**Full Title:** Molecular Detection and Parasite Load Determination of *Leishmania donovani* in Dogs at Humera and Sheraro, Western Tigray, Ethiopia

**Short Title:** *Leishmania donovani* Detection and Parasite Load Determination in Dogs
Authors

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Author Contributions

Designed the study and laboratory work: HFT, GVDA. Performed the sample collection and laboratory work: HFT, BHA, KW, IM. Analyzed the data: HFT, GVDA. Contributed reagents/materials/analysis tools: BH, GVDA. Wrote the paper: HFT, GVDA, BHA.
Molecular Detection and Parasite Load Determination of *Leishmania donovani* in Dogs at Humera and Sheraro, Western Tigray, Ethiopia

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Abstract

Background
Leishmaniasis is a vector borne disease of the tropics and subtropics causing major public health concern in underdeveloped countries. Human visceral leishmaniasis, caused by *Leishmania donovani* is endemic in different parts of Ethiopia. Humera and Sheraro, two districts of north western Ethiopia, are among visceral Leishmaniasis endemic areas. The source of infection and reservoirs of the parasite however are not well studied despite previous reports of anti-*Leishmania* antibodies in different animals in these areas.

Methodology and findings
The study was conducted in two districts of western Tigray (Humera and Sheraro), with the objective of molecular detection and parasite load determination of *Leishmania donovani* in dogs, a means to suggest parasite transmission possibility to sand fly from October 2019 to April 2020. Purposive and systematic sampling techniques employed to select symptomatic and asymptomatic dogs respectively (n=90 in total) including one negative control (from non-endemic area). Blood sample was collected in EDTA containing vacutainer tube from cephalic vein of each dog, and lesion scraping from 14 dogs with clinical signs. Blood samples processed to plasma and buffy coat. Plasma used for rK39ITLeish dipstick test. DNA was extracted from all sample types (whole blood, plasma, buffy coat and skin lesion) of 47 dogs for RT-qPCR analysis. Anti-*Leishmania* antibodies detected in six dog samples using an rK39 rapid diagnostic test. *Leishmania donovani* DNA detected in three dogs through RT-qPCR. Dogs were; one apparently healthy and negative for rK39, one apparently healthy but positive for rK39 and one clinically suspected and positive for rK39.

Conclusion/significance
This is the first report of *Leishmania donovani* DNA detection in dogs in the study areas. Low parasite DNA was detected and was difficult to quantify parasite load. Hence, dog’s reservoir potential for transmission to sand fly remained unclear at the area. Use of enough sample size of dogs, follow up study on confirmed canine leishmaniasis cases, xenodiagnosis, looking at skin parasite load which may be more relevant for infectivity to sand-fly is suggested in order to explore relevance of dogs as reservoir for transmission of *Leishmania donovani* in Ethiopia.
Author Summary

Leishmaniasis is a neglected vector-borne disease of tropics and subtropics endemic to many countries across the world. It is caused by obligate intracellular protozoa of the genus *Leishmania* and is a disease of both humans and animals. In the present study areas, Humera and Sheraro, human visceral leishmaniasis is endemic. However, the source of infection is not yet well known. There are some seroprevalence reports of the parasite in some animal species in these areas but not confirmed with parasitological and or molecular techniques. In this study, the authors examined blood samples from dogs living at human visceral leishmaniasis endemic area with serological and molecular (quantitative PCR) techniques. DNA of the parasite was detected in some clinically symptomatic and asymptomatic dogs suggesting dogs could be potential reservoirs for the parasite at the area. The attempt for parasite load quantification was not successful due to low level of DNA detected. Confirmation of the presence of the parasite in dogs will help in designing appropriate prevention, treatment and control measures of the parasite in the study areas.

WITHDRAWN see manuscript DOI for details
1. Introduction

Leishmaniases are a group of diseases with a broad range of clinical manifestations caused by several species of parasites belonging to the genus *Leishmania* (Family: Trypanosomatidae). *Leishmania* parasite, a haemo-flagellate protozoan organism, is exclusively transmitted by the bite of female sand-fly of the genus *Phlebotomus* or *Lutzomyia* (1). It is a vector-borne tropical disease with major public health concern in poor countries and yet not given the attention it deserves.

*Leishmania* parasite gets into human population when human, sand fly and the reservoir hosts share the same environment (2). *Leishmania* is transmitted to humans and other mammals by the bite of an infected sand fly vector (3). There are two main sources of human leishmaniases; zoonotic leishmaniases, in which the reservoir hosts are primarily wild animals, commensals or domestic animals, and anthroponotic leishmaniases, in which the reservoir host is primarily human (4).

*Leishmania (L.) donovani* and *L. infantum* are species of the *L. donovani* complex and are the main causative agents of visceral leishmaniasis (VL). *L. infantum* is responsible for zoonotic VL, with dogs as the main reservoir hosts in the Mediterranean area, the Middle East, Asia, and South America (5). On the other hand, *L. donovani* is thought to be anthroponotic, and responsible for VL both in the Indian subcontinent and eastern Africa (6).

According to world health organization (WHO) and Pan American Health Organization (7), leishmaniasis can present in three clinical forms. Cutaneous leishmaniasis is one form of the disease, manifested by localized or multiple skin ulcers. Visceral leishmaniasis is the most severe form of the disease manifested by fever, weight loss, inflammation of the spleen and liver, and anemia, and can be fatal in 90% of cases, if not properly treated). Mucosal leishmaniasis is another form of the disease in which lesions occur on mucous membranes of the nose, mouth, throat cavities, and surrounding tissues.
Globally, both cutaneous and visceral leishmaniasis are endemic to many countries (8). An estimated 50,000 to 90,000 new VL cases occur each year across the globe (9). More than 90% of the global reported VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (8).

Leishmaniasis is endemic to Ethiopia. Plain areas of Humera (western Tigray) and Metema share 60% of the burden (10). According to a recent “review by (11)”, the overall random pooled prevalence of leishmaniasis in Leishmania endemic areas of Ethiopia from 34 previous studies done on 52,706 humans and animals was 16% (95% CI: 12%–20%) in humans and 21% (95%, CI: 15%–27%) in animals with major occurrence in the northern part of Ethiopia. The author of this review also stated, VL as the most dominant type of leishmaniasis in Ethiopia (11).

In different areas in Ethiopia, anti-Leishmania donovani antibodies detected in dogs (12), domestic animals (13) and rodents (14). In northern Ethiopia, studies have detected L. donovani antibodies in domestic and wild animals (15). In a study conducted by (13) on exposure to Leishmania species to domestic animals in Humera, Sheraro and Adis alem, 18.9% of animals were seropositive for anti- L. donovani IgG with highest sero-positivity in dogs (55.9%) followed by sheep (10.5%) and goat (10%).

However, whether dogs could potentially be a reservoir for transmission of the parasite to sand flies or not is yet not clearly stated. The hypothesis was, L. donovani can be detected in clinically symptomatic and asymptomatic dogs in an area endemic for human VL caused by L. donovani and dogs could have sufficient parasitemia /skin parasite load to be potential reservoirs for transmission to sand fly.

Hence, detecting the parasite DNA, determining the parasitemia level and skin parasite load in dogs could suggest parasite transmission possibility from clinically diseased and asymptomatic dogs to sand flies. Therefore, the primary objective of the study was to detect the L. donovani parasite in symptomatic and asymptomatic dogs living in human VL endemic areas, Western Tigray through real time quantitative PCR (qPCR) and quantify the parasite load.
2. Material and Methods

2.1. Study Area

The study was conducted in two districts of western Tigray (Humera and Sheraro) (Fig. 1) in the northern part of Ethiopia from October 2019 to April 2020. Humera has a hot semi-arid climate with fertile land used for agricultural production. Human visceral leishmaniasis is endemic to the area (11). Human visceral leishmaniasis incidence is more common during weeding and harvesting season (July-December) than in dry season (January-June) (16). Previous research reports have also shown the detection of anti-*L. donovani* anti-bodies in various domestic animals among which dogs are the main ones in these areas (13, 15, 17). Moreover, phlebotomine sand flies are believed to be present especially in Humera where they prefer to live in hot low land areas (18). Sheraro is also near to Humera, in a relatively higher elevation, some 1246 meters above sea level.

Fig 1. Map showing the present study areas (Humera and Sheraro)
2.2. Ethical Clearance and Nagoya Compliance

For sampling and animal handling, ethical approval was obtained from Animal Ethics and Experimentation Committee (AEEC) of Mekelle University (S1 Appendix). Material (DNA) export permit letter was obtained from Ethiopian Biodiversity Institute (EBI). Nagoya protocol on access and benefit sharing of the biological material was also agreed between the provider country Ethiopia (through Ethiopian Biodiversity Institute and Mekelle University) and Belgium (Institute of Tropical Medicine) and an assurance letter was received from Institute of Tropical Medicine stating that “the genetic material will not be utilized in any other research program or commercial purposes or for obtaining intellectual rights and if any, Ethiopian Biodiversity institute will be informed”.

2.3. Study Design

Cross sectional study was employed in dogs living in human VL endemic areas with the objective of detecting *L. donovani* and determining the parasitemia and skin parasite load as an indication of reservoir potential for transmission to sand fly. Study sites were selected purposively based on previous disease prevalence and incidence rates. Sampling period was in the autumn (October-November), 2019.

2.4. Sampling

A total of 90 (Humera, n= 44; Sheraro, n=45; Mekelle, n=1) dogs were selected from 303 dogs that visited veterinary clinics for rabies vaccination (in Humera), scheduled deworming (in Sheraro) and in Mekelle (for negative control). Thirty six (36) dogs (n=20 from Humera and n=16 from Sheraro) with suspected symptoms ((weight loss, alopecia, dermatitis, abnormally long or brittle nails) (19), (20), (21)) were purposively selected. The remaining 53 dogs with no clinical symptoms were selected through systematic sampling frame of one every five dogs. One additional apparently healthy dog selected from Mekelle for negative control. Selected dogs were registered on structured dog information recording format (S2 Appendix). All dogs were owned, local breed and always spent 24 hours outdoor.
Six milliliter (ml) blood sample was withdrawn from cephalic vein of all selected dogs (n=90) with syringe, immediately reconstituted to 10 ml blood collection vacutainer tube with EDTA and processed as per the sample processing layout (S3 Appendix). Additional lesion scraping samples were also collected from 14 dogs using sterile blade, placed in 1.5ml capped tubes with no buffer.

2.5. rK39ITLeish Dipstick Test

After separation of plasma from whole blood through centrifugation at 1500 revolution per minute (rpm) for 10 minutes (S3 Appendix), rk39ITLeish dipstick test (from InBios International, Inc. Seattle, WA USA 98104) which is Kalazar Detect Rapid Test used for leishmaniasis diagnosis in humans was done on plasma samples of all 90 dogs to detect antibodies of *Leishmania donovani* parasite according to manufacturer’s test procedure. Some studies have shown that the test has been done in dogs before (22, 23). Test results were scored qualitatively according to manufacturer’s result interpretation guideline as indicated in (Fig. 2).

![Fig 2. Kalazar Detect Rapid test interpretation](image)

appearance of test line and control line is interpreted as positive, control line with no visible test line interpreted as negative, no test line and control line indicate invalid test, abbreviations: L=test line, C=control line
2.6. DNA Extraction

QIAamp DNA Blood Mini Kit (for whole blood, buffy coat and plasma) and QIAamp DNA Mini Kit for lesion scraping (Qiagen, Germany) were used for DNA extraction as per the manufacturer’s protocols except elution in 50µl elution buffer AE (10 mM Tris-Cl, 0.5Mm EDTA; pH 9.0) where elution in 200 µl is recommended by the manufacturer. DNA extraction was made in five extraction rounds. One positive control (190µl whole blood or buffy coat from dog of non-endemic area spiked with 10µl containing 100pg (concentration measured by Qubit fluorometer (instrument used for quantification of DNA, RNA and Protein)) known *Leishmania donovani* DNA of strain BPK282/0cl04/12, one negative control (200µl whole blood from dog of non-endemic area) and one negative extraction control (200µl distilled water) were also included in every extraction round. Extracted DNA samples were kept at -20°C until shipped to The Institute of Tropical Medicine (ITM), Antwerp (Belgium) in ice box for detection and quantification of the parasite DNA using real time quantitative PCR (qPCR).

2.7. Quantitative Real Time PCR (RT-qPCR)

Quantitative real time PCR was done for all DNA extracts and controls. Amplification was made in a final volume of 25µl PCR reaction targeting kinetoplast minicircle DNA (kDNA) of *Leishmania donovani* parasite (25). PCR mixture components included in the PCR reaction and sequence of primers are presented in (table 1 and 2) respectively. No-template controls (nuclease free water), positive extraction control (2µl *Leishmania donovani* DNA of strain BPK282/0cl04/12), negative extraction control (an extract of distilled water) were included in each run, as well as serial dilutions from 22.5 ng/µl DNA concentration of parasite BPK282/0cl04/12 to make standard curve. Starting from a 22.5 ng/µl DNA solution in TE, a stock of 22.5pg/µl was prepared. This stock was used to prepare dilutions of 10,000 femtogram (fg) down to 0.01 fg in steps of 10, in TE supplemented with 0.1 mg/ml acetylated bovine serum albumin (AcBSA) (Fig. 3). TE with 0.1 mg/ml AcBSA was prepared by mixing 50µl of 10 mg/ml acetylated bovine serum albumin (AcBSA) with 4950µl of TE.
Table 1: PCR reaction mixture components

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Volume/reaction</th>
<th>Final con.</th>
<th>Company and lot numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>HotStarTaq master mix kit Qiagen</td>
<td>12.5μl</td>
<td>1x</td>
<td>QIAGEN, ln. 160035620</td>
</tr>
<tr>
<td>Cat No. 203443 (250 U) 2X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylated bovine serum albumin AcBSA (10 mg/ml)</td>
<td>0.25μl</td>
<td>0.1 mg/ml</td>
<td>Promega, ln. 0000311319</td>
</tr>
<tr>
<td>kDNA-CMF (100 μM)</td>
<td>0.15μl</td>
<td>0.6 μM</td>
<td>MAES, ln. 197290825</td>
</tr>
<tr>
<td>kDNA-CMR (100 μM)</td>
<td>0.15μl</td>
<td>0.6 μM</td>
<td>MAES, ln. 197290826</td>
</tr>
<tr>
<td>kDNA-CMP (100 μM)</td>
<td>0.1μl</td>
<td>0.4 μM</td>
<td>MAES, ln. 197830955</td>
</tr>
</tbody>
</table>

nuclease free water

DNA template 2μl or 4μl

Total 25μl

Abbreviations: kDNA-CMF= kinetoplast DNA-charles mary forward primer, kDNA-CMR=kinetoplast DNA-charles mary reverse primer, kDNA-CMP= charles mary probe, AcBSA= acetylated bovine serum albumin and Ln=lot number (26).

Table 2: Primers and Probe

<table>
<thead>
<tr>
<th>Primers and Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDNA-CMF (100 μM)</td>
<td>CTTTTCTCGGTCTCCGCGGTAGG</td>
</tr>
<tr>
<td>kDNA-CMR (100 μM)</td>
<td>CCACCGGGCCCTATTATTACACCAA</td>
</tr>
<tr>
<td>kDNA-CMP (100 μM) (Probe)</td>
<td>FAM-TTTTTCGAGAACGCCCTACCACC-GC-TAMRA</td>
</tr>
</tbody>
</table>

Source: (26)

Fig 3. Serial dilution: dilutions of 10,000 fg down to 0.01 fg in steps of 10, in 0.1% AcBSA TE
X= BPK282/0cI04/12 from concentration 22.5pg/μl, Y= 0.01% AcBSA TE,
After having ready all the preparations, master mix was first aliquoted to plate wells. Then, DNA samples added to aliquoted plate wells. Finally, serial dilution of standard curve added to the plates. Plate wells were then spin down by centrifuge at 1500 revolution per minute (rpm) for 3 minutes before running in Light Cycler machine (http://icob.sinica.edu.tw/pubweb/bio-chem/Core%20Facilities/Data/R401-core/LightCycler480%20II_Manual_V1.5.pdf). Light cycler 480 programed as in (Table 3). TaqMan probe (kDNA-CMP) was already included in the PCR reaction mixture to hybridize on to the target sequence during annealing and then later cleaved by Taq DNA polymerase at extension that lead to the separation of the reporter dye from the quencher dye to give a fluorescent signal. The intensity of this fluorescence is proportional to the amount of amplicon produced.

Analysis was made by absolute quantification/2nd derivative Max for all samples. Standard curve was plotted with cycle threshold (ct) values as a function of logarithm of DNA amount of the serial dilution. Excitation-emission filter combinations of the light cycler were set at 465-510 for fluorophore fluorescein (flous/FAM) in detection format of hydrolysis probe. Standard curve (In run) and high sensitivity were selected to produce the standard curve for absolute quantification and to detect even data curves with a weak rise in fluorescence respectively. The standard curve was then used to calculate y-intercept, the slope of the curve and efficiency of the PCR from which the linear dynamic range was calculated. DNA samples with ct values within the linear dynamic range were considered for quantification.

**Table 3**: Light cycler 480 computer program

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
<th>Ramp rate</th>
<th>Optics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>15 min</td>
<td>1</td>
<td>4.4</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5 sec</td>
<td></td>
<td>4.4</td>
<td>Off</td>
</tr>
<tr>
<td>Cycling</td>
<td>58</td>
<td>20 sec</td>
<td>50x</td>
<td>2.2</td>
<td>Single SYBRGreen I</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>30 sec</td>
<td></td>
<td>4.4</td>
<td>Off</td>
</tr>
<tr>
<td>Cooling</td>
<td>40</td>
<td>30 sec</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**2.8. Data Analysis**

All data related to diagnosis results were stored in Microsoft access and database was created (S4 Appendix) for analysis. Excel was employed to summarize results of clinical examination and
rK39ITLeish dipstick test. RT-qPCR results were analyzed using linear regression for calculating efficiency of PCR, R square value, limit of detection, slope, intercept, and to construct the linear relationship between logarithmic DNA and ct values. Kappa test agreement was also used as a measurement of agreement between diagnostic test results. Sensitivity and specificity of diagnostic techniques were calculated using MedCalc’s diagnostic test evaluation calculator taking RT-qPCR as a reference.
3. RESULTS

3.1. Clinical Examination, rK39 Serology

Of the 89 clinically examined dogs, 36 were clinically suspected. Clinical symptoms noticed were skin lesions (alopecia, dermatitis), weight loss, emaciation and long and brittle nails. The rest look apparently healthy. Only 6.74% (6/89) were positive for rK39ITLeish dipstick test. The sample from non-endemic area of one dog was negative for rK39ITLeish dipstick test and PCR.

3.2. Real Time Quantitative PCR (RT-qPCR)

3.2.1. Technical validation of standard PCR curves

Samples were analyzed in five PCR runs. Standard curves made from Serial dilution of known Leishmania donovani DNA (BPK282/oocl04/12) starting from 10000 femtogram (fg) down to 0.01 fg in steps of 10 were included in all the five PCR runs. Efficiency of the PCR is 2.04 with limit of detection being 0.01fg DNA in all PCRs. The linear relationship between the logarithmic DNA amount in femtogram and ct values is $Y = aX + b$, i.e. $-3.2262X + 38.311$ with an R square value of 0.994. Details of linear dynamic range and connected data points in five PCR runs are presented in (Fig.4 and 5) respectively and used as a reference for quantification in all PCRs.

![Fig 4. Linear regression of ct values as a function of DNA amount in femtogram used in all PCRs. It is used as a reference for quantification in all PCRs. Data points within the circle above are detected DNAs that cannot be quantified.](https://example.com/fig4.png)
Fig 5. Connected data points of Standard curves included in 5 PCR runs.
The Ct values are shown in function of the amount of DNA of strain BPK282/oclo4/12 added to the PCR mix, on a logarithmic scale.

3.2.2. Technical validation of positive control in PCRs
All (n=5) positive extraction controls (PEC), included one in each PCR run with 2μl volume and expected 3.2pg known DNA in it (80% DNA extraction efficiency assumed, QIAamp DNA Mini and Blood Mini Handbook, 2016), show signals that can be quantified as indicated in (Fig. 6) but output DNA in PCR is reduced in all PCR runs as compared to the input DNA (table 4). Positive control extracted with QIAamp DNA Mini Kit give even a lesser DNA amount than positive controls extracted with QIAamp DNA Blood Mini Kit in PCR (Table 6).

Table 4: Summary of input DNA before extraction, output DNA in PCR, and kit used

<table>
<thead>
<tr>
<th>Input DNA</th>
<th>Output DNA (PCR)</th>
<th>ct value</th>
<th>Kit used</th>
</tr>
</thead>
<tbody>
<tr>
<td>100pg in 200μl sample,</td>
<td>1070 fg</td>
<td>28.09</td>
<td>QIAamp DNA Blood Mini Kit</td>
</tr>
<tr>
<td>Eluted in 50μl AE buffer, with 80% efficiency=80pg,</td>
<td>1890fg</td>
<td>27.57</td>
<td>QIAamp DNA Blood Mini Kit</td>
</tr>
<tr>
<td>Then, 2μl DNA included in PCR=3.2pg</td>
<td>63.7fg</td>
<td>32.99</td>
<td>QIAamp DNA Mini Kit</td>
</tr>
<tr>
<td></td>
<td>1890fg</td>
<td>27.57</td>
<td>QIAamp DNA Blood Mini Kit</td>
</tr>
<tr>
<td></td>
<td>1070fg</td>
<td>28.09</td>
<td>QIAamp DNA Blood Mini Kit</td>
</tr>
</tbody>
</table>
3.2.3. Technical validation of no template controls (NTCs) in extraction and PCRs

PCR signal was detected in two no template control (NTC) wells included in extraction and PCR with ct values of 18.1 and 40.32 respectively. 14 NTC wells in extraction and 14 NTC in PCR show no amplification detection (Fig. 7).

Fig 6. Technical validation of positive control in PCRs: Blue diamonds in the above figure represent input DNA before extraction in all extraction rounds, red squares represent output DNA in PCR.

Fig 7. PCR results of no template controls. Ct value of 50 is an artificial value for negative results. Blue diamonds in the above figure represent negative results of no template controls, red squares represent positive results of no template control.
3.2.4. Technical validation of negative control and negative extraction controls in PCRs

No signal detected in all the five negative control (2μl DNA of whole blood from dog of non-endemic area) and five negative extraction control (extract of distilled water) included in all PCRs (Table 5).

**Table 5:** PCR results of negative control and negative extraction controls

<table>
<thead>
<tr>
<th>Negative Extraction Control</th>
<th>volume</th>
<th>Number (n) included</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>2μl</td>
<td>Five, one in each PCR</td>
<td>all negative</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td>Number (n) included</td>
<td>PCR result</td>
</tr>
<tr>
<td>whole blood from dog of non-endemic area</td>
<td>2μl</td>
<td>Five, one in each PCR</td>
<td>all negative</td>
</tr>
</tbody>
</table>

Negative control and negative extraction controls, all tested negative

3.2.5. PCR results of dog samples

For real time qPCR analysis, 47 dog samples including seropositive samples were used. PCR signal detected in 3/47 dog samples (3.37%); in whole blood, buffy coat and plasma of n=2 dogs and in whole blood of the other dog. No signal detected in lesion scraping samples. In positive dog samples, there was variation in detection and when detected, there was variation in amount of DNA between sample types (whole blood, buffy coat and plasma) of different PCR runs. Positive samples were spiked with 10fg DNA in PCR tube to see if the variation was due to PCR inhibition. Cycle of threshold (Ct) values and DNA amount (in fg) of spiked samples were compared with ct value and DNA of 10 fg in standard curve (Table 6).

**Table 6:** DNA amount in fg in 2μl of positive dog samples, Ct values, DNA amount (in fg in PCR) before and after spiking

<table>
<thead>
<tr>
<th>PCR signal Detected in</th>
<th>animal code</th>
<th>sample type</th>
<th>PCR run 1 and 2</th>
<th>PCR run 5</th>
<th>Ct value and DNA amount (in fg before spiking)</th>
<th>Ct value and DNA amount after spiked with 10 fg DNA</th>
<th>Ct value in 10 fg DNA of standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>dog 31</td>
<td>whole blood</td>
<td>37.25 (6.39E+00)</td>
<td>ND</td>
<td>36.02 (5.15E+00)</td>
<td>35.59 (6.85E+00)</td>
<td>36.02 (5.16E+00)</td>
<td>34.95 (1.06E+01)</td>
</tr>
<tr>
<td>dog 31</td>
<td>buffy coat</td>
<td>ND</td>
<td>40.25 (&lt;1fg)</td>
<td>35.97 (5.34E+00)</td>
<td>35.12 (9.40E+00)</td>
<td>36.02 (5.16E+00)</td>
<td></td>
</tr>
<tr>
<td>dog 31</td>
<td>plasma</td>
<td>ND</td>
<td>40.81 (&lt;1fg)</td>
<td>35.05 (9.86E+00)</td>
<td>36.02 (5.16E+00)</td>
<td>35.56 (7.02E+00)</td>
<td></td>
</tr>
<tr>
<td>dog 88</td>
<td>whole blood</td>
<td>26.1(3.51E+03)</td>
<td>40.52 (&lt;1fg)</td>
<td>35.56 (7.02E+00)</td>
<td>35.02 (1.01E+01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7: Summary of correlation between different diagnostic technique results

<table>
<thead>
<tr>
<th>Correlation between rK39 and RT-qPCR</th>
<th>RT_qPCR</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
<td>total</td>
<td></td>
</tr>
<tr>
<td>rK39ITLeish dipstick test</td>
<td>positive</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>1</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>3</td>
<td>44</td>
<td>47</td>
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</table>

<table>
<thead>
<tr>
<th>Correlation between rK39 and clinical examination</th>
<th>Clinical examination</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>clinically suspect</td>
<td>apparently healthy</td>
<td>total</td>
<td></td>
</tr>
<tr>
<td>rK39ITLeish dipstick test</td>
<td>positive</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>34</td>
<td>49</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>36</td>
<td>53</td>
<td>89</td>
</tr>
</tbody>
</table>
Table 8: Summary of positivity of PCR positive samples in clinical exam, rK39ITLeish dipstick test

<table>
<thead>
<tr>
<th>PCR positive dogs</th>
<th>Clinical examination</th>
<th>rK39ITLeish dipstick test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 19</td>
<td>Apparently healthy</td>
<td>Positive</td>
</tr>
<tr>
<td>Dog 31</td>
<td>Clinically suspected</td>
<td>Positive</td>
</tr>
<tr>
<td>Dog 88</td>
<td>Apparently healthy</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Abbreviations: exam=examination

3.4. Sample Type and PCR Positivity

Positive PCR signal was detected in three dog samples of whole blood and buffy coat and in two plasma samples with varying DNA amount. There was no signal detected in skin lesion scraping samples (Fig. 8).

Fig 8. Sample type and PCR positivity
4. Discussion

In the present study, a total of eighty nine (89) dogs from human VL endemic areas and one dog from non-endemic area (as negative control) used for leishmaniasis diagnosis with clinical examination and rK39 serology. RT-qPCR employed on samples of 47 dogs.

Clinically, 36 of the dogs had *Leishmania* related symptoms. Skin lesions (alopecia, dermatitis) were major clinical symptoms (in 18/36) followed by long and brittle nails (in 11/36). Six dogs had skin lesion, weight loss and long and brittle nails; one dog with skin lesion and emaciation. However, only one dog from those with *Leishmania* related clinical symptoms were positive in RT-qPCR.

The seroprevalence of 6.74% detected in this study using rK39ITLeish dipstick test was lower than the 14.8% (from N=162) reported by (15) using kalazar detect test (KDRT) but slightly higher than 4.35% (from N=46) observed by (27) using ITLeish rapid detection test. This could be due to differences in sensitivity and specificity of serological tests (28), infection status of the patient (23) and differences in sample size. Another reason could also be due to differences in the type of test kit used where rK39ITLeish dipstick test used in this study was the one designed for leishmaniasis diagnosis in humans. Herrera and colleagues suggested that designing of tests with antigens of the circulating strains in humans and dog would improve diagnostic utility (22).

Presence of rK39 antibodies in dogs in the present study areas indicated possible contact of *L. donovani* with dogs. However, this doesn’t always reflect true infection due to i) low specificity of the serologic test and or presence of other cross reacting parasites in sampled dogs (29), ii) inability of the serologic test to differentiate active infection from cured as circulating antibodies may persist longer after cure (30).

The most important finding of the study was detection of *L. donovani* DNA in dog samples (whole blood, buffy coat and plasma) through RT-qPCR. In order to detect and quantify, standard PCR curve was made for each PCR run. To validate the extraction process and possible contamination, positive control, negative control and negative extraction controls were included in each extraction round. All positive controls included in each extraction round become positive in PCR but with reduced DNA yield than input DNA in extraction. With inclusion of 100pg
DNA positive control in extraction and assumed 80% extraction efficiency in 50\(\mu\)l elution buffer from which 2\(\mu\)l DNA template included in PCR, an output of 3.2 pg DNA was expected in PCR. However, only 1070 fg and 1890 fg DNA was detected in QIAamp blood mini kit and 63.7 fg DNA in QIAamp mini kit. QIAamp mini kit is originally designed for DNA extraction of tissue samples but in this study, it was used for DNA extraction from whole blood of positive control and this could be the reason for the low DNA yield in PCR (63.7 fg DNA). All negative and negative extraction controls tested negative. No template controls (NTCs) included in PCRs tested negative except in two NTCs with possible contamination. These two positive NTCs could be due to either possible contamination in extraction room or error in pipetting from serial dilution of higher concentration.

Three of the 47 dog samples tested positive for RT-qPCR with varying DNA concentration in different PCR runs. This is the first report of molecular detection of \textit{L. donovani} DNA in dogs in the study areas where human VL is endemic in Ethiopia.

PCR results of positive dog samples show discrepancy in DNA amount (fg) in different PCR runs of same and different sample types (whole blood, buffy coat and plasma). Reasons for this variation could be: (i.), variation in the amount of parasite DNA within the 2\(\mu\)l sample DNA included in RT-qPCR. When there are few circulating parasites or DNA in the blood, there is a small chance that they are picked up however, why variation in ct or DNA amount from the same sample in repeated PCR runs is not clear. (ii.), variation in the amount of parasite DNA in different sample types (whole blood, buffy coat and plasma). Buffy coat is the portion of blood that contains concentrates of white blood cells. Buffy coat contains more parasite DNA than whole blood and plasma therefore PCR is more sensitive for buffy coat than for whole blood (33). In two dog (dog 31 and dog 19) samples, there was PCR signal in whole blood, buffy coat and plasma at least in one PCR run of repeated PCRs. However, there was still variation in ct even with diplo PCR runs of same DNA template. PCR inhibition test employed in all positive dog samples spiked with 10 fg DNA each, show very little inhibition when compared with standard curve of 10 fg DNA, (only one ct value difference that is half to what is expected of 10 fg DNA in PCR).
Low DNA concentration detected in different PCR runs and different sample types make it difficult to calculate parasite load in blood and chance of the parasite to be picked up by the sand-fly. In principle, if there is at least one parasite in a sample, eluted in 50μl extraction buffer from which 2μl template DNA is included for PCR, it should also give positive result in PCR with 4 fg DNA (provided 100% extraction efficiency). However, with less than 1 fg DNA detected in most PCR runs of positive samples from 200μl sample extract, one can extrapolate that the chance of a sand-fly picking up a parasite in a blood meal would be less than 1 in 100 times considering a sand-fly to pick 2-3μl blood in one sting. *Leishmania* is not a true blood parasite thus more DNA would be expected to be extracted from tissue samples than blood samples by conducting a follow up study identifying DNA in skin samples which is more relevant to sandfly transmission. Though role of dogs as a reservoir for transmission was not confirmed in this study, presence of parasite DNAs in blood could be an indication that dogs could play a role in transmission as in other regions of east Africa (34, 35) and India (36) where dog is considered a possible reservoir for transmission of the parasite *L. donovani*.

Only two of the 36 dogs with clinical symptoms were positive for rK39 antibodies whereas, of the apparently healthy 53 dogs, 4 were positive with detectable rK39 antibody. Absence of clinical symptoms in rK39 antibody positive dogs could either be due to recovery from infection but persistent circulating antibodies or resistance to the parasitic infection. Kalayou and colleagues also reported detection of *Leishmania donovani* antibodies in 14.8% asymptomatic dogs in the same study area (15).

Of clinically suspected 36 dogs, only one dog tested positive for RT-qPCR. The other two PCR positive dogs were asymptomatic. Reasons for asymptomatic infection could be that; dogs could be resistant to the parasite, diagnosis could have been done at early infection before clinical symptoms develop. Therefore, asymptomatic dogs can still have parasites. Observed clinical symptoms in dogs tested negative for RT-qPCR could be due to other diseases as other infections can cause similar clinical presentations too. This shows that, all symptomatic dogs are not truly infected with the parasite as *Leishmania* infection does not show pathognomonic clinical symptoms.
Only 33.33% (2/6) dogs with rK39 antibodies tested positive by RT-qPCR. Four seropositive dogs tested negative for PCR. This could be due to the reason that dogs recovered from infection but still have circulating antibodies tested positive for rK39. This is the major limitation of serological tests; inability to differentiate active infection from relapsed ones (30).

Taking RT-qPCR as a definitive diagnostic technique, Diagnostic accuracy of rK39 was 89.36% (95% CI: 76.90% - 96.45%) and sensitivity and specificity was 66.67% (95% CI: 9.43% - 99.16%), and 90.9% (95%, CI: 78.33% - 97.47%) respectively, slightly lower than sensitivity (72 to 77%) but higher than specificity (61 to 75%) reports of (37) in Brazil.

In conclusion, Leishmania donovani DNA was detected in one symptomatic and two asymptomatic dogs living in human VL endemic areas. DNA was detected in different sample types; whole blood, buffy coat and plasma with varying DNA concentration but not in lesion scraping. Parasitemia and skin parasite load were not calculated because of high ct values of positive dog samples in repeated PCR runs and sample types. Small sample size of dogs used in this study and use of rapid diagnostic test not designed for dogs may partially contribute for some inconclusive results. Use of enough sample size of dogs, and follow up study on confirmed canine leishmaniasis cases from the area, xenodiagnosis, looking at skin parasite load which may be more relevant for infectivity to sand-fly is suggested in order to explore relevance of dogs as reservoir for transmission of Leishmania donovani in Ethiopia.
Acknowledgments

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5. REFERENCES

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