Comparative evaluation of nucleic acid stabilizing reagents for RNA- and DNA-based Leishmania detection in blood as proxy for visceral burdens

Full title: Comparative evaluation of nucleic acid stabilizing reagents for RNA- and DNA-based *Leishmania* detection in blood as proxy for visceral burdens.

Short tile: Improved sampling for simultaneous *Leishmania* RNA and DNA detection

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Abstract

Background: Molecular detection techniques using peripheral blood are preferred over invasive tissue aspiration for the diagnosis and post-treatment follow-up of visceral leishmaniasis (VL) patients. This study aims to identify suitable stabilizing reagents to prevent DNA and RNA degradation during storage and transport to specialized laboratories where molecular diagnosis is performed.

Methodology: The stabilizing capacities of different commercially available reagents were compared using promastigote-spiked human blood and peripheral blood of Syrian golden hamsters subjected to experimental infection, treatment (miltefosine of aminopyrazole DNDi-1044) and immunosuppression. The impact of various storage temperature conditions was tested in combination with an established kinetoplast DNA (kDNA) qPCR and a recently developed spliced leader RNA (SL-RNA) assay for *Leishmania* detection.

Principal Findings: Irrespective of the blood type and stabilizer used, threshold (cT) values obtained with the SL-RNA qPCR were systematically lower than those obtained with the kDNA assay, confirming the advantage of the SL-RNA assay over the widely used kDNA assay for low-level *Leishmania* detection. Peripheral blood parasite levels correlated relatively well with hepatic burdens. RNA Protect Cell Reagent provided the most optimal simultaneous DNA and RNA stabilization in both human and hamster blood. However, this stabilizer requires an erythrocyte lysis step, which can be challenging under field conditions. DNA/RNA Shield provides a good alternative for downstream kDNA and SL-RNA assays, especially if sample storage capacity at 4 °C can be guaranteed.

Conclusions/Significance: The recommended stabilizing reagents are compatible with RNA- and DNA-based *Leishmania* detection in peripheral blood in the VL hamster model and spiked human blood. Since molecular detection techniques using peripheral blood are less invasive than microscopic assessment of tissue aspirates, the findings of this study may be applied to human VL clinical studies.

Introduction

Visceral leishmaniasis (VL) is one of the clinical manifestations of leishmaniasis – a group of neglected tropical diseases - that is caused by *Leishmania infantum* and *L. donovani* parasites (1) responsible for over 20,000 deaths annually (WHO, http://www.who.int/mediacentre/factsheets/fs375/en/, last accessed 09/08/2019). Active disease is progressive and characterized by fever, weight loss, hepatosplenomegaly and pancytopenia (2) and is generally fatal within two years if left untreated (3). However, most infections remain asymptomatic or subclinical (2) with their role in transmission still to be elucidated (4-6). Treatment failures are also becoming more frequent (7-9), underlining the need for more specific and sensitive detection methods for *Leishmania* infection. VL in patients can be diagnosed in the laboratory in three ways: (*i*) microscopic detection of *Leishmania* parasites in tissue aspirates, (*ii*) serological detection of *Leishmania* antibodies, or (*iii*) molecular detection of *Leishmania* DNA or RNA in tissues and blood (10).

Due to its high sensitivity and specificity, PCR has become an important tool for diagnosis and test of cure of VL in clinical research and can also detect asymptomatic infections (3, 10). Many (q)PCR assays target minicircle kinetoplast DNA (kDNA) that is present in high copy numbers (up to 10^4 per parasite) (11) enabling low-level *Leishmania* detection in dogs (12-14), mice (15, 16), hamsters (17) and humans (18, 19). However, the heterogeneity of kDNA minicircles in terms of number and sequence prevent its use as a universal *pan-Leishmania* qPCR assay (19). Recently, we developed a novel RNA qPCR assay (20) targeting spliced-leader RNA sequences that are attached to the 5' end of all mature nuclear mRNAs during *trans*-splicing and are required for RNA processing, transcript stability and initiation of translation (21). This assay proved superior to the kDNA assay both in infected hamster tissues and in clinical blood samples of VL patients, without any false positives – a problem frequently occurring in kDNA assays – and enabled detection of all *Leishmania* species (20).

In patients, (q)PCR also permits non-invasive parasite detection in venous blood (10, 22, 23). Ideally, RNA and DNA are extracted immediately after blood sampling to prevent degradation and achieve optimal detection and quantification. However, in local hospitals in disease endemic countries, it is not generally possible to perform nucleic acid extractions adequately. In most cases, the samples are stabilized at local hospitals and shipped to specialized laboratories for extraction and (q)PCR. Various stabilizing agents for DNA and RNA have been developed by different companies for on-site sample stabilization, storage and transport to molecular diagnostic laboratories. In this study, different commercially available reagents were compared in a search for a practical and cheap protocol for sample storage in suboptimal conditions that ideally simultaneously stabilizes

DNA and RNA. This is of particular interest because qPCR using peripheral blood is increasingly being explored as an alternative to microscopy of tissue aspirates in dogs and humans (13, 14, 22, 23). In a second part of this study, the use of blood as a proxy for visceral organ burdens was evaluated in the Syrian golden hamster model for VL which closely mimics symptomatic disease (24).

Methods

Ethics statement: The use of laboratory rodents was carried out in strict accordance with all mandatory guidelines (European Union directive 2010/63/EU on the protection of animals used for scientific purposes and the Declaration of Helsinki) and was approved by the ethical committee of the University of Antwerp (UA-ECD 2011-74). The collection of human blood from healthy volunteers was approved by the ethical committee of Antwerp University Hospital (17/42/472) and was following written informed consent.

Animals: Female Syrian golden hamsters (body weight 80 to 100 g) were purchased from Janvier (Genest-Saint-Isle, France) and kept in quarantine for at least 5 days before infection. Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available *ad libitum*.

***Leishmania* parasites and animal infection:** *L. infantum* (MHOM/MA[BE]/67/ITMAP263) was maintained *in vitro* in HOMEM promastigote medium (Gibco®, Life technologies, Ghent, Belgium) at 25°C and sub-cultured twice weekly. *Ex vivo* amastigotes were isolated from the spleens of heavily infected donor hamsters and quantified by determining the Stauber index (25). Six hamsters were infected intracardially under isoflurane inhalation anesthesia with 2 x 10^7 spleen-derived amastigotes in 100 µL phosphate buffered saline (PBS, Gibco®, Life technologies).

Stabilizing promastigote-spiked human blood samples: 40 mL blood was collected from a healthy volunteer by venipuncture and divided in 2.5 mL aliquots to which 100 µL containing 1 x 10^5 *L. infantum* promastigotes were added.. Six different commercially available reagents and methodologies were explored following the manufacturers' instructions: (*i*) DNAgard, (*ii*) DNA/RNA shield (available in two different concentrations depending on the inclusion of erythrocyte lysis), (*iii*) RNAgard blood reagent, (*iv*) RNA protect cell reagent, (*v*) RNAgard blood tubes and (*vi*) PAX gene RNA blood tubes. A further distinction was made between methods that stabilize whole blood and those in which the red blood cells are first lysed during an erythrocyte lysis (EL) step.

1. *DNAgard (Biomatrica):* 625 µL DNAgard was added to 2.5 mL (1:4) whole human blood spiked with 1 x 10^5 promastigotes (100 µL), after which the sample was mixed and divided into 6 aliquots.
2. *DNA/RNA shield (2x concentrate, Zymo Research):* 2.5 mL of stabilizer was added (1:2) to 2.5 mL whole human blood spiked with 1 x 10^5 promastigotes, after which the sample was mixed and divided into 6 aliquots.
3. *RNAgard blood reagent (Biomatrica):* 6.25 mL reagent was added to 2.5 mL (1:4) whole human blood spiked with 1 x 10^5 promastigotes. The sample was mixed and kept at room temperature for at least 2 hours before storing at lower temperatures. The sample was then divided into 6 aliquots. Prior to DNA/RNA extraction, the stabilized cells were precipitated using the precipitation buffer supplied.
4. *RNAgard blood tube (Biomatrica):* the tubes were equilibrated at room temperature for at least 2 hours prior to blood collection, according to the manufacturers' instructions. 2.5 mL whole blood was added to an RNAgard blood tube prefilled with 6.65 mL of RNA stabilization reagent, after which the tube was spiked with 1 x 10^5 promastigotes and divided into 6 aliquots.
5. *PAX gene RNA blood tube (Qiagen):* 2.5 mL whole blood was added to a prefilled PAX gene RNA tube prior to spiking with 1 x 10^5 promastigotes and subsequent dividing into 6 aliquots.
6. *DNA/RNA shield on pelleted leukocytes (Zymo Research):* 2.5 mL blood was divided into 6 aliquots to which 2100 µL EL buffer was added (EL was performed according to the QIAamp RNA blood mini kit, Qiagen, Hilden, Germany). All aliquots were centrifuged at 400 x *g* for 10 minutes, after which the supernatant was removed and 16.7 µL (1/6 of 100 µL) promastigotes were added together with fresh EL buffer. The samples were centrifuged at 1000 x *g* for 10 minutes to pellet the promastigotes and the supernatant was removed, after which 300 µL DNA/RNA shield was added to each pellet.
7. *RNA protect cell reagent on pelleted leukocytes (Qiagen):* EL and promastigote spiking was performed as described for 'DNA/RNA shield on pelleted leukocytes'. The resulting 6 pellets were stabilized each by adding 300 µL RNA protect cell reagent.
8. *Control extractions:* one DNA and one RNA extraction were performed immediately after spiking, *e.g.* without addition of stabilizing reagents. EL and promastigote-spiking were performed as described for 'DNA/RNA shield on pelleted leukocytes'. Upon removal of the supernatant, spiking and the second EL, buffer RLT (RNA) or AL (DNA) were added for further extraction.
9. *Boom method:* This crude reference method for nucleic acid extraction was performed on two independent samples as described previously (26). The samples were stored at -20°C for two weeks prior to Boom extraction of both DNA and RNA.

Except for the Boom method, different storage conditions were compared: two of the 6 aliquots were stored at room temperature (RT), two at 4°C and two at -20°C. After 2 weeks, nucleic acids were extracted. This experiment was repeated once independently, including the entire spiking human blood procedure, sample stabilization, storage at various temperatures, extraction and qPCR.

Hamster treatment, blood sampling and stabilization: To follow changes in parasitaemia, 500 µL of blood was collected sublingually at 7, 21, 35, 49 and 60 days post-infection (dpi) and stabilized using either DNA/RNA shield (2x concentrate) or RNA protect cell reagent (on pelleted leukocytes). For DNA/RNA shield, 250 µL stabilizer was added to 250 µL whole blood, prior to mixing and dividing in 2 aliquots of 250 µL (one for DNA and one for RNA extraction). For the RNA protect cell reagent, EL was performed (QIAamp RNA blood mini kit) on 2 aliquots of 125 µL, and 300 µL RNA protect cell reagent was then added to the 2 resulting leukocyte pellets (1 for DNA and 1 for RNA extraction). All aliquots were stored at 4°C and DNA and RNA extractions were performed 2 weeks later.

At 21 dpi, all hamsters were treated orally for 5 consecutive days with either miltefosine (MIL) at 40 mg/kg in water, once daily or with DNDi-1044 (27) at 25 mg/kg in 1% (w/v) methylcellulose and 5% $Tween_{80}$ in water, twice daily. Starting from 35 dpi, all animals were immune-suppressed to stimulate relapse by intraperitoneal (IP) cyclophosphamide (CPA)-treatment at 150 mg/kg once weekly. At 60 dpi, all animals were euthanized with a CO_2 overdose and spleens and livers were collected. An imprint was made of each tissue, which was fixed with methanol for 2 minutes and stained with a 1:5 Giemsa-dilution (Sigma-Aldrich, Belgium). Intracellular amastigote burdens were assessed microscopically by determining the 'Leishman donovan units' (LDU) (25):

$$\frac{\#\ amastigotes\ counted}{500\ nuclei}\ x\ weight\ organ\ (mg)$$

Nucleic acid extractions: All DNA and RNA extractions were performed according to the manufacturers' instructions using the QIAamp DNA mini kit (200 µL elution volume) and QIAamp RNA blood mini kit (60 µL elution volume) respectively (both from Qiagen). For reasons of compatibility, RNA from blood stabilized with the PAX gene RNA blood tube was extracted using the recommended PAX gene blood miRNA kit.

Real-time PCR

SL-RNA qPCR assay: This high sensitivity assay targeting the highly abundant and conserved spliced leader (SL)-RNA sequence (28) was recently developed for pan-*Leishmania* detection (20). To prevent PCR inhibition, all samples were diluted 1:10 in PCR-grade water. A 20 µL reaction mixture consisted of 10 µL

2× SensiFAST SYBR Hi-ROX One-Step mix, 0.8 µL of each primer, 0.2 µL of reverse transcriptase, 0.4 µL of RNAse inhibitor, 4 µL of 1:10 diluted sample and 3.8 µL of PCR water. All primers (Table 1) were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium). The PCR reaction (see conditions in Table 1) was run on the Step One Plus real-time PCR system (Applied Biosystems, California, USA). A positive control sample (extracts from *L. infantum* promastigote-spiked human blood prepared in another study), a negative control sample (extracts from uninfected human blood) and a blank control (qPCR mastermix) were included in each plate. Melting temperatures of 66.9 ± 0.5°C were considered specific amplification products in the SL-RNA qPCR reaction (20).

Multiplex kDNA/18S DNA qPCR assay: The assay targeting multicopy kDNA is frequently used due to its high sensitivity (14, 19), while the *18S* gene (29) was included in this multiplex assay as an additional confirmation of parasite presence, although this low copy target is hampered by low sensitivity. To prevent PCR inhibition, the standard operating procedure included a 1:10 dilution of samples in PCR-grade water. A 20 µL TaqMan reaction mixture contained 10 µL of 2 × TaqMan Universal Master Mix II (Applied Biosystems), 4 µL of 1:10 diluted sample, 1 µL of each primer, 0.5 µL of each probe and 1 µL of PCR water. The same positive, negative and blank control samples were included as described above for the SL-RNA qPCR assay. The cT cut-off values for false-positivity were determined previously (20): cT<34 for kDNA and cT<35.5 for *18S* DNA. All primers and probes (Table 1) were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium) and qPCR reactions (see conditions in Table 1) were performed on the Step One Plus real-time PCR system (Applied Biosystems).

Statistical analysis and graphical data presentation: Statistical analyses and preparation of graphs were performed using Graphpad-Prism V6.00 software. Correlation analyses were by Pearson analysis, from which the correlation coefficients and *P*-values are reported.

Results

Comparison of different nucleic acid stabilizers in promastigote-spiked human blood

To optimize downstream SL-RNA and kDNA detection, the performance of the different stabilization methods for promastigote-spiked human blood were compared. The results depended on (*i*) the molecular target to be stabilized (DNA or RNA), (*ii*) the temperature during the 2-week storage period and (*iii*) the inclusion of an erythrocyte lysis step prior to stabilization. cT values from the two independent repeats for all stabilizing agents at different storage temperatures are shown in Figure 1 and are available in Table S1. cT values for control promastigote-spiked samples (extractions at D_0 without stabilization represented as dotted lines in Fig. 1; Table S1) or Boom-extracted samples were consistently lower in the SL-RNA assay than in the kDNA assay, which confirmed that the pan-*Leishmania* SL-RNA assay for parasite detection in blood was superior. The difference was much larger than could be accounted for by the different elution volumes in the DNA and RNA extraction procedures (200 versus 60 µL respectively).

Even though all stabilizers tested are designed for RT storage and shipping, several performed better at 4°C. In most cases, the optimal storage condition was irrespective of the molecular target, although suboptimal storage affected RNA more given its relatively lower stability compared to DNA. The different stabilizers with their optimal storage temperatures for both SL-RNA and kDNA are ranked in Table 2. For RNA protect cell reagent (with prior EL) and DNA/RNA shield (2× concentrate, without EL), 4°C was generally the most optimal. For both SL-RNA and kDNA, RNA Protect Cell Reagent provided the best stabilization with similar or even slightly lower cT values compared to extraction performed immediately after spiking (without stabilization). This product, however, requires EL while DNA/RNA shield performs almost equally well at 4°C for both DNA and RNA without the need for EL. When comparing products for sample storage at RT without prior EL, RNAgard blood reagent and PAX gene RNA tubes yielded the best results for separate downstream RNA and DNA detection respectively.

Comparison of different nucleic acid stabilizers in infected hamster blood as a proxy for visceral *Leishmania* burden

The two most efficient reagents for simultaneous DNA and RNA stabilization were compared in hamsters subjected sequentially to an experimental infection, treatment with an antileishmanial drug (MIL or aminopyrazole DNDi-1044) and CPA-based immune-suppression to experimentally trigger post-treatment

relapse. In all MIL- and DNDi-1044-treated hamsters, parasite dynamics reminiscent of drug-induced reductions and CPA-induced relapse could be detected in the blood with both kDNA and SL-RNA qPCR assays (Fig 2A-B).

Similarly to the case for promastigote-spiked human blood, the SL-RNA qPCR assay appeared to yield consistently lower cT values than the kDNA assay, enabling low-level *Leishmania* detection (Fig 2A-B). RNA protect cell reagent with EL provided superior results compared to DNA/RNA shield. DNA/RNA shield resulted in slightly higher cT values, especially for the SL-RNA target at time points with high parasite levels in the blood (21 and 60 dpi, Fig 2A-B). Hence, cT values obtained with the SL-RNA and kDNA assay correlated best upon optimal stabilization with RNA protect cell reagent following EL (Pearson $r = 0.85$, Fig 1C). When using DNA/RNA shield, the congruence between the two molecular targets was less (Pearson $r = 0.65$, Fig 2D). In general, the results for kDNA and SL-RNA correlated acceptably well and differences between both stabilizers were rather minor (Pearson $r = 0.87$ and $r = 0.81$, Fig 2E-F).

Using RNA protect cell reagent, blood parasitaemia levels detected by SL-RNA qPCR at 60 dpi correlated to those in the liver determined by microscopy at necropsy (Pearson $r = -0.72$, Fig 3A). The spleen burdens deviated more from those detected in blood (Pearson $r = -0.65$, Fig 3A). Similarly, for kDNA qPCR on peripheral blood, a better correlation was found with liver than spleen burdens (Pearson $r = -0.61$ compared to $r = -0.54$ respectively, Fig 3B), although both correlations were slightly inferior to those with SL-RNA qPCR.

Discussion

Molecular parasite detection in peripheral blood is preferable for diagnosis and clinical follow-up of VL patients to conventional parasitological detection, which requires invasive tissue aspiration (22, 23). Molecular diagnosis would benefit from the addition of stabilizing reagents to prevent DNA and RNA degradation during storage and transport to specialized laboratories where extraction and (q)PCR are performed. The present study compared six different commercially available reagents using promastigote-spiked human blood, combined with *Leishmania* detection using a multiplex kDNA/*18S* DNA and SL-RNA qPCR assay (20): (*i*) DNAgard, (*ii*) DNA/RNA shield, (*iii*) RNAgard blood reagent, (*iv*) RNA protect cell reagent, (*v*) RNAgard blood tubes and (*vi*) PAX gene RNA blood tubes. The two reagents found to perform best on spiked human blood were subsequently evaluated using blood from amastigote-infected hamsters.

As we reported previously for VL clinical samples subjected to Boom extraction (20), cT values obtained with the SL-RNA qPCR were systematically lower than those obtained with the kDNA assay, both on promastigote-spiked human blood and on amastigote-infected hamster blood. These results strongly support the use of the newly developed SL-RNA qPCR for low-level *Leishmania* detection in patients. Given possible limitations of RNA-based qPCR in field conditions, stabilizers could serve as an approach to circumvent this limitation. Using a reagent that simultaneously stabilizes DNA and RNA enables clinical samples to be subjected to both the SL-RNA and the widely used kDNA assay.

As expected, RNA was slightly more affected by suboptimal storage than DNA due to its relatively lower stability. In promastigote-spiked human blood, RNA protect cell reagent provided the most optimal simultaneous stabilization for *Leishmania* DNA and RNA, but required EL and ideally subsequent storage at 4°C. If for practical reasons inclusion of EL in the field is discouraged, DNA/RNA shield is an acceptable alternative where storage at 4°C is possible. In cases where only storage at ambient temperatures is possible, more expensive reagents are indicated for separate RNA and DNA stabilization: the RNAgard blood reagent and PAX gene RNA blood tubes, respectively.

Additionally, in infected hamster blood, RNA protect cell reagent in combination with EL provided the most optimal stabilization and downstream detection of both *Leishmania* DNA and RNA. Use of this reagent in combination with the kDNA and SL-RNA assays enabled dynamic monitoring of experimentally induced fluctuations in blood parasitaemia following onset of infection, treatment and relapse. Although DNA/RNA shield performed similarly to the kDNA assay, this reagent gave slightly inferior results for RNA storage and detection. If it is difficult to include EL in the sampling protocol, DNA/RNA shield can be used in combination

with the kDNA and SL-RNA molecular detection assays to monitor parasite fluctuations in peripheral blood. It would be of interest for experimental studies to investigate whether this also applies to self-healing mouse models. It has already been shown that blood from *Leishmania*-infected mice is kDNA qPCR positive [16], but this has not been evaluated for different VL species, and blood parasitaemia levels have also not been compared to internal organ burdens.

Despite the small number of animals used in this study, blood parasitaemia levels detected by both assays correlated quite well with the liver parasite burdens but slightly less well with spleen burdens. This is most likely to be because overall spleen burdens were lower following drug treatment and the artificially induced relapse. In drug efficacy studies and post-treatment monitoring of patients, RNA is considered the better marker for viability (30), whereas DNA might remain intact for a longer period of time after parasite death (31). In summary, this study establishes a sampling protocol for peripheral blood compatible with two complementary and effective molecular *Leishmania*-detection tests for estimating parasite burdens in the major organs. Studies with larger group sizes and patient sampling are now required to confirm these results, but the proposed protocols could be used as alternatives to tissue aspiration, an invasive intervention that is still current practice in dogs (13, 14) and human patients (22, 23). qPCR could also be integrated in clinical trials with new chemical entities, where it may help differentiate study arms with higher chances of success.

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Tables

Table 1. Primer sequences and qPCR settings. [1] Primers and probe adopted from Lachaud *et al.* (2002) * Assays combined in a multiplex qPCR.

Target	*Leishmania* gene	Sequence of primers and probes	Activation step	Amplification (40 cycles)	Melting curve
DNA	**kDNA** [1] *	**TaqMan**			
	FP (250nM)	5' - CTTTTCTGGTCCTCCGGGTAGG - 3'			
	RP (250nM)	5' - CCACCCGGCCCTATTTTACACCAA - 3'	95°C 10min	95°C 15s 60°C 1min	/
	Probe (250nM)	5' - 56-JOEN/TTTTCGCAG/ZEN/ AACGCCCCTACCCGC/3IABkFQ/ -3'			
	18S DNA *	**TaqMan**			
	FP (500nM)	5' - TTGGGGATCTTATGGGCCG - 3'			
	RP (500nM)	5' - GAGGATATTCCCGTGGGTGG - 3'	95°C 10min	95°C 15s 60°C 1min	/
	Probe (500nM)	5' - 56-FAM/AGGGTTTACCCTGTGTCA GCACCGCG/3BHQ_1 - 3'			
RNA	**SL-RNA**	**One-Step**			
	FP (400nM)	5'-AACTAACGCTATATAAGTAT-3'	45°C 10min (cDNA synthesis)	95°C 15s 56°C 15s	95°C 15s, 45°C 1min, 95°C 15s
	RP (400nM)	5'-CAATAAAGTACAGAAACTG-3'	95°C 2min	60°C 15s	(step & hold with 0.3°C increments)

Table 2: Stabilizing capacities of the different reagents with their optimal storage temperature ordered from 1 (high stabilization) to 5 (low stabilization) for both targets. The reagent elected to provide the best results for simultaneous *Leishmania* DNA and RNA stabilization is indicated in red. An alternative reagent that does not require EL is indicated in blue. As the optimal storage temperature for DNA/RNA shield in combination with EL varied greatly between experiments, its use was not recommended (indicated in italics and grey). RT = ambient, room temperature.

Stabilizing capacities (1 = highest / 5 = lowest)	**SL-RNA**	**kDNA**
1	**RNA protect cell reagent (+EL), 4°C**	**RNA protect cell reagent (+EL), 4°C** PAX gene RNA blood tube, RT *DNA/RNA shield (+EL), -20°C or RT*
2	*DNA/RNA shield (+EL), -20°C or 4°C*	**DNA/RNA shield (2x), RT** DNAgard , -20°C
3	**DNA/RNA shield (2x), 4°C** RNAgard blood reagent, RT	RNAgard blood reagent, RT
4	RNAgard blood tube, RT PAX gene blood tube, RT	RNAgard blood tube, 4°C
5	DNAgard, -20°C	

Figures

Fig. 1

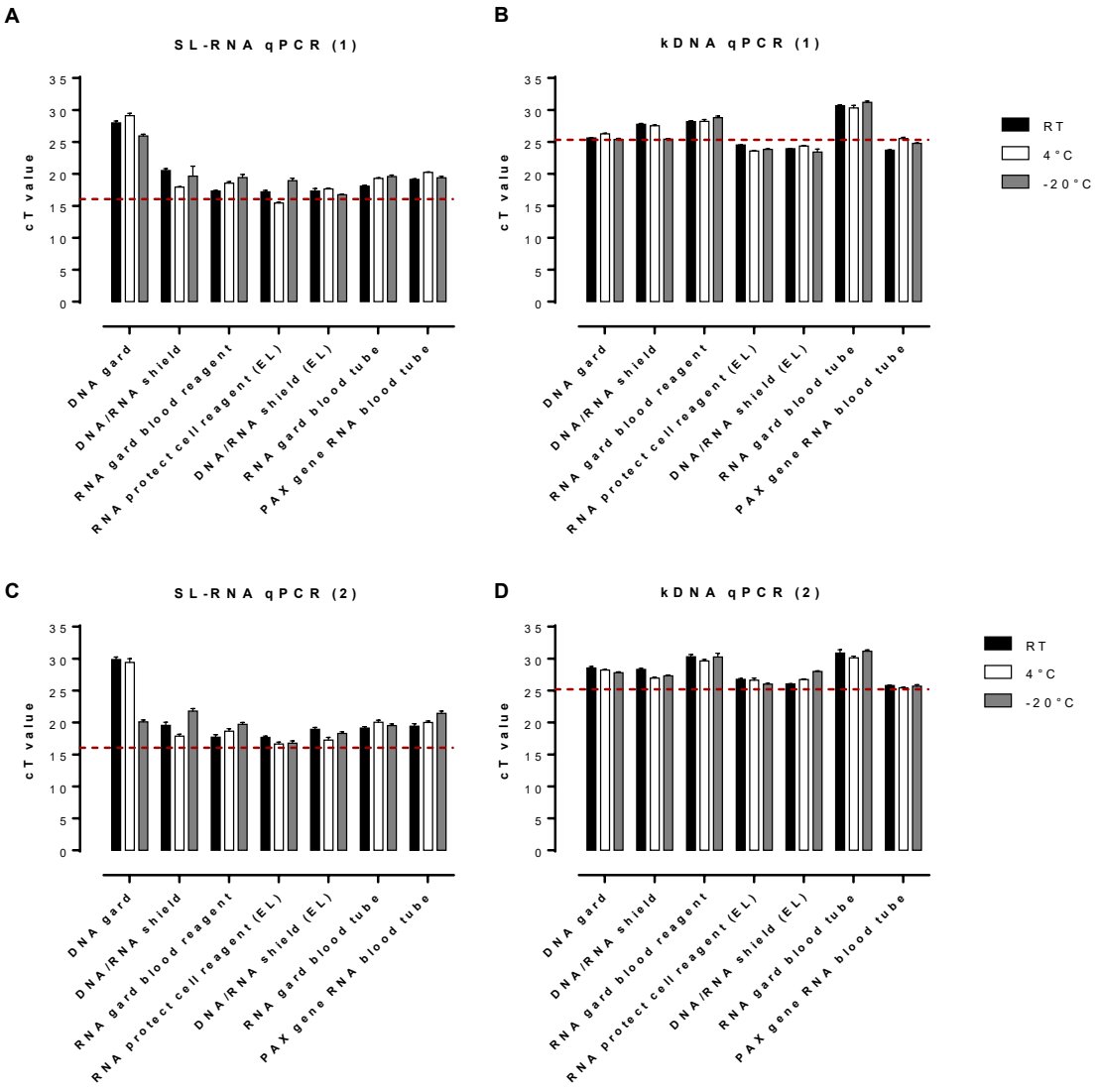

Fig 1. Comparison of various nucleic acid stabilizing reagents for downstream *Leishmania* SL-RNA and kDNA detection in spiked human blood. cT values of SL-RNA qPCR (**A**, **C**) and kDNA qPCR (**B**, **D**) of two independent experiments for all stabilizing agents at the various storage temperatures tested. In each graph, the average cT ± S.D. is shown for two separate qPCR assays per experiment, each containing two technical repeats for each condition. The average cT value for D_0 (control) extraction is indicated by the red dotted line.

Fig. 2

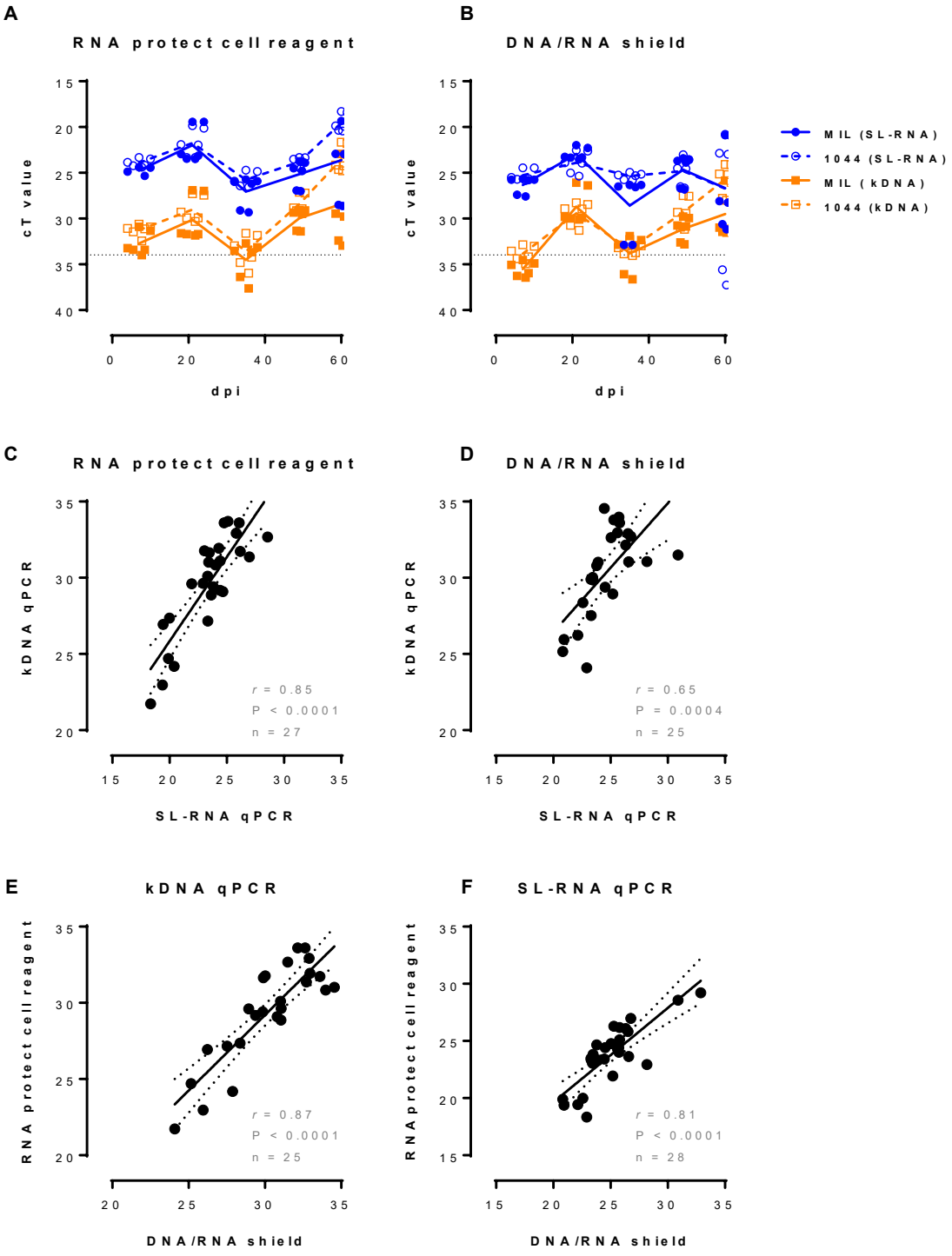

Fig 2. Comparison of RNA protect cell reagent and DNA/RNA shield for longitudinal follow-up of parasite burdens in peripheral blood of hamster using SL-RNA and kDNA detection. All hamsters were treated orally at 21 dpi for 5 consecutive days with either 40 mg/kg s.i.d. miltefosine (MIL) or 25 mg/kg b.i.d. DNDi-1044. Starting from 35 dpi, all animals were immune-suppressed by weekly CPA-treatment at 150 mg/kg I.P. Sampling was at 7, 21, 35, 49 and 60 days post-infection. **A** and **B:** Mean cT values of all MIL-

(filled symbols) and DNDi-1044-treated hamsters (empty symbols) obtained with the SL-RNA assay (blue lines) and kDNA assay (orange lines) using RNA protect cell reagent (**A**) or DNA/RNA shield (**B**). The cT cut-off value of 34 for the kDNA assay is depicted as a dotted line. For both stabilizers, cT values obtained with the SL-RNA qPCR were lower than those obtained with the kDNA qPCR. **C** and **D**: Correlation between cT values obtained with the two molecular assays using RNA protect cell reagent (**C**) and DNA/RNA shield (**D**). As RNA protect cell reagent provided the most optimal DNA and RNA stabilization, the correlation for this stabilizer was better **(C)** than for DNA/RNA shield **(D)**. **E** and **F:** Considering the kDNA (**E**) and SL-RNA (**F**) molecular targets, cT values correlated well across the two reagents used. Sample size (*n*), *P*-values and Pearson correlation coefficients (*r*) are indicated in the graphs.

Fig. 3

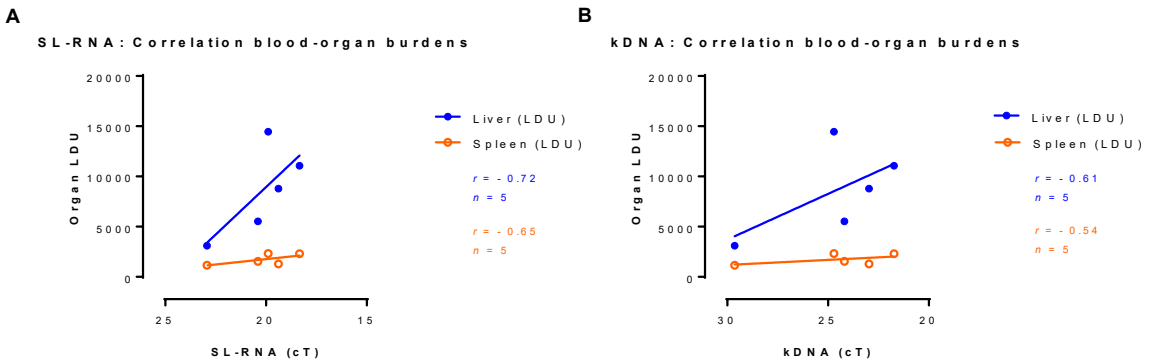

Fig 3. Correlation of liver and spleen parasite burdens with detection of SL-RNA (A) and kDNA (B) in peripheral blood stabilized with RNA protect cell reagent. Treated and subsequently immunosuppressed hamsters were euthanized at 60 dpi to determine hepatic and splenic parasite burdens (LDU) by conventional Giemsa stain microscopy and parasite levels in peripheral blood by SL-RNA (**A**) and kDNA (**B**) detection (cT values). Correlations of cT values with LDU burdens in both organs were, in general, better for the SL-RNA than for the kDNA assay. For both assays, the correlation was better with hepatic than with splenic burdens. Sample size (*n*) and Pearson correlation coefficients (*r*) are indicated in the graphs.

Supplementary Table S1

		SL RNA (Avg cT)		kDNA multiplex (Avg cT)	
		Exp 1	Exp 2	Exp 1	Exp 2
	Boom 1		17.56 ± 0.49		21.14 ± 0.12
	Boom 2		18.14 ± 0.32		22.17 ± 0.12
	Control: extract day 0	16.05 ± 0.27	18.14 ± 0.30	25.32 ± 0.39	25.19 ± 0.07
Product	**Storage condition**				
DNAgard	**RT**	28.00 ± 0.24	29.86 ± 0.33	25.60 ± 0.04	28.52 ± 0.22
	4°C	29.11 ± 0.32	29.41 ± 0.50	26.27 ± 0.10	28.20 ± 0.10
	- 20°C	**25.95 ± 0.23**	**20.12 ± 0.23**	**25.39 ± 0.14**	**27.79 ± 0.11**
DNA/RNA shield (2x)	**RT**	20.55 ± 0.24	19.56 ± 0.41	27.76 ± 0.10	28.30 ± 0.15
	4°C	**17.95 ± 0.04**	**17.89 ± 0.23**	27.52 ± 0.13	**26.96 ± 0.14**
	- 20°C	19.66 ± 1.35	21.79 ± 0.35	**25.41 ± 0.10**	27.31 ± 0.11
RNAgard blood reagent	**RT**	**17.32 ± 0.07**	**17.70 ± 0.35**	**28.18 ± 0.11**	30.30 ± 0.31
	4°C	18.56 ± 0.20	18.66 ± 0.34	28.21 ± 0.25	**29.65 ± 0.19**
	- 20°C	19.43 ± 0.40	19.71 ± 0.26	28.78 ± 0.22	30.26 ± 0.50
RNA protect cell reagent	**RT**	17.21 ± 0.19	17.66 ± 0.19	24.52 ± 0.01	26.75 ± 0.11
	4°C	**15.45 ± 0.09**	**16.66 ± 0.25**	**23.59 ± 0.06**	26.63 ± 0.27
	- 20°C	18.93 ± 0.33	16.77 ± 0.29	23.83 ± 0.08	**25.99 ± 0.12**
DNA/RNA shield on cell pellet	**RT**	17.33 ± 0.34	18.93 ± 0.25	23.92 ± 0.04	**26.03 ± 0.05**
	4°C	17.66 ± 0.11	**17.26 ± 0.35**	24.37 ± 0.06	26.71 ± 0.10
	- 20°C	**16.75 ± 0.07**	18.30 ± 0.23	**23.41 ± 0.39**	27.98 ± 0.10
RNAgard blood tube	**RT**	**18.07 ± 0.12**	**19.15 ± 0.18**	30.67 ± 0.12	30.87 ± 0.45
	4°C	19.30 ± 0.09	20.04 ± 0.29	**30.33 ± 0.30**	**30.12 ± 0.23**
	- 20°C	19.58 ± 0.21	19.54 ± 0.23	31.18 ± 0.17	31.17 ± 0.17
PAXgene RNA blood tube	**RT**	**19.14 ± 0.08**	**19.42 ± 0.34**	**23.69 ± 0.08**	25.78 ± 0.09
	4°C	20.23 ± 0.06	20.00 ± 0.21	25.53 ± 0.14	**25.43 ± 0.08**
	- 20°C	19.40 ± 0.18	21.43 ± 0.30	24.78 ± 0.05	25.69 ± 0.18

Table S1. Average cT values of two independent experiments for all qPCR assays and stabilizing reagents at different storage temperatures. For each assay and experiment (exp. 1 and 2), the average cT values from two separate qPCR assays are listed. The Boom extraction method was only included as a reference nucleic extraction in the second experiment. The lowest cT values for each condition per assay are marked in bold and green. Averages are the result of 2 independent qPCR assays per experiment, each with two technical repeats for each condition.