

# Quantitative monitoring of experimental and human leishmaniasis employing amastigote-specific genes

## Research Article

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### Abstract

The gold standard for diagnosis of leishmaniasis is the microscopic detection of amastigotes/Leishman Donovan (LD) bodies, but its moderate sensitivity necessitates the development of molecular approaches. This study aimed to quantify in experimental animal models and human leishmaniasis the expression of amastigote-specific virulence genes, *A2* and *amastin* by droplet digital polymerase chain reaction (ddPCR). Total RNA was isolated from *L. donovani*-infected hamsters or murine peritoneal macrophages and lesional biopsies from patients with post kala-azar dermal leishmaniasis (PKDL). Following cDNA conversion, EvaGreen-based ddPCR was performed using specific primers for *A2* or *amastin* and parasite load expressed in copies per  $\mu\text{L}$ . Assay was optimized and the specificity of amastigote-specific *A2* and *amastin* was confirmed. In hepatic and splenic tissues of *L. donovani*-infected hamsters and peritoneal macrophages, ddPCR demonstrated a greater abundance of *A2* than *amastin*. Treatment of *L. donovani*-infected peritoneal macrophages with conventional anti-leishmanials, miltefosine and amphotericin B translated into a dose-dependent reduction in copies per  $\mu\text{L}$  of *A2* and *amastin*, and the extrapolated  $\text{IC}_{50}$  was comparable with results obtained by counting LD bodies in Giemsa-stained macrophages. Similarly, in dermal biopsies of patients with PKDL, *A2* and *amastin* were detected. Overall, monitoring of *A2* by ddPCR can be an objective measure of parasite burden and potentially adaptable into a high throughput approach necessary for drug development and monitoring disease progression when the causative species is *L. donovani*.

## Introduction

Leishmaniasis is caused by the digenetic parasite *Leishmania* which are flagellated promastigotes within the sandfly vector and aflagellated amastigotes within susceptible host macrophages (Sunter and Gull, 2017; Bates, 2018). Leishmaniasis has a diverse disease spectrum that ranges from a self-limiting cutaneous lesion to the fatal form of visceral leishmaniasis (VL), while has a dermal sequel, post kala-azar dermal leishmaniasis (PKDL; Chappuis *et al.*, 2007; Zijlstra *et al.*, 2017; Gedda *et al.*, 2020). The study of human leishmaniasis is facilitated using Syrian golden hamsters as it shares pathological features and immunological responses comparable with humans (Melby *et al.*, 2001; Garg and Dube, 2006; Saini and Rai, 2020; Moulik *et al.*, 2021a).

Animal models are an invaluable resource to screen for potential anti-leishmanials using *in vivo/ex vivo* approaches (Vermeersch *et al.*, 2009). Generally, the initial screening is in *Leishmania*-infected macrophages and the number of amastigotes in host cells is a direct measure of drug activity (Escobar *et al.*, 2002; Yardley *et al.*, 2005). This requires microscopic counting and being a subjective approach, is susceptible to operator bias. To obviate this subjectivity, molecular approaches are the need of the hour and preferably those that can identify amastigotes.

A molecular target should have high abundance and this criterion is best achieved by kinetoplast mini-circle DNA (kDNA), present as 1000 copies per cell in all *Leishmania* spp. (Salotra *et al.*, 2001; Mary *et al.*, 2004). In fact, the kDNA-based qPCR or real-time PCR has stood the test of time in determining parasite load (Mary *et al.*, 2006; Verma *et al.*, 2010; Sudarshan *et al.*, 2011; Abbasi *et al.*, 2013; Hossain *et al.*, 2017; Moulik *et al.*, 2018, 2021b). However, for *ex vivo* drug screening, there is no assay that can selectively detect amastigotes, reflecting the need to further optimize molecular methods of detection.

In terms of amastigote-specific genes, the most well studied is the *A2* gene family first identified in *L. donovani* amastigotes translating into a family of *A2* proteins ranging from 45 to 100 kDa (Charest and Matlashewski, 1994; Avishek *et al.*, 2018). The *A2* protein is also expressed at high levels in axenic amastigotes (Matlashewski, 2001). Another important amastigote-specific gene is *amastin* present as a surface protein (Johnston *et al.*, 1999). It contains a unique, highly conserved 11-amino acid signature at the N terminus. The presence of *amastin* has been reported in *Trypanosoma cruzi* (McCall and Matlashewski, 2010) and *Leishmania infantum* amastigotes (Rochette *et al.*, 2005). The latex agglutination test (LAT), a rapid diagnostic test for detection of anti-*Leishmania* antibodies, utilizes the *A2* antigen and exhibited accuracy comparable to direct agglutination test (Akhoundi *et al.*, 2013). The

amastigote-derived A2 protein has also been investigated for antibody responses in India, Sudan and South America with variable sensitivities (Carvalho *et al.*, 2002). Similarly, amastin peptides have been employed for the serodiagnosis of active VL (Rafati *et al.*, 2006).

The droplet digital polymerase chain reaction (ddPCR) detects samples with a low copy number of the target nucleic acid and additionally allows direct quantification without the need for a standard curve (Belmonte *et al.*, 2016). The traditional qPCR uses an analogue approach as it quantifies samples by comparing their cycle threshold ( $C_t$ ) to a standard curve generated by well-defined samples. However, development of such a standard curve for detection of amastigotes is challenging as they are present within host macrophages and it is not feasible to quantify the exact burden of amastigotes per macrophage. The advent of the analytical ddPCR assay and it not requiring a standard curve makes it a value addition in the field of nucleic acid quantification (Gobert *et al.*, 2018). The principle for ddPCR is based on the reaction mixture being divided into hundreds to millions of partitions (droplets in oil emulsion), and each droplet being capable of initiating an independent PCR reaction. At the end-point, the partitions are scored as positive or negative and these values are used to calculate the target concentration using binomial Poisson statistics (Pinheiro *et al.*, 2012). Using statistical software, the target gene copy number is automatically determined based on the number of droplets positive for amplification of the target nucleic acid (Cai *et al.*, 2014; Floren *et al.*, 2015). In view of limited molecular assays to quantify the disease burden in terms of amastigotes, this study aimed to develop a ddPCR-based semi-automated approach for quantifying the expression of amastigote-specific genes A2 and *amastin* adaptable for parasitological detection in (i) drug screening assays and (ii) PKDL.

## Materials and methods

### Reagents

All reagents were of analytical grade and obtained from Bio-Rad Laboratories (Hercules, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), except cDNA Reverse Transcription kit and SYBR Green Quantitative PCR (qPCR) Master Mix from Applied Biosystems (Grand Island, NY, USA), Trizol reagent and RNALater (Ambion, Austin, TX, USA) and fetal bovine serum (FBS, Gibco, Thermo Fischer Scientific, Waltham, MA, USA).

### Parasite culture

*Leishmania donovani* (MHOM/IN/1983/AG83) promastigotes were maintained at 24°C in M199 medium supplemented with 10% heat-inactivated FBS, penicillin G (50 IU mL<sup>-1</sup>) and streptomycin (50 µg mL<sup>-1</sup>); parasites were sub-cultured every 48–72 h, inoculum being  $1 \times 10^6$  cells mL<sup>-1</sup>.

### Experimental models of visceral leishmaniasis

Hepatic and splenic tissues were obtained from *L. donovani*-infected male golden hamsters (*Mesocricetus auratus*, 45–50 g,  $n = 10$ ) maintained in the Division of Parasitology, Central Drug Research Institute (CDRI), Lucknow, India, and kindly provided by Dr Anuradha Dube, CDRI, Lucknow. Tissue samples were stored in RNALater at –80°C.

Peritoneal macrophages were isolated from male/female Swiss albino mice (6–10 weeks old, 20–25 g) housed in the animal facility, IPGME&R, Kolkata, India under controlled temperature (22 ± 5°C), humidity (60 ± 5%) and a 12 h light/dark cycle. Stationary 5–7 days old promastigote cultures were used for

*ex vivo* infection. Briefly, following an IP administration of 10–15 mL chilled phosphate-buffered saline (0.02 M phosphate, pH 7.2, PBS), the peritoneal exudate was aspirated and resuspended in RPMI medium (Phenol Red+) supplemented with 10% FBS, penicillin G (50 IU mL<sup>-1</sup>) and streptomycin (50 µg mL<sup>-1</sup>).

The peritoneal exudate was seeded ( $\sim 2 \times 10^6$  mL<sup>-1</sup>) in 12-well plates; following an overnight incubation (37°C, 5% CO<sub>2</sub>), the non-adherent cells were removed and adherent macrophages infected with stationary phase promastigotes at a multiplicity of infection of 10:1 (parasite: macrophage) for 48 h (Karmakar *et al.*, 2019). Subsequently, anti-leishmanials, miltefosine (HePC, 0–5 µM) or amphotericin B (AmphoB, 0–50 nM) were added and after an additional 48 h, the cells were recovered by gentle scraping, and the cell pellets stored in RNALater at –80°C. Alongside, infected peritoneal macrophages with and without drug treatments were stained with Giemsa and independently examined in a blinded manner under a bright-field light microscope (EVOS FL Cell Imaging System, Waltham, MA, USA), the % infectivity and number of intracellular amastigotes was counted in at least 100 macrophages/slide.

### Study population

Patients with a clinical diagnosis of PKDL ( $n = 10$ ) were recruited either from Dermatology Outpatient Departments of School of Tropical Medicine/Calcutta Medical College/IPGME&R, Kolkata or from active field surveys conducted in endemic districts of West Bengal (Malda, Dakshin Dinajpur, Murshidabad and Birbhum) by a camp approach, using standard case definitions and defined risk factors, for example, living in an endemic area and having an epidemiological link (past history of VL). The diagnosis of PKDL was primarily based on clinical features and a history of VL, rK-39 strip test positivity and/or confirmed by demonstration of LD bodies by Giemsa staining, and/or ITS-1 PCR (Sengupta *et al.*, 2019). Informed consent was taken for a 4 mm punch biopsy. Cases with hypopigmented macules were considered as macular PKDL, whereas an assortment of papules, nodules, macules and/or plaques was termed as polymorphic PKDL. None suffered from any co-infection or pre-existing disease, and pregnant women were excluded. Samples were collected at disease presentation. For controls, skin biopsies ( $n = 10$ ) from foreskin of males undergoing voluntary circumcision were obtained. Dermal biopsies were stored in RNALater at –80°C.

### Optimization and determination of copy number of target genes using droplet digital PCR

The assay was performed using total RNA isolated by the Trizol method from *Leishmania*-infected murine peritoneal macrophages, hepatic or splenic tissues from hamsters ( $n = 10$ ) and dermal biopsies of patients with PKDL ( $n = 10$ ). The concentration of RNA was measured in Nanodrop™ One/One<sup>C</sup> Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific, MA, USA) and converted to single-stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, MA, USA), according to the manufacturer's instructions. The final concentration of prepared cDNA was 1 µg (for a 20 µL reaction) and detection of amplicons was done using primers (sourced from NCBI Primer – BLAST, <https://www.ncbi.nlm.nih.gov/tools/primer-blast>) whose specificity was confirmed by UCSC In-Silico PCR for amastigote-specific genes A2 (F, 5'-CTGCAGGCTGTTGACGTTTC-3'; R, 5'-AAGGTTTGCCTCGTCACCAT-3') and *amastin* (F, 5'-GTGCATCGTGTTCATGTTCC-3'; R, 5'GGGCGGTAGTCGTAATTGTT-3'). Absolute quantification was performed by ddPCR using primers for A2 and *amastin*, and expression determined as copies per µL using QuantaSoft software (version 1.7.4, Bio-Rad Laboratories).

Briefly, the optimized reaction mixture containing cDNA (50 ng) was added to a ddPCR EvaGreen Supermix (Bio-Rad Laboratories, CA, USA) containing  $100 \text{ nM mL}^{-1}$  of each forward and reverse primer, final volume being  $20 \mu\text{L}$ . For negative controls (NC),  $10 \mu\text{L}$  nuclease-free water was added along with  $10 \mu\text{L}$  of ddPCR Eva Green Super mix, and in case of non-template control (NTC), nuclease-free water was used instead of cDNA. Each reaction was then loaded into a sample well of an 8-well disposable cartridge (DG8), along with  $70 \mu\text{L}$  of droplet generation oil. Droplets were formed using QX200 droplet generators which were then transferred to a 96-well PCR plate to perform PCR of 40 cycles at  $95^\circ\text{C}$  for 5 min,  $95^\circ\text{C}$  for 30 s, annealing ( $58\text{--}62^\circ\text{C}$ ) for 1 min, with a final extension at  $90^\circ\text{C}$  for 5 min. In ddPCR, the reaction mixture is partitioned into tens of thousands of droplets, with each droplet being an independent PCR reaction. The target template copy number is determined based on the number of droplets positive for amplification of the target template and these values help to calculate the target concentration using binomial Poisson statistics (Pinheiro *et al.*, 2012). The resultant products were scanned on a QX200 Droplet Reader, and data analysed using QuantaSoft software (version 1.7.4, Bio-Rad Laboratories; Dighal *et al.*, 2020) and expressed as copies per  $\mu\text{L}$  with confidence intervals of 95%. The threshold was adjusted based on clear demarcation of positive droplets from the negative cluster with respect to individual wells (Supplementary Fig. S1). The background signals were eliminated using an NC and NTC.

Optimization of ddPCR reaction conditions was performed with (a) varying amounts of target cDNA obtained from infected liver and splenic tissues of hamsters (12.5–100 ng), (b) annealing temperature ( $58\text{--}62^\circ\text{C}$ ) and (c) primer concentration ( $100\text{--}500 \text{ nM mL}^{-1}$ ). The optimal expression of 6.0 and 1.8 copies  $\mu\text{L}^{-1}$  for A2 and *amastin*, respectively, was detected with 50 ng template,  $100 \text{ nM mL}^{-1}$  of primer,  $T_m$  at  $58^\circ\text{C}$  (Supplementary Figs S2–S4 and Table S1). A dramatic drop in expression was observed when the template concentration was  $100 \text{ ng } \mu\text{L}^{-1}$ , attributable to excess of template causing inhibition of PCR reaction. Additionally, with increasing concentration of template, there may be exhaustion of products like oligos, primer/probe, etc., accounting for the drop in signal amplification. It may also result in the formation of packed DNA structures within the confined space of the reaction tube, thereby increasing the chance of false priming and cause a poor yield (<https://www.caister.com/highveld/pcr/pcr-troubleshooting>). These standardized parameters were considered as optimal conditions and subsequently, the detection of amastigote load was performed.

#### Controls for droplet digital PCR

Both NC and NTC were used to define the cut-off. NC, i.e. template negative for amplification by A2 and *amastin*, included (i) uninfected hepatic and splenic tissues, (ii) uninfected peritoneal macrophages, (iii) preputial skin biopsies from males undergoing voluntary circumcision and (iv) promastigotes. NTC included the use of nuclease-free water as template.

#### Determination of the cut-off for droplet digital PCR

The ddPCR assay determined NTC as ‘No Call’ with a single threshold line for A2 and *amastin* based on no copies detected using the QuantaSoft™ software (black droplets). Samples were marked as positive (blue droplets) if more than 4 fluorescent signal events were shown above the threshold line to avoid false positivity. The cut-off for the ddPCR assay was defined based on negative samples as they did not show more than 2 fluorescent signals. The assay sensitivity was validated by determining the

absolute limit of detection (aLOD), i.e. the lowest target copy number in a sample that can be reliably detected, but not necessarily quantified. The absolute limit of quantification (aLOQ) is the lowest target copy number in a sample that can be reliably quantified with an acceptable level of precision and accuracy (Holst-Jensen *et al.*, 2003); in ddPCR, the aLOQ was equivalent to aLOD. The sensitivity of the performed assay was evaluated by the receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) values with 95% confidence interval. The linearity of the optimized ddPCR assay was determined and the linear regression was established as  $R^2$ ; values  $>0.7$  were considered as a strong and effective coefficient parameter.

#### Measurement of parasite load by real-time polymerase chain reaction (qPCR)

DNA extraction was performed according to the manufacturer’s instructions (QIAamp DNA Mini Kit, Qiagen, NW, Germany) from tissue biopsies collected in phosphate-buffered saline (20 mM, pH 7.4), excised into small pieces and eluted in  $50 \mu\text{L}$  of DNA elution buffer. For measurement of parasite load in hepatic and splenic tissues sourced from infected hamsters and in patients with PKDL, a standard curve was generated and real-time PCR was performed (Ghosh *et al.*, 2015; Moulik *et al.*, 2018). The parasite number when  $<10$  reported a cycle threshold ( $C_t$ ) value almost equivalent to NTC, and was accorded an arbitrary value of 1.

#### Statistical analysis

Data were analysed either by Mann–Whitney *t* test (in case of 2 groups) and Kruskal–Wallis test (more than 2 groups) for non-parametric data using GraphPad Prism software version 8.4.2 (GraphPad Software, La Jolla, California, USA),  $P < 0.05$  was considered significant. ddPCR data were expressed as mean  $\pm$  S.E.M. (standard error of mean). Correlation was calculated using Pearson’s correlation and the coefficient of correlation ( $r$ ) when  $>0.4$  was considered as relevant.

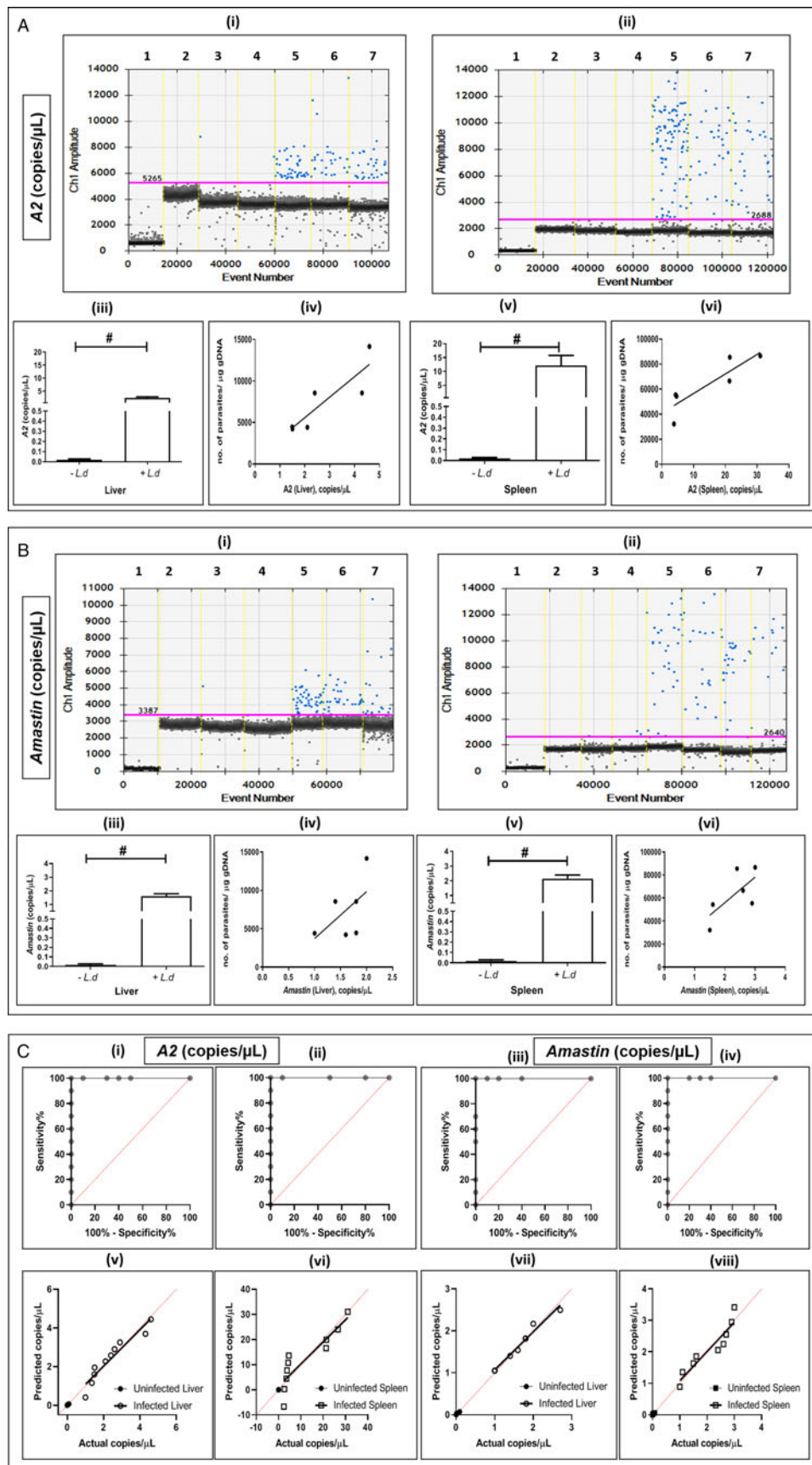
## Results

#### Measurement of parasite load

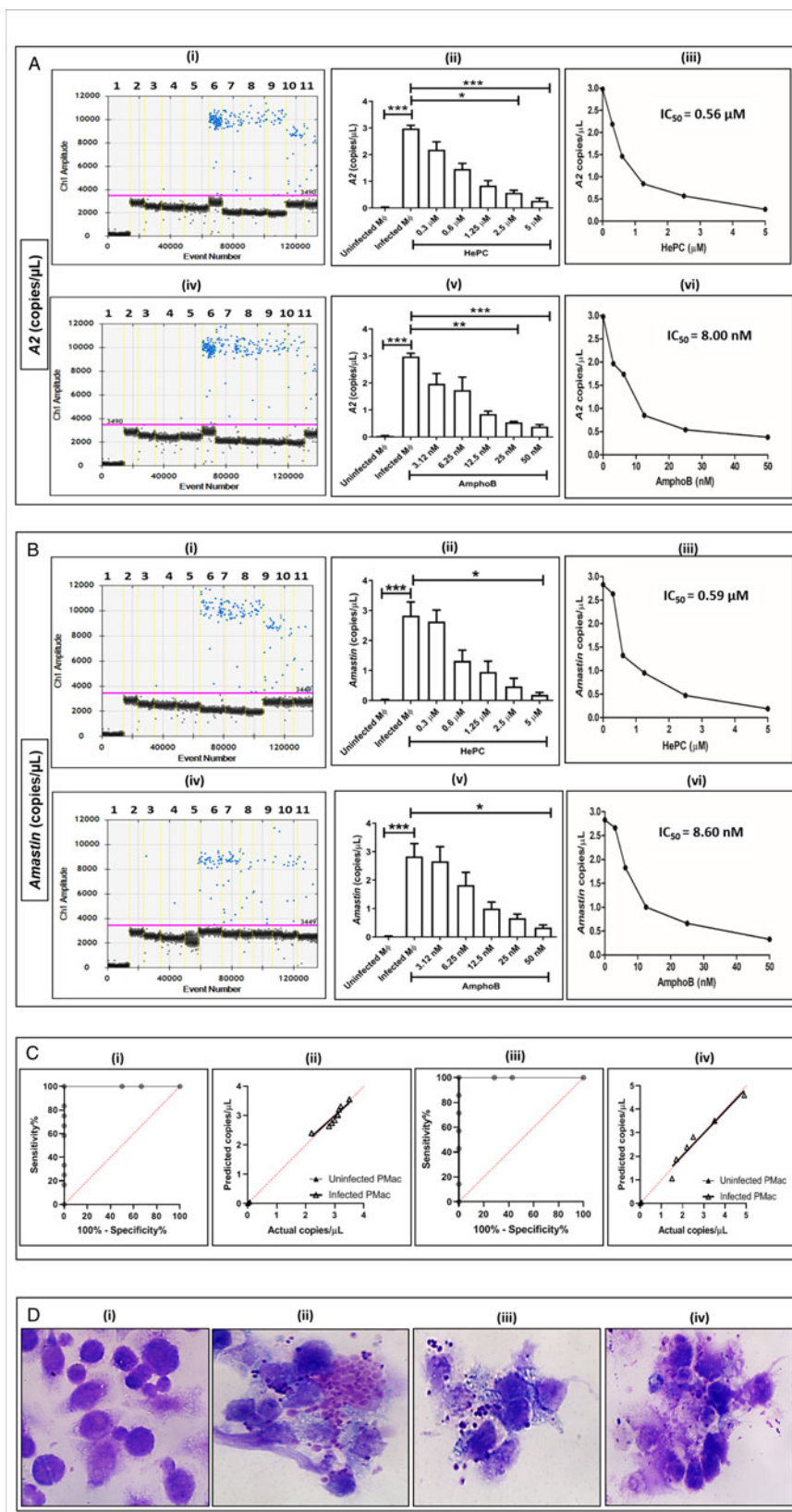
The parasite load was measured in *Leishmania*-infected hamsters by qPCR using specific primers for minicircle kDNA and was quantified using a standard curve. During the initial phase of *Leishmania* infection, parasites rapidly multiply both in the liver and spleen, but the latter demonstrates a higher parasite burden upon establishment of infection (Moreira *et al.*, 2012; Moulik *et al.*, 2021a). This was reflected in this study as the parasite load in hepatic and splenic tissues was 6512 (4369–9957) and 60 982 (48 862–85 820) parasites per  $\mu\text{g}$  of genomic DNA, respectively.

#### Measurement of amastigote-specific genes in animal models of visceral leishmaniasis

In liver tissues from infected hamsters, the expression of A2 was detected as compared to their uninfected counterparts, being  $2.42 \pm 0.38$  vs  $0.01 \pm 0.00$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$  (Fig. 1A, i, iii). Similarly, in splenic tissues, A2 was substantially raised being  $12.15 \pm 3.62$  vs  $0.02 \pm 0.00$  copies  $\mu\text{L}^{-1}$  (Fig. 1A, ii, v). Importantly, A2 correlated positively with parasite load, in hepatic ( $r = 0.87$ ) and splenic ( $r = 0.87$ ) tissues (Fig. 1A, iv, vi). With regard to *amastin*, it was raised in liver and splenic tissues, being  $1.61 \pm 0.17$  vs  $0.01 \pm 0.01$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$  (Fig. 1B, i, iii), and  $2.15 \pm 0.24$  vs  $0.01 \pm 0.01$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$ , respectively (Fig. 1B,



**Fig. 1.** Status of *A2* and *amastin* in hepatic and splenic tissues of infected Syrian golden hamsters. (A, B, i–ii) Representative 1-dimensional plots of droplets measured for fluorescence signals (amplitude indicated on y-axis) emitted from *A2* (A) and *amastin* (B) in infected ( $n=10$ ) and non-infected ( $n=10$ ) liver and splenic tissues of hamsters. Lane 1: negative controls; lane 2: non-template controls; lanes 3–4: non-infected liver and non-infected splenic tissues; lanes 5–7: infected hepatic (i) and splenic tissues (ii) respectively. EvaGreen-bound positive droplets are shown in blue while negative droplets are shown in black, with expression of the genes quantified as copies per  $\mu\text{L}$ . (A, B, iii, v) Bar graphs showing mean mRNA expression of *A2* (A) and *amastin* (B) in infected hepatic (iii) and splenic tissues (v) of hamsters. Each horizontal bar represents mean  $\pm$  s.e.m. of 10 animals in duplicates.  $^{\#}P < 0.001$  as compared to uninfected counterparts. (A, B, iv, vi) Correlation between no. of parasites per  $\mu\text{g}$  gDNA with expression of *A2* (A) and *amastin* (B) in infected liver tissues (iv) and splenic tissues (vi) respectively; (C) receiver operating characteristic (ROC) curve for *A2* and *amastin* in hamster liver (i, iii) and splenic (ii, iv) tissues. Linear regression between predicted and actual ddPCR output (copies per  $\mu\text{L}$ ) was observed for all tested targets and measured as  $R^2$  in hamster liver (v, vii) and splenic (vi, viii) tissues.



**Fig. 2.** Status of A2 (A) and amastin (B) in *L. donovani*-infected murine peritoneal macrophages. (A, B. i, iv) Representative 1-dimensional plots of droplets measured for fluorescence signals (amplitude indicated on y-axis) emitted from A2 (A) and amastin (B) in murine peritoneal macrophages infected with *L. donovani* followed by treatment with HePC (i) or AmphoB (iv) as described in Materials and methods section. Lane 1: negative control, lane 2: non-template control, lanes 3, 4: log phase and stationary phase promastigotes respectively, lane 5: control, non-infected peritoneal macrophages, lane 6: infected peritoneal macrophages, lanes 7–11: HePC (0.3–5  $\mu$ M) [i] and AmphoB (3.12–50 nM) [iv] treated peritoneal macrophages. EvaGreen-bound positive droplets are shown in blue while negative droplets are shown in black, with expression of the genes quantified as the copies per  $\mu$ L. (A, B. ii, v) Bar graphs showing mRNA expression of A2 (A) and amastin (B) in peritoneal macrophages infected with *L. donovani* and treated with HePC (ii) or AmphoB (v). Each horizontal bar represents mean  $\pm$  s.e.m. of at least 3 different experiments in duplicates; \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  as compared to infected macrophages. (A, B. iii, vi) Anti-amastigote activities of HePC (iii) and AmphoB (vi) were evaluated as described in Materials and methods section. (C) Receiver operating characteristic (ROC) curve for A2 (i) and amastin (iii) in infected peritoneal macrophages. Linear regression between predicted and actual ddPCR output (copies per  $\mu$ L) was observed for A2 (ii) and amastin (iv). (D) Representative images showing the anti-amastigote activity of HePC and AmphoB in AG83-infected peritoneal macrophages. Murine peritoneal macrophages (i) infected with *L. donovani* promastigotes (ii) treated with 0.6  $\mu$ M HePC (iii) or 12.5 nM AmphoB (iv); Bar-10  $\mu$ m, magnification 1000 $\times$ .

ii, v). There was a robust positive correlation with hepatic ( $r = 0.56$ ) and splenic parasite load ( $r = 0.68$ , Fig. 1B, iv, vi).

#### Assay specificity and sensitivity

The assay specificity was confirmed using cDNA from target sources (amastigotes in experimental models or dermal lesions

from patients with PKDL) while non-target samples included promastigotes, uninfected hamster tissues and peritoneal macrophages along with skin biopsies from healthy individuals. The control arms exhibited the expression of A2 and amastin similar to NTC. The LOQ was equivalent to LOD and for both A2 and amastin was 0.15 copies/ $\mu$ L<sup>-1</sup>. AUC for A2 and amastin in the liver (Fig. 1C, i, iii) and the spleen (Fig. 1C, ii, iv) was 1.000 (95% CI);  $R^2$  for

**Table 1.** Study population

Characteristics	Patients with PKDL ( <i>n</i> = 10)	Healthy controls ( <i>n</i> = 10)
Age (years) <sup>a</sup>	21.50 (16.50–43.75)	3.05 (1.05–5.57)
Sex (male: female)	2:3	Only males
Lesion type (macular: polymorphic)	2:3	NA
Disease duration (years) <sup>a</sup>	2.00 (0.95–4.50)	NA
Lag period (interval between cure of VL and onset of PKDL, years) <sup>a</sup>	3.00 (1.00–5.50)	NA
ITS-1 PCR +ve	10/10, 100%	NA
Parasite load (parasites per $\mu\text{g}$ of genomic DNA) <sup>a</sup>	6652 (3896–28,556)	NA

ITS-1 PCR, internal transcribed spacer-1 polymerase chain reaction; NA, not applicable; PKDL, post kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

<sup>a</sup>Values given in median (IQR).

A2 in the liver and the spleen was 0.91 and 0.80, while for *amastin*, it was 0.97 and 0.89, respectively (Fig. 1C, v–viii).

### Measurement of amastigote-specific genes A2 and *amastin* in *Leishmania*-infected peritoneal macrophages

In an *ex vivo* model of infection wherein murine peritoneal macrophages were infected with stationary phase promastigotes, A2 was enhanced as compared to uninfected macrophages, being  $2.97 \pm 0.12$  vs  $0.02 \pm 0.01$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$  (Fig. 2A). Importantly, A2 in log and stationary phase promastigotes (AG83) was comparable with NTC, confirming assay specificity. HePC/miltefosine caused a dose-dependent decrease in A2 from  $2.97 \pm 0.12$  to  $2.18 \pm 0.29$  ( $0.3 \mu\text{M}$ ),  $1.46 \pm 0.20$  ( $0.6 \mu\text{M}$ ),  $0.84 \pm 0.18$  ( $1.25 \mu\text{M}$ ),  $0.57$  ( $2.5 \mu\text{M}$ ) and  $0.09$  ( $P < 0.05$ ,  $2.5 \mu\text{M}$ ) and  $0.27 \pm 0.10$  copies  $\mu\text{L}^{-1}$  ( $P < 0.001$ ,  $5 \mu\text{M}$ ) and the  $\text{IC}_{50}$  was graphically extrapolated to be  $0.56 \mu\text{M}$  (Fig. 2A, i–iii). Similarly, AmphoB caused a progressive dose-dependent decrease in A2 from  $2.97 \pm 0.12$  to  $1.96 \pm 0.38$  ( $3.12 \text{ nM}$ ),  $1.74 \pm 0.48$  ( $6.25 \text{ nM}$ ),  $0.85 \pm 0.10$  ( $12.5 \text{ nM}$ ),  $0.54 \pm 0.03$  ( $P < 0.01$ ,  $25.0 \text{ nM}$ ) and  $0.38 \pm 0.07$  copies  $\mu\text{L}^{-1}$  ( $P < 0.001$ ,  $50 \text{ nM}$ ) and the  $\text{IC}_{50}$  was  $8.0 \text{ nM}$  (Fig. 2A, iv–vi).

Similarly, *amastin* was significantly raised in infected macrophages as compared to their non-infected counterparts, being  $2.82 \pm 0.45$  vs  $0.02 \pm 0.01$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$  (Fig. 2B), whereas in log and stationary phase promastigotes, it remained similar to NTC, corroborating assay specificity. HePC caused a dose-dependent decrease in *amastin* from  $2.82 \pm 0.45$  to  $2.63 \pm 0.38$  ( $0.3 \mu\text{M}$ ),  $1.32 \pm 0.35$  ( $0.6 \mu\text{M}$ ),  $0.95 \pm 0.35$  ( $1.25 \mu\text{M}$ ),  $0.47 \pm 0.27$  ( $2.5 \mu\text{M}$ ) and  $0.18 \pm 0.08$  copies  $\mu\text{L}^{-1}$  ( $P < 0.05$ ,  $5 \mu\text{M}$ , Fig. 2B, i–iii), the derived  $\text{IC}_{50}$  being  $0.59 \mu\text{M}$ . Similarly, AmphoB demonstrated a dose-dependent decrease being  $2.82 \pm 0.45$ ,  $2.66 \pm 0.51$  ( $3.12 \text{ nM}$ ),  $1.82 \pm 0.44$  ( $6.25 \text{ nM}$ ),  $1.00 \pm 0.22$  ( $12.5 \text{ nM}$ ),  $0.66 \pm 0.14$  ( $25 \text{ nM}$ ) and  $0.32 \pm 0.09$  copies  $\mu\text{L}^{-1}$  ( $P < 0.05$ ,  $50 \text{ nM}$ ), and the  $\text{IC}_{50}$  extrapolated was  $8.6 \text{ nM}$  (Fig. 2B, iv–vi).

For A2 and *amastin*, AUC was 1.000 (95% CI),  $P < 0.0001$  (Fig. 2C, i, iii);  $R^2$  for A2 and *amastin* in infected peritoneal macrophages was 0.90 and 0.95, respectively (Fig. 2C, ii, iv). In parallel, Giemsa-stained macrophages were microscopically examined (Fig. 2D), and the  $\text{IC}_{50}$  of HePC and AmphoB was  $0.62 \mu\text{M}$  and  $8.4 \text{ nM}$ , respectively.

### Measurement of amastigote-specific genes in patients with post kala-azar dermal leishmaniasis

This study objectively quantified the parasite burden in skin lesions sourced from patients with PKDL (Table 1). In PKDL,

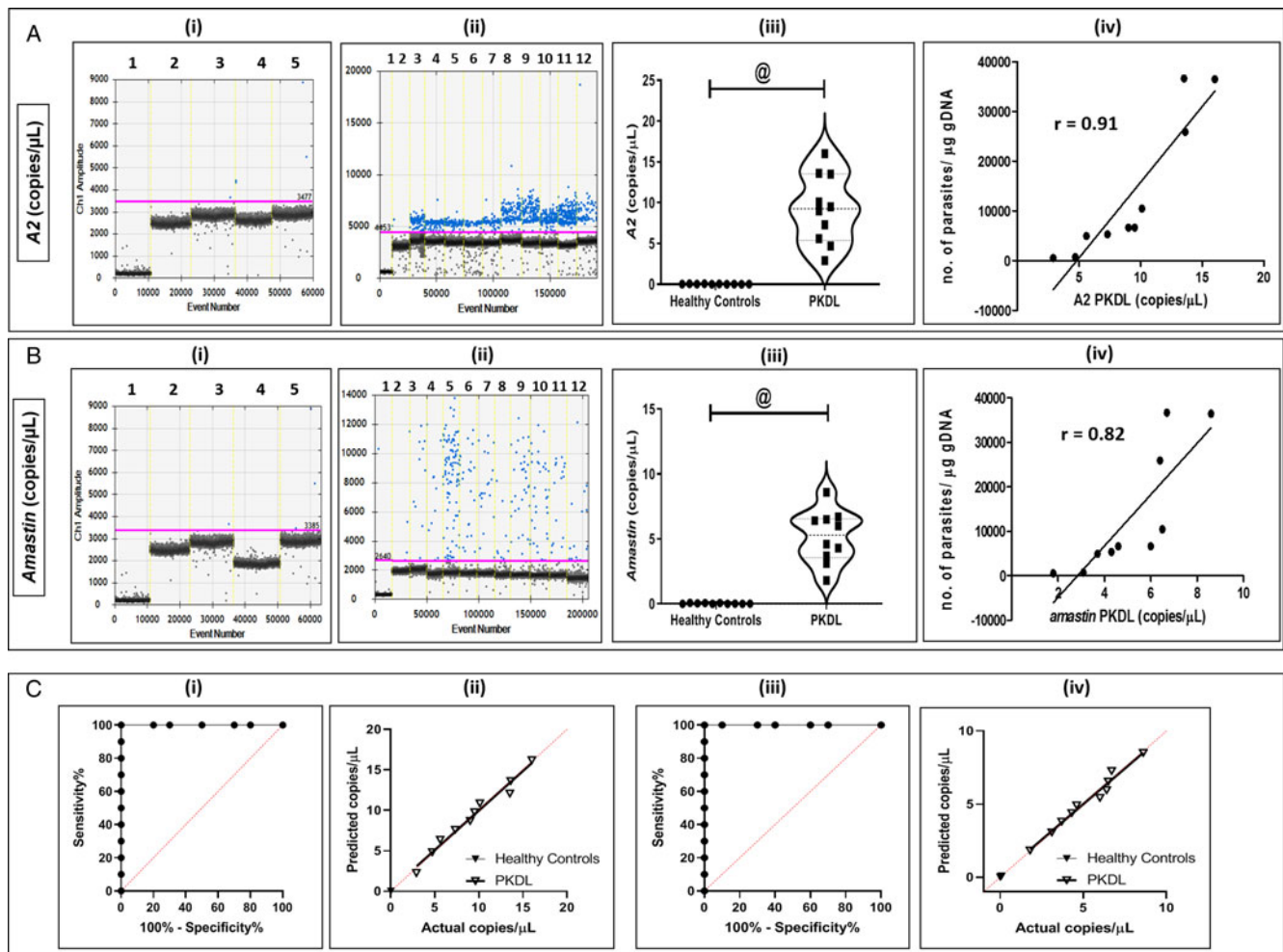
A2 was significantly enhanced in PKDL cases with respect to healthy controls, being  $9.22 \pm 1.34$  vs  $0.03 \pm 0.00$  copies  $\mu\text{L}^{-1}$  respectively,  $P < 0.001$  (Fig. 3A, i–iii) as was *amastin*,  $5.17 \pm 0.64$  vs  $0.03 \pm 0.01$  copies  $\mu\text{L}^{-1}$ ,  $P < 0.001$ , respectively (Fig. 3B, i–iii). Both A2 and *amastin* correlated positively with parasite load,  $r = 0.91$  and  $r = 0.82$ , respectively (Fig. 3A, B, iv). The AUC is 1.000 (95% CI 1.000–1.000) for A2 (Fig. 3C, i) and *amastin* (Fig. 3C, iii) and the  $R^2$  for A2 and *amastin* was 0.98 and 0.97, respectively (Fig. 3C, ii, iv).

## Discussion

Distinct morphological and biochemical changes occur during the differentiation of promastigotes into infective amastigotes (Holst-Jensen *et al.*, 2003; Pinheiro *et al.*, 2012). In view of A2 and *amastin* being exclusively present in amastigotes, it suggested their critical role in supporting parasite survival within host macrophages. Parasites like *T. cruzi* or *Leptomonas* are expected to demonstrate positivity, but *amastin* is more abundant in *Leishmania* spp. (Jackson, 2010). To obviate this cross-reactivity, the primers used in this study were exclusively designed for *Leishmania* spp. and therefore would not show any amplification for other parasites. The initial standardization for A2 and *amastin* endorsed the potential of ddPCR to monitor parasite load, and also emphasized that the optimal concentration of template was important (Supplementary Table S1 and Figs S2–S4). Moreover, their positive correlation with kDNA in qPCR and statistically evaluated AUC values established the assay sensitivity (Fig. 1). Importantly, as the non-target samples yielded a negative amplicon for A2 and *amastin*, it unequivocally confirmed assay specificity. Furthermore, the stage-specific expression of A2 and *amastin* was validated by its negative expression in promastigotes, and with the establishment of infection and transformation into amastigotes, it clearly correlated with an enhanced expression of A2 and *amastin* (Fig. 2).

Drug development in leishmaniasis till date depends on the quantification of amastigotes in *ex vivo*-infected macrophages, the classical method being counting of Giemsa-stained macrophages. However, this approach has an inherent operator bias, is time consuming and laborious, and cannot be applied for high-throughput screening. This limitation can be circumvented by employing objective approaches, e.g. kDNA-based qPCR. This is feasible within an established animal model of *Leishmania* infection wherein the expression of amastigotes can be quantified in terms of kDNA on the assumption that only amastigotes are present (Moulik *et al.*, 2021a). However, in short-term *ex vivo* drug screening methods as in peritoneal macrophages, quantification by kDNA qPCR approach maybe inadequate as despite numerous washing steps, a substantial proportion of promastigotes can remain loosely adherent to macrophages and would result in a false positivity. This can be obviated if quantification of amastigotes with A2 and *amastin* is adopted. Additionally, detection by ddPCR eliminated the need for standard curves for A2 and *amastin*. The generation of amastigotes *in vitro* would be needed to create a standard curve for qPCR but this is not feasible. Also, quantifying the precise number of amastigotes in *Leishmania*-infected peritoneal macrophages cannot be tailor-made for standard curve as the degree of infectivity can vary. Therefore, in this light, this ddPCR approach has a strong potential to be translated into a high-throughput drug screening platform. In view of the  $\text{IC}_{50}$  of miltefosine (HePC) and amphotericin B (AmphoB) in parasitized macrophages, as measured by conventional microscopy matching with the  $\text{IC}_{50}$  quantitatively determined by ddPCR (Fig. 2A–D), it endorsed the implementation of ddPCR.

Patients with PKDL can present with papulonodular (polymorphic) or hypomelanotic lesions (macular). These cases are



**Fig. 3.** Status of amastigote-specific genes A2 (A) and *amastin* (B) in PKDL. (A, B) Representative 1-dimensional plots of droplets measured for fluorescence signals (amplitude indicated on y-axis) emitted from A2 (A) and *amastin* (B) in skin biopsies sourced from PKDL cases ( $n=10$ ) and healthy controls ( $n=10$ ). EvaGreen-bound positive droplets are shown in blue while negative droplets are shown in black, with expression of genes quantified as copies per  $\mu\text{L}$ . (i) Lane 1: negative controls, lane 2: non-template controls, lanes 3–5: healthy controls for A2 (A) and *Amastin* (B) respectively. (ii) Lane 1: negative controls, lane 2: non-template controls, lanes 3–12: PKDL cases tested for expression of A2 (A) and *Amastin* (B) respectively. (iii) Violin plots showing mRNA expression of A2 (A) and *amastin* (B) in PKDL cases and healthy controls ( $n=10$ ). Each horizontal bar represents mean  $\pm$  s.e.m. of 10 individuals in duplicates;  $^{\circledast}P < 0.001$  as compared to healthy controls. (iv) Correlation between number of parasites per  $\mu\text{g}$  gDNA with A2 (A) and *amastin* (B) in PKDL cases. (C) Receiver operating characteristic (ROC) curve for A2 (i) and *amastin* (iii) in PKDL cases. Linear regression between predicted and actual ddPCR output (copies per  $\mu\text{L}$ ) was observed for all tested targets in PKDL for A2 (ii) and *amastin* (iv).

considered as the disease reservoir, especially during inter-epidemic periods, and therefore have an immense epidemiological significance (de Paiva *et al.*, 2015; Zijlstra *et al.*, 2017; Gedda *et al.*, 2020). Sero-diagnostics for kala-azar/VL involves ELISA-based assays employing crude or soluble antigens sourced from *Leishmania* spp. and rK39-immunochromatographic test is the most robust assay (Saha *et al.*, 2005; Sundar *et al.*, 2006; Mondal *et al.*, 2019). Additionally, the heightened anti-leishmanial IgG and its subclasses have helped identify and monitor active VL and PKDL cases (Mukhopadhyay *et al.*, 2012; Bhattacharyya *et al.*, 2014; Sengupta and Chatterjee, 2021). Other important *Leishmania*-specific antigens include recombinant GP63, recombinant cysteine protease A (Didwania *et al.*, 2020) and *Leishmania* membrane antigen (Ejazi *et al.*, 2019). Alternatively, antigen detection in urine can be expected to broadly correlate with the parasite load (Ejazi *et al.*, 2016). The A2 amastigote antigen-based LAT was performed in VL cases (Akhoundi *et al.*, 2013), but no similar study has been performed to identify PKDL cases.

A major limitation in the diagnosis of PKDL is that (a) serological tests are of limited value as positivity can be attributed to a past episode of VL and (b) detection of parasites in

Giemsa-stained skin biopsies is a major challenge especially for macular PKDL which presently constitute almost 50% of the PKDL disease burden (Sengupta *et al.*, 2019). Additionally, the limited availability of a standardized ‘test of cure’ tool for guiding clinicians is a major limitation, as till date the resolution of clinical features is considered as the efficacy parameter. Therefore, this study may find value in PKDL-related clinical trials, an area where chemotherapy regimens till date remain empirical.

Interestingly, A2 has been implicated only in visceralization during establishment of infection, and was considered to be selectively expressed in VL, with negligible expression in CL and PKDL lesions (Sharma *et al.*, 2010). However, as this study has demonstrated the presence of A2 and *amastin* in PKDL (Fig. 3), it may be attributed to a lower sensitivity of the assay employed in that study *vis-a-vis* the more sensitive ddPCR used in this study. The presence of A2 in PKDL has opened new avenues in understanding the potential dermatotropic role of A2 in the pathogenesis of PKDL (Fig. 3). Also, it would be interesting to study the effectiveness of using A2 as a monitoring tool for CL, where interpretation of lesion healing is challenging.

Overall, the optimized ddPCR assay was demonstrated to be sensitive as the linear regression curves exhibited strong goodness

of fit. However, the reagents and ddPCR machines are expensive and perhaps difficult to use as a common tool in laboratories. Irrespective of the study models, the expression of A2 was consistently greater than *amastin*, and would be the preferred biomarker in future. The availability of such a molecular diagnostic platform involving precise detection of amastigote burden will be an invaluable asset for drug screening as also for conducting clinical trials, and thus provide strong support for the ongoing leishmaniasis elimination programme in South Asia.

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**Conflict of interest.** None.

**Ethical standards.** All animal-related experiments were approved by the Institutional Animal Ethics Committee of IPGME&R, Kolkata (CPCSEA Registration No. 544/PO/c/02/CPCSEA). All human-related work included repurposed DNA after receiving approval from the Institutional Ethics Committee of School of Tropical Medicine, Kolkata and Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata. Written informed consent was obtained from all individuals, and for a minor, their legally accepted representative provided the same.

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