results from dry-LAMP mixes stored at RT for less than 7 days were compared to mixes stored for over 30 days, and no significant differences were found between long-term and short-term stored dry-LAMP mixes (Figure 4). Overall, a 90% (CI95: 83.4–96.6)

positive predictive value (PPV) and a 90% (CI95: 83.4–96.6) negative predictive value (NPV) were obtained for dry-LAMP mixes stored under 7 days at RT. Storage over longer periods of time did not significantly affect predictive values: 88.1% (CI95: 78.3–97.9) PPV and 92.1 NPV (CI95: 83.5–100). Subtle differences between LAMP assays were detected, with the SMART-LAMP showing slightly better predictive values for the SARS-CoV-2 and *S. haematobium* assays than the *S. mansoni* and *Strongyloides* spp. Thus, the reactions with faster kinetics (SARS-CoV-2 and *S. haematobium*) showed slightly improved PPV and NPV, regardless of the time of storage. The device, as discussed below, has shown good diagnostic capabilities. Nevertheless, predictive values obtained could probably be improved. The primary improvement would come from a reduction in variability of color measurements. This could be achieved via a standardized montage of SMART-LAMP devices. Additionally, although providing a single positivity threshold allows for a straightforward use of the device and stabilized mixes for the diagnosis of new pathogens, it can also limit the predictive values, when compared to individually optimized positivity thresholds.

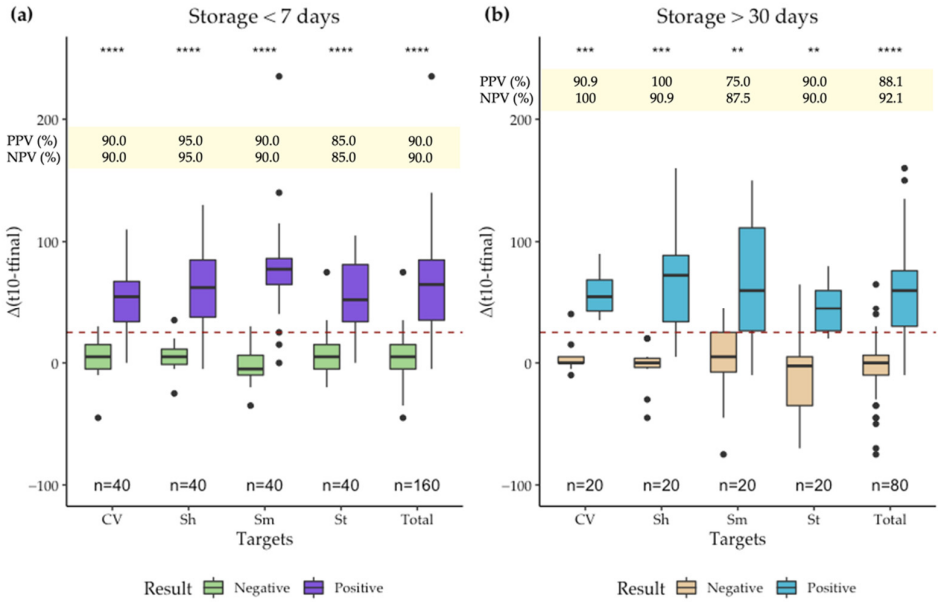

Figure 4. PTC and NTC analyzed with the SMART-LAMP for different dry-LAMP assays and storage times: (**a**) Differences between values of the R component of RGB 10 min after the start of the reaction and final value at reaction-end for different LAMP assays stored less than 7 days at RT after desiccation; (**b**) Differences between values of the R component of RGB 10 min after the start of the reaction and final value at reaction-end for different LAMP assays stored over 30 days at RT after desiccation. In yellow boxes, positive predictive values (PPV) and negative predictive values (NPV) of the SMART-LAMP are indicated. Targets: SARS: SARS-CoV-2; Sh: *S. haematobium*; Sm: *S. mansoni*; St: *Strongyloides* spp. Mann-Whitney U test: **: *p*-value ≤ 0.01, ***: *p*-value ≤ 0.001, ****: *p*-value ≤ 0.0001.

Conclusively, it was demonstrated that SMART-LAMP enabled the detection of color changes associated with DNA amplification. The device proved to be useful for the detection of different infectious agents: *S. mansoni*, *S. haematobium*, *Strongyloides* spp., and SARS-CoV-2. Moreover, the results evidenced that a single positivity threshold could be used, regardless of the LAMP assay performed. Thus, the adaptation of this device for the diagnosis of other diseases should be foreseeably straightforward. Examples of color-based detection with a smartphone are available in the literature, using hydroxynaphthol blue. Although they are able to perform real-time detection of amplification in a similar timeframe to our SMART-LAMP (60 min) [22,23], they still rely on a microfluidic chip. It

is clear that microfluidics offers some advantages over a design such as the SMART-LAMP, particularly in terms of reduced reaction volumes and integration, achieving controlled transport, mixing and reaction in specific microchambers [41]. In addition, some of their challenges, such us clinical validation [28], are shared between both approaches. However, the small scale and sometimes close to single-molecule approaches of microfluidics magnify subtle interactions that can result in inaccurate results. The interactions between plastics, adhesives and other materials can profoundly affect diagnostic performance [42]. This can mean that microfluidic designs are not cost-effective on a large scale, whereas the common laboratory materials used in the SMART-LAMP are more easily acquired, and repaired if, or when, necessary.

3.4. Analytical Sensitivity

Ten-fold serial dilutions of PTC were analyzed in triplicates for each of the dry-LAMP assays, both measuring fluorescence signal with Eva Green (EG) dye in the commercial Genie III device and color turn of MG via RGB in the SMART-LAMP device (Figure 5). Limit of detection (LoD) was proven to be equal for fluorescence-based detection and RGB-based detection. Sensitivity was not reduced by the stabilization process in the case of dry-LAMP reactions for SARS-CoV-2 and *Strongyloides* spp. amplification [31,32]. However, in the case of dry-LAMP for the amplification of *S. haematobium* and *S. mansoni*, the sensitivity was slightly lower than that obtained in fresh-LAMP reactions: 0.1 pg vs. 10 fg for *S. haematobium* [30] and 1 pg vs. 10 fg for *S. mansoni* [29]. According to previous results obtained by our group [33] and others [43], sensitivity tends to decrease after stabilization, especially after extended periods of storage at room temperature. Those effects have been observed not only in the LAMP reactions, but also in other isothermal amplifications, such as rolling circle amplification (RCA) [44]. In some cases, a reduction in signal intensity may occur, which has been observed after stabilization of the reagents via lyophilization in RT-qPCR [45].

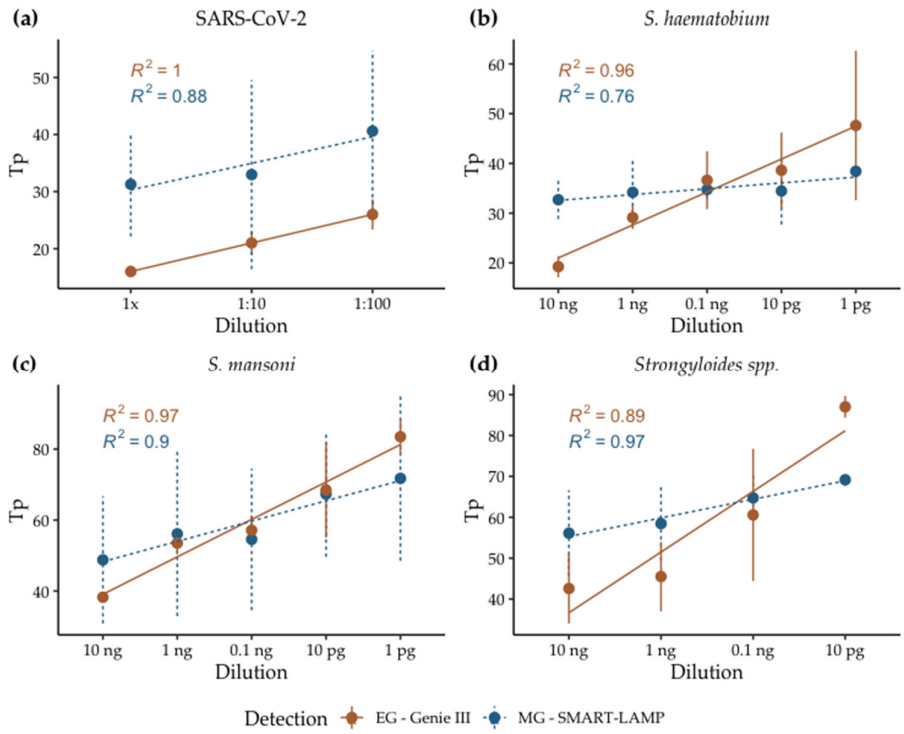

Figure 5. Sensitivity of real-time dry-LAMP assays performed in the Genie III (EG) and the SMART-LAMP (MG) devices: (**a**) SARS-CoV-2; (**b**) *S. haematobium*; (**c**) *S. mansoni*; (**d**) *Strongyloides* spp.

The kinetics of all the reactions correlated well with the analyzed concentrations. The time to positivity (Tp) of the dilutions analyzed by real-time dry-LAMP on the Genie III device fitted linear regression models correctly, with R^2 coefficients ranging from 1 for SARS-CoV-2 to 0.89 for *Strongyloides* spp. On the other hand, for SMART-LAMP results, linear regression ranged from $R^2 = 0.97$ for *Strongyloides* spp. to 0.78 for *S. haematobium*. Of note, for high concentrations of the target, flourescence-based amplification detection was more efficient in all cases. However, at low concentrations, SMART-LAMP could detect amplification earlier than the Genie III device. The reaction time slightly increased when utilizing dried mixes compared to fresh ones. The reasons behind this phenomenon are not clear, but a possible explanation could be the action of the trehalose matrix in retarding conformational dynamics in dehydrated protein systems [46]. In a trehalose system, guest molecules (*Bst* polymerase in this case) are homogeneously integrated into a hydrogen-bond network of water and trehalose, strongly limiting their mobility [47].

Evidently, when comparing EG and MG, an important caveat must be considered, as the amplification monitoring is performed via different mechanisms. While EG directly binds to the DNA [48], MG monitors amplification via the concentration of free Mg^{2+}, which is reduced as amplification takes places and it binds to the pyrophosphate liberated [49]. Notwithstanding this consideration, both are valid approaches for amplification monitoring widely used in LAMP. Interestingly, EG has been shown to have an inhibitory effect, that can reduce both reaction rates, as well as final amplification levels [50]. This effect could partly explain the differences observed at low concentrations of template between EG and MG.

3.5. Proof of Concept: COVID-19 Patients Sample Analysis

RNA from 80 patients with suspected COVID-19 (20 negative and 60 positive) were analyzed by RT-qPCR, fresh-RT-LAMP, and dry-RT-LAMP (in the commercial device Genie III) as well as in the SMART-LAMP to evaluate the applicability of the device in "real-world" conditions. Taking RT-qPCR results as the diagnostic standard [51], fresh-RT-LAMP showed a sensitivity of 93.3% (CI95: 87.0–99.6) and a specificity of 90.0% (CI95: 76.9–100). No deleterious effect was observed due to the desiccation of the RT-LAMP components, as dry-RT-LAMP showed a sensitivity of 95.0% (CI95%: 89.5–100) and a specificity of 90.0% (76.9–100). A slight decrease in sensitivity was observed when performing the analysis in the SMART-LAMP, resulting in 88.3% (80.2–96.5). Nevertheless, specificity was improved slightly to 95.0% (85.4–100). If only RNA samples with a $Ct < 33$ value in RT-qPCR, (samples with an estimated viral load over 500 copies [52]) were considered, then sensitivity of fresh-RT-LAMP increased to 100%, that of dry-RT-LAMP to 97.9% and that of SMART-LAMP to 95.9% (Figure 6).

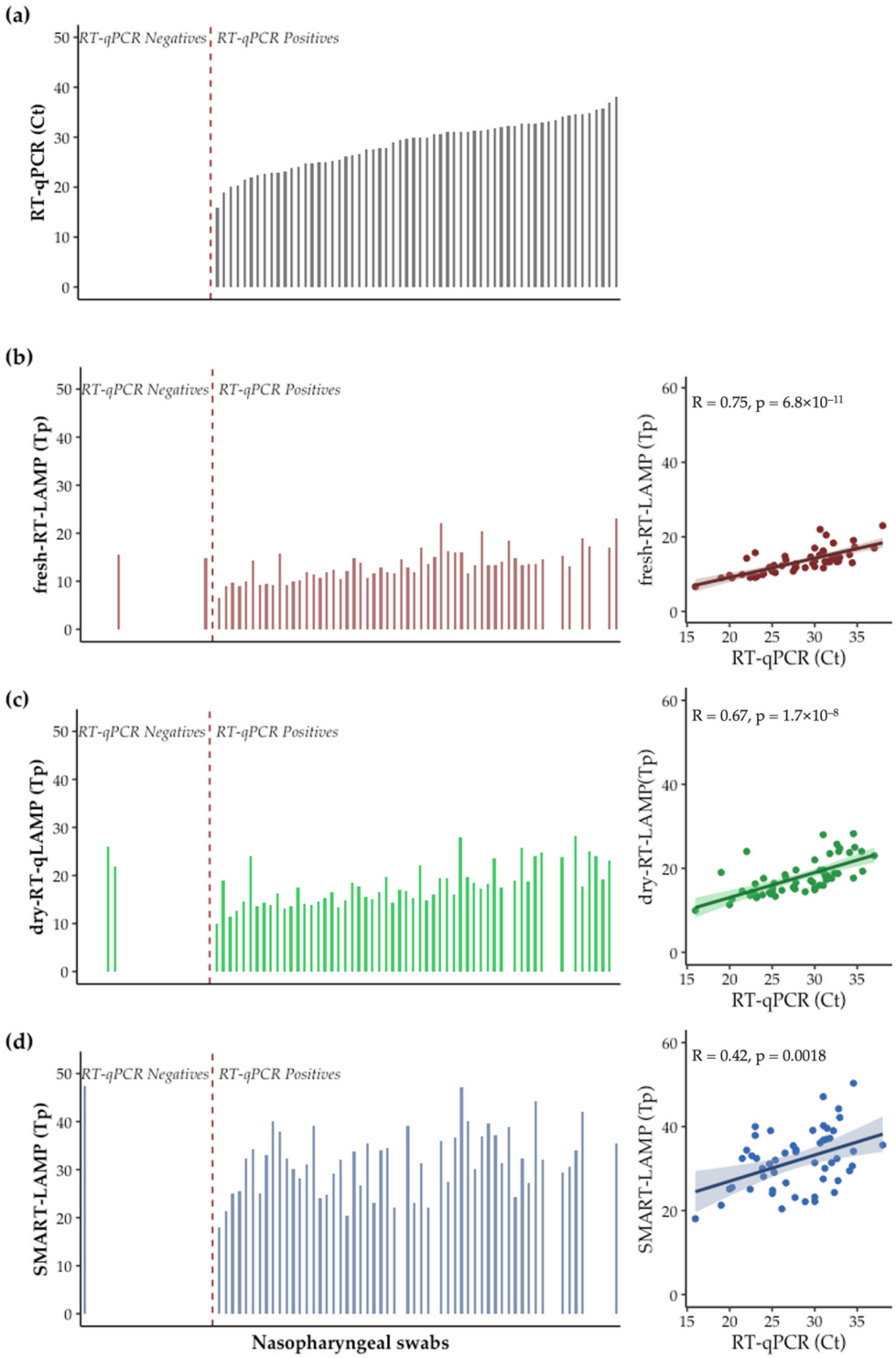

Figure 6. Analysis of RNA samples from patients suspected of having COVID-19. On the left panels, results from the analysis of the 80 samples are shown ordered from low to high RT-qPCR Ct values obtained. On the right panels, correlation plots between Ct and Tp values in RT-qPCR and LAMP assays, respectively, are represented. R and *p* values obtained by Pearson's correlation are shown in each graph: (**a**) RT-qPCR Ct values of the 80 samples analyzed; (**b**) Fresh-RT-qLAMP Tp values of the 80 analyzed samples and their correlation with RT-qPCR results; (**c**) Dry-RT-qLAMP Tp values of the 80 analyzed samples and their correlation with RT-qPCR results; (**d**) SMART-LAMP Tp values of the 80 samples analyzed and their correlation with RT-qPCR results.

Regarding reaction kinetics, a clear decrease in correlation between RT-qPCR Ct values and LAMP Tp values was observed when using the SMART-LAMP (Pearson's correlation coefficient; R = 0.42). The decrease in correlation was not a consequence of the desiccation process as fresh-RT-qLAMP and dry-RT-qLAMP assays showed highly similar Pearson's coefficients (0.75 and 0.67, respectively), and when comparing SMART-LAMP results to either fresh-RT-qLAMP or dry-RT-qLAMP, the Pearson's coefficient in both cases was 0.45. Still, the SMART-LAMP resulted in a moderate correlation, with a single analysis of the samples (Figure 6b–d).

The sensitivity and specificity obtained in our study were comparable to those obtained with other POCT approaches for SARS-CoV-2 but performed in a much simpler manner. Soares et al. [18] tested 162 nasopharyngeal swabs, collected from patients with COVID-19 symptoms, on a centrifugal microfluidic platform. They detected amplification with a bead-based approach, obtaining a sensitivity of 96.6% for samples with a low Ct (Ct < 26) and a specificity of 100%. In the work of Rodríguez-Manzano et al. [17], a lab-on-chip RT-LAMP assay was developed on the basis of semiconductor technology paired with a smartphone for result visualization. As a benchtop technique, when analyzing 183 clinical samples (including 127 positives) the assay showed a LoD of 10 RNA copies per reaction with 91% sensitivity and 100% specificity. The average Tp was 15.45 ± 4.43 min. By testing a sample subset on the POCT platform developed, the authors showed comparable results with the benchtop instrument, with an average Tp of 12.68 ± 2.56 min for positive samples (n = 34). Other approaches have combined a magnetic-based purification of RNA samples with a smart-phone based detection approach, obtaining reduced false positive rates and being able to detect positive samples within large pools of patients bronchoalveolar lavages [53]. Chen et al. [54] developed a 3D-printed portable station to detect amplification at the end of the reaction. They tested 7 positive and 3 negative respiratory swab samples of SARS-CoV-2, obtaining 100% sensitivity and 100% specificity.

In all, we present a novel handheld device for the diagnosis of infectious diseases via colorimetric real-time LAMP assay. Only a few examples of similar platforms have been described, which rely either on pH indicators [55,56] or Hydroxynaphtol blue dye [56], but, to the best of our knowledge, none of them are based on MG. Additionally, those devices do not make use of ready-to-use mixes, thus making them potentially less applicable to POC settings. Moreover, we have demonstrated that the SMART-LAMP is applicable, without a specific optimization, for the detection of a number of different pathogens using a single positivity threshold for all the assays, hence providing straightforward applications for new diagnostics. Regarding SARS-CoV-2 RNA detection in clinical samples, the platform presented by Papadakis et al. [56] showed a 97% sensitivity and 100% specificity when analyzing a total of 89 samples. On the other hand, Diaz et al. [55] used the commercial BioRanger diagnostic platform modified for colorimetric LAMP for COVID-19 testing in a limited number of simulated samples spiked with either synthetic RNA or inactivated SARS-CoV-2 virus (n = 20; 10 positives and 10 negatives), correctly detecting the 10 positive samples and 9/10 of the negatives. In both studies, the results obtained were comparable with those yielded by our SMART-LAMP device.

We acknowledge the limitations of our study. First, there was a lack of standardization in the manufacturing of the devices, which increased the production complexity and made the manufacturing process labor intensive. In this regard, the limitations presented by other POCT approaches were not remedied [57]. Additionally, only moderate correlation was achieved between RT-qPCR results and SMART-LAMP results, but this was not caused by the desiccation protocol, as correlation values were highly similar between fresh-RT-qLAMP and dry-RT-qLAMP assays (0.75 vs. 0.67, respectively). Thus, the real-time measurements of amplification obtained with the SMART-LAMP were less precise. Considering the results obtained for sensitivity measurements, where correlation values were much higher (R ranging 0.82 to 0.99), we could expect that triplicate measurements would noticeably improve correlation. Finally, one of the main challenges when converting laboratory molecular assays into POC tests was the need to extract nucleic acid as the

first step of sample preparation and then to perform the LAMP reaction. However, this could be solved by combining the SMART-LAMP with a number of POC nucleic acid extraction technologies already described that have demonstrated applicability and robustness for the isolation of high-quality nucleic acid from complex raw human samples, including blood, saliva, sputum, nasal swabs, and urine [58].

4. Conclusions

The task of bringing affordable molecular diagnostics to the field has been hindering public health improvement for many years [1]. Nevertheless, the increased sensitivity and specificity of molecular methods is needed to address diagnostic challenges in many resource-limited areas. In recent years, the medical field has focused on the development of microfluidic POCT molecular diagnostics. The approaches present numerous advantages, from sample-to-answer design to high sensitivity, specificity or reduced volume [59]. However, some disadvantages must also be highlighted: limited automation selectivity and stability of sensing moduli, as well as poor scalability and clinical validation [28]. Considering the REASSURED criteria [8] as the benchmark to assess POCT, the SMART-LAMP is a real-time connected, affordable solution for sensitive and specific NAAT analysis. It is based on LAMP technology, which has already largely proven its speed and robustness [12,60]. Relying on ready-to-use reaction mixes and a simple smartphone app interface, it is user-friendly and highly deliverable. More extensive studies are required for clinical application and validation; however, this proposal has the potential to become a valuable alternative to the currently available diagnostic options and could be easily adapted to the diagnosis of many other infectious diseases.

5. Patents

The SMART-LAMP device is protected under a Utility Model granted by the Spanish Ministry of Energy, Tourism and Digital Agenda, with registry number U202032679, with authors Juan García-Bernalt Diego, Pedro Fernández-Soto, Moncef Belhassen-García, Antonio Muro and Juan M. Corchado Rodríguez listed as inventors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bios12060424/s1, Figure S1: Single-line electronic circuit sketches of the different modules of the SMART-LAMP; Figure S2: Printed Circuit Board (PCB) of SMART-LAMP; Figure S3: SMART-LAMP app screen workflow; Figure S4: Comparison of fresh-LAMP and dry-LAMP results using malachite green and the effect of storage; Table S1: List of components of SMART-LAMP.

Author Contributions: Conceptualization, P.F.-S., A.M., J.G.-B.D., S.M.-S., D.S.S. and J.M.C.R.; methodology, J.G.-B.D.; software, S.M.-S., D.S.S. and J.M.C.R.; validation, J.G.-B.D. and D.S.S.; formal analysis, J.G.-B.D.; investigation, J.G.-B.D., D.S.S., B.F.-S. and B.C.-V.; resources, P.F.-S., A.M., M.B.-G., J.L.M.-B. and J.M.C.R.; data curation, J.G.-B.D.; writing—original draft preparation, J.G.-B.D., P.F.-S. and S.M.-S.; writing—review and editing, J.G.-B.D., P.F.-S., S.M.-S., J.M.C.R. and A.M.; visualization, J.G.-B.D.; supervision, P.F.-S., A.M. and J.M.C.R.; project administration, P.F.-S., A.M. and J.M.C.R.; funding acquisition, P.F.-S., A.M. and J.M.C.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of UNIVERSITY HOSPITAL OF SAMALANCA, SPAIN (protocol code CEIMC 2020.06.530 and date of approval 19 October 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The authors declare that data related to this research are available from the authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

SMART-LAMP: A smartphone-operated handheld device for real-time colorimetric point-of-care diagnosis of infectious diseases via loop-mediated isothermal amplification

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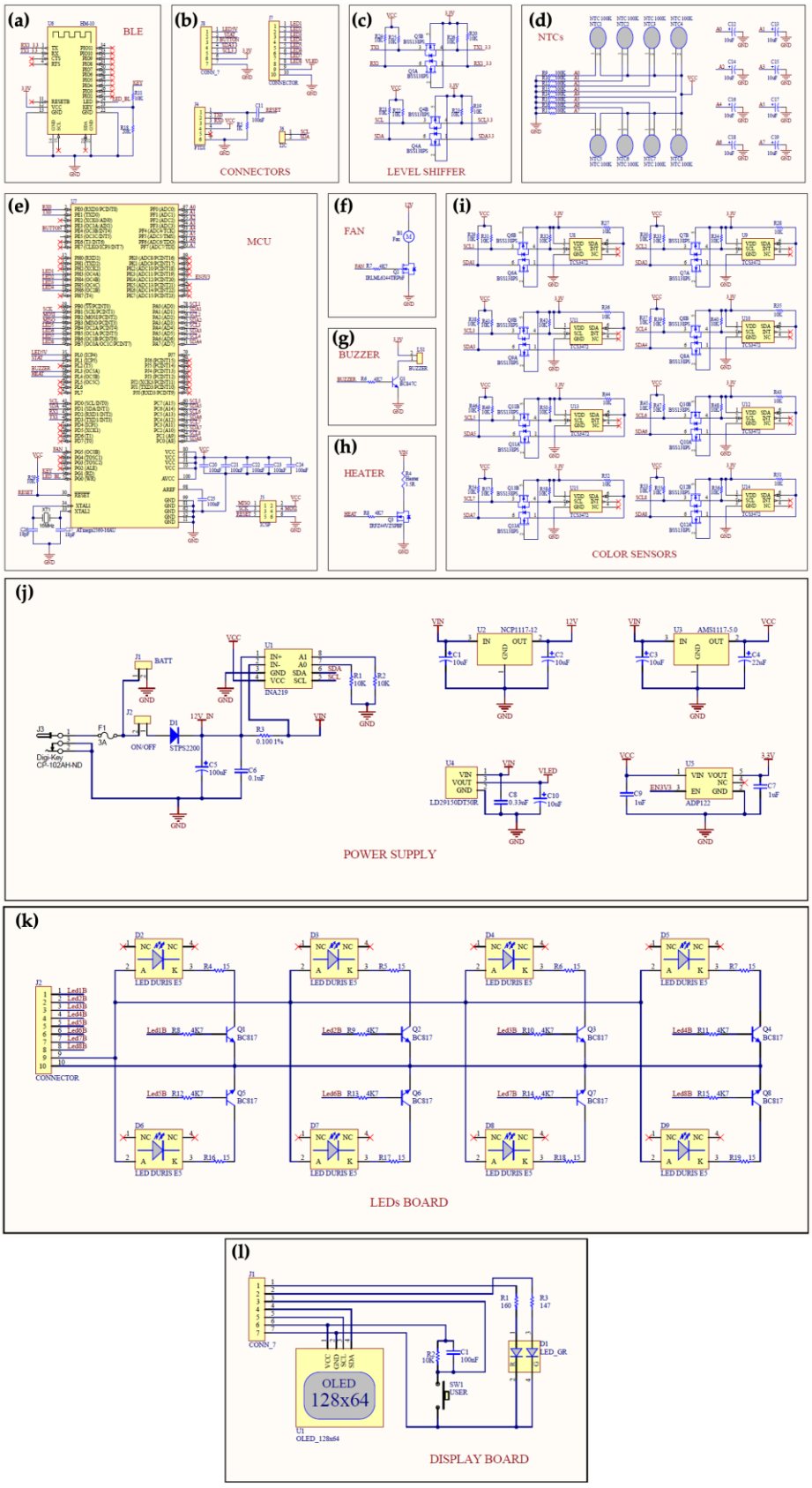


Figure S1. Single-line electronic circuit sketches of the different modules of the SMART-LAMP: **(a)** Bluetooth Low Energy Circuit (BLE); **(b)** Connectors; **(c)** Level Shiffer; **(d)** NTC Thermistors; **(e)** Microcontroller Unit (MCU); **(f)** Fan; **(g)** Buzzer; **(h)** Heater; **(i)** Color sensors; **(j)** Power supply; **(k)** LEDs Board; **(i)** Display Board.

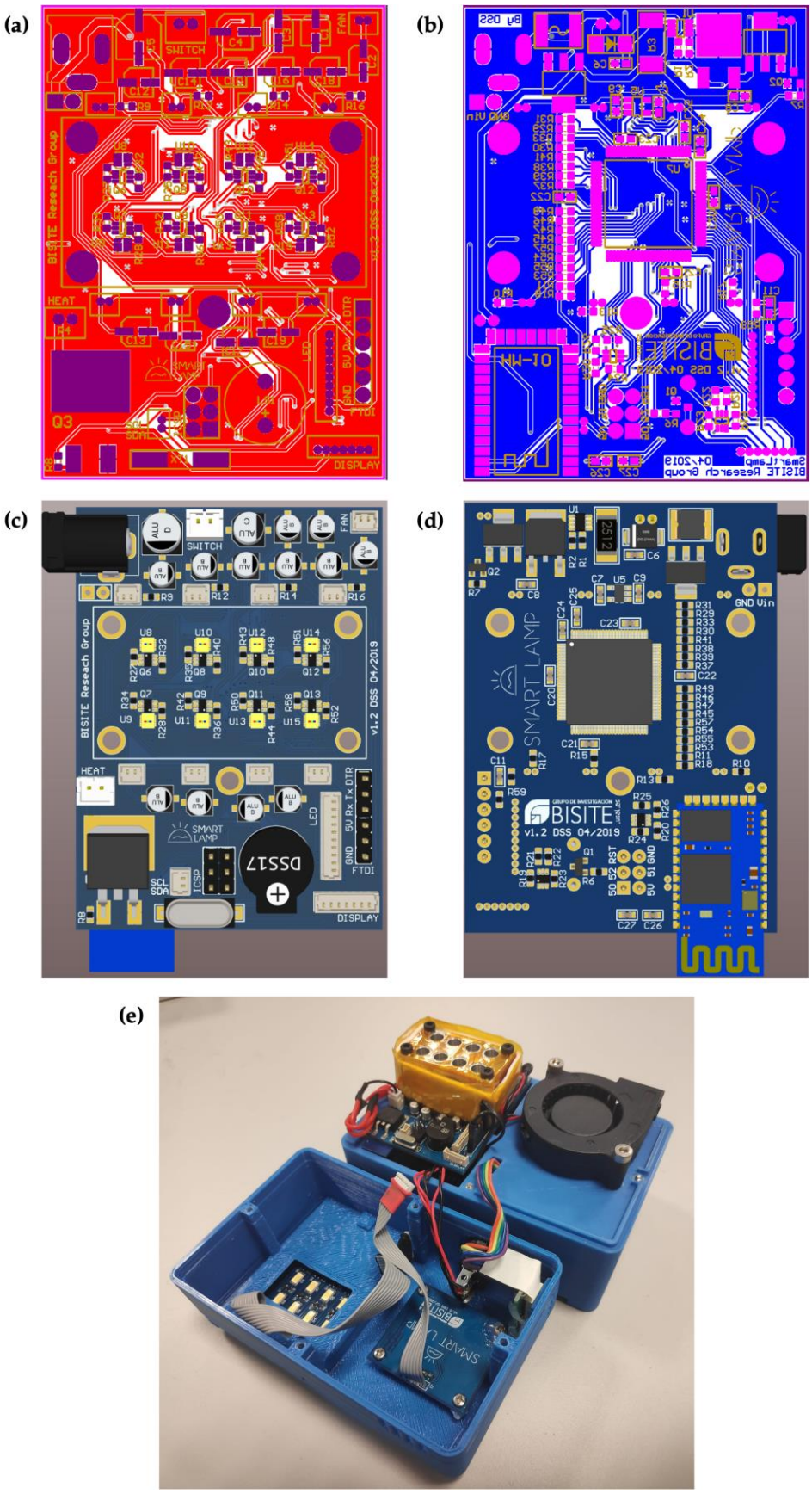


Figure S2. Printed Circuit Board (PCB) of SMART-LAMP: **(a)** Upper face of the PCB; **(b)** Bottom face of the PCB; **(c)** 3D render of the upper face of the PCB; **(d)** 3D render of the bottom face of the PCB; **(e)** Interior of the SMART-LAMP device.

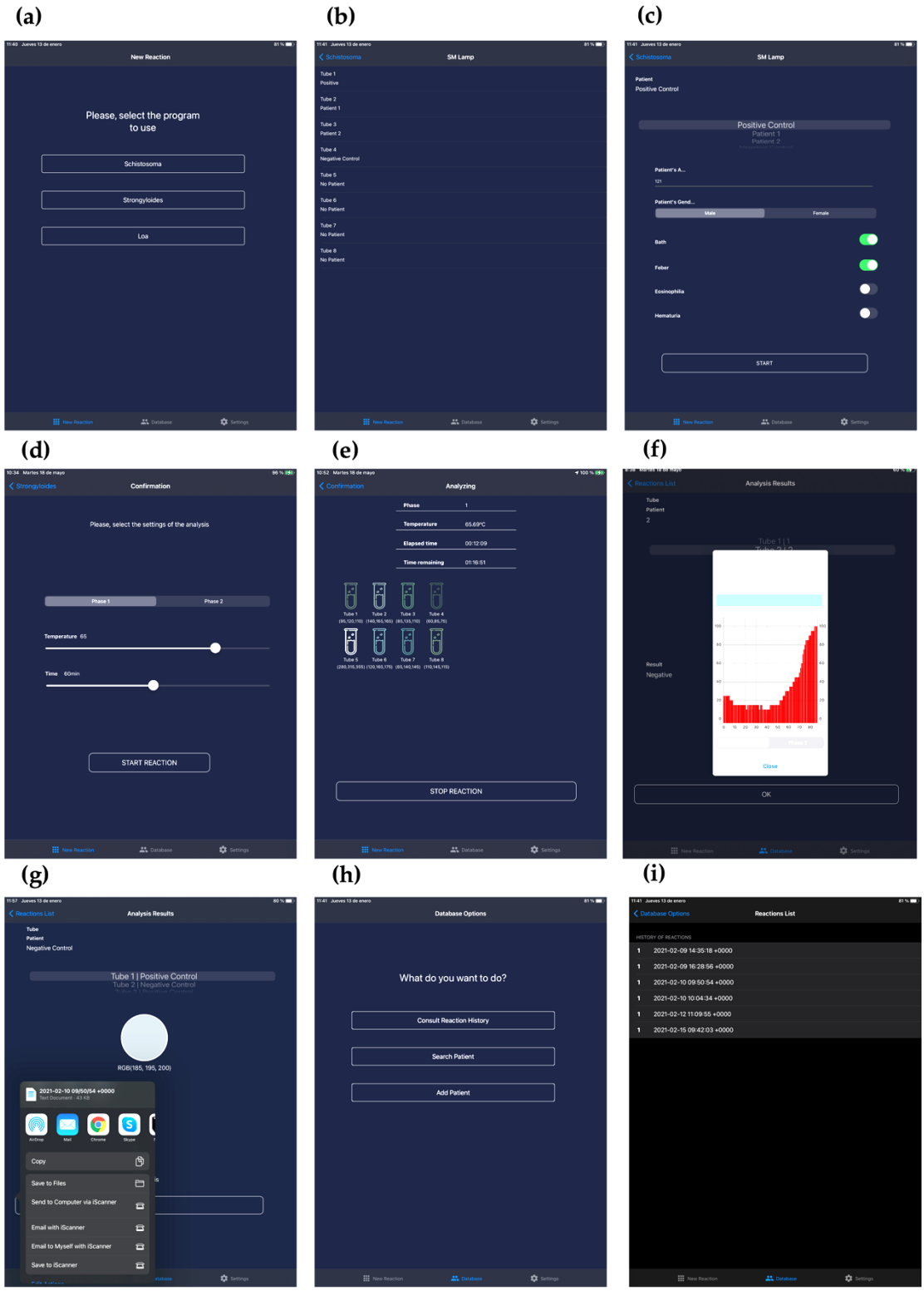


Figure S3. SMART-LAMP app screen workflow: **(a)** Initial screen for the selection of the scheduled reaction conditions; **(b)** Selection of the samples to be analyzed and the position of each sample in the device; **(c)** Screen to add relevant information that has been acquired during sample collection; **(d)** Modification of reaction conditions in terms of temperature and time; **(e)** Real-time visualization of the reaction progress; **(f)** Real-time results for one sample, visualized at the end of the reaction; **(g)** .csv file can be exported at the end of the reaction. (h) Screen to access patient database and previous reactions; **(i)** Past reactions database.

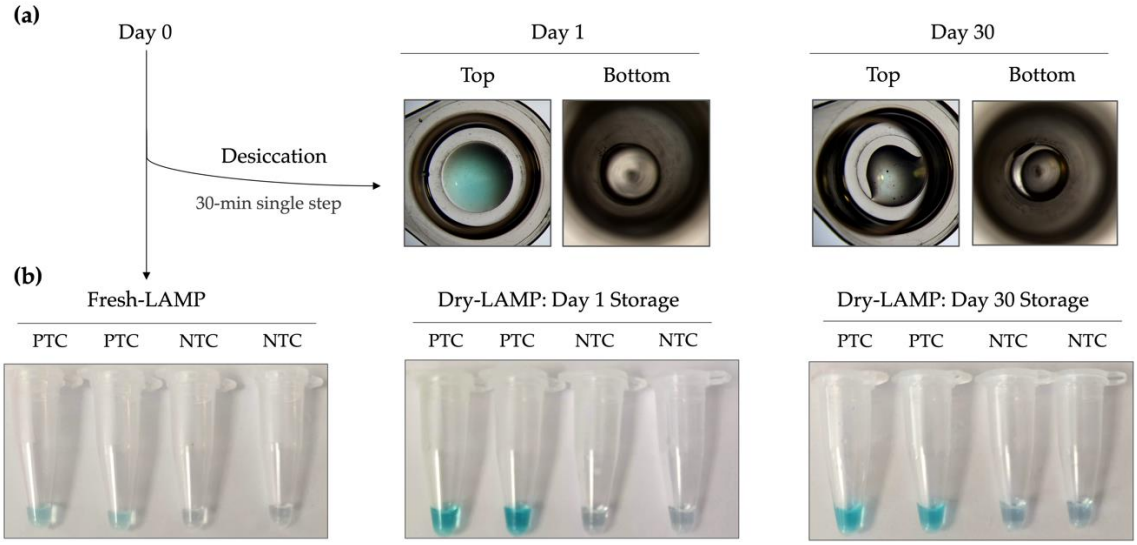


Figure S4. Comparison of fresh-LAMP and dry-LAMP results using malachite green and the effect of storage: **(a)** A view under a magnifying glass of the top (left) and bottom (right) partial mix pellets at day 1 and 30 post-desiccation; **(b)** Colorimetric change of 0.008% w/v MG between positive (PTC) and negative (NTC) results for a LAMP assay targeting *Schistosoma haematobium* as example, using fresh-LAMP and dry-LAMP at day 1 and 30 post-desiccation.

Table S1. List of components of SMART-LAMP.

Modules	Component	Quantity
Main Board	ATmega2560-16AU MCU	1
	HM-10 Bluetooth module	1
	TCS3472 RGB Sensor	8
	NTC Resistor 100K	2
	SMD Resistor 100K 0603	2
	Electrolytic Capacitor 10uF 16V	6
	INA219 Sensor	1
	SMD Resistor 0,1 ohms 1% 2512	1
	Electrolytic Capacitor 100uF 16V	1
	Regulator NCP1117-12	1
	Regulator AMS1117-5.0	1
	Regulator LD29150DT50R	1
	Regulator ADP122	1
	Mosfet BSS138PS	10
	Mosfet IRLML6244TRPbF	1
	Mosfet IRFZ44VZ	1
	BC847C Transistor	1
	SMD Resistor 10K 0603	44
	Ceramic Capacitor 100nF 0603	8
	Ceramic Capacitor 1uF 0603	2
	16MHz crystal	1
	Ceramic Capacitor 18 pF 0603	2
	SMD Resistor 4K7 0603	3
	Electrolytic Capacitor 22uF 16V	1
	Ceramic Capacitor 330nF 0603	1
	3A fuse	1
	STPS2200 diode	1
	PicoBlade PCB Header 10 Circuits	1
	PicoBlade PCB Header 7 Circuits	1
	PicoBlade Receptacle Crimp Housing 10 Circuits	1
	PicoBlade Receptacle Crimp Housing 7 Circuits	1
	PicoBlade Female Crimp Terminal	19
	Active buzzer	1
	6x1 male pin header	1
	3x2 male pin header	1
	PicoBlade PCB Header 2 Circuits	1
	JTS-PHX 2-pin connector female	2
	JTS-PHX 2-pin connector male	2
	12V Radial Fan	1
	FTDI module	1
	Conector DC Hembra	1
	Power Switch	1

Display Board	OLED Display 128x64 I2C	1
	SMD Button	1
	Ceramic Capacitor 100nF 0603	1
	SMD Resistor 10K 0603	1
	SMD Resistor 147 ohms 0603	1
	SMD Resistor 160 ohms 0603	1
	Bicolor SMD LED	1
	PicoBlade Female Crimp Terminal	7
	PicoBlade PCB Header 7 Circuits	1
	PicoBlade Receptacle Crimp Housing 7 Circuits	1
LED Board	LED Duris E5 SMD	8
	BC817 Transistor	8
	SMD Resistor 4K7 0603	8
	SMD Resistor 15 ohms 0603	8
	PicoBlade Female Crimp Terminal	10
	PicoBlade PCB Header 10 Circuits	1
	PicoBlade Receptacle Crimp Housing 10 Circuits	1
Heater	Aluminum block	1
	Polyamide heaters	2
	Thermal insulation cm2	36
Case	PLA gr	416
	Magnets	6
	Screws	20

CAPÍTULO IV

Conclusiones

I. Se ha optimizado una metodología sencilla y rápida de estabilización de reactivos LAMP que permite su almacenamiento a temperatura ambiente en formato listo para el uso.

II. Se ha desarrollado y patentado el dispositivo portátil SMART-LAMP que permite realizar reacciones de amplificación isotérmica tipo LAMP a tiempo real con monitorización colorimétrica, controlado mediante una aplicación móvil que permite el registro, gestión y análisis de los resultados.

III. Se han diseñado ensayos RT-LAMP para la detección colorimétrica y a tiempo real de ARN de SARS-CoV-2, con elevada sensibilidad y especificidad y se ha comprobado su aplicabilidad en diferentes muestras clínicas.

IV. Se ha evaluado el funcionamiento del dispositivo SMART-LAMP con diferentes ensayos LAMP para la detección de *Schistosoma mansoni*, *S. haematobium*, *Strongyloides* spp. y SARS-CoV-2, valorándose, para este último, su utilidad diagnóstica en muestras clínicas, y obteniendo resultados comparables con las técnicas moleculares de referencia y otros dispositivos comerciales.

I. *A simple and rapid methodology has been optimized for the stabilization of LAMP reagents that allows their storage at room-temperature in a ready-to-use format.*

II. *The SMART-LAMP portable device has been developed and patented, which allows the performance of real-time LAMP isothermal amplification reactions via colorimetric monitorization, controlled by a mobile application that enables the registration, management and analysis of results.*

III. *RT-LAMP assays have been designed for colorimetric and real-time detection of* SARS-CoV-2 *RNA, with high sensitivity and specificity, and their applicability has been tested in different clinical samples.*

IV. *The performance of the SMART-LAMP device has been evaluated with different LAMP assays for the detection of Schistosoma mansoni, S. haematobium, Strongyloides* spp. *and the new* SARS-CoV-2*, assessing, for the latter, its diagnostic utility in clinical samples, and obtaining comparable results with reference molecular techniques and other commercial devices.*

ANEXO I

Anexo metodológico

AI.1. Muestras humanas

En los distintos artículos de investigación recogidos en este trabajo se utilizaron distintas muestras de origen humano.

En el **primer artículo de investigación** (*Progress in loop-mediated isothermal amplification assay for detection of Schistosoma mansoni DNA: towards a ready-to-use test*), se utilizó ADN obtenido de muestras de tejido de tres pacientes con infección confirmada por *S. mansoni*. Una biopsia cutánea y otra hepática, cedidas por el Hospital Universitario Vall d'Hebron (Barcelona, España), así como una biopsia de apéndice, cedida por el Departamento de Parasitología del Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid, España).

En el **segundo artículo de investigación** (*A Simple, Affordable, Rapid, Stabilized, Colorimetric, Versatile RT-LAMP Assay to Detect SARS-CoV-2*), se trabajó con exudados nasofaríngeos de veinte pacientes ingresados por COVID-19 recogidos como parte de la rutina diagnóstica del Hospital Universitario de Salamanca (Salamanca, España). Tanto para este estudio y los siguientes se solicitó y se obtuvo permiso del comité de Bioética del Hospital Universitario de Salamanca (CEIMC 2020.06.530) aprobado el 19 de octubre de 2020. Todos los protocolos incluidos en los artículos se realizaron de acuerdo con los estándares descritos en la Declaración de Helsinki revisada en 2013. Los datos de todos los pacientes se anonimizaron.

En el **tercer artículo de investigación** (*Detection of SARS-CoV-2 RNA in urine by RT-LAMP: a very rare finding*), se utilizaron tanto muestras de exudado nasofaríngeo como de orina de 300 pacientes incluidos en el estudio obtenidas como parte del protocolo diagnóstico del Hospital Universitario de Salamanca (Salamanca, España).

En el **cuarto artículo de investigación** (*SMART-LAMP: A smartphone-operated handheld device for real-time colorimetric point-of-care diagnosis of infectious diseases via loop-mediated isothermal amplification*), se incluyeron 80 muestras de exudados nasofaríngeos de pacientes con sintomatología compatible con COVID-19 recogidos como parte del protocolo diagnóstico del Hospital Universitario de Salamanca (Salamanca, España).

AI.2. Procesamiento de muestras y extracción de ácidos nucleicos

Durante la realización de este trabajo, se han utilizado diferentes kits de extracción de ácidos nucleicos (ADN o ARN) para diferentes muestras (Tabla AI.1.).

Tabla AI.1. Tipos de muestra y kits utilizados para la extracción de ácidos nucleicos.

Tipo de muestra	Ácido nucleico	Kit de extracción
Parásito	ADN	NucleoSpin Tissue Kit (Macherey-Nagel, GmH & Co., Düren, Alemania)
Tejido	ADN	NucleoSpin Tissue Kit (Macherey-Nagel, GmH & Co., Düren, Alemania)
Exudado nasofaríngeo	ARN	NZY Viral RNA Isolation Kit (NZYTECH, Lisboa, Portugal)
Orina	ARN	NZY Viral RNA Isolation Kit (NZYTECH, Lisboa, Portugal)

A continuación, se describen de manera detallada los diferentes protocolos utilizados.

AI.2.1. Purificación de ADN genómico de parásitos

Durante el transcurso de esta Tesis Doctoral se extrajo ADN genómico de tres parásitos: *Schistosoma mansoni*, *S. haematobium* y *Strongyloides venezuelensis*. En el caso de *S. mansoni* y *S. haematobium*, se utilizan como material de partida gusanos adultos almacenados congelados. Los adultos de *S. mansoni* y las larvas infectivas en estadio 3 (iL3) de *S. venezuelensis* se obtuvieron gracias al mantenimiento del ciclo de vida de los parásitos de forma experimental en el laboratorio de Parasitología, CIETUS, Universidad de Salamanca

La extracción del ADN de todos los parásitos se realizó utilizando el kit NucleoSpin Tissue Kit (Macherey-Nagel, GmH & Co., Düren, Alemania) siguiendo el protocolo que se describe a continuación:

a. Para pre-lisar las muestras, se seleccionaban entre 20 y 40 adultos de *S. mansoni* o *S. haematobium* o entre 10.000 y 20.000 iL3 de *S. venezuelensis.* Se diluían en 180 µL de *buffer* T1 y se añadían 25 µL de Proteinasa K. Todo se mezcló utilizando el vórtex.
b. Posteriormente se incubaban a 56 ºC *overnight.* El tiempo de incubación puede reducirse hasta un mínimo de 3 h, pero se obtienen mejores resultados si se incuba durante toda la noche.
c. Tras la incubación se llevaba a cabo la lisis de los gusanos, añadiendo 200 µL de *buffer* B3, mezclando mediante un vórtex e incubando a 70 ºC durante 10 min.

d. Para ajustar las condiciones de la muestra para la unión del ADN a la membrana, se añadían 210 µL de etanol al 96 % y se mezclaban mediante vórtex.
e. Una vez se habían ajustado las condiciones la mezcla se pasaba a través de la columna de purificación, donde el ADN se une a la membrana. Se llevaba a cabo una centrifugación de 1 min a 11.000 x *g* y se descartaba el eluido.
f. Tras la unión del ADN a la membrana, se realizaron dos lavados, el primero con 500 µL de *buffer* BW y el segundo con 600 µL de *buffer* B5. Ambos se siguieron de centrifugaciones de 1 min a 11.000 x *g* tras las que se descarta el eluido.
g. Idealmente, la membrana debería secarse al máximo posible para obtener el mayor grado de pureza del ADN. Para ello, se realizaba una última centrifugación, sin añadir ningún *buffer* de lavado de 1 min a 11.000 x *g*.
h. Por último, se eluía el ADN en 100 µL de *buffer* de elución o bien de agua ultrapura, a través de una centrifugación durante 1 min a 11.000 x *g*.

Tras la extracción del ADN genómico, se midió por triplicado su concentración y pureza en un Nanodrop (ND-1000; THERMO FISHER SCIENTIFIC, Estados Unidos) y se diluyó a la concentración de trabajo.

AI.2.2. Purificación de ADN de muestras de tejido de pacientes

Se utilizó el ADN extraído de tres muestras de tejido de pacientes con infección confirmada por *S. mansoni.* Las muestras correspondían a una biopsia cutánea y otra hepática, cedidas por el Hospital Universitario Vall d'Hebron (Barcelona, España) y una biopsia de apéndice, cedida por el Departamento de Parasitología del Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid, España). Para todas ellas, se obtuvo el ADN utilizando el kit NucleoSpin Tissue Kit (Macherey-Nagel, GmH & Co., Düren, Alemania), siguiendo el protocolo descrito en el apartado AI.2.1, con una modificación en el paso (a): se utilizaron 25 mg de tejido que se cortaron en pequeños trozos antes de proceder con el siguiente paso. Este proceso se recoge en el **primer artículo de investigación**.

AI.2.3. Purificación de ARN viral de muestras de pacientes

AI. 2.3.1. Muestras nasofaríngeas

El ARN viral utilizado tanto para el análisis de muestras realizado en los **artículos de investigación 2, 3 y 4**, como control positivo en las reacciones de RT-qPCR y RT-LAMP de SARS-CoV-2, se extrajo de muestras de exudados nasofaríngeos.

Tras la recogida de las muestras, estas se mantenían en *Sample Preservation Solution* (MOLE BIOSCIENCE, SUNGO Europe B.V., Ámsterdam, Países Bajos). Después, si el ARN era extraído en las instalaciones del Servicio de Microbiología de Hospital Universitario de Salamanca, se hacía utilizando el kit NUCLISENS EASYMAG (BIOMÉRIEUX, Francia) o bien en la plataforma integrada de purificación y RT-PCR COBAS 6800 (ROCHE, Suiza).

Si las muestras se trasladaban al CIETUS, el ARN era extraído mediante el kit NZY Viral RNA Isolation Kit (NZYTECH, Lisboa, Portugal) mediante el siguiente protocolo:

a. Para la lisis viral, a 200 µL de muestra se añadían 350 µL de *buffer* NVL y se mezclaba mediante un vórtex. Después se realizaba una incubación a temperatura ambiente durante 10 min.
b. Tras la incubación, para ajustar las condiciones de la mezcla se añadían 350 µL de etanol (96 %) y se mezclaba mediante pipeteo.
c. Posteriormente, se depositaban 700 µL en la columna de purificación, se centrifugaban durante 1 min a 8.000 x *g* y se descartaba el eluido. Este proceso se repetía hasta que el volumen completo de muestra se había añadido a la columna.
d. Después, se añadían 200 µL de *buffer* NV a la columna, se centrifugaban durante 1 min a 8.000 x *g* y se descartaba el eluido.
e. Entonces se hacían dos lavados con el *buffer* NVW, uno con 600 µL y otro con 300 µL. Después de añadir el *buffer* en las dos ocasiones se realizaba una centrifugación durante 1 min a 8.000 x *g* y se descartaba el eluido.
f. Tras los lavados se secaba la membrana de la columna mediante una nueva centrifugación, en este caso de 2 min a 8.000 x *g*.
g. Finalmente, se eluía el ARN purificado en 50 µL de agua libre de RNasas

AI.2.3.2. Muestras de orina

Para la purificación de ARN de SARS-CoV-2 a partir de muestras de orina se utilizó el protocolo descrito en el apartado anterior, pero con un paso previo de concentración de la muestra. En este caso, 1,5 mL de orina se centrifugaban a 4.000 x *g* durante 15 min. Tras la centrifugación, se descartaba el sobrenadante hasta un volumen final de 200 µL con el que se iniciaba el protocolo descrito previamente.

AI.3. Selección de secuencias y diseño de cebadores

Durante el desarrollo de esta Tesis Doctoral se han realizado ensayos LAMP para la detección de *Schistosoma mansoni*, *S. haematobium*, *Strongyloides* spp. y SARS-CoV-2. Para ello, se han utilizado tanto cebadores diseñados por nuestro grupo como otros utilizados por otros autores. Las secuencias, su longitud y la diana específica para todos ellos se recogen en la tabla AI.2

Tabla AI.2. Cebadores utilizados en el desarrollo de la Tesis Doctoral.

Diana	Cebador	Secuencia	Longitud (bases)	Referencia
S. mansoni	F3	TTATCGTCTATAGTACGGTAGG	22	Fernández-Soto *et al.* (2014)[1]
	B3	ATACTTTAACCCCCACCAA	19	
	FIP	GCCAAGTAGAGACTACAAACATCTT-TGGGTAAGGTAGAAAATGTTGT	47	
	BIP	AGAAGTGTTTAACTTGATGAAGGGG-AAACAAAACCGAAACCTACTA	45	
Strongyloides spp.	F3	ACACGCTTTTTATACCACATT	21	Fernández-Soto *et al.* (2016)[2]
	B3	GTGGAGCCGTTTATCAGG	18	
	FIP	ACCAGATACACATACGGTATGTTTT-GGATTTGATGAAACCATTTTTTCG	49	
	BIP	ATCAACTTTCGATGGTAGGGTATTG-CCTATCCGGAGTCGAACC	43	
S. haematobium	LF	GGTGCGCTTTGTTTTCCGT	19	Gandasegui *et al.* (2015)[3]
	LB	ACCATGTGTAAAGCGCGTCAAA	22	
	F3	CTTTCTAAGCCCGCGATA	18	
	B3	GCGCATTACACTTGGTCT	18	
	FIP	TACCCCTAACTTCGTGGTCTCC-CCCCCTTATTTTAGGGTGC	41	

	BIP	CTCCCTATATAACATGGCGAGTAAG-ACTATGAAATCAGTGTTTTTCGG	48	
SARS-CoV-2 ORF1ab	LF	CTGCACTTACACCGCAA	17	El-Tholoth *et al.* (2020)[4]
	LB	GTAGCTGGTTTTGCTAAATTCC	22	
	F3	TGCTTCAGTCAGCTGATG	18	
	B3	TTAAATTGTCATCTTCGTCCTT	22	
	FIP	CAGTACTAGTGCCTGTGCCGCACAAT CG-TTTTTAAACGGGT	41	
	BIP	TCGTATACAGGGCTTTT-GACATCTATCTTGGAAGCGACAACAA	43	
SARS-CoV-2 ORF1b	F3	CACAGACTTTGTGAATGAGTT	21	García-Bernalt Diego *et al.* (2021)[5]
	B3	GTCAGTCTCAGTCCAACAT	19	
	FIP	CTATTGAAACACACAACAGCATCGCA TATTTGCGTAAACATTTCTCA	47	
	BIP	TATGCATCTCAAGGTCTAGTGGCTAT GCTTCAGACATAAAAACATTG	47	
SARS-CoV-2 S447	LF	TCACGGACAGCATCAGTAGTG	21	García-Bernalt Diego *et al.* (2021)[5]
	LB	CAGGAACAAATACTTCTAACCAGGT	25	
	F3	GTTTCTGCCTTTCCAACAA	19	
	B3	AACAGGGACTTCTGTGCA	18	
	FIP	TCAAGAATCTCAAGTGTCTGTGGTGG CAGAGACATTGCTGA	41	
	BIP	ACCATGTTCTTTTGGTGGTGTCAACA TCCTGATAAAGAACAGC	43	
SARS-CoV-2 S*555*	F3	CTATGCAAATGGCTTATAGGTT	22	García-Bernalt Diego *et al.* (2021)[5]
	B3	AGTTGTTTAACAAGCGTGTT	20	
	FIP	GCACTATTAAATTGGTTGGCAATCAT AATGGTATTGGAGTTACACAGA	48	
	BIP	ATTGGCAAAATTCAAGACTCACTTTT GTGCATTTTGGTTGACC	43	
SARS-CoV-2 E	F3	TCATTCGTTTCGGAAGAGA	19	García-Bernalt Diego *et al.* (2021)[5]
	B3	AGGAACTCTAGAAGAATTCAGAT	23	
	FIP	TGTAACTAGCAAGAATACCACGAAAC AGGTACGTTAATAGTTAATAGCG	49	
	BIP	GCTTCGATTGTGTGCGTACTCGAGAG TAAACGTAAAAAGAAGG	43	
SARS-CoV-2 M	F3	GTTTCCTATTCCTTACATGGATT	23	García-Bernalt Diego *et al.* (2021)[5]
	B3	AGCCACATCAAGCCTACA	18	
	FIP	CCATAACAGCCAGAGGAAAATTAACC TTCTACAATTTGCCTATGCC	46	

	BIP	AACTTTAGCTTGTTTTGTGCTTGCACA AGCCATTGCGATAGC	42	
SARS-CoV-2 N5	LF	ATTATTGGGTAAACCTTGGGGC	22	García-Bernalt Diego *et al.* (2021)[5]
	LB	ATTAACACCAATAGCAGTCCAGATG	25	
	F3	CCAGAATGGAGAACGCAGTG	20	
	B3	CCGTCACCACCACGAATT	18	
	FIP	AGCGGTGAACCAAGACGCAGGGCGC GATCAAAACAACG	38	
	BIP	AATTCCCTCGAGGACAAGGCGAGCT CTTCGGTAGTAGCCAA	41	
SARS-CoV-2 N15	LF	GCAATGTTGTTCCTTGAGGAAGTT	24	García-Bernalt Diego *et al.* (2021)[5]
	LB	CCTCATCACGTAGTCGCAACAG	22	
	F3	AGATCACATTGGCACCCG	18	
	B3	CCATTGCCAGCCATTCTAGC	20	
	FIP	TGCTCCCTTCTGCGTAGAAGCCAATG CTGCAATCGTGCTAC	41	
	BIP	GGCGGCAGTCAAGCCTCTTCCCTACT GCTGCCTGGAGTT	39	

La selección de dianas adecuadas y el diseño de cebadores para la técnica LAMP es un punto crítico para el éxito de la amplificación. Hay numerosos factores que influyen tanto en la selección de secuencias como en el diseño de los cebadores. En el caso de la selección de las secuencias encontramos cuatro factores principales[6]:

a. Grado de especificidad: valoración de la potencial reactividad cruzada de la secuencia con otras secuencias de especies cercanas, así como con las secuencias del hospedador.
b. Grado de conservación: la secuencia no debe presentar variaciones frecuentes entre cepas.
c. Nivel de estudio de la secuencia: la secuencia de referencia utilizada para el diseño ha de ser profundamente conocida y haber pasado numerosas correcciones.
d. Repetición en el genoma: la probabilidad de detección de cualquier secuencia aumenta cuanto mayor es su grado de repetición en el genoma.

Para el diseño de los cebadores, hay que tener en cuenta cinco factores principales[7]:

a. Temperatura de anillamiento (Tm, del inglés *melting temperature*): se estima utilizando el método estadístico Nearest-Neighbour, el método estadístico que ofrece un valor de Tm más similar al real. Entre los factores que influyen en la Tm de los cebadores encontramos la composición de su secuencia, su concentración y la concentración de algunas sales en el medio de reacción. Por ello, se debe calcular la Tm para unas condiciones experimentales concretas. Las Tm de las distintas secuencias de los cebadores han de ser:
    - F1c y B1c: 64 ºC- 66 ºC
    - F2 y B2: 59 ºC- 61 ºC
    - F3 y B3: 64 ºC - 66 ºC
b. Estabilidad del extremo terminal del cebador: El final de cada cebador sirve como punto de partida para la síntesis de una nueva hebra de ADN. Por ello se busca que los extremos 3' de F2/B2, F3/B3 y LF/LB y el 5' de F1c/B1c sean lo más estables posibles. Esa estabilidad se mide a través del incremento de energía libre de Gibbs (ΔG). Cuanto más negativo es este incremento, mayor estabilidad. Así, los cebadores se diseñan para que su energía libre sea igual o menor a -4 kcal/mol.
c. Contenido en G+C: ha de estar entre el 40 % y 65 %, aunque un porcentaje de entre 50 % y 60 % tiende a dar los mejores resultados.
d. Estructuras secundarias: particularmente importante para los cebadores internos es evitar la formación de estructuras secundarias, por ello hay que evitar secuencias auto-complementarias dentro de los cebadores. También es importante que los extremos 3' de los distintos cebadores no sean complementarios para evitar anillamientos de tipo cebador-cebador.
e. Distancia entre los cebadores: los cebadores se diseñan de tal forma que desde el final del cebador F2 al final del B2 haya entre 120 y 160 bases. Además, la distancia entre los extremos 5' de las secuencias F1 y F2 ha de ser de entre 40 y 60 bases. Por último, la distancia máxima entre las secuencias F2 y F3 ha de ser de 60 bases.

El proceso seguido para el diseño de todos los juegos de cebadores nuevos presentados en este trabajo para la detección de SARS-CoV-2 es análogo, por lo que

se utilizará como ejemplo metodológico el diseño de los cebadores que anillan en el gen *N* para la detección de SARS-CoV-2.

AI.3.1. Selección y obtención de la secuencia

El diseño de cebadores LAMP comenzó en todos los casos con una revisión bibliográfica en PubMed para la búsqueda de secuencias del genoma del virus SARS-CoV-2, y específicamente, aquellas que previamente se hubiesen utilizado en técnicas de amplificación de su genoma. Si bien los ejemplos eran aún limitados, algunas secuencias seleccionadas eran de interés ya en el momento de la realización del estudio. Para el diseño de los cebadores, se partió de la secuencia del virus original de Wuhan, cuyo número de acceso de GenBank era MN908947.3 (Fig. AI.1). A continuación, se procedió a descargar la secuencia. Como se indica en la Fig. AI.1, 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.

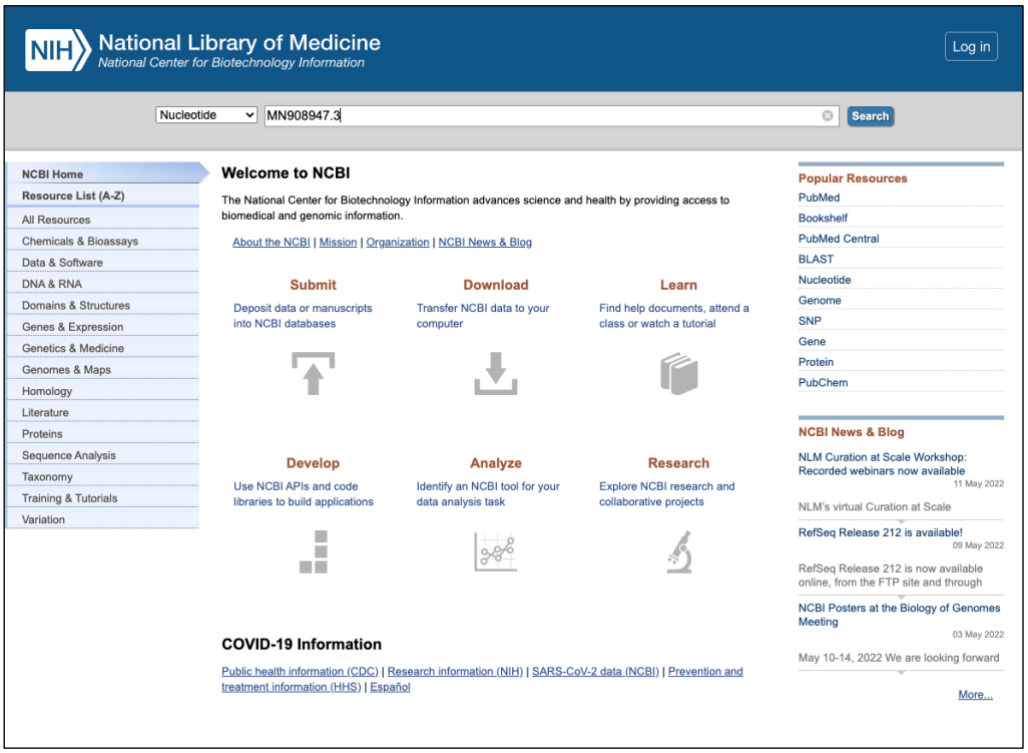


Figura AI.1. Búsqueda de la secuencia MN908947.3 en la base de datos *Nucleotide* de NCBI.

Al realizar la búsqueda, apareció una página con toda la información correspondiente a la secuencia (código del GenBank, publicación de la secuencia, organismo al que pertenece, la secuencia de ADN, de proteína, genes, …). La descarga

de la secuencia se hizo en formato FASTA, el más habitual para el manejo de secuencias nucleotídicas y proteicas (Fig. AI.2).

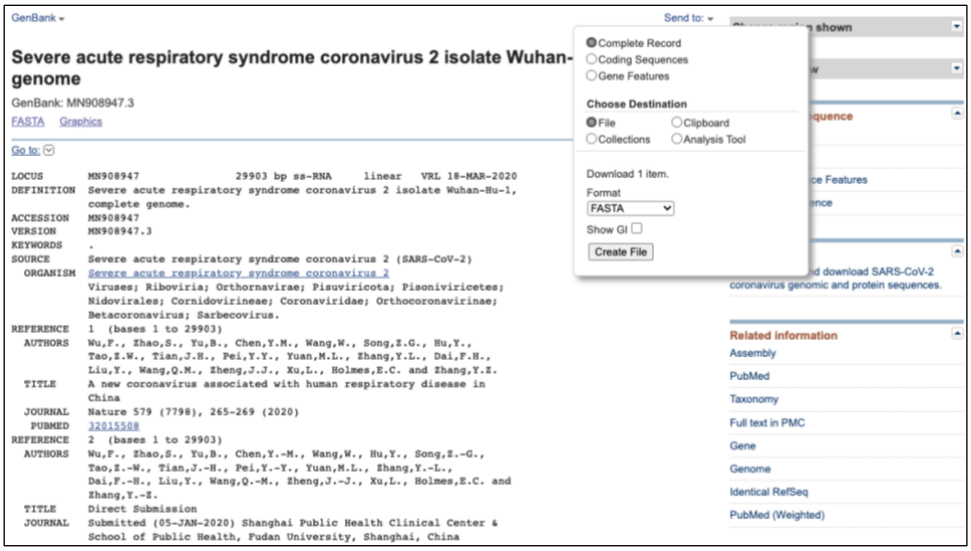


Figura AI.2. Descarga de la secuencia MN908947.3 en la base de datos *Nucleotide* de NCBI.

AI.3.2. Diseño de cebadores

Una vez descargada, la secuencia puede visualizarse y editarse a través de distintos programas. En este caso, el programa de trabajo habitual fue SnapGene® Viewer (v 5.2.3) (https://www.snapgene.com/snapgene-viewer), pero otros programas como BioEdit (disponible únicamente para usuarios de Windows) (https://bioedit.software.informer.com/Descargar-gratis/) o Integrative Genomics Viewer (IGV) (https://software.broadinstitute.org/software/igv/download), pueden realizar funciones similares. Tras el procesamiento de la secuencia, la introducimos en el software PrimerExplorer V5 (https://primerexplorer.jp/e/) y comenzamos el diseño y selección de los cebadores. Para ello, en Seleccionar archivo, seleccionamos nuestra secuencia desde su ubicación en el ordenador y posteriormente se usa la función *Primer design* para obtener nuestros resultados (Fig. AI.3).

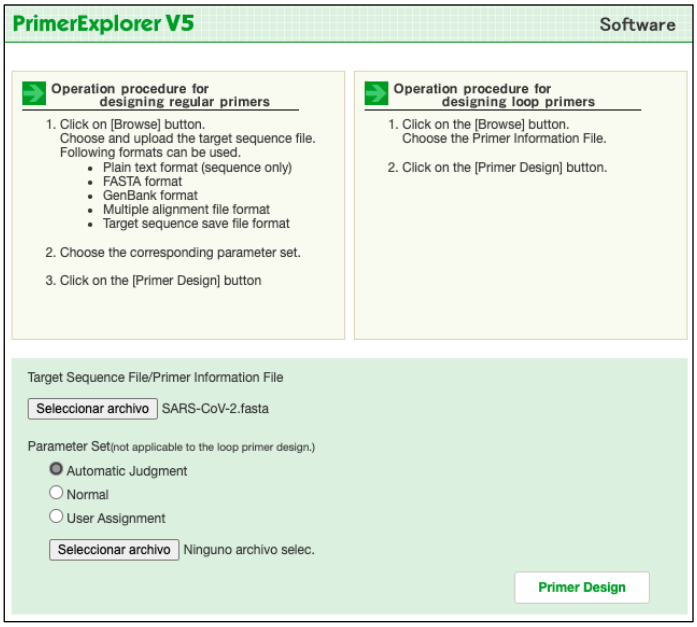


Figura AI.3. Interfaz inicial del software Primer Explorer v5

El software nos permitió en este punto modificar las condiciones estándar de diseño en *Detail settings*. Esto solo sería necesario si en la secuencia seleccionada no se generaran cebadores con la configuración estándar, aunque no fue necesario en este caso. El número de sets de cebadores generados lo conocimos a través de *Generate*. En este ejemplo se generaron 5 juegos de cebadores distintos (Fig AI.4). Para poder conocer los detalles de estos cebadores, accedimos a través de *Display*.

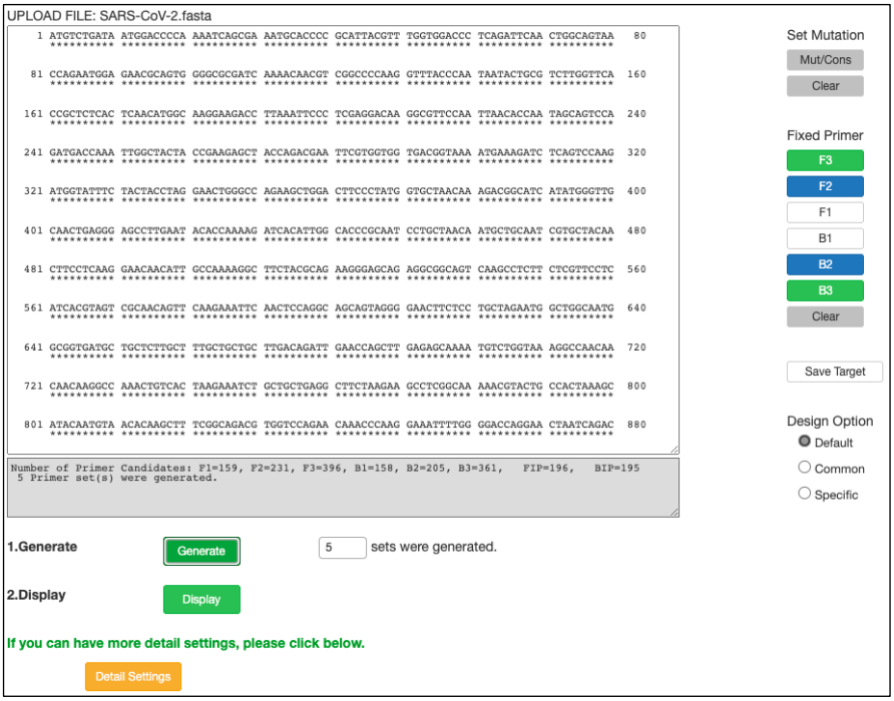


Figura AI.4. Generación de set de cebadores LAMP dentro del gen *N* de SARS-CoV-2.

AI.3.3. Análisis de los cebadores obtenidos

En este punto se pudo observar el sitio de anillamiento concreto de los diferentes juegos de cebadores diseñados, así como el ΔG total de cada set, que nos permite seleccionar los más adecuados. Como norma general, rechazamos los juegos de cebadores que presenten una ΔG superior a -2,00 kcal/ml. En este caso, se seleccionaron los juegos N5 y N15 (Fig. AI.5). A continuación, mediante la función *Confirm* pudimos evaluar las características de cada cebador dentro de cada juego.

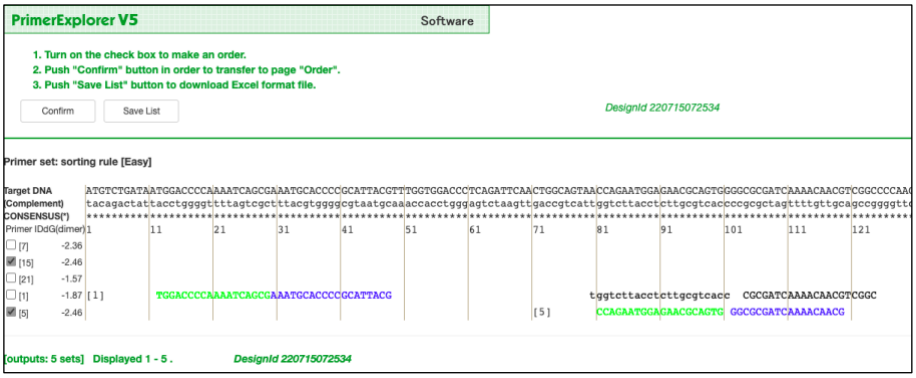
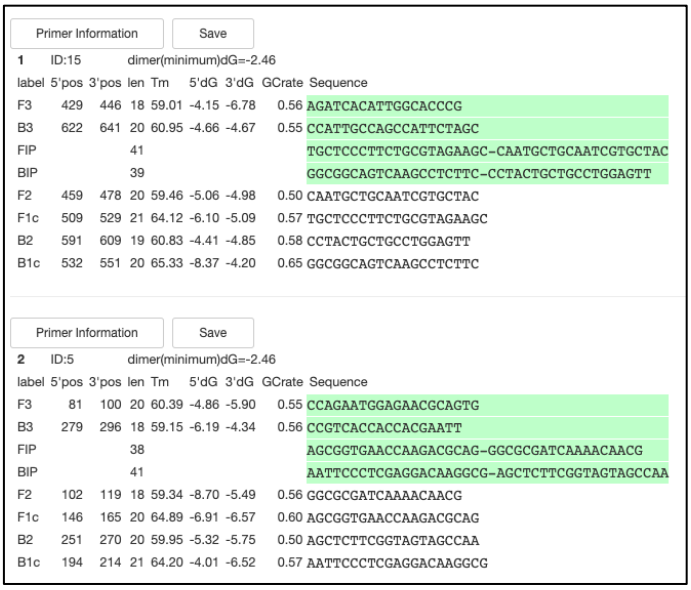


Figura AI.5. Posición de anillamiento y ΔG de los sets de cebadores LAMP diseñados dentro del gen *N* de SARS-CoV-2.

Finalmente, obtuvimos toda la información de cada uno de los cebadores de los juegos seleccionados, incluyendo su posición, longitud, temperatura de anillamiento (Tm), contenido en G+C (*GC rate*) y ΔG en los extremos 3' y 5' (Fig. AI.6). Con toda esta información, la selección definitiva del juego de cebadores se hará atendiendo a los criterios descritos previamente en este anexo, que se pueden encontrar también en el manual "*A Guide to LAMP primer designing*"[7], disponible en la misma página web de Primer Explorer.



Primer Information | Save

1 ID:15 dimer(minimum)dG=-2.46

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	429	446	18	59.01	-4.15	-6.78	0.56	AGATCACATTGGCACCCG
B3	622	641	20	60.95	-4.66	-4.67	0.55	CCATTGCCAGCCATTCTAGC
FIP			41					TGCTCCCTTCTGCGTAGAAGC-CAATGCTGCAATCGTGCTAC
BIP			39					GGCGGCAGTCAAGCCTCTTC-CCTACTGCTGCCTGGAGTT
F2	459	478	20	59.46	-5.06	-4.98	0.50	CAATGCTGCAATCGTGCTAC
F1c	509	529	21	64.12	-6.10	-5.09	0.57	TGCTCCCTTCTGCGTAGAAGC
B2	591	609	19	60.83	-4.41	-4.85	0.58	CCTACTGCTGCCTGGAGTT
B1c	532	551	20	65.33	-8.37	-4.20	0.65	GGCGGCAGTCAAGCCTCTTC

Primer Information | Save

2 ID:5 dimer(minimum)dG=-2.46

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	81	100	20	60.39	-4.86	-5.90	0.55	CCAGAATGGAGAACGCAGTG
B3	279	296	18	59.15	-6.19	-4.34	0.56	CCGTCACCACCACGAATT
FIP			38					AGCGGTGAACCAAGACGCAG-GGCGCGATCAAAACAACG
BIP			41					AATTCCCTCGAGGACAAGGCG-AGCTCTTCGGTAGTAGCCAA
F2	102	119	18	59.34	-8.70	-5.49	0.56	GGCGCGATCAAAACAACG
F1c	146	165	20	64.89	-6.91	-6.57	0.60	AGCGGTGAACCAAGACGCAG
B2	251	270	20	59.95	-5.32	-5.75	0.50	AGCTCTTCGGTAGTAGCCAA
B1c	194	214	21	64.20	-4.01	-6.52	0.57	AATTCCCTCGAGGACAAGGCG

Figura AI.6. Características de los cebadores incluidos en los juegos N5 y N15.

AI.3.4. Ensayo in silico de especificidad

Finalmente, antes de sintetizar las secuencias de los cebadores diseñados, es conveniente hacer un análisis *in silico* para comprobar que la secuencia seleccionada y los cebadores diseñados, no generan reactividad cruzada con secuencias de otros posibles patógenos o con secuencias del hospedador. Para ello se utiliza la herramienta Nucleotide BLAST (Nucleotide Basic Local Alignment Search tool: https://blast.ncbi.nlm.nih.gov/Blast.cgi). En esta herramienta se pueden introducir la secuencia diana, la secuencia de los cebadores diseñados, y compararlas con las secuencias de todos los genomas y genes disponibles en la base de datos de NCBI. Siguiendo con el ejemplo de la secuencia *N* de SARS-CoV-2 la introducimos en *Enter Query Sequence*. Además, excluimos las secuencias del organismo que pretendemos detectar (ya que obtendríamos 100% de similitud) introduciéndolo en la categoría de *Organism* y seleccionando *Exclude* (Fig AI.7).

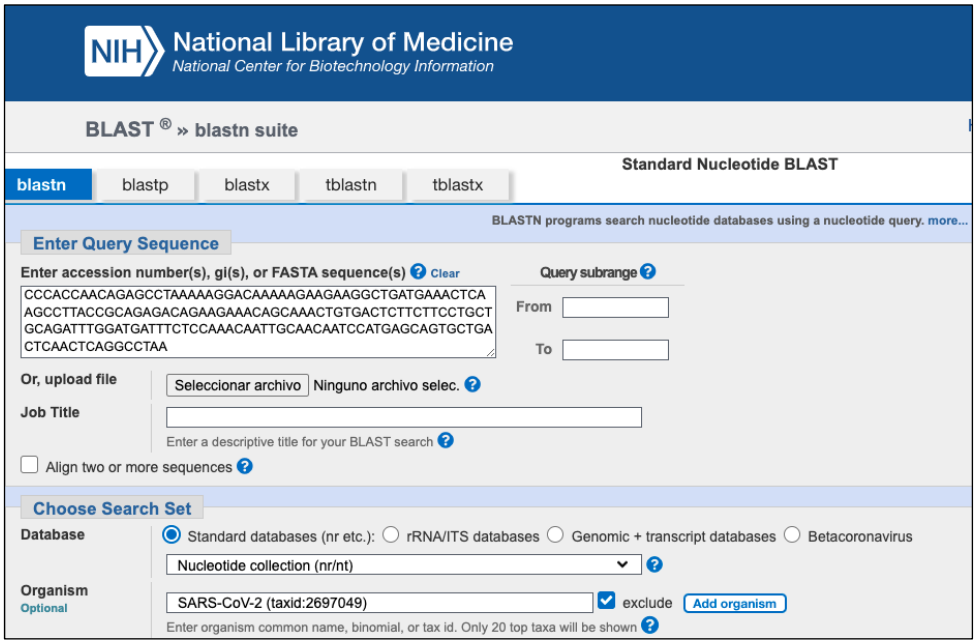


Figura AI.6. Nucleotide BLAST de todos los organismos en comparación con la secuencia *N* de SARS-CoV-2.

AI.4. Amplificación de ADN

Para el trabajo realizado con cualquier técnica de amplificación de ADN se siguieron normas de buenas prácticas de laboratorio. Así, siempre se realizó una limpieza con etanol al 70 % todo el material a utilizar y las áreas de trabajo. Se trabajó en áreas totalmente separadas para cada procedimiento: las mezclas de reacción se prepararon en una cabina de flujo laminar previamente descontaminada e irradiada con luz ultravioleta durante 15 min, el ADN se añadió en una zona de extracción y la manipulación de los productos amplificados se realizó en la zona de amplificación. El material de uso en la cabina es exclusivo de la misma y no se extrae ni emplea para otra actividad.

AI.4.1. Reverse-transcription–quantitative polymerase chain reaction (RT-qPCR)

En los **artículos de investigación 2, 3 y 4** de este trabajo se incluye el uso de RT-qPCR como técnica de referencia para el diagnóstico de SARS-CoV-2.

En el **segundo artículo de investigación** el procesamiento de la muestra, la extracción del ARN y la RT-qPCR se realizaron en el Servicio de Microbiología del Hospital Clínico Universitario de Salamanca. Brevemente, en todos los casos las muestras se procesaron realizando la extracción del ARN (NUCLISENS EASYMAG,

BIOMÉRIEUX, Francia) y la RT-qPCR (VIASURE SARS-CoV-2 Real-Time PCR Detection Kit, CERTEST BIOTECH, España) por separado o de forma integrada en una plataforma automatizada (COBAS 6800, ROCHE, Suiza) siguiendo las instrucciones del fabricante. La RT-qPCR para la detección del ARN del SARS-CoV-2 se realizó utilizando kits comerciales y reactivos dirigidos al *ORF1ab* y al gen *N* (VIASURE SARS-CoV-2) o al *ORF1ab* y al gen *E* (COBAS SARS-CoV-2 Test). Las alícuotas de las muestras de ARN purificado se almacenaron a -80 ºC hasta su posterior análisis. Para las muestras de ARN analizadas, los valores del umbral de ciclo (Ct, del inglés *Cycle treshold*) de la RT-qPCR para las distintas dianas amplificadas (*ORF1ab*, *E* o *N*) se utilizaron como referencia para los ensayos de RT-LAMP.

Este mismo procesamiento y análisis se llevó a cabo para las muestras nasofaríngeas analizados en el **tercer artículo de investigación**. La RT-qPCR del ARN extraído de muestras de orina del grupo de pacientes positivos (grupo 1) se llevó a cabo en el laboratorio e-INTRO. En este caso, se concentró 1,5 mL de cada muestra de orina hasta un volumen final de 200 µL que se utilizó como volumen de partida para la extracción de ARN utilizando el kit de purificación de ARN viral NZY Viral RNA Isolation kit (NZYTECH, Lisboa, Portugal) como se ha descrito previamente. A continuación, el ARN extraído de las 100 muestras de orina del grupo de positivos se analizaron mediante RT-qPCR utilizando el kit de RT-qPCR de un solo paso SARS-CoV-2 One-Step RT-PCR Kit (NZYTECH, Lisboa, Portugal) siguiendo las instrucciones del fabricante. Se utilizaron 2 µL del ARN extraído en cada caso, para poder comparar los resultados obtenidos con los del RT-LAMP. El volumen de los distintos reactivos usados se encuentra en la Tab. AI.3 y los ciclos de temperatura en la Tab. AI.4. Este mismo kit de extracción y RT-qPCR se utilizó en el **cuarto artículo de investigación**, para el análisis de las muestras nasofaríngeas del estudio piloto. Tras la RT-qPCR, se calculó el número de copias virales según las instrucciones del fabricante.

Tabla AI.3. Mezcla de reacción de la RT-qPCR para SARS-CoV-2.

Componente[a]	**1 reacción** (µL)	**N reacciones** (µL)[b]
SARS-CoV-2 MMix	10	N x 10,5
SARS-CoV-2 PPMix	2	N x 2,1
H_2O mQ	6	N x 6,3
Muestra	2	Añadido individualmente
Volumen final	**20**	**N x 18,9**

[a]MMix (master mix); PPMix (*primer* mix); H_2O mQ (agua ultrapura). [b]Se añade un 5% más del volumen necesario para compensar posibles errores de pipeteo.

Tabla AI.4. Ciclos de la RT-qPCR para SARS-CoV-2.

Ciclos	**Temperatura** (ºC)	**Tiempo**	**Etapa**
1	50	20 min	Retrotranscripción
1	95	2 min	Activación de la polimerasa
40	95	5 seg	Desnaturalización
	60	30 seg	Anillamiento/Extensión

Todas las reacciones fueron realizadas en el dispositivo PCRmax ECO 48 real time PCR system (PCRmax, Stone, Reino Unido).

AI.4.2. LAMP colorimétrico

Aunque a lo largo de este trabajo se realizan diferentes ensayos LAMP, las mezclas de reacción son análogas para todos ellos, por lo que se describirán de forma general, y se harán especificaciones particulares cuando sea necesario. La mezcla de reacción LAMP convencional se compone de los siguientes reactivos: betaína 1 M. (SIGMA, Estados Unidos); *Isothermal buffer* 1X (20mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM MgSO4, 0.1 % Tween20), $MgSO_4$ suplementario (6 mM) y 8U de polimerasa *Bst* 2.0 WarmStart (NEW ENGLAND BIOLABS, Reino Unido); dNTPs (1,4 mM de cada uno); 0,4 µM LF/LB (de cada uno, si se incluyen), 1,6 µM FIP/BIP (de cada uno) y 0,2 µM F3/B3 (de cada uno) (BIORON). Detallados en la Tab. AI.5 se encuentran los volúmenes de reacción para cada uno de los reactivos.

Cabe destacar que anteriormente se añadía a esta mezcla de reacción betaína (1M), que se utiliza también como potenciador de reacciones de PCR. Sin embargo, en el **primer artículo de investigación** de esta Tesis Doctoral, demostramos que la betaína tiene un efecto negativo en la cinética del SmMIT-LAMP, por lo que se eliminó de la reacción en los trabajos posteriores. Además, se observa también en ensayos en el laboratorio (Fig. AI. 7), que una reducción de volumen de reacción de 25 µL a 15 µL no afecta ni a la cinética ni a la sensibilidad de los ensayos, por lo en los

artículos de investigación 2, 3 y 4 también se reduce el volumen de reacción de 25 µL a 15 µL.

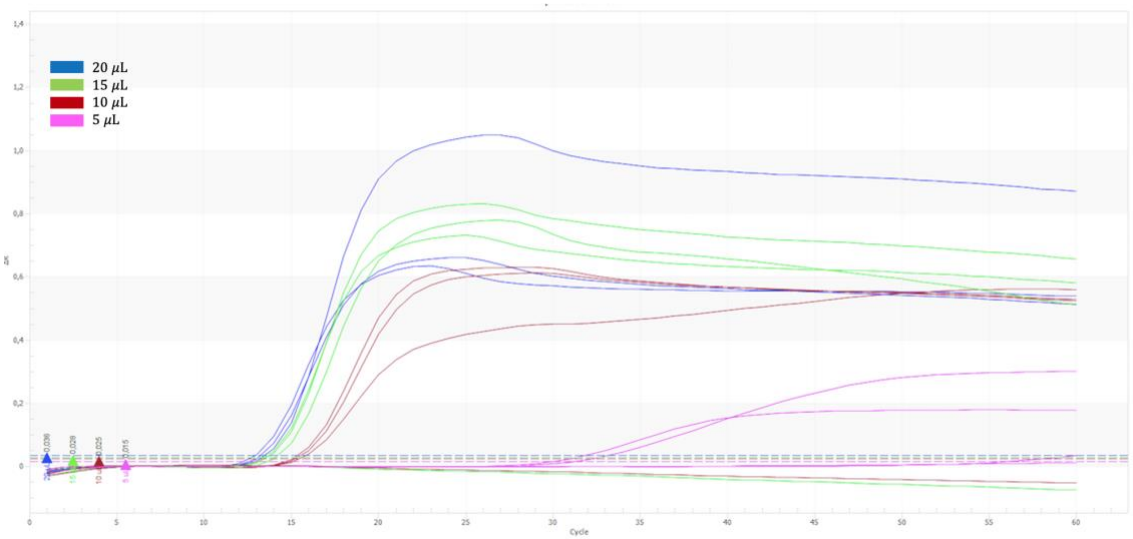


Figura AI.7. Efecto de la reducción del volumen en la amplificación LAMP

Tabla AI.5. Componentes de una reacción LAMP colorimétrica. VR: volumen de reacción

Componente	Volumen en µL (VR: 15 µL)	Volumen en µL (VR: 25 µL)
H_2O mQ	Hasta completar 15 µL	Hasta completar 25 µL
dNTPs	2,1	3,5
***Isothermal buffer* 10x**	1,5	2,5
$MgSO_4$ adicional	0,9	1,5
LF/LB	0,6	1
F3/B3	0,3	0,5
FIP/BIP	0,24	0,4
Bst 2.0	0,6	1
Muestra/Control	2	2

VR: volumen de reacción

La incubación de los tubos de reacción se realizó a 65 ºC durante 60 min y posteriormente 5-10 min a 80 ºC para inactivar la enzima y finalizar la reacción, tanto para el LAMP de *S. mansoni* como *Strongyloides* spp. El LAMP de *S. haematobium*, de mejor cinética, se incubó a 65 ºC durante 50 min. La detección de los productos amplificados de reacción en el LAMP colorimétrico se realiza de manera visual una vez la reacción ha concluido, bien sea a través de la adición de 2 µL de SYBR Green I 1000x (INVITROGEN), que cambia de naranja a verde cuando hay un resultado positivo o mantiene el color naranja cuando hay un resultado negativo, o bien a través del patrón de bandas de amplificación en escalera que puede observarse en un gel de agarosa al 1,5 %.

AI.4.3. LAMP a tiempo real

La reacción LAMP a tiempo real es análoga a la convencional, pero la detección de la amplificación se realiza añadiendo al inicio de la reacción un colorante fluorescente, como, por ejemplo, EvaGreen 20x en agua (BIOTIUM, Fremont, Estados Unidos). La señal de fluorescencia generada por la unión del colorante al ADN amplificado se monitorizó a lo largo de la reacción, bien en el dispositivo portátil Genie III (OPTIGENE Ltd., Horsham, Renio Unido) o en el dispositivo de qPCR PCRmax ECO 48 real time PCR system (PCRmax, Stone, Reino Unido).

La detección a tiempo real realizada por el dispositivo SMART-LAMP, incluida en el **artículo de investigación 4**, se consiguió a través de la monitorización de cambios colorimétricos, no fluorescentes. En este caso se añadió al inicio de la reacción el colorante verde malaquita al 0,008 % de peso en volumen. Este colorante, al interaccionar con el ion Mg^{2+} no genera color, pero a medida que se produce la amplificación el Mg^{2+} interacciona con el pirofosfato liberado y el verde de malaquita generando un color azul-verdoso. El viraje de color se monitoriza mediante sensores RGB en el dispositivo SMART-LAMP, como se detallará más adelante.

AI.4.4. Reverse-transcription–LAMP (RT-LAMP)

La detección de ARN mediante LAMP (RT-LAMP) se puede conseguir de dos maneras: con una única enzima con actividad tanto polimerasa como retrotranscriptasa (*Bst* 3.0, NEW ENGLAND BIOLABS Ltd., Ipswich, Estados Unidos) o con una polimerasa (*Bst* 2.0 WarmStart) y una retrotranscriptasa (RTx, NEW ENGLAND BIOLABS Ltd., Ipswich, EEUU). Tras realizar ensayos con una o la combinación de dos enzimas, se obtuvieron mejores resultados con las dos enzimas combinadas y, por tanto, esta metodología fue la que se utilizó en todos los experimentos descritos en esta Tesis Doctoral. Las reacciones son análogas al LAMP convencional, añadiendo a la mezcla de reacción 0,3 µL de la enzima *RTx*. En el caso de las reacciones RT-LAMP incluidas en este trabajo (*ORF1ab*, *E*, *N5* y *N15*) se realizan todas a 63 ºC durante 40 min, a excepción de la E-RT-LAMP que se realiza durante 1 h.

AI.5. Estabilización de reactivos LAMP

Los diferentes protocolos de estabilización de reactivos LAMP desarrollados durante este trabajo se llevaron a cabo en un Concentrator Plus (EPPENDORF, Alemania) por simple aplicación de vacío (modo D-AQ) o bien combinándolo con centrifugación a 1400 rpm (V-AQ). Se utilizaron crioprotectores para conservar en condiciones óptimas los distintos componentes de reacción. Después de probar diversas alternativas (sacarosa, trehalosa y pululano) se decidió trabajar con trehalosa (2M), que mostraba resultados óptimos para el secado, presentando una viscosidad suficientemente baja que permitía mantener medidas de volumen precisas en las mezclas de reacción.

En el **primer artículo de investigación** se detallan diversos métodos de estabilización para los reactivos del SmMIT-LAMP que se presentan a continuación y en la Fig.AI.8.

a. *Estabilización por concentración*: tubos abiertos de 1,5 mL que contenían los reactivos de SmMIT-LAMP se sometieron a vacío en un Concentrator Plus (EPPENDORF, Alemania) en centrifugación (modo V-AQ) a 1400 rpm a temperatura ambiente. En este modo se llevaron a cabo los siguientes procedimientos de secado
    - *Estabilización parcial en dos etapas*: en primer lugar, se colocó en el fondo de los tubos una mezcla que contenía 1,8 µL de cebadores, 0,56 µL de trehalosa 2M (SIGMA, EEUU) y 0,14 µL de glicerol al 50 % y se concentró durante 30 min obteniendo así un *pellet* seco. A continuación, se incorporó una mezcla que contenía 3,5 µL de dNTPs, 1 µL de polimerasa *Bst* y 1,5 µL de trehalosa (2M) sobre el *pellet* para concentrarlo durante 15 min más. Para la reacción LAMP, la reconstitución de los *pellets* se realizó en 2,5 µL de tampón, 1,5 µL de $MgSO_4$ y 19 µL de agua (y en 2 µL de ADN, si procede) mezclados hasta un volumen final de 25 µL.
    - *Estabilización parcial en un paso*: se colocó en el fondo de los tubos una mezcla que contenía 1,8 µL de cebadores, 3,5 µL de dNTPs, 1 µL de polimerasa *Bst*, 2,06 µL de trehalosa y 0,14 µL de glicerol al 50 % y se concentró durante 30 min. La rehidratación de los *pellets* formados se

realizó como se indica en el apartado anterior (y 2 µL de ADN, en su caso).

- *Estabilización completa en un paso*: todos los reactivos SmMIT-LAMP necesarios se colocaron en el fondo de los tubos en presencia de 2 µL de trehalosa (sin glicerol) y luego se concentraron durante un único paso de 30 min. Para la rehidratación posterior, sólo se añadió agua hasta un volumen final de 25 µL (y 2 µL de ADN, si procede).

b. *Estabilización por desecación*: este procedimiento se realizó en un solo paso de secado. Los tubos abiertos de 1,5 mL que contenían todos los reactivos SmMIT-LAMP necesarios en el fondo en presencia de 2 µL de trehalosa (sin glicerol) se expusieron al vacío en un Concentrator Plus (EPPENDORF, Alemania) sin centrifugación (modo D-AQ) a temperatura ambiente durante 30 min. Para la reacción posterior, sólo se añadió agua hasta un volumen final de 25 µL (y 2 µL de ADN, en su caso).

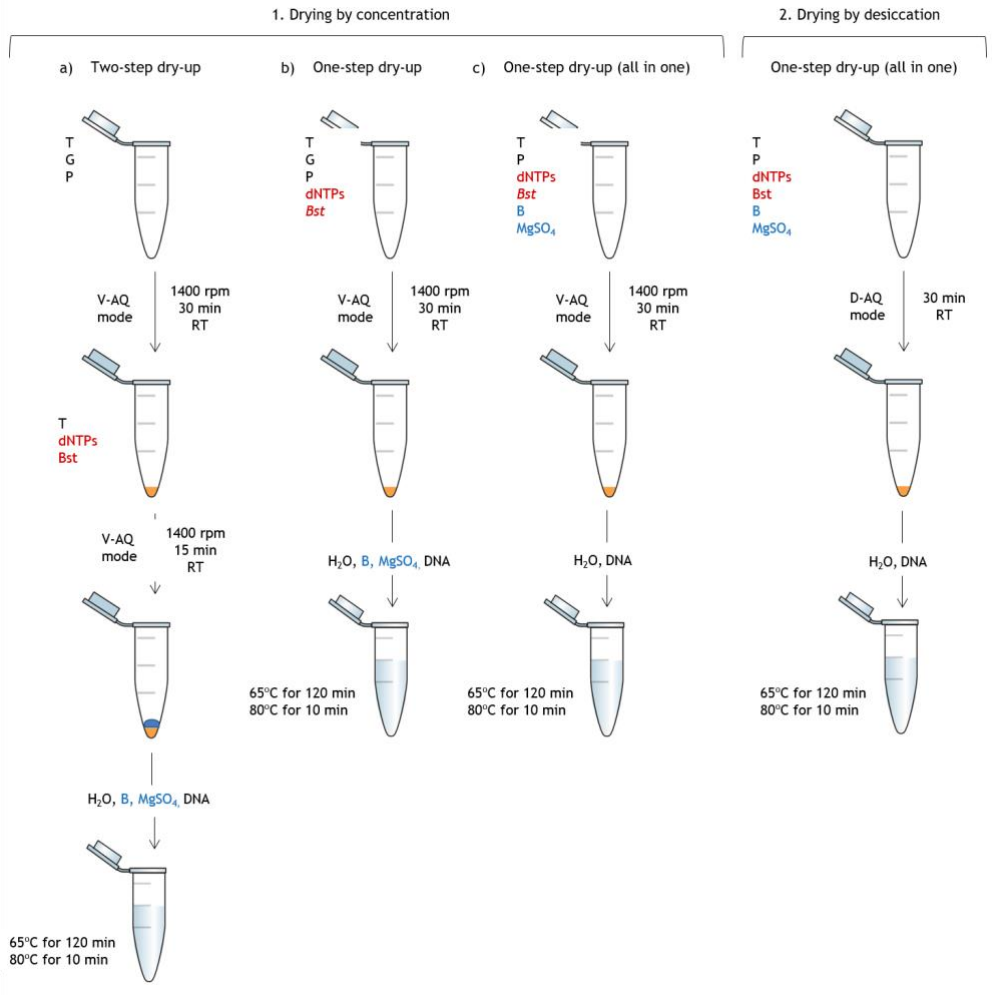


Figura AI.8. Procesos de estabilización de reactivos SmMIT-LAMP. Tomada del material suplementario del **artículo de investigación 1.** García-Bernalt Diego *et al.* (2019)[8].

Este protocolo provocaba una ralentización de la reacción SmMIT-LAMP, que pasaba de 60 min a 120 min. Por ello, el protocolo se modificó en los siguientes trabajos, separando los reactivos entre la tapa y el fondo del tubo. En resumen, mediante un único paso de secado por desecación de 30 min las mezclas de reacción de LAMP y RT-LAMP se secaron por separado en dos mezclas parciales: una que contenía cebadores, dNTPs y la(s) enzima(s) colocadas en el fondo del tubo en presencia de 1,8 µL de trehalosa 2M, y otra que contenía el *isothermal buffer* 10x, $MgSO_4$ y el colorante (ya fuera *EvaGreen* o verde malaquita) en la tapa del tubo en presencia de 2,25 µL de trehalosa 2M. El procedimiento de desecación produjo dos *pellets* estables y bien adheridos tanto en la tapa como en el fondo de los tubos. Para rehidratar las mezclas, simplemente se añadía el agua o la muestra correspondiente y los tubos se colocaban boca abajo durante 2 min para favorecer el proceso de rehidratación (ver Fig AI.10).

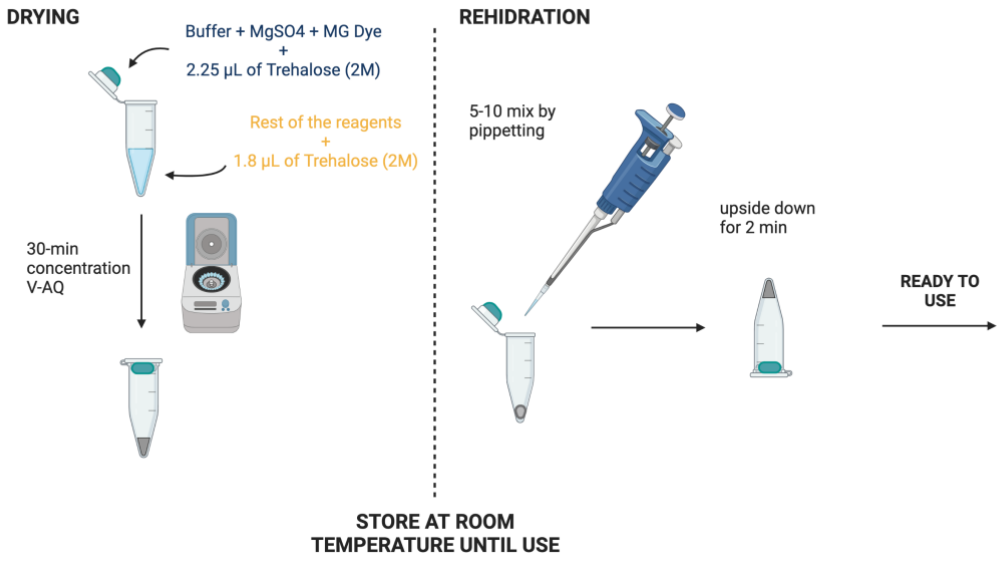


Figura AI.9. Estabilización (drying) y rehidratación (rehydration) de reactivos LAMP en la tapa y el fondo del tubo. Creada con Biorender.com

Este protocolo permitía realizar reacciones LAMP y RT-LAMP para *Schistosoma haematobium* y SARS-CoV-2, respectivamente, en menos de 1 h. Para *S. mansoni* y *Strongyloides* spp. permitía realizarlas en menos de 90 min.

AI.5.2. Evaluación de la estabilidad de los reactivos almacenados y método Q_{10}

Para evaluar la estabilidad de las mezclas de reacción almacenadas, sin tener que recurrir a largos tiempos de espera, en el **artículo de investigación 2** se aplicó el método Q_{10}. Este método fue definido por Clark en 1991[9] y se conoce también como la técnica de envejecimiento acelerado. La vida útil de las mezclas de reactivos puede determinarse mediante un ensayo de envejecimiento en tiempo real o acelerado, en el que se aplica la Ley de Arrhenius en un entorno simulado. El método se llevó a cabo exponiendo las mezclas de reactivos secos RT-LAMP estabilizados por desecación a diferentes temperaturas (25 ºC, 37 ºC, 45 ºC) durante 28 días y evaluando la funcionalidad de los reactivos RT-LAMP secos periódicamente, a los 0, 1, 7, 14, 21 y 28 días después de la desecación. Los datos obtenidos se utilizaron para calcular la estabilidad del ensayo a temperatura ambiente (25 ºC) con las siguientes fórmulas:

$$AF = Q_{10}^{[0.1\times(Te-Ta)]}$$

$$AG = t_e \times AF$$

$$Estimated\ Shelf-life = AG + t_e$$

donde AF es el factor de aceleración utilizado para correlacionar la vida útil del producto a una temperatura inferior a la utilizada para realizar el experimento; el factor Q_{10} mide la sensibilidad a la temperatura de una tasa de reacción enzimática debido a un aumento de 10 ºC; Te representa la temperatura elevada; AG es la edad acelerada; te es el tiempo de almacenamiento a temperatura elevada, y Ta es la temperatura ambiente. Dado que para la mayoría de las reacciones biológicas el Q_{10} se encuentra entre 2 y 3, establecimos un valor conservador de Q_{10} = 1,9 para realizar todos los cálculos. La evaluación de la vida útil estimada se realizó para las mezclas maestras secas mencionadas anteriormente durante un máximo de 28 días.

Tras comprobar que las mezclas de reacción podían conservarse almacenadas a temperatura ambiente durante más de dos meses, las mezclas utilizadas en el **cuarto artículo de investigación** se almacenaron a temperatura ambiente hasta su análisis (o bien menos de una semana para los experimentos de almacenamiento a corto plazo o bien más de un mes para los experimentos de almacenamiento a largo plazo) con la única precaución de mantenerlas alejadas de la luz.

AI.6. Sensibilidad y especificidad

AI.6.1. Sensibilidad

En el **segundo artículo de investigación** se evalúa la sensibilidad analítica en los ensayos RT-LAMP para la detección de SARS-CoV-2. Para ello se utilizó como control positivo una muestra de un paciente de COVID-19 con un resultado positivo por RT-qPCR (Ct = 25 para *ORF1ab*) de la que se realizaron diluciones seriadas en agua ultrapura desde 1x a 1:10^4.

En el **cuarto artículo de investigación** se evalúa la sensibilidad de los ensayos RT-LAMP en el SMART-LAMP de manera análoga para SARS-CoV-2, mientras que para el resto de los ensayos se partió de ADN genómico de los distintos parásitos a una concentración de 5 ng/µL y se hicieron diluciones seriadas 1:10 hasta una concentración de 10 fg/µL.

La sensibilidad analítica de los otros métodos LAMP utilizados, descritos para la amplificación de ADN de *Schistosoma mansoni*[1], *S. haematobium*[3] y *Strongyloides* spp.[2] se establece en las publicaciones originales de nuestro grupo de investigación.

AI6.2. Especificidad

La especificidad de los ensayos RT-LAMP de SARS-CoV-2 se evaluó en el **segundo artículo de investigación** frente a ARN de diversos virus respiratorios, cedidos generosamente por la Dr. Marta Domínguez-Gil, del Servicio de Microbiología del Hospital Río Hortega (Valladolid, España). Estos ARN procedían de virus tales como: Coronavirus NL63, Coronavirus OC43, Bocavirus, Rinovirus, Metapneumovirus, Respiratory Syncytial Virus A, Respiratory Syncytial Virus B, Enterovirus, Parainfluenzae 1, Influenza H1N1, Influenza A H3, Influenza A H1 e Influenza B.

La especificidad de los otros métodos LAMP utilizados, descritos para la amplificación de *Schistosoma mansoni*[1], *S. haematobium*[3] y *Strongyloides* spp.[2], se establece en las publicaciones originales de nuestro grupo de investigación.

AI.7. Diseño y desarrollo del dispositivo SMART-LAMP

El dispositivo SMART-LAMP ha pasado por numerosas fases de desarrollo a lo largo de estos años de trabajo. Desde un simple sensor RGB conectado por un cable USB a un ordenador, pasando por un pequeño dispositivo con capacidad para el análisis de un único tubo, un dispositivo sin baterías extraíbles controlado por una aplicación móvil y con capacidad de analizar ocho muestras, hasta la versión final más elaborada (Fig. AI.10).

Figura AI.10. Diferentes prototipos SMART-LAMP a lo largo del tiempo

A continuación, se definirán únicamente las características de la versión final del SMART-LAMP, así como los ensayos utilizados para la evaluación de su funcionalidad.

AI.7.1. Características del SMART-LAMP

En el **modelo de utilidad** y en el **artículo de investigación 4** se define el dispositivo SMART-LAMP como una plataforma diagnóstica portátil impresa en 3D, fabricada mediante la tecnología de deposición fundida (FDM, del inglés *fused deposition modelling*) en material de ácido poliláctico (PLA, del inglés *polylactic acid*). Fue desarrollado en colaboración con el grupo BISITE de la Universidad de Salamanca. Su función es la incubación simultánea y la adquisición de datos en tiempo real de ocho muestras. Tiene un diseño modular que comprende: (a) un módulo de incubación de muestras, (b) un módulo de lectura de muestras y, (c) un módulo de transmisión.

a. El *módulo de incubación* de muestras las mantiene entre 60 ºC y 70 °C durante un período de hasta 120 min. Se han diseñado algoritmos inteligentes que

permiten a este módulo modificar tanto la temperatura (desde 40 °C a 80 °C) como el tiempo de procesamiento (que va de 5 a 120 min). También incluye indicadores LED y acústicos que proporcionan información sobre el estado del dispositivo (por ejemplo "no preparado", "listo para usar", "conectado" o "en uso"), la temperatura y los tiempos de reacción transcurridos y restantes. Para estabilizar la temperatura se aplicó un algoritmo de control de temperatura proporcional integral y derivativo (PID)[10] que mantiene la temperatura con una precisión de ±0,9 °C. El PID se implementó a través de la biblioteca PID_v1 de Brett Beauregard[11] con valores de Kp = 4, Ki = 1 y Kd = 1, ajustados mediante prueba y error. Debido al amortiguamiento térmico causado por el termobloque y su aislamiento, el PID se desactivaba cuando la temperatura alcanzaba el objetivo. El mantenimiento de la temperatura se realizó manualmente para aliviar la carga computacional del microcontrolador (MCU, del inglés *Microcontroller unit*), ya que los resultados fueron los mismos que los obtenidos mediante el control PID. La temperatura se calcula con la ecuación de Steinhart-Hart. Se realizaron tres mediciones para obtener los coeficientes A, B y C de la ecuación. El sistema supervisa continuamente la temperatura para garantizar la seguridad y evitar incendios, por lo que se activan alarmas sonoras a través del software en caso de aumentos de temperatura excesivos o no controlados.

b. El *módulo de lectura* de muestras consta de fotorresistencias y elementos de fibra óptica. Como detector se utilizó un sensor de color TCS3472, que proporciona un retorno digital de valores de detección de luz roja, verde, azul (RGB) y claridad. La alta sensibilidad, el amplio rango dinámico y el filtro de infrarrojos (filtro IR Block) convierten al TCS3472 en un sensor de color ideal para su uso en condiciones de iluminación variables y a través de materiales atenuantes. Además, las luces LED blancas situadas sobre los tubos que contienen las muestras proporcionan la luz suficiente y homogénea para una lectura eficaz.
c. El *módulo de transmisión* incluye cuatro sistemas necesarios para la recepción y protección de los resultados de las muestras y otra información relevante: recepción de datos, protección de datos, transmisión y almacenamiento.
    - En cuanto a la *recepción de datos*, junto con los resultados de la muestra, el sistema incorpora otra información complementaria relacionada con el contexto en el que se toma la muestra, como la posición GPS,

información sobre el paciente, profesional sanitario responsable de la toma de muestra, información del equipo de medición, incidencias, etc. La información se almacena de acuerdo con las especificaciones HL7[12]

- La *protección de datos* incluye todos los elementos necesarios para su encriptación, salvaguardar la identidad de los usuarios y certificar tanto el origen como la corrección de los mismos, de forma que se cumplan todas las leyes internacionales de protección de datos.
- En relación con la *transmisión*, la organización virtual facilita la comunicación a través de *Bluetooth Low Energy* (BLE) para enviar los datos a un dispositivo conectado, siguiendo los estándares de la arquitectura *Edge Computing*.
- Para el *almacenamiento*, el módulo almacena los datos recogidos mediante una memoria interna *Process Context Block* (PCB) para facilitar la retransmisión en diferido, en caso de que no haya cobertura de señal[13].

Además de los tres módulos fundamentales, el SMART-LAMP está diseñado para ser utilizado en zonas sin conexión eléctrica e internet mediante dos características principales

a. *La generación y recarga de la batería*: el dispositivo utiliza el MC73831T y tres baterías de iones de litio (NCR186508), elegidas para alimentar todo el sistema. Estas ofrecen un buen rendimiento en cuanto a la capacidad de descarga y al número de recargas posibles[14].
a. *La aplicación móvil del SMART-LAMP*: permite al dispositivo visualizar y validar los resultados, enviar información, realizar copias de seguridad, etc. La interfaz está diseñada tanto a nivel del sistema de medición de datos, como a nivel del sistema de análisis y almacenamiento de información. La información generada puede visualizarse tanto en ordenadores personales como en tabletas o smartphones, y es adaptable a diferentes formatos de pantalla. La visualización también se muestra en el dispositivo SMART-LAMP en una pantalla LCD alfanumérica de 16 caracteres y 2 líneas. La interfaz puede analizar la información teniendo en cuenta parámetros geográficos, locales y/o personales. Además, se ha creado una aplicación móvil para mostrar los resultados en tiempo real a través de una interfaz amigable en el sistema operativo iOS (Apple Inc., Los Altos, California, USA), donde se puede acceder

a los datos de las mediciones. La aplicación también permite interactuar con el dispositivo SMART-LAMP, iniciando o forzando la detención de una medición, así como registrar un usuario, enlazar con un dispositivo cercano y controlar el nivel de batería del dispositivo. Aunque la aplicación para teléfonos inteligentes aún no es de uso público, se ha creado un sitio web donde se podrá descargar en un futuro https://smartlamp.es/en.

AI.7.2. Evaluación del SMART-LAMP

En el **cuarto artículo de investigación** de esta Tesis Doctoral, se llevó a cabo una evaluación de la utilidad y capacidad diagnóstica del dispositivo SMART-LAMP. Esta evaluación se estructuró en tres niveles:

a. *Lectura de color y perfil de temperatura:* para la evaluación de la lectura del color, se midieron diluciones seriadas 1:2 del colorante verde malaquita en agua con los tres prototipos de SMART-LAMP, desde el 0,1% p/v hasta el 7,81 × 10-4 % p/v. Cada dilución se midió por triplicado en cada pocillo de cada prototipo. Tras esto los resultados obtenidos se analizaron estadísticamente a través de un test ANOVA, realizado en el software R (v.3.6.3). El perfil de temperatura se evaluó midiendo los tiempos que tardaba el dispositivo en ascender entre temperatura ambiente y la temperatura de reacción (25 °C a 65 °C), entre la temperatura de reacción y la temperatura de inhibición (65 °C a 80 °C), y de vuelta a la temperatura de reacción (80 °C a 65 °C). Para evaluar la influencia de las baterías en estos tiempos, se realizaron mediciones con el 100 %, el 75 %, el 50 %, el 25 % y por debajo del 25 % de carga de las baterías. La estabilidad de la temperatura se evaluó durante 1 h a 65 °C, con mediciones realizadas cada 5 min.

b. *Valores predictivos positivos y negativos:* se utilizaron cuatro controles positivos y cuatro controles negativos de cada ensayo de LAMP en fresco y LAMP en seco para establecer la mejor medición para discriminar los resultados positivos y negativos. Los valores RGB se controlaron en tiempo real con el SMART-LAMP. Se consideraron los valores absolutos de cada componente al final de la reacción (t_{final}), así como las diferencias relativas entre los componentes de color en t_{final} y los tiempos de reacción 0 min, 5 min, 10 min y 15 min, calculados desde el archivo tipo .csv, exportable al final de cada reacción. Una vez establecida la medida más adecuada, se analizaron 40 controles (20

positivos y 20 negativos) para cada LAMP estabilizado con el fin de establecer el valor umbral de positividad y calcular el valor predictivo positivo (PPV, del inglés *Predictive positive value*) y el valor predictivo negativo (NPV, del inglés *Negative predictive value*) para el SMART-LAMP. Para tener en cuenta cualquier posible efecto del almacenamiento a temperatura ambiente a largo plazo de las mezclas secas, se analizaron 20 controles (10 positivos y 10 negativos) después de más de 30 días de almacenamiento a temperatura ambiente.

c. *Sensibilidad analítica:* para evaluar la sensibilidad de las mezclas estabilizadas de LAMP, se prepararon y ensayaron diluciones seriadas 1:10, partiendo de 5 ng/µL de cada ADN diana, y se midió utilizando EvaGreen en el dispositivo Genie III y el verde de malaquita en los prototipos SMART-LAMP. Para cada ensayo LAMP, se prepararon diluciones hasta el límite de detección obtenido en sus descripciones originales: 10 fg en el caso de *Schistosoma mansoni* y *S. haematobium*[1,3], y 10 pg para *Strongyloides* spp.[2]. Para la amplificación por LAMP del SARS-CoV-2, se utilizó como control positivo el ARN de una muestra nasofaríngea de un paciente con un valorde Ct = 25 en la RT-qPCR para la región *ORF1ab*. Asimismo, para evaluar la sensibilidad se prepararon diluciones seriadas de 10 veces, hasta el límite de detección de 1:100 descrito originalmente[5], para evaluar la sensibilidad. Además, se evaluó la cinética de la reacción a través de una regresión lineal (realizada en R) para comprobar la fuerza de la correlación entre las diluciones seriadas y el tiempo de amplificación, tanto en el dispositivo comercial Genie III como en nuestro SMART-LAMP.

AI.8. Análisis estadístico

Todos los análisis estadísticos incluidos en esta Tesis Doctoral fueron realizados en el software R (v. 3.6.3). Para la realización de las pruebas estadísticas y la representación gráfica de los datos se hizo uso de los paquetes ggplot2, ggbeeswarm, ggpubr, grid, EnvStats y dplyr.

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ANEXO II

Otras publicaciones científicas, comunicaciones en congresos, publicaciones docentes premios de investigación y estancias en el extranjero

AII.1. Otras publicaciones científicas

Se recogen en esta sección, otros artículos de investigación y un capítulo de libro en los que también se ha colaborado durante el tiempo de formación predoctoral en la Universidad de Salamanca. Se detalla una lista de los mismos y se incluye la primera página de cada uno de los artículos, en la que se encuentra su resumen; también la primera página del capítulo de libro, en la que se encuentra el índice de contenidos.

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PLOS ONE

RESEARCH ARTICLE

Detection of *Schistosoma mansoni*-derived DNA in human urine samples by loop-mediated isothermal amplification (LAMP)

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Abstract

Background

Schistosoma mansoni is the main species causing hepatic and intestinal schistosomiasis in Sub-Saharan Africa, and it is the only species in South America. Adult stages of the parasite reside in the mesenteric venous plexus of infected hosts, and eggs are shed in feces. Collecting patient stool samples for *S. mansoni* diagnostic purposes is difficult in large-scale field trials. Urine samples would be an alternative approach for molecular *S. mansoni* detection since they have several advantages over stool samples, including better handling, management and storage. Additionally, loop-mediated isothermal amplification (LAMP) technology is a powerful molecular diagnostic tool for infectious diseases, particularly under field conditions in developing countries. The present study aimed to assess the effectiveness of our previously developed LAMP assay (SmMIT-LAMP) for *S. mansoni*-specific detection in clinical urine samples.

Methodology/Principal findings

The sensitivity of SmMIT-LAMP in urine was established in simulated fresh human urine samples artificially spiked with genomic DNA from *S. mansoni*. LAMP for 120 min instead of 60 min improved the sensitivity, reaching values of 0.01 fg/μL. A set of well-defined frozen stored human urine samples collected from Sub-Saharan immigrant patients was selected from a biobank to evaluate the diagnostic validity of SmMIT-LAMP. The set included urine samples from patients with microscopy-confirmed infections with *S. mansoni*, *S. haematobium* and other nonschistosome parasites, as well as urine samples from patients with microscopy-negative eosinophilia without a confirmed diagnosis. The SmMIT-LAMP was incubated for 60 and 120 min. A longer incubation time was shown to increase the LAMP-positive results in patient urine samples. We also tested urine samples from mice experimentally infected with *S. mansoni*, and LAMP-positive results were obtained from the third

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Loop Mediated Isothermal Amplification: Towards Point-of-Care Diagnostic

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Perspective

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Abbreviations: POCD: Point-of-Care Diagnostics; SDI: Sexually Transmitted Diseases Diagnostics Initiative; ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable; LAMP: Loop-Mediated Isothermal Amplification; iRBCs: Infected Red Blood Cells; COC: Cyclic Oleofin Copolymer.

Introduction

In 2002, Daar AS, et al. [1] listed the top ten challenges that biotechnology faced to improve health in developing countries. On the top of that list we could find: "Modified molecular technologies for affordable, simple diagnosis of infectious diseases". This idea has been materialized over the following years into the principle of point-of-care diagnostics (POCD). Several definitions have been published since then. A good example of that is the one given by Schito M, et al. [2], which says: "a diagnostic test that is performed near the patient or treatment facility, has a fast turnaround time, and may lead to a change in patient management". Moreover, the test should not require trained laboratory personnel, clinical laboratory or other infrastructural support. Over ten years ago, this basic concept was divided into specific criteria that must be achieved with any POCD. Those criteria were proposed by the World Health Organization Sexually Transmitted Diseases Diagnostics Initiative (SDI) in the acronym ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable) [3]. This term has been revisited recently by Land KJ, et al. [4], updating it to the newest technological innovations. Thus, they established the new acronym REASSURED that now also includes: Real-time connectivity and Ease of specimen collection and Environmental friendliness.

The challenge that Daar AS, et al. [1] recognized often face strong limitations, as classic molecular techniques, such as PCR and its variants, require specialized and expensive equipment and a high degree of expertise to perform them. Thus, their arrival and establishment into low-income developing countries is constantly set back. At present, there is a nucleic acid amplification method named loop-mediated isothermal amplification (LAMP) [5]. Compared to PCR-based techniques, the simplicity of the LAMP method makes it suitable for field testing in developing countries [6,7] and an ideal candidate to be used as a POCD tool. LAMP is a highly specific, sensitive and efficient DNA amplification method based on strand displacement reaction and a stem-loop structure under isothermal condition [5]. It uses the *Bacillus stearothermophilus* DNA polymerase and a set of 4 (or 6) primers that hybridize to 6 (or 8) different regions of the target DNA sequence. The LAMP reaction does not require expensive devices and is completed in a short time. Since its emergence, LAMP technology has been successfully used for the detection of a great number of pathogens, including viruses, bacteria, fungi and parasites [8] and, over the past 10 years the number of publications involving the use of LAMP has showed a multiplied exponentially.

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Research Article

A *Trypanosoma cruzi* Genome Tandem Repetitive Satellite DNA Sequence as a Molecular Marker for a LAMP Assay for Diagnosing Chagas' Disease

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Guest Editor: Marcos Vinícius da Silva

Chagas' disease is a neglected tropical disease caused by *Trypanosoma cruzi* which is endemic throughout Latin America and is spread by worldwide migration. Diagnosis is currently limited to serological and molecular techniques having variations regarding their sensitivity and specificity. This work was aimed at developing a new sensitive, applicable, and cost-effective molecular diagnosis technique for loop-mediated isothermal amplification-based detection of *T. cruzi* (Tc-LAMP). The results led to determining a highly homologous satellite repeat region (231 bp) among parasite strains as a molecular marker for diagnosing the disease. Tc-LAMP was performed correctly for detecting parasite DNA (5 fg for the CL Brener strain and 50 fg for the DM28, TcVI, and TcI strains). Assay results proved negative for DNA from 16 helminth species and 7 protozoa, including *Leishmania* spp. Tc-LAMP based on the highly repeated *T. cruzi* satellite region is thus proposed as an important alternative for diagnosing *T. cruzi* infection, overcoming other methods' limitations such as their analytic capability, speed, and requiring specialized equipment or highly trained personnel. Tc-LAMP could be easily adapted for point-of-care testing in areas having limited resources.

1. Introduction

American trypanosomiasis, or Chagas' disease, is a zoonotic disease, usually consisting of chronic parasitic infection caused by the kinetoplastid protozoan *Trypanosoma cruzi*. The World Health Organization (WHO) recognizes Chagas' disease as one of the 20 neglected tropical diseases (NTD) [1] and one of the 13 most NTD worldwide [2]. Chagas' disease was considered a strictly rural disease for many decades; however, socioeconomic changes, rural exodus, deforestation, and urbanization have transformed the disease's epidemiological profile, making it an increasingly urban phenomenon and a major public health problem [3]. The disease can currently be found in 21 Latin American countries, and it has been estimated that at least 8 million people are infected worldwide. Migration has increased the disease's incidence, and it has been spread to other continents [2, 4].

Chagas' disease diagnosis depends on the phase in which a patient is found to be. Parasitemia is high during the acute phase and the congenital form, as well as in reactivations

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Research Article

Molecular Markers for Detecting *Schistosoma* Species by Loop-Mediated Isothermal Amplification

Pedro Fernández-Soto,[1] Catalina Avendaño,[2] Anna Sala-Vizcaíno,[1] Beatriz Crego-Vicente,[1] Begoña Febrer-Sendra,[1] Juan García-Bernalt Diego,[1] Ana Oleaga,[3] Julio López-Abán,[1] Belén Vicente,[1] Manuel A. Patarroyo,[4,5] and Antonio Muro[1]

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3 FDFJWFE 0 DUPCFS 3 FWJTFE +VOF " DDFQUFE +VMZ 1 VCMJTIFE +VMZ

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Schistosomiasis is considered a neglected parasitic disease. Around 280,000 people die from it annually, and more than 779 million people are at risk of getting infected. The schistosome species which infect human beings are *Schistosoma mansoni*, *Schistosoma haematobium*, *Schistosoma intercalatum*, *Schistosoma japonicum*, *Schistosoma guineensis*, and *Schistosoma mekongi*. This disease is also of veterinary significance; the most important species being *Schistosoma bovis* since it causes the disease in around 160 million livestock in Africa and Asia. This work was aimed at designing and developing a genus-specific loop-mediated isothermal amplification (LAMP) method for detecting the most important schistosome species affecting humans and for the species-specific detection of *S. bovis*. Bioinformatics tools were used for primer design, and the LAMP method was standardised for detecting the ITS-1 region from *S. intercalatum*, *S. haematobium*, *S. mansoni*, *S. japonicum*, and *S. bovis* DNA (generic test) and the NADH 1 gene for specifically detecting *S. bovis* (at different DNA concentrations). Detection limits achieved were 1 pg DNA for *S. mansoni*, 0.1 pg for *S. haematobium*, 1 pg for *S. intercalatum*, and 10 pg for *S. bovis*. No amplification for *S. japonicum* DNA was obtained. The LAMP designed for the amplification of *S. bovis* NADH-1 worked specifically for this species, and no other DNA from other schistosome species included in the study was amplified. Two highly sensitive LAMP methods for detecting different *Schistosoma* species important for human and veterinary health were standardised. These methods could be very useful for the diagnosis and surveillance of schistosome infections.

1. Introduction

Schistosomiasis is a parasitic disease caused by several species of trematode worms of the genus *Schistosoma*. It is one of the 20 tropical diseases on the World Health Organization's (WHO) list of Neglected Tropical Diseases (NTDs) [1]. The disease affects at least 240 million people worldwide and more than 779 million are at risk of contracting it [2]. The infection is endemic in 78 countries, mainly in tropical and subtropical areas, although it predominates in Sub-Saharan Africa where more than 80% of the cases occur, leading to around 280,000 deaths annually. The Global Burden of Disease study attributed 1.43 million disability-adjusted life years (DALYs) to it in 2017 [2–5].

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Parasites & Vectors

RESEARCH Open Access

Whip-LAMP: a novel LAMP assay for the detection of *Trichuris muris*-derived DNA in stool and urine samples in a murine experimental infection model

Pedro Fernández-Soto*†, Carlos Fernández-Medina†, Susana Cruz-Fernández, Beatriz Crego-Vicente, Begoña Febrer-Sendra, Juan García-Bernalt Diego, Óscar Gorgojo-Galindo, Julio López-Abán, Belén Vicente Santiago and Antonio Muro Álvarez*

Abstract

Background: *Trichuris trichiura* (human whipworm) infects an estimated 477 million individuals worldwide. In addition to *T. trichiura*, other *Trichuris* species can cause an uncommon zoonosis and a number of human cases have been reported. The diagnosis of trichuriasis has relied traditionally on microscopy. Recently, there is an effort to use molecular diagnostic methods, mainly qPCR. LAMP technology could be an alternative for qPCR especially in low-income endemic areas. *Trichuris muris*, the causative agent of trichuriasis in mice, is of great importance as a model for human trichuriasis. Here, we evaluate the diagnostic utility of a new LAMP assay in an active experimental mouse trichuriasis in parallel with parasitological method by using stool and, for the first time, urine samples.

Methods: Stool and urine samples were collected from mice infected with eggs of *T. muris*. The dynamics of infection was determined by counting the number of eggs per gram of faeces. A LAMP based on the *18S* rRNA gene from *T. muris* was designed. Sensitivity and specificity of LAMP was tested and compared with PCR. Stool and urine samples were analysed by both LAMP and PCR techniques.

Results: *Trichuris muris* eggs were detected for the first time in faeces 35 days post-infection. LAMP resulted specific and no cross-reactions were found when using 18 DNA samples from different parasites. The detection limit of the LAMP assay was 2 pg of *T. muris* DNA. When testing stool samples by LAMP we obtained positive results on day 35 p.i. and urine samples showed amplification results on day 20 p.i., i.e. 15 days before the onset of *T. muris* eggs in faeces.

Conclusions: To the best of our knowledge, we report, for the first time, a novel LAMP assay (Whip-LAMP) for sensitive detection of *T. muris* DNA in both stool and urine samples in a well-established mice experimental infection model. Considering the advantages of urine in molecular diagnosis in comparison to stool samples, should make us consider the possibility of starting the use urine specimens in molecular diagnosis and for field-based studies of human trichuriasis where possible. Further studies with clinical samples are still needed.

Keywords: *Trichuris trichiura*, LAMP, *Trichuris muris*, Human trichuriasis, Urine

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Journal of *Clinical Medicine*

MDPI

Article

Application of a Genus-Specific LAMP Assay for Schistosome Species to Detect *Schistosoma haematobium* x *Schistosoma bovis* Hybrids

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Abstract: Schistosomiasis is a disease of great medical and veterinary importance in tropical and subtropical regions caused by different species of parasitic flatworms of the genus *Schistosoma*. The emergence of natural hybrids of schistosomes indicate the risk of possible infection to humans and their zoonotic potential, specifically for *Schistosoma haematobium* and *S. bovis*. Hybrid schistosomes have the potential to replace existing species, generate new resistances, pathologies and extending host ranges. Hybrids may also confuse the serological, molecular and parasitological diagnosis. Currently, LAMP technology based on detection of nucleic acids is used for detection of many agents, including schistosomes. Here, we evaluate our previously developed species-specific LAMP assays for *S. haematobium*, *S. mansoni*, *S. bovis* and also the genus-specific LAMP for the simultaneous detection of several *Schistosoma* species against both DNA from pure and, for the first time, *S. haematobium* x *S. bovis* hybrids. Proper operation was evaluated with DNA from hybrid schistosomes and with human urine samples artificially contaminated with parasites' DNA. LAMP was performed with and without prior DNA extraction. The genus-specific LAMP properly amplified pure *Schistosoma* species and different *S. haematobium-S. bovis* hybrids with different sensitivity. The *Schistosoma* spp.-LAMP method is potentially adaptable for field diagnosis and disease surveillance in schistosomiasis endemic areas where human infections by schistosome hybrids are increasingly common.

Keywords: LAMP; schistosomiasis; schistosome hybrids; *Schistosoma haematobium*; *Schistosoma bovis*; molecular diagnosis; species-specific LAMP; genus-specific LAMP

1. Introduction

Environmental changes due to ecosystem decline, biodiversity loss and climate change are some issues with potential ecological risk that we are facing as human beings. These changes driven by increasing economic development, migration, agricultural and livestock practices and deforestation have consequences in emerging infectious diseases (EIDs) [1–6]. Changes in biodiversity have the potential to either increase or reduce the incidence of infectious disease in humans because they involve interactions among species. The

Journal of *Clinical Medicine*

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Article

Seroprevalence of SARS-CoV-2 Antibodies and Factors Associated with Seropositivity at the University of Salamanca: The DIANCUSAL Study

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Abstract: Background: Systematic screening for antibodies against SARS-CoV-2 is a crucial tool for surveillance of the COVID-19 pandemic. The University of Salamanca (USAL) in Spain designed a project called "DIANCUSAL" (Diagnosis of New Coronavirus, COVID-19, in University of Salamanca) to measure antibodies against SARS-CoV-2 among its ~34,000 students and academic staff, as the influence of the university community in the spread of the SARS-CoV-2 pandemic in the city of Salamanca and neighboring towns hosting USAL campuses could be substantial. Objective: The aim of this study was to estimate the prevalence of SARS-CoV-2 antibodies among USAL students, professors and staff and to evaluate the demographic, academic, clinical and lifestyle and behavioral factors related to seropositivity. Methodology: The DIANCUSAL study is an ongoing university population-based cross-sectional study, with the work described herein conducted from July–October 2020. All USAL students, professors and staff were invited to complete an anonymized questionnaire. Seroprevalence of anti-SARS-CoV-2 antibodies was detected and quantified by using chemiluminescent assays for IgG and IgM. Principal findings: A total of 8197 (24.71%) participants were included. The mean age was 31.4 (14.5 SD) years, and 66.0% of the participants were female. The seroprevalence was 8.25% overall and was highest for students from the education campus (12.5%) and professors from the biomedical campus (12.6%), with significant differences among faculties ($p = 0.006$). Based on the questionnaire, loss of smell and fever were the symptoms most strongly associated with seropositivity, and 22.6% of seropositive participants were asymptomatic. Social distancing was the

diagnostics

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Colorimetric and Real-Time Loop-Mediated Isothermal Amplification (LAMP) for Detection of *Loa loa* DNA in Human Blood Samples

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Abstract: Loiasis, caused by the filarial nematode *Loa loa*, is endemic in Central and West Africa. *Loa loa* has been associated with severe adverse reactions in high *Loa*-infected individuals receiving ivermectin during mass drug administration programs for the control of onchocerciasis and lymphatic filariasis. Diagnosis of loiasis still depends on microscopy in blood samples, but this is not effective for large-scale surveys. New diagnostics methods for loiasis are urgently needed. Previously, we developed a colorimetric high-sensitive and species-specific LAMP for *Loa loa* DNA detection. Here, we evaluate it in a set of 100 field-collected clinical samples stored as dried blood spots. In addition, *Loa loa*-LAMP was also evaluated in real-time testing and compared with microscopy and a specific PCR/nested PCR. A simple saponin/Chelex-based method was used to extract DNA. Colorimetric and real-time LAMP assays detected more samples with microscopy-confirmed *Loa loa* and *Loa loa*/*Mansonella perstans* mixed infections than PCR/nested-PCR. Samples with the highest *Loa loa* microfilariae counts were amplified faster in real-time LAMP assays. Our *Loa loa*-LAMP could be a promising molecular tool for the easy, rapid and accurate screening of patients for loiasis in endemic areas with low-resource settings. The real-time testing (feasible in a handheld device) could be very useful to rule out high-microfilariae loads in infected patients.

Keywords: *Loa loa*; loiasis; colorimetric LAMP; real-time LAMP; PCR; nested-PCR; dried blood spots; saponin/Chelex; microscopy; molecular diagnosis

1. Introduction

Loa loa is a parasitic nematode that causes loiasis (commonly known as African eye worm). The parasite is transmitted to humans by Tabanid flies of the genus *Chrysops* and affects between 3 and 13 million people in the west and central regions of Africa [1]. Human loiasis is known to be endemic in eleven countries, including Angola, Chad, the Democratic Republic of the Congo, Cameroon, the Central African Republic, Equatorial Guinea, Ethiopia, Gabon, Nigeria, Republic of Congo and Sudan [2]. The main specific clinical manifestations include subcutaneous edema (Calabar swelling) and pruritus. Additionally, the ocular passage of the adult worm under the conjunctiva may be noticed. However,

Manuscript under preparation

Breakthrough infections by SARS-CoV-2 variants of concern boost both cross-reactive cellular and humoral immune responses in mRNA-vaccinated Golden Syrian Hamsters

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ABSTRACT

Syrian Golden Hamsters were vaccinated once with a suboptimal dose of Pfizer/BioNTech COVID-19 mRNA vaccine (5mg/animal). Four weeks post vaccination, animals were challenged with different variants of SARS-CoV-2. Although vaccination resulted in detectable antibody ELISA titers against the ancestral SARS-CoV-2 spike protein, antibody titers were too low for efficient neutralization of the antigenically matching USA-WA1/2020 or the drifted variants of concern (Alpha, Beta, Delta and Mu) in *in vitro* microneutralizaton assays. Despite absence of virus-neutralizing antibodies, suboptimal vaccination resulted in reduced morbidity for USA-WA1/2020 and Alpha-challenged animals and complete control of lung virus titers for USA-WA1/2020, Alpha and Delta but with breakthrough infection for the antigenically more distant Beta and Mu. Moreover, T cell responses in the spleen were higher in vaccinated animals compared to unvaccinated animals at 5 days post infection, suggesting vaccination was able to efficiently prime T cell responses that were recalled during infection with vaccine-matched and mismatched virus. Infection with different SARS-CoV-2 variants also back-boosted neutralizing antibody responses against the challenge virus and in the case of the variants of concern, against the antigenically distant but vaccine-matched USA-WA1/2020. Transcriptomic analysis of host immune responses to infection reflects both vaccination status and disease course, is further compared with lung pathology data and suggests a role for interstitial lung macrophages in vaccine-mediated protection. In summary, we show that suboptimal vaccination protects against SARS-CoV-2 challenge with different variants of concern, using the hamster SARS-CoV-2 vaccination and challenge model. This protection correlates with recall of vaccine-induced T cell responses during virus infection in the absence of neutralizing antibody titers. These data show the importance of vaccine-mediated protection against infection with SARS-CoV-2 variants of concern, even in the absence of high neutralizing antibody titers.

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CONTENTS

AII.2. Comunicaciones en congresos

Se recogen en esta sección las comunicaciones en congresos nacionales e internacionales presentadas por el doctorando para la difusión del trabajo científico incluido en esta Tesis Doctoral y de otras en las que también se ha participado durante el periodo de formación predoctoral. Se detalla una lista de las comunicaciones, ordenadas por congresos, incluyendo si fueron presentadas como comunicación oral o como póster, y el resumen o el certificado de comunicación de cada una de ellas.

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4. Crego-Vicente B, Fernández Soto P, Sempere MA, **García-Bernalt Diego J**, Febrer-Sendra B, Ramos Montesdeoca M, Saugar JM, Rubio JM, Gómez Sanchez A, López-Abán J, Vicente B, Muro A. Detección de ADN de *Schistosoma mansoni* mediante LAMP en un paciente asintomático con eosinofilia y quiluria. XXI Congreso SOCEPA. Pontevedra (España). 2019. **Póster.**

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7. Crego-Vicente B, Fernández Soto P, Sempere MA, Febrer-Sendra B, **García-Bernalt Diego J**, Saugar JM, Rubio JM, Gómez Sanchez A, López-Abán J, Vicente B, Muro A. *Schistosoma mansoni* en paciente con quiluria y eosinofilia: detección molecular y seguimiento del tratamiento por LAMP. XI Congreso Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Retos en Salud Global. Ávila (España). 2019. **Póster.**

8. Fernández Soto P, **García-Bernalt Diego J**, Santos Santos D, Crego-Vicente B, Febrer-Sendra B, Vicente Santiago B, López-Abán J, Corchado JM, Muro A. SMART-LAMP: un nuevo dispositivo portátil para el diagnóstico molecular *point-of-care*. XI Congreso Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Retos en Salud Global. Ávila (España). 2019. **Comunicación oral**.

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11. Crego-Vicente B, Fernández-Soto P, Febrer-Sendra B, **García-Bernalt Diego J**, Soto López JD, Murcia X, Muro Álvarez A. Desarrollo de tecnología LAMP

múltiple para el diagnóstico de eosinofilias importadas de difícil tipificación. XII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Pamplona (España). 2022. **Comunicación oral.**

12. **García-Bernalt Diego J**, Fernández-Soto P, Márquez-Sánchez S, Santos Santos D, Febrer-Sendra B, Crego-Vicente B, Muñoz Bellido JL, Belhassen-García M, Corchado Rodríguez JM, Muro Álvarez A. SMART-LAMP: diagnóstico molecular a pie de paciente mediante un dispositivo portátil y tecnología móvil. XII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Pamplona (España). 2022. **Comunicación oral.**

13. Febrer-Sendra B, Fernández-Soto P, Crego-Vicente B, **García-Bernalt Diego J**, Negredo Antón A, Sánchez Seco Fariñas MP, Vieira Lista MC, López Bernús A, Belhassen-García M, Muñoz Bellido JL, Muro Álvarez A. Diseño, desarrollo y aplicación de un nuevo RT-LAMP para la detección del Virus de la Fiebre Hemorrágica de Crimea-Congo. XII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Pamplona (España). 2022. **Comunicación oral.**

14. **García-Bernalt Diego J**, Fernández-Soto P, Márquez-Sánchez S, Santos Santos D, Febrer-Sendra B, Crego-Vicente B, Corchado Rodríguez JM, Muro A. Diagnóstico molecular de enfermedades parasitarias a pie de paciente mediante un dispositivo portátil y tecnología móvil: SMART-LAMP. XXII Congreso SOCEPA. 2022. Madrid (España). **Comunicación oral.**

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Strong-LAMP assay as a molecular method for the diagnosis of strongyloidiasis in field conditions: a preliminary study in Puerto Iguazú, Misiones, Argentina

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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. Several techniques are used in diagnosing the disease, but definitive diagnosis is accomplished by parasitological examination of stool samples for morphological identification of parasite. The present study aimed to evaluate the use of our previously designed molecular LAMP assay for detection of *Strongyloides* spp. (named, Strong-LAMP) in field conditions for diagnosis of strongyloidiasis in periurban and sylvatic areas of Puerto Iguazú, Misiones, Argentina. Stool samples were collected from individuals living in the studied areas and tested for the presence of intestinal parasites by sedimentation, floating, Baermann and Kato-Katz. Additionally, urine samples were collected and tested by LAMP method as alternative to stool samples for diagnosing strongyloidiasis. In stool samples, several parasites were identified including, hookworms, *Trichuris trichiura* and *S. stercoralis.* The Strong-LAMP method showed to be more sensitive than classical parasitological methods for strongyloidiasis diagnosis using both stool and urine samples for analysis. After further validation, the Strong-LAMP assay has the potential to be used as an effective molecular large-scale screening test for strongyloidiasis-endemic areas.

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Sociedad Española de Parasitología

Universidad de Vigo

[196] Estabilización de reactivos para la aplicación de la técnica SmMIT-LAMP en el diagnóstico de la esquistosomosis a pie de paciente

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La tecnología LAMP aúna las características ideales para el diagnóstico molecular a pie de paciente (*Point-of-care test*, POCT). Sin embargo, la necesidad de mantenimiento de la cadena de frío para los reactivos, limita su utilización en condiciones de campo. Nuestro grupo desarrolló un método LAMP para la detección de ADN de *S. mansoni* (SmMIT-LAMP) efectivo en el diagnóstico de la esquistosomosis aguda en modelo experimental murino. Recientemente, se comprobó su utilidad diagnóstica con muestras de pacientes (heces y orina), y epidemiológica, con muestras de caracoles en zona endémica. En este trabajo presentamos la adaptación del método SmMIT-LAMP a un formato kit listo para su uso mediante la estabilización de los componentes de la reacción en presencia de trehalosa. Para dicha estabilización se utilizaron métodos de desecado y concentración, tanto para reacciones en formato convencional (en termobloque) como en pruebas a tiempo real (utilizando un dispositivo portátil). Se evaluaron distintos colorantes (añadidos pre o post-amplificación) para la visualización directa de los resultados, disminución del volumen de reacción y el mantenimiento de la funcionalidad de las mezclas de reacción almacenadas a temperatura ambiente y a 4 °C. También se prepararon mezclas estabilizadas conteniendo ADN de *S. mansoni* como control positivo de amplificación listas para su uso. El rehidratado de las mezclas de reacción se llevó a cabo a distintos tiempos post-desecación. El método SmMIT-LAMP ofreció resultados positivos con mezclas de reacción estabilizadas tras 5 meses de almacenamiento a 4 °C y hasta 1 mes almacenadas a temperatura ambiente. En reacciones a tiempo real, se observó un retraso en la amplificación de las mezclas estabilizadas, aunque no se detectó reducción en la calidad de la amplificación final. Se observaron diferencias importantes en los virajes de color entre mezclas estabilizadas y frescas, para una misma concentración de colorante. Además, se rebajó el volumen de reacción hasta 15 µL. En conclusión, hemos establecido un protocolo simple y rápido para estabilizar mezclas de reacción SmMIT-LAMP para su uso potencial como formato kit en el diagnóstico de la esquistosomosis. Este procedimiento es adaptable a diferentes métodos LAMP para su posible aplicación como POCT de diferentes Neglected Tropical Diseases (NTDs).

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Palabras clave: LAMP, esquistosomosis, *point of care*, trehalosa.

Sociedad Española de Parasitología

Universidad de Vigo

[239] Detección de ADN de Loa loa en muestras clínicas mediante LAMP (*Loop-Mediated Isothermal Amplification*) a tiempo real

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La loasis es una enfermedad parasitaria humana causada por el nematodo *Loa loa*, transmitido por las picaduras de tábanos del género *Chrysops*. Es endémica de regiones centrales y del oeste de África, afectando entre 3 y 13 millones de personas. Clínicamente suele cursar de forma asintomática, con eosinofilia y microfilaremia. Cuando produce sintomatología, aparece angioedema transitorio (edema de Calabar) y migración transconjuntival de los vermes adultos causando inflamación y edema conjuntival. Si hay complicaciones, aparecen tardíamente y asociadas a fenómenos de hipersensibilidad. Generalmente, el diagnóstico se establece mediante observación microscópica de las microfilarias de preparaciones hemáticas directas o tras concentración. Los tests inmunodiagnósticos y moleculares útiles en el diagnóstico de loasis no están comercializados y solo están disponibles en laboratorios especializados de referencia. Nuestro grupo de investigación diseñó y desarrolló un nuevo método molecular basado en la tecnología LAMP para la detección sensible y específica de ADN genómico de *Loa loa* en muestras simuladas de sangre preparadas artificialmente. En este trabajo evaluamos de forma preliminar el método LAMP desarrollado para la detección de ADN de *Loa loa* en muestras clínicas de pacientes. Se utilizó el ADN extraído de 10 muestras de sangre de pacientes con confirmación parasitológica y resultado positivo por PCR de infección por *Loa loa* procedentes de Guinea Ecuatorial. Las muestras se analizaron mediante el LAMP convencional (en termobloque) y a tiempo real (en un dispositivo portátil Genie III). Se obtuvo amplificación en las 10 muestras analizadas mediante los dos ensayos. Se observaron claramente los resultados colorimétricos del LAMP convencional y se obtuvieron valores de fluorescencia a tiempo real a los 15 minutos de reacción. El método LAMP desarrollado para la detección sensible y específica de ADN de *Loa loa* ha resultado eficaz por primera vez en el análisis de muestras clínicas. La posibilidad de realizar la técnica en un dispositivo portátil a tiempo real de forma fácil y rápida hace de nuestro LAMP una herramienta potencialmente útil en el diagnóstico de la loasis tanto en consulta clínica como en condiciones de campo en zonas endémicas de la enfermedad.

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Palabras clave: Loasis, *Loa loa*, LAMP, LAMP a tiempo real.

Sociedad Española de Parasitología

Universidad de Vigo

[252] Detección de ADN de *Schistosoma mansoni* mediante LAMP en un paciente asintomático con eosinofilia y quiluria

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Paciente masculino de 15 años de edad, español, residente en Marbella, que se presenta asintomático en consulta. Se confirma quiluria evidente y eosinofilia del 24% (1090 eosinófilos/mm^3). Refiere como antecedente un viaje de una semana a Puerto Rico hospedado en hotel donde permanece salvo un día lluvioso que hace excursión a zona de vegetación frondosa. En su domicilio está en contacto con animales (tortugas, perros, ratones, etc) y ha tenido pecera con peces. Intervención de varicocele hace dos años. Ante la quiluria y eosinofilia, la primera sospecha es la presencia de microfilarias. Se realiza linfografía sin apreciar anomalías. Análisis de sangre (frotis, gota gruesa, Knott) y PCR negativo para filarias. PCR en heces para *Strongyloides stercoralis* negativa. ELISA para *Loa loa*, *Onchocerca*, *Wuchereria*, *Toxocara* y *Strongyloides* negativos. Serología para VIH, VHS-1, VHS-2 y *Borrelia* negativos. ELISA para *Schistosoma* positivo (IgG 2.05). Ante un diagnóstico probable de esquistosomosis (serología positiva y eosinofilia) se solicita prueba molecular para la detección de *Schistosoma mansoni*. Se realiza LAMP específico (convencional y a tiempo real) para *S. mansoni* en muestras de orina y heces del paciente con resultado positivo. Se administra tratamiento con dosis única de praziquantel al paciente. Un mes después del tratamiento el paciente presenta eosinofilia del 26,7% (1700 eosinófilos/mm^3). Se realiza de nuevo LAMP para la detección de *S. mansoni* en muestras de orina y heces recogidas seis semanas después del tratamiento con resultado positivo. El análisis mediante LAMP para la detección de *S. mansoni* indica persistencia de la infección tras el tratamiento. El método LAMP puede ser una herramienta molecular útil para la evaluación de la efectividad del tratamiento en infecciones por *S. mansoni*.

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Palabras clave: *Schistosoma mansoni*, LAMP, quiluria, eosinofilia.

When comparing the results according to the educational level (elementary, secondary and higher education, the average percentages of correct answers about ways of transmission were 2.2, 3.2 and 4.1 respectively ($p<0.001$), and about heart involvement were 0.7, 1.1 and 1.7 (p=0,001).
Conclusions: Knowledge about CD among Latin-American migrants was poor, especially regarding vertical transmission of CD, which is the most relevant mechanism of transmission in Europe.

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STABILIZATION OF SmMIT-LAMP REAGENTS FOR APPLICATION IN POINT-OF-CARE DIAGNOSTIC OF SCHISTOSOMIASIS

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Introduction: Human schistosomiasis is one of the most important NTDs. A LAMP method for the detection of *S. mansoni* DNA (SmMIT-LAMP) was established by our group to perform early diagnosis of schistosomiasis in a murine model. Recently, the SmMIT-LAMP was successfully tested in human stool samples, snails and in human urine samples. LAMP technology has been proposed as the ideal tool for a molecular POC diagnosis. However, one of the drawbacks is to maintain the cold chain to preserve reagents for application in low-resources endemic areas.
Aim: The goal is to develop a LAMP kit in a ready-to-use format with dried reagents useful for easy application in resources limited settings of schistosomiasis.
Methods: Both concentration and desiccation procedures, based on the use of trehalose, were studied to stabilize SmMIT-LAMP reaction mixes over. These procedures were applied to reaction mixes used in real-time LAMP assays (using a portable device) or in end-point conventional colorimetric detection (using a thermoblock) adding different dyes pre- (malachite green/calcein) or post-amplification (SYBR Green I). Reaction volumes ranging 5-25 µL were also tested. Desiccated mixes were stored up to 3 months both at RT and 4°C to evaluate. DNA from *S. mansoni* was also included from the beginning of the desiccation procedure as positive control. The rehydration of the mixtures and subsequent LAMP reactions were performed at different times post-desiccation.
Results: The SmMIT-LAMP reactions gave positive results with desiccated mixes after 1 month stored at RT and 3 months stored at 4°C. A notable delay in amplification time (30 to 60 min) was observed in stabilized mixes in comparison to fresh liquid ones, although not significant reduction in final amplification levels took place. We find out differences in color turns between fresh and desiccated LAMP mixes for the same pre-amplification dyes concentration. We also obtained functional LAMP reactions with reaction mix volumes down to 15 µL.
Conclusion: We established a simple 30 min one-step desiccation procedure to stabilize SmMIT-LAMP reaction mixes for potential ready-to-use application. This procedure is adaptable to different LAMP assays to a POCT set-up for many NTDs.
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AN UNUSUAL CASE OF RELAPSING VISCERAL AND CUTANEOUS LEISHMANIASIS IN AN IMMUNOCOMPROMISED PATIENT

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Introduction: We present the case of a 43-year-old man with refractory visceral leishmaniasis.
Background: This gentleman of Portugese origin had a background of treated hepatitis C infection and of HIV-1 infection diagnosed over 20 years ago, with intermittent anti-retroviral therapy adherence leading to development of resistance mutations. He initially presented in 2012 in Portugal with visceral leishmaniasis diagnosed on bone marrow morphology. He required three consecutive courses of treatment with ambisome due to persistently abnormal bone marrow morphology on repeat sampling. He represented in March 2018 with symptoms of fever and weight loss and was given a further course of ambisome treatment.
His latest presentation was in August 2018 with suspicious skin lesions and incidental mild pancytopenia. His CD4 was <100 at the time due to continued non-adherence to HIV therapy. Amastigotes were seen in the skin biopsy, and in a subsequent bone marrow biopsy. Bone marrow PCR was positive for Leishmania donovanii. He was treated with a full course of ambisome as well as concurrent miltefosine, followed by 3 weekly prophylactic pentamidine due to multiple relapses. He also re-engaged with HIV services with satisfactory viral load suppression.
Discussion: Literature suggests that unusual manifestations of visceral leishmaniasis are more common in HIV co-infected patients, as is the incidence of relapses or refractory leishmaniasis. Predictive factors are poorly known, although low CD4 count and absence of secondary prophylaxis against visceral leishmaniasis contribute to a higher relapse rate. Diagnostics are more difficult as serology sensitivity is much lower; indeed, diagnosis was made first on bone marrow biopsy and finally on skin biopsy in the above case.
This presents a reminder in clinical practice that HIV and visceral leishmaniasis coinfection is on the increase, with 5-7% of cases occurring in southern European countries and can present with atypical symptoms. The relapse rate is higher in HIV co-infected individuals, and this is compounded by low CD4 count and lack of secondary prophylaxis post treatment. Finally, cutaneous leishmaniasis in an immunosuppressed patient should always prompt investigation for visceral leishmaniasis even in the absence of typical clinical symptoms. Serology often has low sensitivity in these patients.

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VALIDATION OF A PORTABLE THREE-DIMENSIONAL IMAGING SYSTEM FOR MEASURING LOWER LIMB VOLUME OF PODOCONIOSIS PATIENTS; A PILOT STUDY

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RES0154 Diseño, síntesis y evaluación de amidas y sulfonamidas con actividad tipo colchicina contra *Strongyloides venezuelensis*

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Introducción

La estrongiloidosis es una enfermedad emergente presente en inmigrantes y viajeros de zona endémica. El tratamiento de elección es la ivermectina teniendo como alternativa el mebendazol. La presencia de fallos de tratamiento y posibilidad de desarrollo de resistencias hacen necesarios el desarrollo de nuevos compuestos contra el parasito.

Objetivos

El objetivo de este trabajo es diseñar, sintetizar y evaluar compuestos para interferir en el sitio de unión de la colchicina en un modelo in vitro con larvas de tercer estadio de *Strongyloides venezuelensis*.

Materiales y Métodos

El sitio de la colchicina en tubulina presenta tres zonas diferenciadas (A, B y C) y se han diseñado compuestos basados en amidas y sulfonamidas para ocupar dichos sitios, teniendo en cuenta las diferencias de secuencia que presentan en esas zonas las tubulinas del género Strongyloides y de mamíferos. Los compuestos diseñados se han sintetizado y se han ensayado frente a *S. venezuelenesis* en screening a una concentración 1-20 µM y para determinación de la concentración letal 50% (CL50). Tambien se determino la citotoxicidad en celula eucariota con la tecnica XTT. Se utilizaron como controles DMSO al 1% e ivermectina y albendazol como farmacos de referencia.

Resultados

Los resultados del cribado inicial con la colchicin mostrarón baja actividad frente a larvas de tercer estadio de *Strongyloides venezuelensis* habiendo una viabilidad superior al 90% que podria estar relacionado con las diferencias existentes entre la beta-tubulina de mamiferos y *Strongyloides*. El mebendazol tiene actividad comparable al albendazol. Sin embargo, algunos compuestos sintetizados (amidas y sulfonamidas) sí han mostrado una disminucion de la viabilidad inferior al 50%.

Conclusiones

Solo unas pocas sulfoamidas tienen capacidad para impedir la motilidad de L3 en cultivo que pueden deberse a la especificidad de union al sitio de la colchicina.

Financiación

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RES0155 La sobreexpresión de *Jean 3* en *Leishmania* genera una respuesta inmune protectora y reduce la infectividad del parásito

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Introducción

Alrededor de 300 millones de personas están en riesgo de contraer Leishmaniasis, una enfermedad transmitida por vectores y causada por parásitos de Leishmania. No existe vacuna eficaz y el descubrimiento de nuevos agentes leishmanicidas es una necesidad urgente.

Objetivos

Nuestro grupo ha identificado la Serin-treonin-quinasa Jean3, sin ortólogos en mamíferos y constitutivamente expresada en tripanosomátidos causantes de patologías. Para evaluar su implicación en la infectividad de *Leishmania*, hemos generado parásitos que sobreexpresan *Jean3* (LmJ3OE).

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Conclusiones

La prevalencia de HTLV en la cuenca amazónica es inferior comparado a otros estudios realizados en otras áreas geográficas del Perú (altiplano y Lima) (aprox. 3%).

Financiación

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RES0145 *Schistosoma mansoni* en paciente con quiluria y eosinofilia: detección molecular y seguimiento del tratamiento por LAMP

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Introducción

Varón español, 16 años, residente en Marbella, asintomático en consulta con quiluria evidente. Describe viaje a Puerto Rico alojado en hotel, con excursión en día lluvioso a isla boscosa. Intervención pasada de varicocele y animales en domicilio.

Objetivos

Un primer análisis muestra eosinofilia (1090/mm^3). La quiluria y eosinofilia hacen sospechar de filariasis, descartada tras PCR y ELISA negativas para filarias. PCR y serología para *Strongyloides* spp. negativas. Serología para *Toxocara*, VIH, VHS-1, VHS-2 y *Borrelia* negativas. Serología positiva para *Schistosoma* spp (Índice 2.05). LAMP para *S. mansoni* en heces y orina positivo. Un mes previo al tratamiento desaparece quiluría. Linfografía sin alteraciones que sugieran otras causas. Se administra dosis única de Praziquantel (3.200 mg) según OMS (40 mg/Kg). Un mes post-tratamiento mantiene eosinofilia (1700/mm^3) y LAMP positivo en heces y orina. Cuatro meses después del primer tratamiento mantiene eosinofilia (1030/mm^3) y se administra nuevamente Praziquantel (3.300 mg). Dos meses después se toman muestras de heces, orina y suero para LAMP y serología.

Materiales y Métodos

La linfografía se realiza en La Clínica Universidad de Navarra; microscopía de frotis, gota gruesa, Knott y serología convencional en laboratorio HC Hospital; PCRs y serologías específicas (*Shistosoma, Toxocara, Strongiloides*, filarias) en Laboratorio de Referencia del Centro Nacional de Microbiología del ISCIII; el LAMP para *S. mansoni* (convencional y tiempo real) en el CIETUS.

Resultados

El LAMP positivo en muestras de heces y orina indicó infección por *S. mansoni*, manteniéndole tras el primer tratamiento. El LAMP resultó positivo en heces, orina y suero incluso tras el segundo tratamiento, indicando un posible caso refractario al Praziquantel, como también lo sugiere el incremento del índice serológico (4.59) y la persistente eosinofilia a los 2 meses (970/mm^3) y 4 meses (700/mm^3) post segundo tratamiento.

Conclusiones

El método LAMP para la detección de *S. mansoni* en muestras de heces, orina y suero ha resultado útil en el diagnóstico y en el seguimiento de la efectividad del tratamiento con Praziquantel.

Financiación

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RES0148 Análisis de los casos y valoración de la demora diagnostica en los casos malaria importada (2014-2018)

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Introducción

La Malaria es una patología de obligado estudio en toda persona que vuelva del trópico con fiebre. Aunque sea una enfermedad potencialmente mortal y de rápida evolución muchas veces no la tenemos tan presente como se debería.

Objetivos

Analizar los casos de malaria atendidos en los últimos 5 años en nuestro hospital calculando la proporción de casos que han sido atendidos previamente por sanitarios y no se ha valorado esa posibilidad diagnóstica. Demora diagnóstica secundaria y evolución clínica de los mismos.

Materiales y Métodos

Identificación y revisión retrospectiva de historias clínicas y evolutivos electrónicos de primaria/urgencias de los casos de malaria atendidos en nuestro centro (2014-2018). Descripción de características clínico-epidemiológicas, métodos diagnósticos, especie, grado parasitación, tratamientos, cálculo de demora diagnóstica en los casos de valoración sanitaria "inadecuada" y evolución médica de las mismas.

Resultados

Analizados: 56 episodios (11/15/13/5/12 casos en 2014/2015/2016/2017/2018).

57'1% hombres, mediana de edad 37 años (rango14-72).

Tipo paciente: VFR 64'28%, viajeros 19'64%, inmigrantes 10'71%, otros 5'35%.

Destino: África 92'86%, Asia/Oceanía 7'14%.

Motivo consulta: fiebre 92'86%, otros 7'14% (MEG)

Consejo previo 16'07%, quimioprofilaxis 55'55% (completo 1).

Especies: *P.falciparum* 89'28%, *P.vivax* 7'14%, *P. ovale* 3'58%.

Parasitación: mediana 0'99% (rango 0-5'3%), 14'29% (>2'5%)

Método diagnostico: 83'93% gota gruesa/Ag y PCR (+)

Valorados previamente por sanitarios sin descartar la posibilidad de malaria: 20 (35'71%), media de demora diagnostica 3'45 días (rango 0-11) e ingreso 4'65 días (rango 1-20).

Correctamente evaluados: media demora diagnostica 0'14días (rango 0-4) e ingreso 3'78 días (rango 1-9).

Tratamientos: artesunato intravenoso en 9 casos (16'07%). Derivados orales de artemisina; 25%, proguanil/atuovaquona; 50%, otras pautas; 8'93%. Precisaron asociación de primaquina: 10'71%

Exitus: 1.

Conclusiones

En nuestra cohorte, el porcentaje de casos enfocados erróneamente en la primera valoración es muy alto, 35'7%, a pesar del perfil de los pacientes (VFR 64'2%, origen/destino África 92'8%, motivo de consulta la fiebre 92'86%, *P.falciparum* 89'28%).

La demora diagnóstica media en esos casos fue de 3'45 días frente a 0'14 días en los correctamente enfocados. Afortunadamente sin diferencias significativas en la evolución clínica.

Sólo el 16% habían pedido consejo previo y solo uno realizo correctamente la quimioprofilaxis.

Tanto el diagnostico como la prevencion son areas a mejorar

Financiación

No

RES0149 SMART-LAMP: un nuevo dispositivo portátil para el diagnóstico molecular point-of-care

Pedro Fernández Soto[1], Juan García-Bernalt Diego[1], Daniel Santos Santos[2], Beatriz Crego Vicente[1], Begoña Febrer Sendra[1], Belén Vicente Santiago[1], Julio López Abán[1], Juan Manuel Corchado[2], Antonio Muro Álvarez [1]

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Introducción

Las técnicas de diagnóstico molecular para enfermedades infecciosas se desarrollan exponencialmente. Algunas limitaciones de su aplicación en regiones endémicas siguen siendo la utilización de reacciones complejas en equipos caros, sofisticados y de requerimientos técnicos especializados. Actualmente, la tecnología LAMP puede ser una solución.

Objetivos

Nuestro grupo ha desarrollado métodos LAMP para el diagnóstico de diferentes NTD aplicados con éxito en condiciones de campo de zonas endémicas y ha establecido protocolos de estabilización de reacciones LAMP para su almacenamiento en formato kit *ready-to-use*. El objetivo de este trabajo es la fabricación de un dispositivo portátil capaz de realizar reacciones tipo LAMP con mezclas estabilizadas y monitorizar y gestionar el resultado a tiempo real mediante una aplicación de telefonía móvil.

XI Congreso Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). Retos en Salud Global. Ávila, 28-30 octubre de 2019

Materiales y Métodos

Se diseñó, desarrolló y fabricó un dispositivo económico, portátil y autónomo para realizar LAMP con lectura de amplificación de ADN con lectores RGB y un registro, visualización y gestión a tiempo real en un smartphone mediante una App para sistemas iOs y Android. El funcionamiento se validó con mezclas LAMP estabilizadas a distintos tiempos con trehalosa como crioprotector en presencia de verde de malaquita para la detección colorimétrica de la amplificación de ADN de *Schistosoma mansoni.*

Resultados

El dispositivo permitió la realización del LAMP y la detección colorimétrica por RGB de la amplificación de ADN de *S. mansoni* a tiempo real en muestras estabilizadas. La autonomía de funcionamiento con baterías resultó muy superior a la de otros dispositivos comerciales de mayores dimensiones, peso, y que utilizan para la determinación reactivos fluorescentes económicamente más costosos.

Conclusiones

Nuestro dispositivo, denominado "SMART-LAMP" (Sistema Múltiple de Análisis Remoto de Enfermedades Tropicales mediante LAMP), junto con el uso de mezclas LAMP estabilizadas en formato kit ha demostrado su utilidad como sistema de diagnóstico *point-of-care* potencialmente adaptable a cualquier NTD en condiciones de campo de zona endémica de enfermedad.

Financiación

RICET RD16/0027/0018 y PI16/01784. Subvencionados por el ISCIII. Cofinanciación con fondos FEDER "Una manera de hacer Europa". Contrato pre-doctoral cofinanciado por Universidad de Salamanca y Banco Santander. Ayuda a personal técnico de apoyo a la investigación. Sistema Nacional de Garantía Juvenil. Cofinanciación con Fondo Social Europeo, Iniciativa de Empleo Juvenil. BDNS: 376072.

RES0151 Aplicación del dispositivo SMART-LAMP para el diagnóstico point-of-care en campo: prueba piloto en Cayapas, Ecuador

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Introducción

Las técnicas moleculares tienen aún tres obstáculos para llegar del laboratorio al campo: la extracción del material genético de las muestras, la preparación y transporte de las mezclas de reacción y el equipo tecnológico para realizarlas.

Objetivos

Existe una alta prevalencia de infección por el parásito hepático *Amphimerus* sp. en comunidades Chachi en Río Cayapas (Esmeraldas, Ecuador). Nuestro grupo desarrolló un LAMP útil en la detección molecular de *Amphimerus* sp. en muestras de heces de población indígena (LAMPhimerus). Este estudio utiliza, por primera vez en zona endémica de amphimeriosis, el dispositivo SMART-LAMP para el análisis de muestras de orina utilizando mezclas de reacción estabilizadas en formato kit ready-to-use.

Materiales y Métodos

Se estabilizaron en el laboratorio mezclas de LAMPhimerus con trehalosa y verde de malaquita para la lectura colorimétrica de resultados en SMART-LAMP. Las mezclas se mantuvieron a temperatura ambiente (preparación, transporte y uso en campo). Se recogieron 30 muestras de orina y analizaron en el SMART-LAMP sin extracción de ADN. Además, muestras de sangre y heces se fijaron en papel y se transportaron al CIETUS (España) para realización de LAMP con extracción de ADN para comparar resultados.

Resultados

Utilizando el SMART-LAMP en campo, 18/30 muestras de orina (60%) resultaron positivas. En el laboratorio, 16/30 muestras de sangre y/o heces (53,3%) resultaron positivas mediante LAMP convencional. La coincidencia de resultados fue del 71,4%, llegando al 90,9% en las primeras muestras analizadas.

Conclusiones

Nuestro dispositivo SMART-LAMP ha sido utilizado por primera vez en condiciones de campo utilizando mezclas de reacción en formato kit para un diagnóstico point-of-care. La monitorización y gestión de resultados se realizó con éxito mediante la App para smartphone. El uso del SMART-LAMP con mezclas estabilizadas y muestras sin extracción de ADN es un sistema potencialmente útil en el diagnóstico de enfermedades infecciosas a pie de paciente, incluyendo NTDs para las que nuestro grupo ha desarrollado diferentes métodos LAMP.

Financiación

RICET RD16/0027/0018 y PI16/01784. Subvencionados por el ISCIII. Cofinanciación con fondos FEDER "Una manera de hacer

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Certifica que el trabajo

SMART-LAMP: diagnóstico molecular a pie de paciente mediante un dispositivo portátil y tecnología móvil.

de los autores

Juan García Bernalt Diego, Pedro Fernández Soto, Sergio Márquez Sánchez, Daniel Santos Santos, Begoña Febrer Sendra, Beatriz Crego Vicente, Juan Luis Muñoz Bellido, Moncef Belhassen García, Juan Manuel Corchado Rodríguez, Antonio Muro Álvarez

ha sido presentado como comunicación oral en el **XII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)**, celebrado del 8 al 10 de marzo de 2022 en el Museo Universidad de Navarra de Pamplona.

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Diseño, desarrollo y aplicación de un nuevo RT-LAMP para la detección del Virus de la Fiebre Hemorrágica de Crimea-Congo

de los autores

Begoña Febrer Sendra, Pedro Fernández Soto, Beatriz Crego Vicente, Juan García-Bernalt Diego, Anabel Negredo Antón, María Paz Sánchez Seco Fariñas, María Carmen Vieira Lista, Amparo López Bernús, Moncef Belhassen García, Juan Luis Muñoz Bellido, Antonio Muro Álvarez

ha sido presentado como comunicación oral en el **XII Congreso de la Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI)**, celebrado del 8 al 10 de marzo de 2022 en el Museo Universidad de Navarra de Pamplona.

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JUAN GARCÍA-BERNALT DIEGO [1], PEDRO FERNÁNDEZ-SOTO [1],
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Diagnóstico de parasitosis humana y animal

DIAGNÓSTICO MOLECULAR DE ENFERMEDADES PARASITARIAS A PIE DE PACIENTE MEDIANTE UN DISPOSITIVO PORTÁTIL Y TECNOLOGÍA MÓVIL: SMART-LAMP

KEYWORDS: Diagnóstico Molecular, Esquistosomosis, Estrongiloidosis, LAMP, Diagnóstico A Pie De Paciente.

INTRODUCTION | *RESUMEN*

Trasladar el diagnóstico molecular del laboratorio al campo, supone un gran reto sanitario a nivel global, especialmente en enfermedades parasitarias endémicas en zonas de limitados recursos. Gracias a la tecnología móvil, la posibilidad de analizar, procesar y almacenar resultados diagnósticos en las áreas más remotas se ha vuelto posible.

En este contexto se ha desarrollado el SMART-LAMP, un dispositivo portátil, construido por impresión 3D, alimentado por baterías recargables e intercambiables que permite el diagnóstico molecular de enfermedades parasitarias en unos minutos, mediante colorimetría a tiempo real, gracias a la tecnología de amplificación isotérmica tipo LAMP. A través de su integración con una aplicación en un smartphone, permite al analista gestionar de manera sencilla las condiciones de reacción, la información de pacientes y datos de geoposicionamiento de los análisis, así como la posibilidad de compartir los resultados de forma instantánea con otros investigadores o un laboratorio de referencia. El dispositivo, combinado con la estabilización de los reactivos LAMP, permite disponer de mezclas de reacción listas para su uso en formato kit almacenadas a temperatura ambiente durante varios meses, haciendo posible el diagnóstico molecular en zonas remotas de limitada infraestructura. El SMART-LAMP ha sido evaluado para el diagnóstico molecular de la infección por *Schistosoma mansoni, S. haematobium y Strongyloides spp.* con resultados de sensibilidad y especificidad cercanos o superiores al 90% en todos los casos, ofreciendo resultados a tiempo real comparables con los obtenidos con dispositivos comerciales mediante fluorescencia. En conclusión, la visualización a tiempo real de los resultados del diagnóstico molecular mediante cambio colorimétrico es simple, no requiere de formación avanzada, permite disponer de las mezclas de reacción preparadas listas para el uso, reduciendo significativamente el coste de las reacciones en comparación con dispositivos comerciales basados en fluorescencia. Además, es fácilmente adaptable al diagnóstico molecular de otras enfermedades parasitarias.

FUNDING | *FINANCIACIÓN*

Instituto de Salud Carlos III (Proyecto PI19/1727) y Consejería de Educación de la Junta de Castilla y León (Proyecto COV20EDU/00657). Cofinanciación con fondos FEDER (Fondo Europeo de Desarrollo Regional) "Una manera de hacer Europa". Contrato predoctoral cofinanciado por Universidad de Salamanca y Banco Santander. Programa de Becas Predoctorales de la Junta de Castilla y León cofinanciadas por el Fondo Social Europeo. BDNS (Identif.): 422058; BDNS (Identif.): 487971.

AII.3. Publicaciones docentes

Se recogen en esta sección las publicaciones docentes en las que se ha colaborado durante el tiempo de formación predoctoral en la Universidad de Salamanca. A continuación, se detalla una lista de las publicaciones y se incluye la primera página de cada una de ellas, en la que se encuentra su resumen.

1. Febrer-Sendra B, Crego-Vicente B, **García-Bernalt Diego J**, Fernández-Soto P. Tecnología LAMP: teoría, práctica y aplicación diagnóstica. Educafarma 7.0 Curso 2018/2019. White *papers* sobre innovación aplicada y divulgación científica en el área de las ciencias biosanitarias. 2020. ISBN: 978-84-09-17931-2.

2. Crego-Vicente B, **García-Bernalt Diego J**, Febrer-Sendra B, Fernández-Soto P. Selección de secuencias nucleotídicas y proteicas útiles para el diagnóstico y vacunas de enfermedades parasitarias. Educafarma 8.0. Curso 2019/2020. White *papers* sobre innovación aplicada y divulgación científica en el área de las ciencias biosanitarias. 2021. ISBN: 978-84-09-28512-9.

3. Febrer-Sendra B, Crego-Vicente B, **García-Bernalt Diego J**, Fernández-Soto P. Tecnología LAMP: diagnóstico molecular a pie de paciente. Educafarma 9.0 Curso 2020/2021. White *papers* sobre innovación aplicada y divulgación científica en el área de las ciencias biosanitarias. 2020. ISBN: 978-84-09-32855-0.

4. Febrer-Sendra B, Crego-Vicente B, **García-Bernalt Diego J**, Manzano-Román R, Fernández-Soto P. Diagnóstico molecular en enfermedades infecciosas. Educafarma 10.0 Curso 2021/2022. White *papers* sobre innovación aplicada y divulgación científica en el área de las ciencias biosanitarias. 2022. ISBN: 978-84-09-44271-3.

TECNOLOGÍA LAMP: TEORÍA, PRÁCTICA Y APLICACIÓN DIAGNÓSTICA

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PALABRAS CLAVE: NTD, Neglected Tropical Diseases, LAMP, Loop-mediated isothermal amplification, secuencias, primers

1.- INTRODUCCIÓN

La lucha contra las enfermedades tropicales desatendidas (NTD, del inglés *Neglected Tropical Diseases*), se ve muy afectada, entre otros factores, por las limitaciones que presentan los métodos diagnósticos clásicos. Generalmente, la microscopía carece de la sensibilidad necesaria y la serología, de la especificidad. Las técnicas moleculares clásicas (PCR y sus variantes) aunque poseen características de especificidad y sensibilidad, solo tienen una aplicación realista en laboratorios o clínicas donde se dispone tanto de un equipamiento especializado, como de personal entrenado. Esto sigue dificultando enormemente su utilización en zonas de escasos recursos, donde la incidencia de estas enfermedades es mucho más relevante.

Para solventar este problema, han surgido recientemente un grupo de técnicas isotérmicas de amplificación de ácidos nucleicos. Todas ellas siguen los principios básicos de la PCR, aunque no necesitan de un paso previo de desnaturalización de la doble hebra de ADN, ya que utilizan enzimas con actividad de desplazamiento de cadena que permiten realizar la reacción a temperatura constante sin depender de un termociclador, utilizando un simple termobloque o incluso un baño de agua.

Estas técnicas se dividen en tres grupos, en función de la cinética de reacción: lineales, exponenciales y en cascada (que combinan dos o más técnicas para la amplificación del ADN). Para el diagnóstico se utilizan las exponenciales, que nos permiten obtener una cantidad apreciable de ADN diana a partir de las ínfimas cantidades iniciales que suelen estar presentes en muestras de fluidos o tejidos de los pacientes. Entre ellas destacan: NASBA, SDA, RCA, LAMP, HDA, RPA y EXPAR (Zhao et al., 2015). Hasta la fecha, la más utilizada en el diagnóstico de NTD es la tecnología LAMP (del inglés, *Loop-mediated isothermal amplification*) (Notomi et al., 2000).

SELECCIÓN DE SECUENCIAS NUCLEOTÍDICAS Y PROTEICAS ÚTILES PARA EL DIAGNÓSTICO Y VACUNAS DE ENFERMEDADES PARASITARIAS

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PALABRAS CLAVE: NTD, Neglected Tropical Diseases, LAMP, Loop-mediated isothermal amplification, secuencias, primers

1.- INTRODUCCIÓN. DIAGNÓSTICO MOLECULAR MEDIANTE TECNOLOGÍA LAMP. IMPORTANCIA Y APLICACIÓN

La lucha contra las enfermedades tropicales olvidadas (NTDs) es uno de los principales retos de la OMS. Se trata principalmente de enfermedades infecciosas que proliferan en lugares de escasos recursos, donde es necesario un buen diagnóstico y tratamiento. Los métodos de diagnóstico habituales se centran en técnicas de microscopía y serología con problemas de sensibilidad y especificidad. El surgimiento de técnicas moleculares, como la PCR, son una alternativa más fiable, sin embargo, requieren de equipamiento especial y personal cualificado lo que supone un problema para su aplicación en zona endémica. Una alternativa es el uso de técnicas isotérmicas de amplificación de ácidos nucleicos como la tecnología LAMP (del inglés, *Loop-mediated isothermal amplification*).

Para la aplicación de esta técnica LAMP (Figura 1) basta con un termobloque o baño de agua capaz de mantener una temperatura constante. Su funcionamiento se basa en el uso de 4 o 6 primers capaces de reconocer respectivamente 6 u 8 secuencias específicas dentro de la región de amplificación. Además, se utiliza una *Bst* polimerasa de *Bacillus stearothermophylus* con actividad de desplazamiento de cadena. La cantidad de ADN generada es mayor a la de otras técnicas moleculares y da lugar a fragmentos de distintos tamaños de ADN. Así, la obtención de resultados se puede llevar a cabo mediante diferentes métodos de detección: **electroforesis en gel de agarosa** donde se visualiza un patrón en escalera característico de los productos de reacción; **turbidez** causada por el precipitado de un subproducto de la reacción (pirofosfato de magnesio); **colorantes** que permiten la observación directa de un cambio colorimétrico en función del resultado, donde podemos distinguir entre colorantes intercalantes en el ADN como el SYBR™ Green y EvaGreen®, e indicadores de metal como calceína y verde de malaquita; **florescencia a tiempo real** capaz de cuantificar a medida que avanza la

Tecnología LAMP: diagnóstico molecular a pie de paciente.

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PALABRAS CLAVE: LAMP, Loop-mediated isothermal amplification, point-of-care, multiplex-LAMP, sequences, primers design

INTRODUCCIÓN

Desde el revolucionario desarrollo de la reacción en cadena de la polimerasa (PCR) en la década de 1980, las pruebas de amplificación de ácidos nucleicos (NAAT) se han convertido en una herramienta imprescindible tanto en el laboratorio como en la rutina clínica. Sin embargo, las limitaciones de la PCR, entre las que destacan la necesidad de equipos sofisticados y complejos, así como de un laboratorio equipado y personal capacitado, provocaron una búsqueda de alternativas más simples y asequibles, resultando en el desarrollo de varias NAAT isotérmicas. Estas técnicas, a diferencia de la PCR, no necesitan de un paso previo de desnaturalización de la doble hebra de ADN, ya que utilizan enzimas con actividad de desplazamiento de cadena que permiten realizar la reacción a temperatura constante sin depender de un termociclador, utilizando un simple termobloque. Hasta la fecha, la más utilizada en el diagnóstico de NTD (del inglés, *Neglected Tropical Diseases*) es la tecnología LAMP (del inglés, *Loop-mediated isothermal amplification*) (Notomi *et al.,* 2000).

Esta tecnología, se basa en el uso de 4 o 6 *primers* capaces de reconocer 6 u 8 secuencias específicas dentro de la región de amplificación. Además, la obtención de los resultados se puede llevar a cabo mediante deferentes métodos de detección: **electroforesis en gel de agarosa**; **turbidez** causada por el precipitado de un subproducto de la reacción (pirofosfato de magnesio); **colorantes** que permiten la observación directa de un cambio colorimétrico en función del resultado, donde podemos distinguir entre colorantes intercalantes del ADN (como el SYBR Green y EvaGreen) e indicadores de metal (como calceína y verde malaquita); **fluorescencia a tiempo real,** capaz de cuantificar la señal de florescencia generada en la reacción de amplificación de ADN, gracias al uso de termocicladores convencionales a tiempo real o dede dispositivos portátiles, como por ejemplo el Genie® III (Optigen, UK); **ensayos de flujo lateral,** que combinan la técnica LAMP con el uso de tiras inmunocromatográficas (Wong et al. 2018).

DIAGNÓSTICO MOLECULAR EN ENFERMEDADES PARASITARIAS

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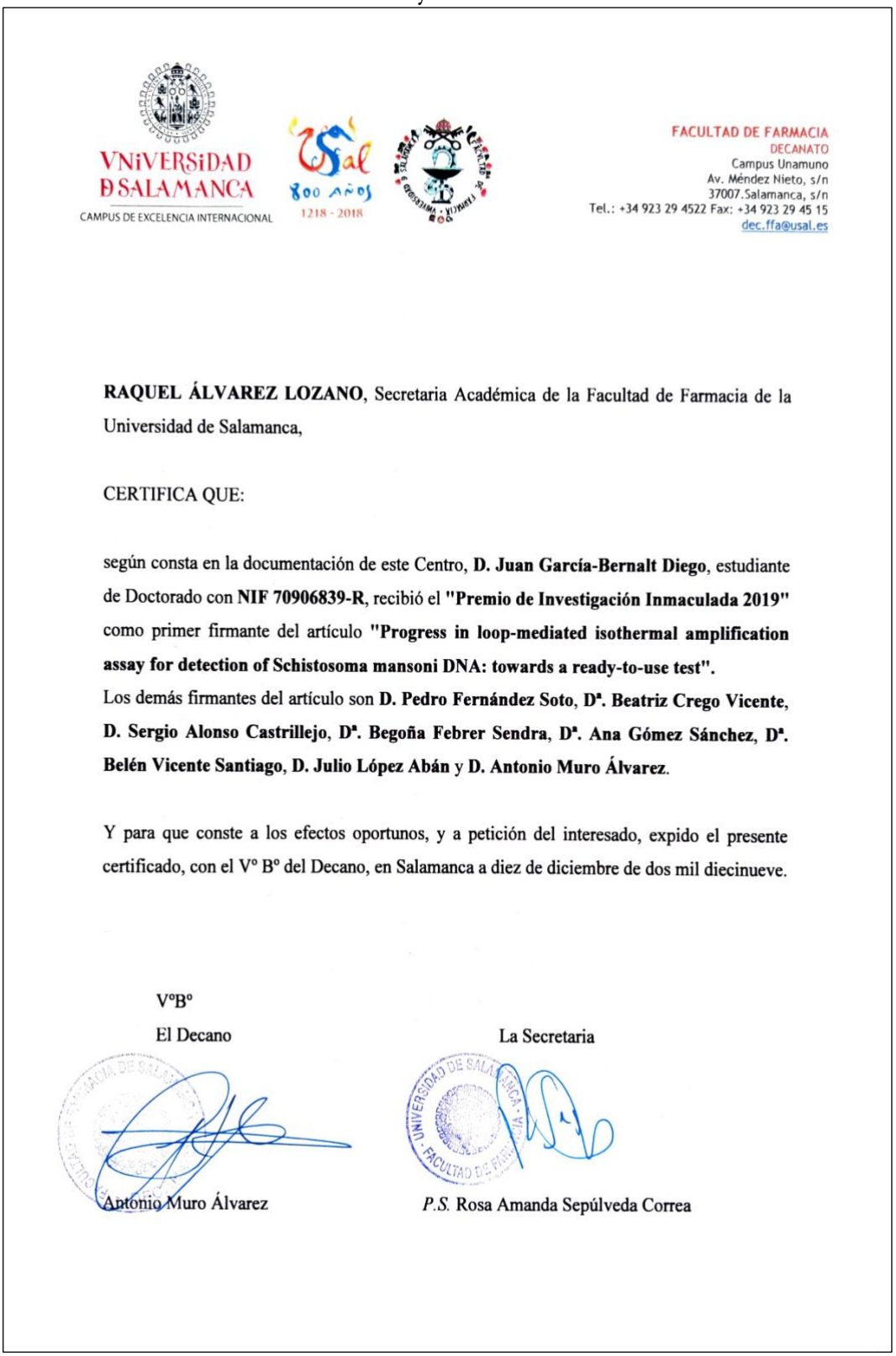
PALABRAS CLAVE: LAMP, Loop-mediated isothermal amplification, point-of-care, multiplex-LAMP, sequences, primers design

1. TÉCNICAS DE AMPLIFICACIÓN ISOTÉRMICA DE ÁCIDOS NUCLEICOS Y LAMP

Desde el revolucionario desarrollo de la reacción en cadena de la polimerasa (PCR) en la década de 1980, las pruebas de amplificación de ácidos nucleicos (NAAT) se han convertido en una herramienta imprescindible tanto en el laboratorio como en la rutina clínica. Sin embargo, las limitaciones de la PCR, entre las que destacan la necesidad de equipos sofisticados y complejos, así como de un laboratorio equipado y personal capacitado, provocaron una búsqueda de alternativas más simples y asequibles, resultando en el desarrollo de varias NAAT isotérmicas. Estas técnicas, a diferencia de la PCR, no necesitan de un paso previo de desnaturalización de la doble hebra de ADN, ya que utilizan distintas enzimas que permiten realizar la reacción a temperatura constante sin depender de un termociclador, utilizando un simple termobloque o baño de agua. Hay numerosos ejemplos de estas técnicas, y nuevos desarrollos amplían regularmente esta lista. Un resumen de las principales técnicas de amplificación isotérmica se puede encontrar en la Tabla 1.

AII.4. Premios de investigación

Se incluyen dos premios de investigación otorgados por el Decanato de la Facultad de Farmacia a las mejores publicaciones científicas presentadas por estudiantes de doctorado en las convocatorias de 2019 y 2021.



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RAQUEL ÁLVAREZ LOZANO, Secretaria Académica de la Facultad de Farmacia de la Universidad de Salamanca,

CERTIFICA QUE:

según consta en la documentación de este Centro, **D. Juan García-Bernalt Diego**, estudiante de Doctorado con **NIF 70906839-R**, recibió el **"Premio de Investigación Inmaculada 2019"** como primer firmante del artículo **"Progress in loop-mediated isothermal amplification assay for detection of Schistosoma mansoni DNA: towards a ready-to-use test".**
Los demás firmantes del artículo son **D. Pedro Fernández Soto**, **Dª. Beatriz Crego Vicente**, **D. Sergio Alonso Castrillejo**, **Dª. Begoña Febrer Sendra**, **Dª. Ana Gómez Sánchez**, **Dª. Belén Vicente Santiago**, **D. Julio López Abán** y **D. Antonio Muro Álvarez**.

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 diez de diciembre de dos mil diecinueve.

Vº Bº
El Decano

Antonio Muro Álvarez

La Secretaria

P.S. Rosa Amanda Sepúlveda Correa

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Dª. ROSA AMANDA SEPÚLVEDA CORREA, Secretaria de la Facultad de Farmacia de la Universidad de Salamanca,

CERTIFICA QUE:

Según consta en la documentación de este Centro, D.ª BEATRIZ CREGO VICENTE, estudiante de Doctorado con NIF 70888306-Y, recibió el "Premio de Investigación Experimental INMACULADA 2021" como primer firmante del artículo "Application of a Genus-Specific LAMP Assay for Schistosome Species to Detect *Schistosoma haematobium x Schistosoma bovis Hybrids*".

Los demás firmantes del artículo son D. Pedro Fernández-Soto, Dª. Begoña Febrer-Sendra, D. Juan García-Bernalt Diego, D. Jerôme Boissier, D. Etienne K. Angora, Dª. Ana Oleaga y D. Antonio Muro.

Y para que conste a los efectos oportunos, y a petición de la interesada, expido el presente certificado, con el V.º B.º de la Decana, en Salamanca a veintiuno de diciembre de dos mil veintiuno.

V.º B.º

LA DECANA

MORALES MARTIN ANA ISABEL - 11764194Q

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Fdo.: Ana Isabel Morales Martín

LA SECRETARIA

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AII.5. Estancias en el extranjero

Se adjunta certificado de estancia de investigación en Icahn School of Medicine at Mount Sinai, New York, Estados Unidos; realizada por el doctorando para cumplir con los requisitos necesarios de mención internacional de esta tesis doctoral.

Icahn School of Medicine at Mount Sinai

Adolfo García-Sastre, Ph.D.
Director
Global Health and Emerging Pathogens Institute
Professor of Microbiology
Fishberg Professor of Medicine, Infectious Diseases
Professor, The Tisch Cancer Institute

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adolfo.garcia-sastre@mssm.edu

May 7, 2021

Re: Visiting Researcher Invitation Letter for Juan García-Bernalt Diego

Dear Juan,

The Department of Microbiology, Icahn School of Medicine at Mount Sinai would like to extend our invitation to you for the duration on or around 09/06/2021 – 12/17/2021 as a Visiting Researcher. Dr. Adolfo García-Sastre will act as your supervisor.

You will take part in research currently being done involving the new SARS-CoV-2 variants and how the new mutations affect immunity. You will perform basic molecular biology as well as virology techniques such as cell culture, virus infection, viral titration, Western Blot, mutagenesis and cloning.

Your participation is solely for the purposes of observation, consultation, teaching, or research. No element of patient care services will be involved.

You will receive funding through a PhD fellowship from the University of Salamanca in the amount of $1,300 per month for the duration of your stay in addition to personal funds. Additionally, Dr. García-Sastre will pay for the cost of housing up to $1,200 per month.

Should the conditions of your J-1 sponsorship change, you must contact International Personnel, Mount Sinai Medical Center before the changes take place.

Sincerely,

Adolfo García-Sastre, Ph.D.