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REAL TIME PCR DIAGNOSIS OF LEISHMANASIS IN CONSTANTINE (ALGERIA)

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Résumé

Compte tenu de la réémergence de la leishmaniose dans le monde, de la variabilité géographique de l'épidémiologie et de l'augmentation du nombre de voyageurs, une étude pilote sur le diagnostic des leishmanioses cutanée et viscérale a été réalisée à Constantine, l'un des foyers de l'Est algérien. 143 prélèvements cutanés et 108 échantillons de moelle osseuse ont été récoltés sur buvards et testés par PCR en temps réel et les résultats comparés à ceux de l'examen direct. Le diagnostic de la LC par PCR a été positif dans 81% des cas pour la PCR contre 48% pour la microscopie. Quant à la LV, le taux de positivité est de 29,6% pour la PCR en temps réel contre 24% pour la microscopie, La PCR en temps réel a montré une différence quantitative significative entre les patients pour lesquels le diagnostic microscopique a été positif et ceux dont l'examen direct a été négatif. Les résultats présentés dans cette étude ont montré l'efficacité et la sensibilité de la PCR dans le diagnostic des leishmanioses cutanée et viscérale à partir des buvards. Avec cette technique, il a été possible de réaliser sur place un prélèvement à chaque malade et d'obtenir des résultats dans un délai court. Une collaboration Nord-Sud basée sur l'utilisation de moyens simples de transmission des échantillons pour le diagnostic moléculaire a permis de créer un partenariat efficace en terme de diagnostic quotidien et favoriser ainsi l'échange de chercheurs afin de préparer le transfert de technologie.

Mots clés : *Leishmaniose cutanée – Leishmaniose viscérale - Diagnostic - PCR en temps réel - Algérie.*

Abstract

Taking into account the re-emergence of leishmaniasis in the world, the geographic variability of its epidemiology and the growing numbers of travellers, a pilot study on the diagnosis of cutaneous leishmaniaasis and visceral leishmaniasis was undertaken in Constantine, one of the outbreak regions in eastern Algeria. A total of 143 cutaneous specimens and 108 bone marrow samples were collected on blotters and tested by real-time PCR. Results were compared with those of direct examination. Diagnosis, in CL, was positive for leishmaniasis in 81% of cases using PCR versus 48% of cases using microscopy. For VL, results were 26.9% for real time PCR versus 24% for microscopy exam. Real-time PCR showed a significant quantitative difference between patients for whom microscopic diagnosis was positive and those for whom direct examination was negative. The results presented in this study demonstrated the effectiveness and sensitivity of PCR in the diagnosis of cutaneous and visceral leishmaniasis from blotter specimens. This technique enabled in-field collection of specimens from each patient and provided prompt results. North- South cooperation based on the use of simple means for transmission of specimens for molecular diagnosis allowed creation of an effective partnership for daily diagnosis and promoted exchange between investigators in preparation for technology transfer

Keyswords: *Cutaneous leishmaniasis – Visceral leishmaniasis – Diagnosis – Real time PCR- Algeria.*

ملخص

نتيجة لعودة ظهور داء اللشمنيات في العالم ، للتغيرات الجغرافية من حيث الإصابات وزيادة عدد المسافرين ، تمت دراسة لتشخيص هذا المرض الجلدي و الحشوي بقسنطينة التي تعتبر إحدى البؤر بالشرق الجزائري .تمت تحليل 338 عينة جلدية و 112 عينة من مخ العظام بواسطة المجهر و PCR في الوقت الحالي .النتائج المحصل عليها بينت بان عدد المرض ذو نتيجة اجابية يقدر بـ 81% بالنسبة لـ PCR في الوقت الحقيقي و 48% بالنسبة تشخيص المجهري لليشمانيا الجلدية .أما داء الليشمانيات الحشوي، أظهرت PCR في الوقت الحقيقي النتائج المحصل عليها : 29,6٪ PCR في الوقت الحقيقي، مقابل 24 ٪ للفحص المجهري.وأظهرت PCR في الوقت الحقيقي فرق كبير بين المرضى الذين الكمية التشخيص المجهري كان ايجابيا وأولئك الذين الفحص المباشر كان سلبيا. وأظهرت النتائج الواردة في هذه الدراسة فعالية بكر في تشخيص الليشمانيا الجلدية والحشوية من السجلات اليومية. مع هذا الأسلوب كان من الممكن جمع العينات لكل مريض والنتائج في وقت قصير وإنشاء تعاون بين الشمال والجنوب على أساس استخدام وسائل بسيطة لنقل العينات للتشخيص الجزيئي شراكة فعالة من حيث التشخيص وبالتالي تشجيع تبادل يوميا من الباحثين لإعداد ونقل التكنولوجيا.

الكلمات المفتاحية : *الليشمانيات الجلدي - - الليشمانيات الحشوي - التشخيص- PCR في الوقت الحقيقي - -الجزائر.*

Leishmaniasis is a parasitic infectious disease comprised from a variety of syndromes, which are different between them in pathogenesis and clinical picture. According to the World Health Organization (WHO), leishmaniasis is considered as one of the most important parasitic diseases [1]. It is endemic in 88 countries in five continents with 12 million people afflicted world wide from it and another 350 million living at risk of infection. About 1-1.5 million new cases of cutaneous leishmaniasis (CL) and 500,000 of visceral leishmaniasis (VL), rise per year [2].

Algeria people, affected by two kinds of leishmaniasis; cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL).

Cutaneous leishmaniasis which is a serious public health problem in Algeria. It has two clinical forms caused by *Leishmania major* and *L. infantum* [3] [4]. *Leishmania infantum* zymodeme MON-24 is the main strain responsible for the sporadic CL distributed throughout the northern part of the country [5]. The vector is *Phlebotomus perfiliewi* [6] and dogs constitute the most likely reservoir [7]. *Leishmania major* zymodeme MON-25 is present in the steppe areas of central and southern parts of the country. *Psammomys obesus* and *Meriones shawi* are its proven reservoir hosts [8] [9] and *P.papatasi* is the main vector [10].

Visceral leishmaniasis due to *L. infantum* is the most sever form. It is fatale if untreated. It occurs mainly in the north where it reaches almost 540 per year. The incidence of visceral leishmaniasis is 0.31 cases per 100,000 inhabitants [11]. The outbreak of the Great Kabylia alone accounts for nearly 50% of cases [12] where the disease is responsible for 6% of deaths [13].

Diagnosis of this disease, in Algeria, is still based on direct examination and *in vitro* culture of bone marrow aspirates or detection of antibodies by immunofluorescence [14].

In recent years, real-time polymerase chain reaction (PCR) has been developed and used successfully for the diagnosis of leishmaniasis [15][16]. This procedure is non-invasive, highly sensitive and specific, and useful in monitoring responses to therapy by quantification of parasitic load [17]. The aim of the present study was to propose a specific real-time PCR approach for the diagnosis of the two forms of leishmaniasis imprinted onto filter paper.

MATERIAL AND METHODS

Parasites samples

Leishmania reference strains

L. infantum MHOM/TN/80/IPT1, *L. major* MHOM/SU/73/5-ASKH were kindly provided by M. Gramiccia, Istituto Superiore di Sanita in Roma, Italy. The strains were cultivated at 25 °C in RPMI medium (Invitrogen, Cergy Pontoise, France) supplemented with 15% foetal calf serum and Penicillin. Parasites were harvested at a density of 10^4 parasites/ml. They were washed twice with PBS buffer, pelleted by centrifugation (5900×*g* for 3 min) and stored at −20 °C.

Human clinical investigations

Cutaneous lesions

A total of 143 skin lesions were collected from143 patients consulting at the parasitological laboratory in the east of Algeria (Constantine Hospital) from the period of September 2004 to May 2006.

The CL are the typical lesions skin not affected with fungi and bacteriological infection and the absence of specific treatment during three months before the consultation. All individuals enrolled in the study provided informed consent.

Bone Marrow samples: A total of 108 bone marrow samples were collected on filter paper 3MM Watmann From children with visceral leishmaniasis in the east of Algeria (Constantine hospital) from the period of 2004 until 2006, on the basis clinical symptoms (anarchic fever, splenomegaly, hepatomegaly, mucocutaneous and pallor) and biological arguments (normocytic anemia, pancytopenia, thrombocytopenia…).

Cutaneous lesions and bone marrow samples have been spotted on 3mm Whatman™ filter paper, air-dried and stored in individual plastic zip-lock bag at room temperature.

All individuals enrolled in the study provided informed consent.

Nucleic acid isolation

Leishmania reference strains

DNA was extracted using QiAamp DNA mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions with minor modifications. The incubation time with proteinase K was increased to 1 h at 56 °C and DNA was eluted from the column with 50 µl of PCR-grade H_20.

Filter paper samples

The DNA was isolated from the dried cutaneous spots using Instagene Matrix® resin (Bio-Rad, Marnes la Coquette, France). A 1-cm paper circle was cut avoiding the inclusion of non-impregnated filter paper. Paper disks were cut in small sectors in a sterile Eppendorf tube and soaked in Saponin 5% during 60 min. The red residue was collected by centrifugation and papers were incubated with 200µl Instagene Matrix® resin at 56 °C for 30 min with vortexing after 15 min and at the end of incubation. Tubes were transferred in a boiling bath for 8 min before

centrifugation and supernatants were decanted carefully to avoid resin carry-over. The extracted DNA was diluted in sterile water to 1/5 and kept at −20 °C before PCR.

Polymerase chain reaction amplification

Each sample has been subjected to real-time PCR assay for the detection of Leishmania DNAas previously described [15]. The samples presenting a crossing point (point which marked the cycle at which the fluorescence of the sample is significantly different from the baseline signal) lower or equal to 25 have been selected for the identification of old world Leishmania species. The primers JW11 (5'-CCTATTTTACACCAACCCCCAGT-3') and JW12 (5'- GGGTAGGGGCGTTCTGCGAAA- 3'), designed on the conserved region of *Leishmania* kDNA minicircle, were used to allow the amplification of the genus Leishmania [18]. Real-time PCR technology was performed with fluorescent SYBR Green I. The extracted DNA (5 µl) was added to 15µl of reaction mixture containing 4mM of MgCl2, 0.5µM of each primer (JW11 and JW12), 2µl of LightCycler FastStart DNA Master SybrGreen I buffer (Roche Applied Science, Meylan, France). Conditions for cycling were 95 °C for 4 min, followed by 35 cycles of 95 °C for 10 s, 62 °C for 10 s, and 72 °C for 10 s with monitoring of fluorescence at the end of elongation step. PCR product identity was confirmed with melting curve analysis. Melting program consists of one cycle of 95 °C for 0 s, 67 °C for 20 s and heating at 98 ◦C. The transition rates were 20°C/s except for the extension step and the final step, which have a temperature transition rate at 1 °C/s and 0.1 °C, respectively.

An uninfected sample was systematically included to control the amplification specificity. DNA isolated from the *Leishmania* reference strains has been used as a positive control in each run. The sensitivity of the PCR assay was assessed on a serial ten fold dilutions ranging from 1000 to 1 parasite/ml of DNA purified from the four *Leishmania* references strains. The detection limit was 10 parasite /ml corresponding to 0.05 parasites per reaction (data not shown).

RESULTS

Human clinical investigations

Cutaneous lesions

During this study, 143 patients were included. The sex ratio is 1.55 (87 men/56 women). The mean ages of men patients were 28.3 ± 16.5 years and for women were 35 ± 8.5. Children (under 16 years) account for 17.5% of all patients. Clinically, the lesions were most frequently on exposed parts: faces (61 patients: 45%) and members (42 patients: 31%). The lesions were multiple and sometimes showed different aspe cts. They ranged between 1 and 4 cm in diameter.

Bone marrow samples

In this present study108 patients were included. The ave rage age of children was from7.20 ±3.53 years (males: 4.86 ± 4.43 years, girls: 5.01 ± 4.28 years) and sex ratio of 1.3.

For both types of leishmaniasis, patients were mostly fr om Constantine and its around (Ibn Ziad, Hamma Bouziane, Ain Abid, Chelghoum Laid).

Molecular diagnosis
Cutaneous samples

The real-time PCR waspositive for 117/143 samples (81.81%). The increased sensitivityof laboratory iagnosis obtained by the use of PCR compared to the direct examination was therefore 46/143 patients (32,16%). All patients with a negative result by PCR had a negative microscopic examination.

The real-time PCR by Light Cycler, showed that there was a significant quantitativedifference between patients whose diagnosis was microscopically positive and those who were negative. Indeed, patients for whom microscopic examination was positive showed a detectionlimit of 19.56 ±0. 108 average PCR cycles with an average of 28.47 ± 0.059 ($p < 0.01$) in patients whose direct examination was negative.

Bone marrow samples

The real time PCR was positive for 32/108 samples, representing 29.6% of all samples analyzed. Cycles of positivity were observed in average of 25.77. In all positive cases, the denaturation curves showed the expected characteristics of specificity.

The results of molecular diagnosis were compared with those obtained by microscopy. It shows that increasing the sensitivity of laboratory diagnosis, obtained by using PCR, compared to the direct examination was 6/108 patients (5.5%). In fact, 6 patients were negative on microscopic examination and positive by real-time PCR. The positive rate of direct examination was about 24%.

DISCUSSION

The yearly incidence of leishmaniasis, estimated at 1.5 millions for CL and 0,5 million for LV, is increasing related to deforestation, urbanization or environmental changes [18].

Given its reemergence in Algeria, it was important to establish a pilot study on the diagnosis of both forms of the disease [19]. Recent data show that the incidence is increasing, and geographic range extension [20] [14]. Visceral leishmaniasis takes, with cutaneous leishmaniasis, the first place in parasitic diseases and accounts for 35% of notifiable diseases [21]. This requires a rapid and sensitive diagnosis.

The current diagnosis is usually based on direct examination and culture which are difficult because of low parasite loads of samples, anddifficult to achieve un der experimental conditions that do not always fulfil all necessary conditions. Obviously, as with most parasitic diseases, the contribution of molecular diagnostics can be considerable [22][23] [24] [25] [26][27]. PCR is a diagnosis method confirmed cutaneous leishmaniasis [15] or mucocutaneous leishmaniasis [28].

The realtimePCR, as new technology has revolutionized moleculardiagnosticsbalancing reliability and timeliness [2 9]. Indeed,early diagnosis bringsPCR canidentify leishmani asis before the clinical picture is complete (suspect). The skin and bone marrow samples are very suitable for detection of Leishmania DNA by PCR [30].

Several authors report its interest in the diagnosis of leis hmaniasis [31].

It was used in the diagnosis of VL of the child in several Mediterraneancountries [16]. It has also shown it s benefits in the detection of asymptomatic carriers of the parasite in patients infected with HIV for whom, VL coinfection is frequently reported in countries where both infections (cutaneous and visceral) are endemic [32]. It has proved useful in monitoring therapy by quantification of parasite load [16].

It can be applies to different types of samples from reservoirs and vectors, hence its interest in epidemiological studies [33]. Quantitatively, the RT-PCR seemsto be the most appropriate in evaluating the succ ess of therapy and new types of treatments [34].

Moreover, the collection of bone marrow samples on filter paper is a good alternative for preserving samples for molecular analysis relocated to a reference laboratory. Its use in the diagnosis of cutaneous leishmaniasis and the identification of different species of Leishmania has been applied with success [35] [36].
In our study, we included all patients with clinical and biological signs suggestive of visceral and cutaneous leishmaniasis.

The real-time PCR increased the number of certain documentation of diagnosis from71 to 117 cases for CL and 26 to 32 cases for VL, in a suspected population with respectively, 143 and 108 patients. As part of this retrospective study, it was not possible to follow the clinical evolution ofpatients and we have no information to confirm absolutely that patients identified have visceral leishmaniasis and that treatment made a specific improvement or even a cure. However, it was not the aim of the study show that the real-time PCR to better care for patients, but rather to demonstrate the concept that this technique, applied to samples preserved on blotting paper andeasilytransportable, can give better results in term s of detection rate of visceral leishmaniasis.

Thisconcept is important since it may allow institutions to mobilize the country to promote the establishment of reference laboratories equipped with real-time PCR technology. The availability of this powerful tool will lead the development of clinical and biological studies more complete systems that provide definitive answers to the conditions for improving the diagnosis of this severe disease.

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