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Usefulness of PCR Method for Detection of *Leishmania* in Poland

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Abstract

Leishmania parasites are the etiological agents of leishmaniosis, with severe course and often fatal prognosis, and the global number of cases has increased in recent decades. The gold standards for the diagnosis of leishmaniosis are microscopic examinations and culture *in vitro* of the different clinical specimens. The sensitivity of these methods is insufficient. Recent development in specific and sensitive molecular methods (PCR) allows for detection as well as identification of the parasite species (subspecies). The aim of the study was to estimate the usefulness of molecular methods (PCR) for detection of *Leishmania* species and consequently for the implementation of such methods in routine diagnostics of leishmaniosis in Polish patients returning from endemic areas of the disease. In our investigations we used 54 known *Leishmania* positive DNA templates (from culture and clinical specimens) received from the CDC (Atlanta, GA, USA). Moreover, 25 samples of bone marrow, blood or other tissues obtained from 18 Polish individuals suspected of leishmaniosis were also examined. In PCR we used two pairs of primers specific to the conserved region of *Leishmania* kinetoplast DNA (kDNA) minicircle (13A/13B and F/R). Using these primers we obtained amplicons in all DNA templates from the CDC and in three Polish patients suspected for *Leishmania* infection. In one sample from among these cases we also obtained positive results with DNA isolated from a blood specimen which was previously negative in microscopic examinations.

Key words: leishmaniosis in Poland, molecular diagnosis (PCR)

Introduction

Leishmaniosis is a parasitic infection that occurs in 88 tropical and subtropical countries except for Australia and Oceania. The disease is caused by several protozoan species of the genus *Leishmania* and is characterized by a wide variety of clinical forms from cutaneous (CL) and mucocutaneous (MCL) to visceral (VL) form with severe course and often fatal prognosis (Berman, 1997; Eddleston *et al.*, 2006; Salata, 1993; Schwartz *et al.*, 2006).

Geographical distribution and the risk of getting infection are different for each form of the disease. The highest number of cutaneous leishmaniosis cases is observed in Afghanistan, Algeria, Iran, Iraq, and Saudi Arabia as well as in countries of Middle America and South America where mucocutaneous form occurs with similar frequency. The visceral form of the disease is often reported on the African continent, in Southeast Asia and Middle America and South America. In Europe cutaneous and visceral leishmaniosis occur in the countries of Mediterranean Sea Basin. It is estimated that about 12 million people are infected worldwide, and there are recorded up to 2 million of new cases every year (Stefaniak *et al.*, 2003). In Poland only a few cases are reported annually. The disease is diagnosed in individuals who had visited endemic areas.

The reservoirs of *Leishmania* spp. are humans as well as domestic and wild animals. In the countries of the Mediterranean Sea Basin dogs are the main source of endemic persistence of the disease. Visceral

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leishmaniosis is caused by *L. donovani*, *L. infantum* or *L. chagasi* species, depending on the region. Cutaneous leishmaniosis is caused mainly by *L. tropica* or *L. major*, while the mucocutaneous form by *L. braziliensis* complex (Guerin *et al*., 2002; Salata, 1993; Schwartz *et al*., 2006).

Parasites are transmitted by mosquitoes of *Phlebotomus* and *Lutzomyia* species, known as sandflies. Especially susceptible to infection are children, elderly people and immunocompromised persons (HIV infected, after organ transplantation, pharmacologically – immunosuppressed patients). Lately, the number of people infected with this parasite has increased among patients with HIV/AIDS. Protozoans of *Leishmania* spp. have a predilection to immune system cells, particularly to the spleen, liver and bone marrow, causing severe damage of these organs. The process often progresses slowly and in an apparent way resulting in deep lesions in affected sites (Berman, 1997; Guerin *et al*., 2002).

Patients with leishmaniosis suffer from fever, which tends to be sustained or intermittent, progressive weakness, hepatosplenomegaly with signs of splenomegaly and lymphadenopathy. Accompanying pancytopenia often leads to misdiagnosis of hematologic malignancy and delays the institution of the appropriate treatment.

The bases of the diagnosis of leishmaniosis are microscopic examinations of the biopsy material form spleen, bone marrow, lymph nodes or skin ulcers. In VL the most valuable is examination of the material from biopsy of the spleen, however it poses for patients a big risk of complications. The sensitivity of this method is insufficient. It is possible to culture the parasites *in vitro* using special media, most often NNN medium, which is more sensitive than the microscopic investigation (Boelaert *et al*., 2007; Reithinger and Dujardin, 2007). Out of immunological methods, the skin test (Montenegro test) is used or serological tests that turn out to be of little value in the case of cutaneous or mucocutaneous leishmaniosis. In order to identify the species isoenzymatic profile or monoclonal antibodies may be used. However, these methods need prior multiplication of the parasites using *in vitro* culture (Reithinger and Dujardin, 2007).

The treatment of leishmaniosis is difficult, long-lasting and expensive. Pentavalent antimonials, amphotericin B, pentamidyne, ketoconazole are still in use together with recently introduced miltefosine. Of prime importance is the treatment of concomitant illnesses such as tuberculosis, HIV/AIDS and others. Untreated visceral leishmaniosis leads to death in 90% of cases. Therefore, early and modern diagnosis is needed in order to implement effective treatment (Guerin *et al*., 2002; Olliario *et al*., 2005; Eddleston *et al*., 2006).

Reithinger and Dujardin (2007) claim that for parasite detection in laboratories in countries of nonendemic areas there is a trend to prefer molecular diagnostics. Recent development in specific and sensitive molecular methods (PCR) allows for detection as well as identification of the parasite species (subspecies) (Belli *et al*., 1998; Marques *et al*., 2001; Reithinger and Dujardin, 2007).

Therefore, the aim of this study was to estimate the usefulness of molecular methods (PCR) for detection of *Leishmania* spp. and consequently for their implementation in routine diagnostics of leishmaniosis in Polish patients arriving from endemic areas of the disease.

Experimental

Material and Methods

Leishmania infantum constantly cultured *in vitro* in Philips medium was used as a positive control in PCR assay.

Specimens. The following known DNA samples received from the CDC (Atlanta, GA, USA) were investigated: 26 templates isolated from *in vitro* culture obtained from infected persons, 28 templates isolated directly from clinical specimens from patients with leishmaniosis and one sample isolated from the blood of an infected dog. These samples were characterized using Isoenzyme Analysis based on the CAE method (Cellulose Acetate Electrophoresis) developed by Kreutzer (Kreutzer *et al*., 1983; 1987; Kreutzer, 1996), using 6PGDH and GPI Isoenzymes. The obtained templates belonged to the most important species of *Leishmania* such as: *L. donovani*, *L. infantum*, *L. chagasi*, *L. major*, *L. tropica* and *L. braziliensis.*

Moreover, 25 samples of bone marrow, blood or other tissues obtained from 18 Polish citizens suspected of leishmaniosis were also investigated. The samples were sent to the Department of Tropical Parasitology (Interfaculty Institute of Maritime and Tropical Medicine of Medical University of Gdańsk) in order to perform routine diagnostic examinations in the laboratory, after which they were frozen at –20°C. Written informed consents were obtained from all patients. In three cases amastigota forms of *Leishmania* spp. were detected in microscopic examination of the smears from bone marrow aspirates.

DNA extraction. DNA was extracted from 100 µl of *Leishmania in vitro* culture (100 µg of tissue) or 100 µl of whole EDTA-stabilized bone marrow (blood) collected from Polish patients (kept frozen at –20°C) with the use of the Genomic Mini Kit or Blood Mini Kit (A&A Biotechnology, Gdynia, Poland), respectively. At the CDC (Atlanta, GA, USA) DNA was isolated using QIAamp DNA Micro Kit (Qiagen, Chatsworth, CA, USA). All DNA tem-

Table I
PCR master mixes (in µl)

Reagent	Primers		
	13A/13B		F/R
PCR buffer for RUN polymerase	2		2
dNTP 2,5 mM	2		2
Primer 1 10 mM	1		1
Primer 2 10 mM	1		1
Tag Polymerase RUN 1U/µl	0.625		1
Distilled water	16.375	13.375	16
DNA template	2 (1:10)	5 (1:5)	2 (1:5 or 1:10)
Total	25		25

In parentheses (used dilutions of templates received from the CDC)

Table II
PCR conditions

Step	Primers			
	13A/13B		F/R	
	Temp. °C	Time m	Temp. °C	Time m
Initial denaturation	94	3	94	5
Denaturation	94	1	94	1
Annealing	52	1	60	1
Extension	72	1	72	0.5
Final extension	72	10	72	5
Number of cycles	40		30	

plates were extracted according to the manufacturer's instructions.

PCR amplification. Two pairs of primers were used in this study: 13A (5' GTG GGG GAG GGG CGT TCT-3') and 13B (ATT TTA CAC CAA CCC CCA GTT-3') (Bell *et al*., 1998) as well as F (5' GGG (G/T)AG GGG CGT TCT (G/C)CG AA-3' and R (5-(G/C)(G/C)(G/C)(A/T)CT AT(A/T) TTA CAC CAA CCC C-3') (Marques *et al*., 2001) that are specific to conserved region of all *Leishmania* species kinetoplast DNA (kDNA) minicircle. The length of amplified PCR products is about 120 bp.

The composition of amplification reaction mixture as well as PCR conditions are presented in Table I and II, respectively. DNA amplifications were performed in the 7600 Gold thermocycler (Applied Biosystems, USA).

Results and Discussion

DNA isolated from promastigota forms obtained from *in vitro* culture of *Leishmania* allowed for optimization of the PCR reaction (data not shown). The DNA templates received from the CDC (Atlanta, USA) were used to perform PCR with different primers in the Polish laboratory. In PCR tests performed with these primer pairs using reference material from the CDC, the presence of appropriate *Leishmania* DNA fragments was detected in all 54 examined samples (Table III). However, a significant imperfection of these primers is their inability to identify *Leishmania* species.

The methodology devised on the base of templates achieved from the CDC (Atlanta, USA) allowed for introducing PCR method into diagnostics of Polish patients.

Examination of clinical samples taken from Polish patients gave positive results of PCR performed with the use of two pairs of primers in 3 cases (Table IV). Those were the patients (returning from Georgia, Turkey and Portugal) seropositive in Indirect Fluorescence Antibody test (IFA) and in which amastigota forms of the parasite were found in smears from bone marrow aspirates. In one sample from these cases we also obtained positive results with DNA isolated from a blood sample which was previously negative in microscopic examinations (Table IV). These patients were previously suspected of hematological neoplasms for several months and treatment was introduced in one case.

In case of the other 15 persons suspected of leishmaniosis, where no parasites were found as well as the results of IFA tests were negative, none of the pair of primers gave a positive result in PCR reaction (Table IV).

In diagnostics of leishmaniosis the most important is detection of the parasite or its DNA. Of prime importance is also identification of a particular species (subspecies), because the therapeutic response is species and, perhaps, even strain specific (Reithinger and Dujardin, 2007). For the routine diagnostics of leishmaniosis in Poland we suggest the adoption of primers encoding kDNA minicircle. It occurs in a high copy number in the cell and in consequence makes

Table III
PCR results of templates obtained from the CDC

Specimen	*Leishmania* species						
	L. donovani	*L. infantum*	*L. infantum/ chagasi*	*L. major*	*L. tropica*	*L. braziliensis*	Species not determined
Culture			2	5	7	8	4
Clinical	4	5	3	5	6	4	1
Total	4	5	5	10	13	12	5

Table IV
PCR results of samples obtained from Polish patients

Culture or Patient	Day from first examination	Microscopic results	DNA isolation from	PCR results with primers		IFA results
				13A/13B	F/R	
positive control		promastigota	*in vitro* culture	+	+	n.d.
DJ	0	–	blood	+	+	1:160
	3	amastigota	bone marrow	+	+	n.d.
	3	–	blood	–	–	n.d.
	38	–	bone marrow	–	–	n.d.
HR	0	amastigota	bone marrow	+	+	1 : 80
	626	–	bone marrow	–	–	1 : 40
CA	0	amastigota	bone marrow	+	+	1 : 80
	33	–	bone marrow	–	–	n.d.
	78	–	bone marrow	–	–	1 : 80
	125	–	bone marrow	–	–	1 : 20–1 : 40
N (7)	0	–	bone marrow	–	–	–
N (5)	0	–	blood	–	–	–
N (3)	0	–	other tissues	–	–	–

+ = positive; – = negative; n.d. = not done; DJ = patients' initials; N = number of patients

the reaction more sensitive. The best are the primers F/R, because primers 13A/13B may give additional unspecific products with templates isolated from clinical specimens.

In conclusion, the results of this study indicate that the tested pairs of primers can be used for routine diagnostics of leishmaniosis in Poland.

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