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Comparison of Various Sample Preparation Methods for PCR Diagnosis of Visceral Leishmaniasis Using Peripheral Blood

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We have compared various sample preparation methods for the PCR diagnosis of visceral leishmaniasis (VL) using peripheral blood samples and tested the influence of these protocols upon sensitivity. Four methods of lysis-DNA extraction were used with two types of blood samples: whole blood (WB) and buffy coat (BC). Comparisons were first carried out with seeded samples at various parasite concentrations. At high concentrations (≥1,000 parasites/ml), there were no significant differences in PCR sensitivity among the methods tested. At concentrations of ≤100 parasites/ml, proteinase K (PK)-based methods proved clearly superior to guanidine-EDTA-based methods. Moreover, a 10-fold increase in sensitivity was observed for BC over that for WB. Thus, the best sensitivity was obtained with the BC prepared with PK-based methods. With this combination, the PCR reliably detected 10 parasites/ml but was inconsistent when the sample contained 1 parasite/ml of blood. The methods that yielded the highest sensitivities were evaluated with seven dogs and four human VL patients. Again, the utilization of the BC prepared with PK-based methods gave the best results. The optimization of each step of the assay (sample preparation, DNA extraction, and PCR conditions) yielded a highly sensitive tool for the diagnosis of VL using patient blood, thus avoiding more invasive diagnostic procedures and allowing the detection of low parasitemia during posttherapeutic follow-up.

Leishmaniases are anthropozoonoses due to infection with species of the protozoan parasite *Leishmania*. They present a wide clinical spectrum (from benign cutaneous lesions to fatal visceral disease) and are distributed throughout 88 countries on four continents. Kala-azar, or visceral leishmaniasis (VL), is essentially caused by *Leishmania donovani* sensu stricto in the Indian subcontinent and eastern Africa and by *L. infantum* (also called *L. chagasi* in Latin America) in the whole Mediterranean area, the Middle East, and Latin America. The reservoir of the latter organism is the dog. Atypical VL (also called viscerotropic leishmaniasis) can also be due to infection by *L. tropica* in the Old World (11, 22) and *L. amazonensis* in the New World (2). VL is fatal in the absence of treatment, and all currently available drug treatments are costly, must be given parenterally, and cause serious side effects (6). During the past 10 years, there has been a steady increase of Mediterranean VL, due to the appearance of this disease as a complication of human immunodeficiency virus infection, particularly reported in southern Europe (3). Until the beginning of the 1990s, the biological diagnosis of leishmaniasis relied on classical microbiological methods (Giemsa-stained smears, in vitro cultivation on blood-agar or axenic media, and serological tests). Over the last decade, several studies have shown PCR to be both highly specific and more sensitive than the classical methods for the diagnosis of VL (reviewed in reference 14). PCR can be performed on any biological sample, including skin tissue, blood, and bone marrow, and has been applied to routine hospital diagnosis in many laboratories all over the world. Unfortunately, *Leishmania* PCR is still far from being standardized. Each laboratory has set up its own assay, differing in the DNA preparation, the PCR target, and the reaction optimization; thus, results vary widely according to the lab. Now, it is well documented for different classes of microorganisms that the sample preparation and DNA extraction methods can greatly influence the outcome and reliability of the test (5, 10, 13, 16, 24). Therefore, one must find a compromise between the rapidity needed for hospital routine and the quality of a high-performance diagnosis necessary for such a severe disease. Optimized PCR condition assays for the diagnosis of VL now attain excellent sensitivity using patient blood (8, 14, 18), thus avoiding invasive procedures such as bone marrow sampling.

Two steps can be considered essential for correct DNA amplification: the disruption of the cell membranes and proteins (lysis) and the extraction of the DNA from sample contaminants and cell proteins and debris. For protozoan parasites, as for some other microorganisms, this process is complicated by the abundance of host DNA, which can compete with the parasite DNA (sometimes one million-fold less abundant) and strongly interfere with the reaction. A great number of lysis and DNA extraction methods have been described in the literature (4, 5, 9, 10, 16, 17, 21). We chose to compare two lysis methods coupled with two DNA extraction methods well known as being efficient for blood preparation (see Discussion), as well as a commercial kit in which both steps are performed. We also compared the use of whole blood (WB) and that of the leukocyte fraction, or buffy coat (BC), for this diagnosis. The comparisons were first carried out with seeded blood samples at various parasite concentrations; then

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the methods yielding the highest sensitivities were applied to dogs and human patients.

MATERIALS AND METHODS

Preparation of seeded samples. Seeded samples were made by adding live *L. infantum* promastigotes either to peripheral blood collected on EDTA-coated tubes (as described in reference 14) or to the BC of the latter (for testing a seeded BC, it was logical to add the parasites directly to the BC since leishmanias are localized within the monocytes during natural infection). The concentrations of parasites tested were 1,000, 100, 10, 5, and 1 parasite/ml of blood, corresponding to the DNA equivalents of 10, 1, 0.1, 0.05, and 0.01 parasite/PCR tube, respectively.

Sample lysis methods. All samples consisted of 5 ml of peripheral blood collected in EDTA-coated tubes. For seeded samples, the blood was collected from two different healthy donors and then pooled before being aliquoted again; then the parasites were added at an adequate concentration either in WB or in the BC as described above.

(i) GE. The guanidine-EDTA (GE) method, based on the use of a chaotropic salt to disrupt cells and inhibit nucleases, was adapted from that described by Avila et al. (4) for the PCR detection of *Trypanosoma cruzi* in blood and modified by Wincker et al. (26). For WB preparation, the tube was centrifuged for 10 min at 1,600 × *g*, two-thirds of the plasma was removed, and 1 volume of GE (6 M guanidine hydrochloride–0.2 M EDTA [pH 8.0]) was added. For the BC, the tube was centrifuged as described above, 500 μl of BC was transferred to another tube, and 1 volume of GE was added. The preparation was then incubated for ≥2 days at room temperature (RT), boiled for 10 min, left again between 1 and 7 days at RT, and stored at +4°C.

(ii) PK. Five hundred microliters of WB or BC was removed from sample tubes without or after centrifugation, respectively. Two volumes of TNNT buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH, 10 mM Tris [pH 7.2]) and 320 μg of proteinase K (PK) per ml were added, and the tube was incubated between 2 and 24 h at 56°C, boiled for 10 min, and then stored at +4°C. For dog blood, the PK was raised to 960 μg/ml.

DNA extraction methods. (i) Phenol-chloroform. WB-GE and BC-GE lysates were processed as follows: 200 μl of lysate with 300 μl of sterile distilled water added was subjected to a simplified phenol-chloroform extraction (ΦC) (i.e., phenol-chloroform followed by chloroform), and the DNA was precipitated with ethanol and resuspended in 150 and 200 μl of sterile distilled water for WB and BC, respectively. We noted empirically that the sensitivity of the assay was improved if this extraction was performed immediately after the boiling procedure. For WB-PK and BC-PK lysates, 500 μl of the lysate was subjected to the same protocol as described above, except that the final resuspension volume was 130 μl for both. For dog blood, a phenol step was added before the simplified ΦC.

(ii) Silica beads. The silica bead method (termed S) being part of a commercial kit using guanidine at high concentrations, it was applicable to GE lysates only: 200 μl of lysate was mixed with 10 μl of silica beads (Organon Teknika), and the tube was then processed according to the supplier's instructions. The final elution volume was 150 μl for WB and 200 μl for the BC.

Sample preparation commercial kit. The DNeasy tissue kit (Qiagen), termed K below, was used according to the supplier's instructions with 100 μl of WB or BC. The lysis was based on PK. The final elution volume was 100 μl.

All DNA samples were stored at −20°C.

PCR amplification. The DNA target for PCR amplification was the gene coding for 18S rRNA, a 20- to 40-fold-repeated sequence specific for the genus *Leishmania* (12, 26). The primers used were 5′-GGT-TCC-TTT-CCT-GAT-TTA-CG-3′ (R221) and 5′-GGC-CGG-TAA-AGG-CCG-AAT-AG-3′ (R332), which produce a 603-bp fragment upon amplification (26). A thorough optimization of the PCR conditions was carried out as described previously (14) for each of the preparation methods. The optimized conditions for samples lysed by the PK method and the commercial kit were the following: 5 μl of 10× buffer, 0.6 mg of bovine serum albumin per ml, 200 μM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 60 pmol of primers R221 and R332, and 3 U of *Taq* DNA polymerase (Goldstar; Eurogentec), in a total reaction volume of 50 μl including 10 μl of sample DNA. For the samples lysed by the GE method, the conditions were identical except that the $MgCl_2$ was present at 5 mM and the *Taq* polymerase was present at 4 U. The hot-start technique was used to increase specificity (Dynawax; Eurogentec). The reaction mixtures were cycled in an MJ Research thermal cycler using the following conditions: 94°C for 4 min and 40 cycles of 94°C for 30 s, 56°C (for GE lysates) or 54°C (for PK lysates) for 30 s, and 72°C for 90 s, followed by 72°C for 10 min. Each sample was tested at least twice in duplicate. Furthermore, in each test, one semi-internal positive control tube for the detection of PCR inhibition and one DNA extraction control tube were included for each sample. The inhibition positive control consisted of purified DNA from *L. infantum* promastigotes equivalent to 0.8 parasite, which was added to 10 μl of the DNA sample. The DNA extraction control procedure consisted of the amplification of a fragment of the human β-globin gene using the primers described by Saiki et al. (23) under particularly stringent conditions ($MgCl_2$, primers, and temperature). Finally, three negative control tubes that each received 10 μl of H_2O instead of DNA were included in each test to detect any amplicon contamination. Contamination by amplicons was completely prevented by using drastic physical measures as outlined in the work of Lachaud et al. (14).

PCR product analysis and hybridization. The reaction products were visualized under UV light after electrophoresis of 20 μl of the reaction solution in a 2% agarose gel. All gels were then Southern blotted and hybridized with an α-^{32}P-labeled PCR product from our reference *L. infantum* strain (MHOM/FR/78/LEM75) in an effort to increase the sensitivity.

Patients. Four AIDS patients diagnosed with VL and presenting at the Centre Hospitalier Universitaire of Montpellier were included in the study. The primary diagnosis of VL was confirmed by in vitro cultivation on blood-agar medium as published elsewhere (14) and by PCR on bone marrow samples.

Dogs. Seven dogs living in the area of endemicity of the Maritime Alps (southern France) were also included as VL patients. The VL diagnosis was classically established from clinical signs and serology and was confirmed by PCR on the bone marrow.

RESULTS

Comparison of several lysis and DNA extraction methods using seeded blood samples. The PCR results obtained with seeded blood samples are shown in Table 1. Our seeded samples are similar to real patient samples, as we added live parasites to freshly drawn peripheral blood instead of simply adding purified parasite DNA to standard amounts of host blood DNA. Concentrations of 1,000 and 100 parasites/ml of blood were each tested in quadruplicate. Concentrations of 10, 5, and 1 parasite/ml were each tested twice in quadruplicate for a better assessment of sensitivity of the PCR assay. Indeed, only a portion of these reactions were positive, probably due to the scarcity of parasite DNA, at these lower concentrations. The sensitivity of each PCR test was assessed from the number of positive reactions together with the intensity (arbitrarily graded from + to ++++) of the banding pattern in ethidium bromide-stained agarose gels. Southern blotting followed by hybridization confirmed but did not improve the PCR product detection. A negative result was always confirmed by the positivity of both the DNA extraction control and the inhibition control (see Materials and Methods). It is noteworthy that the reaction conditions were optimized for each of the methods presented. After optimization, the technical specificity was 100% with all methods tested (i.e., no artifactual bands were observed). Therefore, only sensitivities were compared.

At high concentrations (≥1,000 parasites/ml), there were no significant differences in PCR sensitivity among the eight methods tested (Table 1). The differences between the methods became significant at low concentrations, i.e., ≤100 parasites/ml.

(i) WB. When WB was used as the template, the best method was WB-GE-S, particularly at very low concentrations. However, at 100 parasites/ml, PK-ΦC and K were superior in terms of intensity of the banding patterns.

(ii) Buffy coat. When the BC was used as the template, the best methods were BC-PK-ΦC and K at all concentrations. With these methods, we could reliably detect 10 parasites/ml of blood and inconsistently detect one parasite per ml. GE-based methods were less efficient.

TABLE 1. Comparison of eight sample preparation methods using blood samples

Concn tested[a]	No. of positive PCRs/no. of PCRs performed (intensity of banding pattern in ethidium bromide-stained agarose gels) for sample preparation method[b]							
	WB-GE-ΦC	WB-GE-S	WB-PK-ΦC	WB-K	BC-GE-ΦC	BC-GE-S	BC-PK-ΦC	BC-K
1,000	4/4 (++)	4/4 (+++)	4/4 (++++)	4/4 (++++)	4/4 (+++)	4/4 (+++)	4/4 (++++)	4/4 (++++)
100	2/4 (+)	4/4 (+)	4/4 (+++)	3/4 (+++)	3/4 (++)	3/4 (++)	4/4 (++++)	4/4 (+++)
10	3/8 (+)	4/8 (++)	3/8 (+)	2/8 (+)	3/8 (+)	7/8 (+)	8/8 (+++)	8/8 (+++)
5	0/8	1/8 (+)	1/8 (+)	2/8 (++)	0/8	2/8 (+)	2/8 (++)	2/8 (+)
1	0/8	1/8 (+)	0/8	0/8	1/8 (+)	1/8 (+)	2/8 (++)	2/8 (++)

[a] Number of parasites per milliliter of peripheral blood.
[b] + to ++++, least intense to most intense, respectively.

(iii) WB versus BC. In comparing WB with BC using the same method (Table 1), the BC clearly gave better results than WB, by all methods used and at all concentrations tested. This was particularly obvious with the PK-based methods (PK-ΦC and K), but only a slight superiority of the BC was noted with the GE-based methods. If one compares the best method for each type of sample, i.e., WB-GE-S versus BC-PK-ΦC, the superiority of the latter cannot be in doubt. Overall, a 10-fold gain in sensitivity was achieved using the BC.

(iv) GE versus PK lysates. On the whole, the GE-based methods proved less sensitive and less easy to use (from the DNA extraction down to the PCR product analysis) than the PK-based ones. This is not necessarily evident from the number of positives tubes alone (in Table 1) but can be inferred from our experience. In our hands, the reaction optimization proved more difficult with GE-based methods than with the PK-based ones (e.g., Mg^{2+} concentrations). Also, partial or complete inhibition of PCR (possibly due to high salt concentrations) is more frequent and, generally, results are more erratic with GE-based methods.

Comparison of three selected methods using dog and human samples. We then tested the best methods found for both WB and BC samples with dogs and human VL patients in order to validate the in vitro study for in vivo use. Here, the parasite concentrations in the blood were not known. With dogs, the BC-PK-ΦC and BC-K methods were clearly more sensitive than WB-GE-S (Table 2). In particular, with two dogs out of seven, the latter did not detect *Leishmania* in samples graded ++++ with the BC-PK-based methods. Only four samples from four AIDS patients could be tested by two methods (WB-GE-S and BC-PK-ΦC). For two samples, identical results were obtained with the two methods, but for the two remaining samples, BC-PK-ΦC gave better results in terms of both the number of positive reactions and banding pattern intensity (data not shown).

TABLE 2. Comparison of three blood sample preparation methods for PCR diagnosis of VL in dogs

Dog code no.	No. of positive PCRs/no. of PCRs performed (intensity of banding pattern in ethidium bromide-stained gels) for sample preparation method[a]		
	WB-GE-S	BC-PK-ΦC	BC-K
14	0/4	4/4 (++++)	4/4 (++++)
15	4/4 (+++)	4/4 (++++)	4/4 (++++)
16	4/4 (++)	4/4 (++++)	4/4 (++++)
17	2/4 (++)	4/4 (++++)	4/4 (+++)
20	2/4 (++)	4/4 (++++)	4/4 (++++)
21	4/4 (+++)	4/4 (++++)	4/4 (++++)
24	0/4	4/4 (++++)	4/4 (++++)

[a] ++ to ++++, least intense to most intense, respectively.

DISCUSSION

We have compared several methods of sample preparation for the PCR diagnosis of VL using blood samples. We used peripheral blood only, as we aim at replacing bone marrow sampling by this type of sampling, which is less invasive, is easy to repeat (e.g., for posttherapeutic monitoring), and, in human VL, gives an excellent diagnostic sensitivity with an optimized PCR assay (8, 14, 18). Bearing in mind that the parasite load in the circulating blood is lower than that in the bone marrow, we have tried to develop the most sensitive method possible. We compared these methods using two types of blood samples, WB and BC, as both present advantages. The use of WB is easier than that of BC, particularly in field studies, and it has been reported previously to provide good sensitivity (1, 8, 18, 19, 21). On the other hand, leishmanias are obligate intracellular parasites (in the vertebrate host) and therefore are supposedly more concentrated in the BC. The latter should therefore yield a better sensitivity.

We chose methods which have previously been shown to be efficient for the isolation of protozoan DNA from peripheral blood (8, 9, 10, 20, 21, 27). We have avoided the numerous published methods that do not include a DNA extraction step (e.g., crude cell lysates obtained by lysis plus centrifugation or lysis plus boiling), as their results are not reliable enough for a highly sensitive diagnosis (9, 10, 20). For lysis, we compared GE versus PK. GE has been used with great success for the diagnosis of another blood trypanosomatid, *T. cruzi*, the agent of Chagas' disease (4, 7), and recently for *Leishmania* (21). It also presents several advantages for field studies and difficult working conditions, such as immediate and simple conditioning of the samples and storage at RT for 1 to 2 weeks (27) and at +4°C for at least 1½ (21) to 3 (our unpublished data) years. The PK method is more widely used; it allows the whole DNA preparation to be done within a day and is easily adaptable to all kinds of samples. For DNA extraction, the most widely used method is the classical ΦC, which, even if simplified for routine hospital diagnosis, remains among the most efficient methods (10, 20, 21) and is cheap compared with commercial kits. For extraction from GE lysates, because ΦC gave a poor sensitivity in our hands and because in the initial protocol (4) it was followed by a purification step (Centricon), we tested a simple

extraction method based on silica beads. Finally, these methods were also compared to a commercial lysis and extraction kit (Qiagen), since the latter has the advantages of technical simplicity, speed, and greater safety.

Overall, using the optimized PCR conditions developed in our laboratory, (i) the BC gave a 10-fold increase in sensitivity over that of WB, and (ii) PK-based methods proved clearly superior to GE-based methods. Thus, using PK-based methods with BC samples, the sensitivity of our PCR assay reached 10 parasites/ml of blood (and inconsistently 1 parasite/ml). It is interesting to compare our results to those of a recently published similar study testing WB-GE lysis and different extraction methods with blood samples spiked with *L. peruviana*, a species responsible for cutaneous leishmaniasis in Peru (21). Indeed, using the same R primers, these authors obtained a sensitivity equivalent to ours (actually fourfold lower, i.e., 250 parasites/ml of blood) with the same method (WB-GE-ΦC) and 10-fold lower (1,250 parasites/ml) than ours with a commercial kit (21, 22). Like us, they showed that an additional hybridization step does not improve sensitivity following the WB-GE-ΦC method. However, when they used it following extraction with the commercial kit, sensitivity was increased 100-fold (up to 12.5 parasites/ml). By contrast, when they used different primers targeting the highly repetitive kinetoplast minicircle DNA, the sensitivity before the hybridization step was identical to that obtained with the R primers, but it increased 100- and 10,000-fold after hybridization (i.e., to 2.5 parasites/ml and 1 parasite/10 ml) following the GE-ΦC method and the commercial kit, respectively. With the R primers, technical achievements in the optimization of the different steps might explain the differences observed between the two groups. On the other hand, it clearly appears that the use of kinetoplast DNA primers considerably improved the PCR sensitivity in the assay of Reithinger et al. (21) if followed by a hybridization step. It is noteworthy that we tested other primers specific for the *L. infantum* kinetoplast minicircle but that they were not specific enough to avoid the hybridization step (15; unpublished data). The original GE method had been described for another trypanosomatid with primers also targeting the kinetoplast minicircle (4, 27). This suggests that GE-based DNA preparation methods using WB are extremely effective for kinetoplast minicircle DNA but not for genomic targets. Thus, a moderately efficient sample preparation method may be balanced by the extreme sensitivity given by a highly repetitive DNA target. Since WB retains several advantages outlined above, as do the GE-based methods, this combination (WB-GE plus kinetoplastic primers) remains very interesting for field studies. For routine hospital diagnosis and with other DNA targets, we recommend the utilization of the BC with PK-based preparation methods, which seems to increase sensitivity, in particular for low parasitemia. Indeed, we observed that parasitemias are generally low during *L. infantum* VL and particularly so (<100 parasites/ml) during VL relapses in AIDS patients (unpublished data). This study therefore provides us with a good routine diagnostic tool for VL using patient blood for both primary diagnosis and post-therapeutic monitoring in AIDS patients.

Our results also address the question of whether it is worth aiming at the maximal sensitivity for diagnosing infectious diseases. With our PCR system (detecting 1 to 10 parasites/ml of blood), we can confidently diagnose 99% of the VL cases, including diagnosis early during relapses (14; our unpublished data), but we have not been able to identify healthy carriers. Is it necessary to be able to detect parasitemias as low as one parasite per 20 ml? In the case of Chagas' disease (27), this was an essential achievement, as parasitemias are extremely low. In the case of *L. infantum* VL, it does not seem necessary for routine diagnosis of human disease, but it might be for research use. The determination of the micropathogen load threshold between disease and healthy carriage is certainly one of the most interesting challenges to diagnostic PCR today.

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