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A novel Real-Time PCR-based kit for the diagnosis of *Leishmania* infection: integration into a routine reference laboratory

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Abstract

Introduction. Leishmaniasis is a vector-born disease endemic in more than 98 countries that cause over 1.6 million of new cases annually. The wide range of clinical symptoms makes leishmaniasis a diagnostic challenge, therefore a rapid and accurate diagnosis is essential for effective treatment. Several researches established that Polymerase Chain Reaction (PCR) is a sensitive and rapid method for the diagnosis of leishmaniasis in human samples. The aim of this work is to evaluate the performance of STAT-NAT® Leishmania spp. kit, a novel Real-Time PCR-based assay designed to detect all *Leishmania* species.
Materials and Methods. The assay was developed as a ready-to-use test containing all the required elements for the amplification of both *Leishmania* kinetoplast DNA (kDNA) minicircle fragment and human beta-globin gene as internal control. In the present study, a total of 78 samples obtained from San Matteo Hospital of Pavia, previously diagnosed with the methods considered "Gold Standard", were analyzed with STAT-NAT® Leishmania spp. kit. PCR reactions were performed on nucleic acids extracted from peripheral blood samples. STAT-NAT® Leishmania spp. kit was also used for a clinical evaluation performed by Bambino Gesù Children's Hospital (OPBG). During 2012-2013, a total of 453 samples (peripheral blood samples and bone marrow aspirates) were analyzed for *Leishmania* screening with STAT-NAT® Leishmania spp. kit.
Results. Among the 78 samples, 28 were diagnosed as positive and the remaining 50/78 patients were reported as *Leishmania* negative. The immunocompromised patients were reported as negative (5/50). STAT-NAT® Leishmania spp. kit confirmed all the results with one exception: one of the immunocompromised patients was found positive to the Real-Time PCR assay. In the clinical evaluation, nine of 453 samples, obtained from patients living in endemic areas, were found positive with STAT-NAT® Leishmania spp. kit and the data were totally confirmed with microscopy. STAT-NAT® Leishmania spp. kit was introduced in OPBG diagnostic routine, for its rapidity (about 2 hours versus 5 hours) and reproducibility.
Conclusions. The described Real-Time PCR assay proved its effectiveness for the detection of pathogenic *Leishmania* spp. in various clinical samples. The test showed a sensitivity and a specificity of 100%. The use of the Real-Time PCR-based assay to investigate peripheral blood samples, which can be collected much more easily than bone-marrow aspirates and with much less pain for the subject, can be essential as a confirmatory test but also as a screening test.

Background

Leishmaniasis is a vector-born neglected tropical disease, caused by the infection with *Leishmania* parasites transmitted through the bite of an insect vector, the phlebotomine sandfly. The most severe form is Visceral Leishmaniasis (VL) but it could be also mucocutaneous or cutaneous, the most common form. VL, also known as kala-azar, has emerged as an important opportunistic infection associated with AIDS. The wide range of clinical symptoms makes leishmaniasis a diagnostics challenge, for that reason a rapid and accurate diagnosis is a prerequisite for effective treatment. Recent researches have established the high rapidity, sensitivity and specificity of Polymerase Chain Reaction (PCR) in the detection of the *Leishmania* spp. DNA in a wide range of human samples. This finding recommends the use of a PCR molecular assay in the routine diagnosis.

Materials and Methods

STAT-NAT® Leishmania spp. (IVD CE marked) is a freeze-dried and ready-to-use kit that includes all the required elements for the amplification of nucleic acid targets (Figures 1, 2). Its intended use is for the detection of *Leishmania* parasites.

Figure 1: STAT-NAT® Leishmania spp. components.

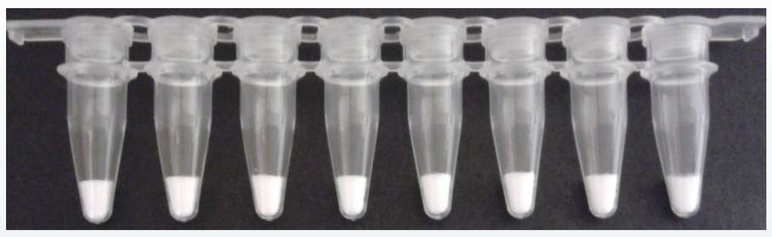

Figure 2: STAT-NAT® Leishmania spp. freeze-dried mix.

*A specific set of primers and probe was designed to identify a fragment in the kinetoplast DNA (kDNA) minicircle; a second set of primers and probe amplify the Human Beta Globin gene fragment, used as an internal amplification control (IC).
The two targets are co-amplified and detected by 7500 and 7300 Real-Time PCR instruments (Life Technologies) (Table A).

Step	Cycle Number	Denaturation	Annealing	Total run time
1	1	95 °C 10 min	-	90 min
2	45	95 °C 15 sec	-	
		-	60 °C 1 min	

Table A: Thermal cycler protocol.

STAT-NAT® Leishmania spp. performances were evaluated on:
- a total of 78 blood samples, 5 of them from immunocompromised patient, obtained from San Matteo Hospital (OSM) of Pavia;
- a total of 453 blood and bone marrow samples collected during 2012-2013 and tested by Bambino Gesù Children's Hospital (OPBG) of Rome.

All samples were diagnosed using different "Gold Standard" methods:
- direct determination of *Leishmania* amastigotes in Giemsa stained bone marrow aspirate;
- microscopical evaluation of various biological specimens;
- in-vitro culture;
- serology test for anti-*Leishmania* IgG titration.

The panel of samples includes strong positives as well as weak positives and negatives (Table B).

	Positives	Negatives
OSM samples	25	50
OPBG samples	9	444

Table B: Samples panel.

DNA samples were extracted both with a silica based system or QiaSimphony instrument (Qiagen).

Results

Among the 78 samples, 28 were diagnosed as positive and the remaining 50/78 patients were reported as *Leishmania* negative. The immunocompromised patients were reported as negative (5/78). 25 patients were suspected for Visceral Leishmaniasis (VL), and the other 3 for Cutaneous Leishmaniasis (CL).

Samples	Positives	Negatives
VL	25	50
CL	3	
TOTAL	**28**	**50**

Table C: OSM samples results.

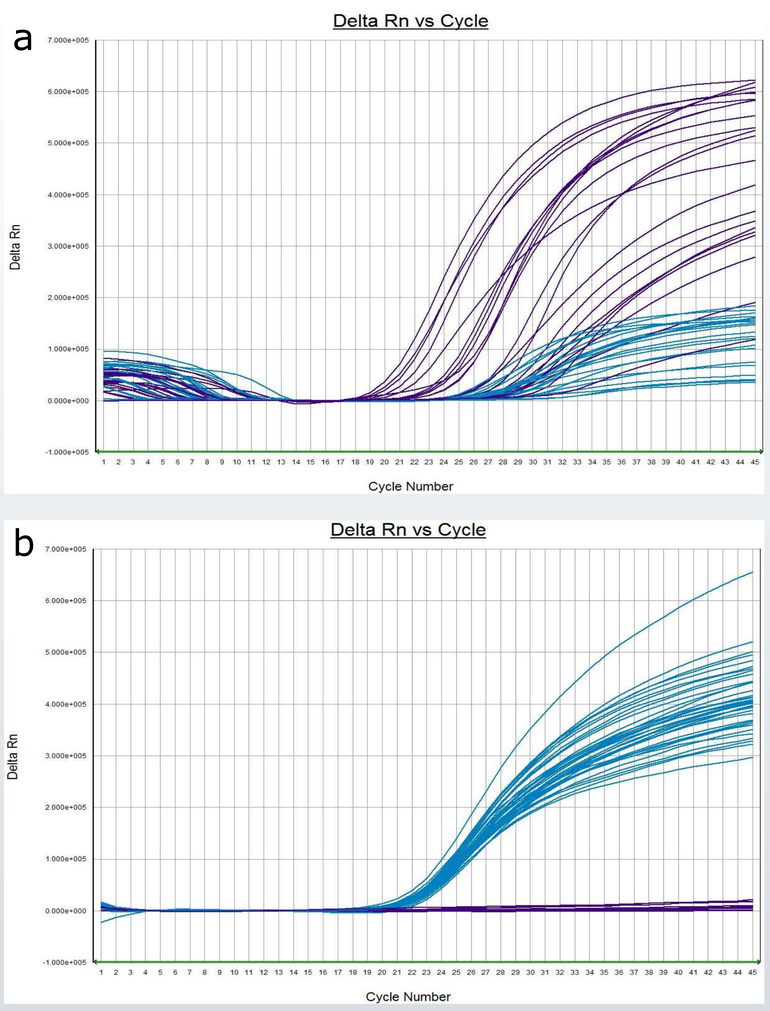

Figure 3: Real-Time PCR amplification plot: Human Beta globin gene (IC) amplification in blue; *Leishmania* amplification in purple. a) *Leishmania* positive samples; b) *Leishmania* negative samples.

The clinical evaluation of 453 samples, obtained from patients living in Italian endemic areas, were performed with STAT-NAT® Leishmania spp. in OPBG laboratory (Figures 4, 5). Obtained data totally agreed with microscopy results and confirmed nine positive samples, collected in Lazio and Abruzzo, and 444 negative samples (Figure 6).

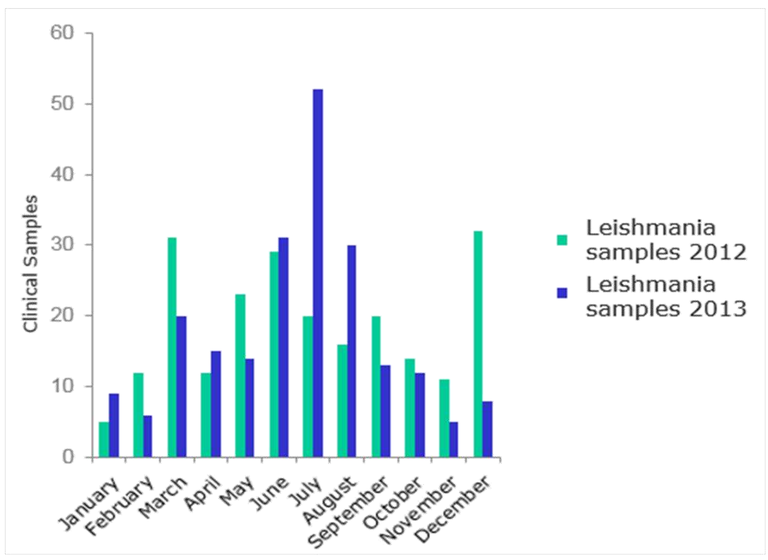

Figure 4: OPBG Clinical *Leishmania* samples tested during 2012-2013.

Figure 5: Map (ArcView GIS 10) showing the distribution of town (in blue) where autochthonous cases of canine leishmaniasis have been recorded, Italy, 2005-2012 (Gramiccia *et al.*, 2013).

	2012	2013
January	0	0
February	1	0
March	1	0
April	0	0
May	1	0
June	0	1
July	1	2
August	0	0
September	0	0
October	1	0
November	0	1
December	0	0

Lazio	Positive samples 2012-2013
ROMA	4
LATINA	4
FROSINONE	0
VITERBO	0
RIETI	0
Abruzzo	**Positive samples 2012-2013**
L'AQUILA	0
TERAMO	0
CHIETI	0
PESCARA	1

Figure 6: Positive *Leishmania* samples found during the 2012-2013 screening.

Conclusions

STAT-NAT® Leishmania spp. is able to detect all the *Leishmania* infections of a samples panel obtained from reference centers. This kit was introduced in OPBG diagnostic routine for its rapidity and reproducibility.

Bibliography

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