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Diagnosis of Mediterranean Visceral Leishmaniasis by Detection of *Leishmania* Antibodies and *Leishmania* DNA in Oral Fluid Samples Collected Using an Oracol Device[▽]

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Current methods for diagnosis of visceral leishmaniasis (VL) require invasive sampling procedures such as visceral aspiration and/or blood drawing. The use of diagnostic tests using oral fluid, which is easier to collect, would be more simple and practical for VL diagnosis, especially under field conditions. Oral fluids from 37 VL patients and 40 healthy controls were collected using Oracol devices. Blood samples and oral fluid specimens from both groups were analyzed by recombinant protein K39 (rK39) enzyme-linked immunosorbent assay and quantitative real-time PCR. Detection of antibodies in the oral fluid had a sensitivity of 100% and a specificity of 97.5%. Antibody levels measured in serum and oral fluid showed a significant positive correlation ($p = 0.655$ and $P = 0.01$). Detection of *Leishmania* DNA in oral fluid had a sensitivity of 94.6% and a specificity of 90%. The median parasite load estimated in blood was 133 parasites/ml (interquartile range [IR], 10 to 1,048), whereas that in oral fluid specimens was 3 parasites/ml (IR, 0.41 to 92). However, there was no significant linear relationship between parasite loads assessed in the two biological samples ($p = 0.31$ and $P = 0.06$). VL diagnosis based on specific antibody detection and *Leishmania* DNA identification using oral fluid samples was equivalent in accuracy to that using blood and therefore is promising for clinical use.

Visceral leishmaniasis (VL) is a life-threatening systemic infection caused by protozoa of the *Leishmania* genus (2). The disease is endemic in the Mediterranean basin, where *Leishmania infantum* is the causative species (10). In Tunisia, VL is primarily a pediatric disease and is responsible for considerably high morbidity and mortality rates (1, 3, 7). Its accurate diagnosis requires the availability of reliable laboratory methods, especially in the early stage of the disease, when clinical features of VL can cause it to be easily mistaken for other febrile illnesses (1, 25).

Parasitological diagnosis remains the gold standard in VL diagnosis owing to its high specificity (26). Parasitological diagnosis is generally based on the detection of *Leishmania* parasites in bone marrow aspirates (26). Enzyme-linked immunosorbent assay (ELISA) is also routinely used in VL serodiagnosis. Its most interesting results were obtained with recombinant protein K39 (rK39) antigen (5, 8, 26). Molecular diagnosis of VL is essentially based on PCR assays. Quantitative real-time PCR (qPCR) technology, using primers designed from kinetoplast DNA (kDNA), has been successfully used on blood samples with 100% sensitivity (4, 20). However, blood collection remains an invasive procedure that demands technical expertise.

The use of diagnostic tests performed on other biological fluids that are more available and easy to collect would be

more simple and more practical for VL diagnosis, especially under field conditions. Interestingly, oral fluid offers distinctive advantages as a biological specimen (15). It does not require special equipment for sampling, conservation, and transport via specialized centers. Furthermore, oral fluid collection eases the diagnostic process in specific population groups, such as children, for whom blood removal is usually difficult.

Oral fluid-based diagnostic tests are already validated for detection of antiviral antibodies (15, 18, 19, 24). They were also used for detection of nucleic acids in viruses and bacteria (9, 15). Recently, this practical sampling has been proved to be a valuable tool for diagnosis of some parasitic infections, namely, hydatidosis, amoebiasis, and malaria (6, 14, 23, 27). As far as we know, there is only one report about detection of anti-*Leishmania* antibodies in saliva (21) and none about detection of *Leishmania* DNA in oral fluid specimens from VL patients. The purpose of this study was to assess the diagnostic performances of both immunological and molecular tests based on oral fluid specimens from VL patients and to eventually investigate the correlation between antibody levels and DNA parasitic loads detected in both blood and oral fluid.

MATERIALS AND METHODS

Patients and controls. The study included 37 Tunisian VL patients and 40 control subjects. VL patients were referred to the Pediatrics Departments of Kairouan Regional Hospital and Zaghuan Regional Hospital. These hospitals are usually involved in VL diagnosis. Patients were hospitalized during the period from October 2009 to September 2010 (1 year). Their ages ranged from 4 months to 6 1/2 years (mean = 20 ± 18 months). They did not present immunosuppressive diseases or risk factors for human immunodeficiency syndrome infection. VL diagnosis was suspected based on clinical signs and confirmed by the microscopic observation of *Leishmania* amastigotes in Giemsa-stained bone marrow smears. Forty matched control patients were also enrolled

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in the study. They were referred to the Kairouan and Zaghouan hospitals during the same period for diseases other than leishmaniasis and did not have a history of VL. Matching was done according to age and geographical origin. The study was reviewed and approved by the Pasteur Institute of Tunis (PIT) Ethics Committee.

Collection and processing of specimens. Blood and oral fluid specimens were collected from the 2 groups. Specimen collection from VL patients was performed before treatment. Standard operating procedures for sampling, processing, and storage complied with human ethical regulations and were approved by the PIT Ethics Committee. Samples were stored at +4°C and sent within the day to PIT.

Blood samples (2 to 5 ml) were collected into tubes containing EDTA. Centrifugation was used to separate cellular and noncellular components. Aliquots of plasma were stored at -80°C for further serological analysis, whereas DNA extraction was performed on the nucleated cells layer. A QIAamp DNA minikit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. Proteinase K digestion in Qiagen lysis buffer was for 1 h. DNA was eluted in 50 µl of AE buffer and stored at -80°C.

Oral fluids were collected using an Oracol device (Malvern Medical Developments, Worcester, United Kingdom). Briefly, the foam swab was removed from the collection device and rubbed over the children's gums until saturated with saliva. One milliliter of preservative medium (phosphate-buffered saline [PBS] [pH 7.4], 10% fetal calf serum, 0.2% Tween 20, 0.5% gentamicin, 0.2% amphotericin B [Fungizone]) was added to the tube containing the swab, and the tube was stored at +4°C until being sent to PIT. At reception, the swab was removed by a twisted movement in order to extract as much liquid as possible, inverted, and replaced in the tube to keep the pink foam at the top, and then the tube was centrifuged at 1,200 rpm for 10 min. The inverted swab was then removed and discarded. Finally, the extracted oral fluid recovered from the tube was divided into aliquots of cellular pellet and supernatant and stored at -80°C.

At the end of the sampling period (at most 1 year), DNA extractions were performed on 200 µl of each cellular pellet and supernatant as described above and stored at -80°C. Anti-*Leishmania* antibody detection was performed only on oral fluid supernatant (OFS).

Detection of anti-*Leishmania* antibodies by ELISA. The recombinant protein K39 (rK39) is a 39-amino-acid repeat sequence derived from a gene cloned from *L. infantum*. It was kindly provided by S. G. Reed, Infectious Disease Research Institute (IDRI), Seattle, WA. The presence of anti-rK39 antibodies was screened using both sera and oral fluid specimens. Two different protocols were optimized for detection of antibodies in sera and oral fluids. A standard indirect ELISA was used with blood samples, whereas a biotin-streptavidin assay was used with oral fluid specimens. The detection threshold of the standard ELISA was too low to be applied for the screening of oral fluid specimens (data not shown).

Antigen was diluted in carbonate-bicarbonate buffer (0.1 M, pH 9.6) at a concentration of 0.25 µg/ml for sera and 1 µg/ml for OFS and then used (100 µl/well) to sensitize microassay plates (Nunc MaxiSorp; Thermo Fisher Scientific, Roskilde, Denmark). The antigen incubation was for 1 h at 37°C and then overnight at 4°C. The wells were washed three times with phosphate-buffered saline with 0.1% Tween 20 (PBS-T) and then blocked with PBS-T supplemented with 5% skim milk for 1 h at 37°C.

All samples (sera or OFS) were tested in duplicate. PBS-T was used as a washing solution as well as a dilution buffer. After washing, the diluted sera (1/1,000) and OFS (1/20) were added (100 µl/well) and incubated at 37°C for 1 h for sera and 2 h for OFS. After 5 washings, conjugates were incubated for 30 min for sera and 2 h for OFS. The anti-human IgG conjugate used for sera was an anti-IgG (Fc-specific) horseradish peroxidase diluted at 1/5,000 (Sigma-Aldrich, St. Louis, MO), and that used for OFS was a biotinylated anti-IgG diluted at 1/5,000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The latter was exclusively used for oral fluid screening in order to increase the signal detection. The wells were then washed 5 times. OFS samples required a further incubation step with streptavidin-horseradish peroxidase conjugate (GE Healthcare) followed by a washing. The substrate solution (0.4 mg/ml *ortho*-phenylenediamine dihydrochloride [Sigma-Aldrich] in 0.1 M citrate buffer [pH 5] and 0.03% H₂O₂) was added at a volume of 100 µl/well. The reaction, developed at room temperature, was stopped with 50 µl of 4 N sulfuric acid (H₂SO₄). The absorbance was measured at 492 nm and 630 nm. The mean optical density (OD) value was recorded.

Real-time quantitative PCR assay. Real time qPCR was conducted as described by Mary et al. using primers designed from *Leishmania* kinetoplast DNA (20). The final mix volume was 25 µl. It included the TaqMan universal master mixture (Roche, Palo Alto, CA) with 100 µM direct primer (5'-CTTTTCGTG TCCTCCGGGTAGG-3'), 100 µM reverse primer (5'-CCACCCGGCCCTATT

TTACACCAA-3'), 50 µM probe (FAM-5'-TTTTCGCAGAACGCCCTACC CGC-3'-TAMRA), and 1 µl of DNA extract. DNA was amplified in an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) for 50 cycles at 95°C and 60°C. Each sample was tested in duplicate, and each run included both positive and negative controls. Samples were checked for inhibition by being retested at a 1:10 dilution and by spiking these samples with 1 µl of the positive control.

A standard curve, plotted from a dilution series of *Leishmania* DNA (extracted from 10⁶ *L. infantum* promastigotes), allowed parasite quantification by PCR. The volume of collected samples and the dilution rates applied during DNA extraction and amplification were taken into account to estimate the parasite load (PL) in each collected specimen.

Statistical analysis. Statistical analysis was done using the MedCalc statistical software (version 11.4.4.0). Receiver operating characteristic (ROC) curves were used to analyze the diagnostic performance of each test in discriminating patients with VL from those without (12) and to assess the sensitivities and specificities of all diagnostic assays. The areas under the ROC curves (AUCs) were compared as described by Hanley and McNeil (13). The Wilcoxon nonparametric test was used to access PL differences between biological fluids. Spearman's nonparametric correlation test was applied to establish the relationship between antibody levels and parasite load in both sera and oral fluids. A test was considered significant if *P* value was less than 0.05.

RESULTS

Immunodiagnostic assays. ROC curves were drawn to assess the clinical value of blood- and oral fluid-based rK39 ELISAs applied for VL diagnosis (Fig. 1). The analysis showed that both assays had an excellent ability to discriminate between VL cases and healthy controls (Fig. 1). The optimal diagnostic specificity was 95% for serum-based versus 97.5% for oral fluid-based ELISA. The optimal diagnostic sensitivity of antibody detection in both sera and oral fluids was 100% (Fig. 1). There was no difference between the AUCs for VL diagnostic assays based on blood and oral fluids ($z = 0.28$; $P = 0.77$). The optical densities measured in sera and oral fluids showed a significant positive correlation ($\rho = 0.828$; $P < 0.0001$), which may suggest a positive correlation of specific antibody levels in the 2 biological fluids (Fig. 1).

Real-time quantitative PCR assays. ROC curves were drawn to assess the clinical value of blood- and oral fluid-based qPCR assays in VL diagnosis (Fig. 2). The analysis showed that qPCR assays using DNA extracted from both blood and oral fluid cells were able to discriminate between VL cases and healthy controls (Fig. 2). The optimal diagnostic sensitivity and specificity of blood-based qPCR assay were 100% and 90%, respectively. The sensitivity of the qPCR assay performed on oral fluid cell extract was 94.6%, with a specificity of 90% (Fig. 2). There was no difference between the AUCs for VL diagnosis using DNA extracted from blood and oral fluid cells ($z = 1.51$; $P = 0.12$).

Leishmania parasite DNA was successfully quantified in both blood and oral fluid cell samples obtained concurrently from 35 patients with VL. The median PL estimated in blood samples was higher than the median PL accessed in oral fluid samples (median, 133 parasites/ml of blood versus 3 parasites/ml of oral fluid; interquartile range [IR], 11.25 to 1,032.5 parasites/ml of blood versus 0.41 to 92 parasites/ml of oral fluid) ($P = 0.001$). However, the PL scatter plot did not show a linear relationship between counts determined in the two biological samples. Blood and oral fluid PLs displayed no significant positive correlation ($\rho = 0.31$ and $P = 0.06$).

Quantitative PCR was also performed on both the cell pellet and supernatant of the oral fluid to determine which compo-

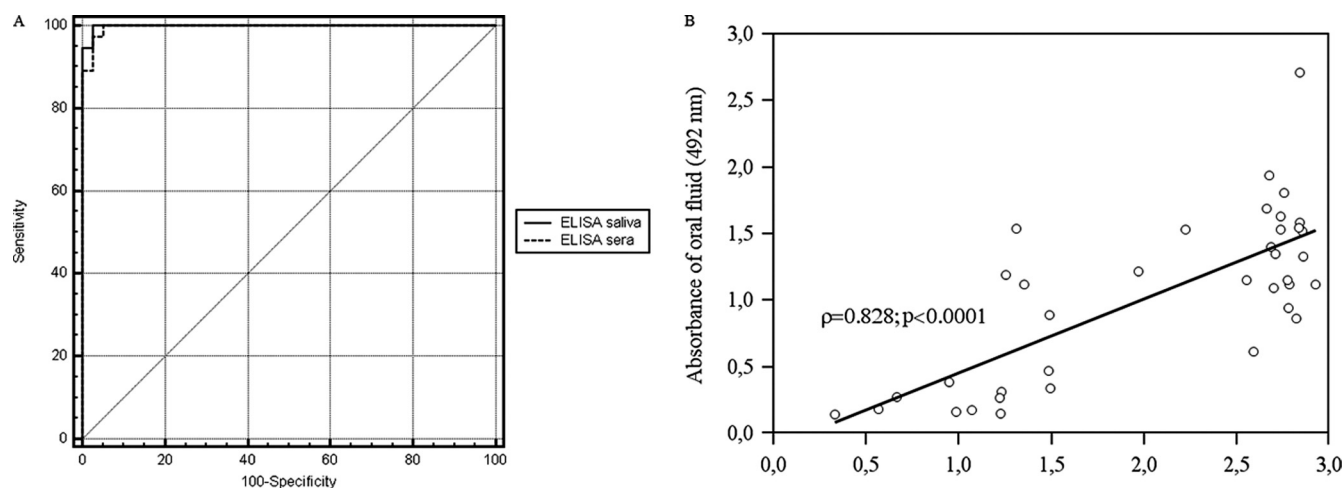


FIG. 1. Detection of anti-rK39 antibodies in sera and oral fluid samples. (A) ROC curves for serum- and oral fluid-based rK39 ELISAs applied to patients with VL versus the control group. For the ELISA with sera, the AUC was 0.997 (standard error [SE], 0.006); for the ELISA with oral fluid, the AUC was 0.999 (SE, 0.004). (B) Correlation between absorbance values obtained with anti-rK39 IgG ELISA in sera and oral fluid samples.

nent provided a higher yield of DNA. qPCR applied to the extracted cells gave better discrimination between VL cases and healthy controls than qPCR applied to extracted supernatant (Fig. 2). A significant difference between the AUCs was observed ($z = 4.6$; $P < 0.0001$). qPCR applied to the extracted supernatant demonstrated a sensitivity of 51.4% and a specificity of 90% (Fig. 2).

It was possible to quantify *Leishmania* DNA in both cell pellets and supernatants of 18 oral fluid samples. Much more *Leishmania* DNA was consistently recovered from the cell pellet compared with the supernatant. In fact, the median PL estimated in the cell pellet (84 parasites/ml; IR, 8 to 420) was significantly higher than that found in the supernatant (5.75 parasites/ml; IR, 1.57 to 12) ($P = 0.0001$). Moreover, the PL scatter plot showed a linear relationship between the counts in the two components. The correlation was high and very significant ($\rho = 0.8$; $P = 0.0001$), which may suggest a concentration-dependent release of parasite material from oral fluid cells.

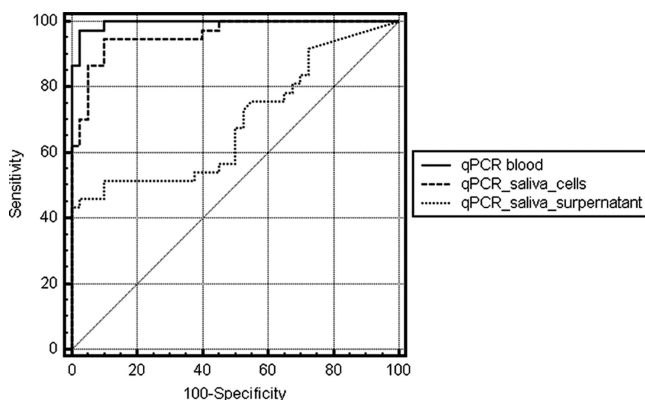


FIG. 2. ROC curves for blood- and oral fluid-based qPCR assays applied to patients with VL versus the control group. For the qPCR with blood, the AUC was 0.995 (SE, 0.008); for the qPCR with oral fluid cells, the AUC was 0.959 (SE, 0.02); and for the qPCR with oral fluid supernatant, the AUC was 0.691 (SE, 0.059).

DISCUSSION

Oral fluid is a complex body fluid consisting of several components, including saliva (19, 29). It contains salivary gland secretion and several components of nonsalivary origin, such as gingival crevicular fluid, serum or blood resulting from oral wounds, bacteria and their products, viruses, fungi, desquamated epithelial cells, and other cellular components (15).

There are several methods for oral fluid collection (29). the Oracol device was preferred in this study owing to its simple use, especially with children. Furthermore, this device collects oral fluid of high quality for antibody testing (29). In fact, the Oracol device is designed specifically to target the gums, the part of the oral cavity most likely to be rich in crevicular fluid. Indeed, the gingival crevicular transudate contains a relatively high IgG concentration compared with the whole saliva, which makes this anatomic site a suitable source of IgG detection (18, 22, 29). In addition, the preservative medium added to samples helps to maintain IgG stability over time at levels similar to the starting ones (18). On the other hand, the Oracol device picks up gingival cellular material rather than strictly saliva fluid, allowing testing of the cellular contribution in specific molecular VL diagnosis.

Previous reports showed the accuracy of serological rK39 ELISA methods in Mediterranean VL diagnosis (17). Other authors established that anti-rK39 antibodies titers correlate directly with active disease and consequently allow prediction of clinical relapse (16). The aim of this study was to optimize and evaluate an oral fluid-based rK39 ELISA for VL diagnosis. A biotin-streptavidin procedure was necessary with oral fluid specimens. This improved the sensitivity of the detection of specific antibodies present at lower concentration than in serum. The biotin-streptavidin assay detecting specific antibodies in oral fluid was equivalent to that detecting antibodies in serum in accuracy, making this test applicable for clinical use. Moreover, the oral fluid anti-*Leishmania* antibody levels of infected individuals appeared to correlate with those of serum

antibodies, making the former assay useful for the follow-up of VL patients.

In addition, the kDNA qPCR used for detection of *Leishmania* in blood from VL patients is already known for its high sensitivity, amplifying a very small number of DNA copies (20). qPCR was also used in the PL follow-up of treated patients (4). In our study, qPCR applied to DNA extracted from oral fluid cells was as accurate in VL diagnosis as qPCR performed with peripheral blood samples obtained simultaneously. Nevertheless, a larger sample size should be screened in order to confirm this result. On the other hand, there was no correlation between the PL in the blood and that in the oral fluid cells. In fact, the amount of cellular DNA that could be extracted from a standard volume of oral fluid varies widely between individuals and in the same subject over time (28). Accordingly, its use for PL follow-up might not be appropriate.

Comparisons of qPCR results from the two oral fluid components (cells and supernatant) suggest an intracellular origin of *Leishmania* DNA in oral fluid sample. In fact, in VL patients, *L. infantum* is found in blood monocytes. These cells may reach the oral fluid via intraoral bleeding as well as the gingival crevicular fluid. However, the presence of *L. infantum* in mucosal tissue could not be excluded (11).

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