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Short Communication

Development of a Digital Droplet Polymerase Chain Reaction (ddPCR) assay to detect *Leishmania* DNA in samples from Cutaneous Leishmaniasis patients

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ABSTRACT

Aim: Here, we evaluate the ddPCR platform using an evaluated qPCR-based diagnostic assay for the detection of *Leishmania* infection in Cutaneous Leishmaniasis patients.
Methods: A standard curve of cultured *Leishmania* parasite material and clinical samples of CL patients were tested with ddPCR to determine the analytical and diagnostic performance.
Results: The limit of detection of the assay on the ddPCR platform was much higher than the published limit of detection of the same assay on the qPCR platform (100 vs 1 parasites/mL, respectively).
Conclusion: While the performance of this assay in ddPCR format was acceptable for research purposes, it is not sufficient for clinical diagnostic purposes. The assay is more suited to the qPCR platform.

Cutaneous leishmaniasis (CL) caused by *Leishmania* species is characterized by the presence of ulcerative lesions leading to disfiguring and/or incapacitating scars (Akhoundi et al., 2017; Desjeux, 2001; Alvar et al., 2012; Jara et al., 2013). Traditionally, microscopic examination is considered the diagnostic routine method for CL. However, its sensitivity is low and molecular methods have shown increased sensitivity. Recently, our group reported the first analytical validation of the L18S target using PCR, qPCR and LAMP platforms for New World *Leishmania* species (León et al., 2017, 2018). Different authors have also conducted this across Latin America (Moreira et al., 2018). Droplet digital PCR (ddPCR) enables the absolute quantitation of nucleic acids in a sample and has subsequently been applied to detect DNA of pathogens. Therefore, the aim of this work was to develop and test a ddPCR assay and compare its results with those obtained by the previously analytically validated qPCR assay.

L. braziliensis, L. panamensis, L. major, L. donovani, L. infantum, L. guyanensis and *L. mexicana* were used to construct Standard curves diluting each parasite culture eluate in ten-fold dilution steps from 10^{5}–10^{-2} parasites/mL. For ddPCR, a new dual-labelled probe was designed using Primer Express 3 software (L18SPr 5′-[6FAM] AGT TCG GGG GAG AAC GTA CT [BHQ1]-3′), and checked using AmpliFx. An endogenous control assay (*Homo sapiens* ribonuclease P/MRP subunit p30 [HsRPP30]) was described by Luo et al., (2005). The L18S primers were previously reported in León et al. (2017)

The *L. braziliensis* standard curve was repeat tested five times within-plate and on five different plates to assess the technical performance of the assay. The analytical limit of detection was determined as the concentration of the lowest step of this series at which all 10 replicates tested positive. In order to test the diagnostic performance of ddPCR, 138 samples previously diagnosed as positive by microscopy and qPCR and 83 samples that were negative by microscopy and qPCR were tested with ddPCR.

During repeat testing of the *L. braziliensis* standard curve, the lower limit of detection was 100 parasites/mL (6 copies/μL by ddPCR). The Pearson Correlation Coefficient between L18S copies/μL (ddPCR) and parasites/mL (qPCR) in the dilution steps where all

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repeat tests were detected was 0.983 and the efficiency of the assay was 106% (Figure 1A). Upon testing of a number of different *Leishmania* species, the L18S assay detected all the different species tested (Figure 1B). The mean between-plate coefficient of variance (CV) across the whole series was 17.8%, and the CV at 100 parasites/mL is much higher than at 100,000 parasites/mL (47.2 compared to 3.6%). The mean within-plate CV was 30.0%, and the CV did not vary very much throughout the curve (range: 27.7–32.6%). The total coefficient of variance across both of these sources of variation was 53.6%. Finally, the ddPCR CV is not significantly different to the CV of qPCR at either the lowest concentration of the dilution series (100 parasites/mL qPCR CV = 28.8% versus ddPCR CV = 27.7%) but was higher than the qPCR CV at the highest concentration of the series (100,000 parasites/mL qPCR CV = 17.4% versus ddPCR CV = 31.3%; Figure 1C).

The ROC plot for the comparison of diagnostic performance at different zeta values is displayed in Figure 1D. A zeta value of 0.85–0.90 resulted in the highest area under the curve in the ROC analysis (0.832), and at that level the optimised sensitivity and specificity of ddPCR compared to qPCR were 84 and 85%, respectively. However, given there are a number of other causes of skin lesions in tropical settings, we decided specificity >90% should be prioritized. We therefore considered a zeta value of 0.95 (95% probability of non-zero load) to be optimal for this test. The area under the curve at that level was 0.822.

In conclusion, we report the first evaluation of the suitability of a ddPCR platform to detect *Leishmania* DNA in specimens obtained from patients with CL. The technical and clinical performance of the ddPCR assay evaluated here does not support its routine usage over qPCR, mainly due to its lack of sensitivity and specificity. In addition, ddPCR is three times more expensive than qPCR and not suitable as a routine diagnostic test. However, ddPCR shows several advantages as quantitation of DNA without the need for calibration curves and therefore exact and reference values could be obtained per sample (higher-order reference measurement method) as has been suggested for various viral diseases (Hayden et al., 2013; Rutsaert et al., 2018). The platform may also to be evaluated with other oligonucleotide assays to further investigate its potential for the molecular diagnosis of CL.

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Conflict of interest

None.

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Ethical approval

The study protocol was approved by the Technical Research Committee and Ethics Research Board at the National Health Institute in Bogotá, Colombia, protocol CTIN-023-17 in the framework of the project “FORTALECIMIENTO Y VIGILANCIA DE LA CAPACIDAD DIAGNOSTICO DE ENFERMEDADES EMERGENTES Y REEMERGENTES EN COLOMBIA”. Participation (Only adults) was voluntary and patients were asked for informed written consent. The diagnostic evaluation protocol was additionally approved by the London School of Hygiene & Tropical Medicine Observational Ethics Committee (protocol number 15515).

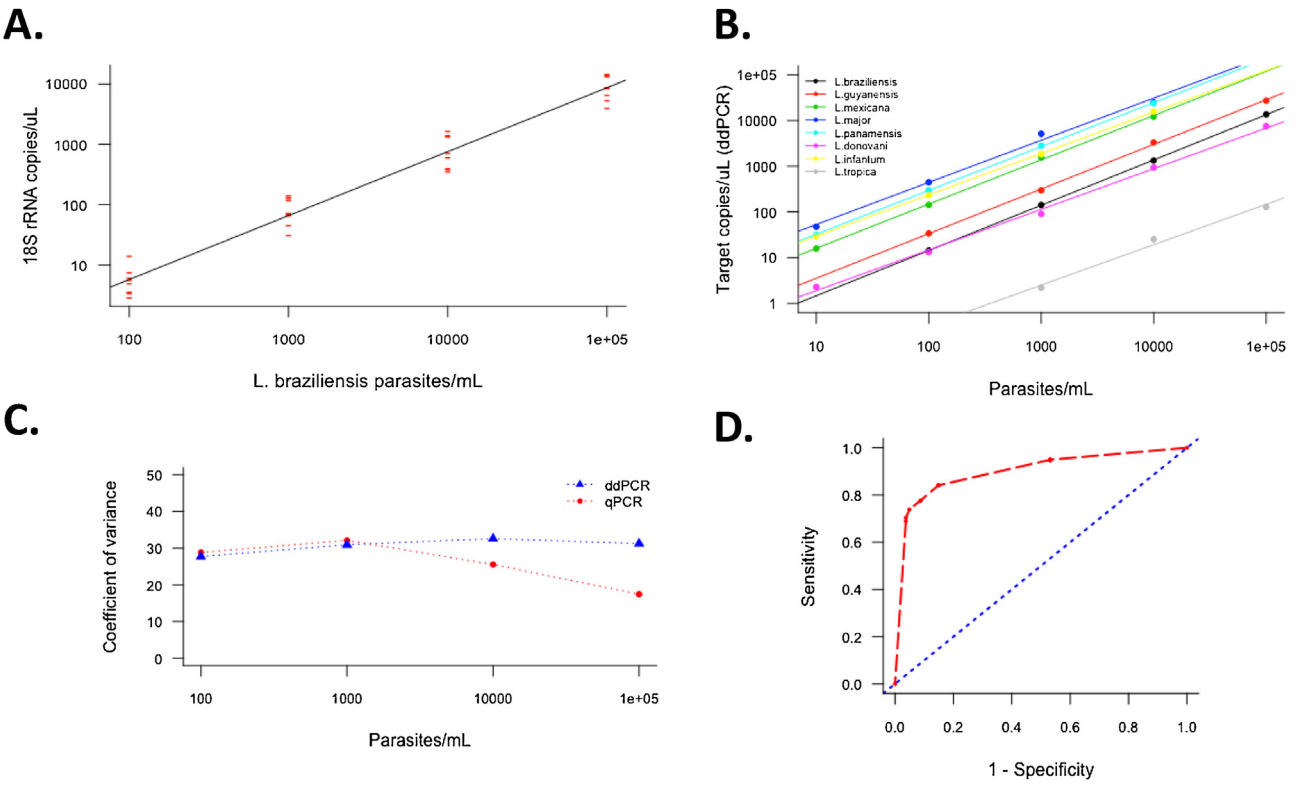

Figure 1. A. Serial ten-fold dilution of *L.braziliensis* repeat tested with L18S assay on ddPCR platform. B. Serial ten-fold dilution of several *Leishmania* species on ddPCR platform. C. Within-plate variance of L18S assay run on qPCR and ddPCR platforms across a range of parasite loads. D. ROC curve results compating ddPCR vs. qPCR.

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