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Development of affordable molecular techniques for the diagnosis of leishmaniasis in Yemen

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ZUSAMMENFASSUNG

Die Leishmaniose ist eine durch einzellige Parasiten der Gattung *Leishmania* verursachte Infektionskrankheit, die durch den Stich von Sandmücken übertragen wird. Als erstes klinisches Symptom bildet sich eine nicht abheilende ulceröse Wunde von bis zu mehreren cm Durchmesser um die Einstichstelle herum. Nach mehreren Monaten kann die Wunde unter Hinterlassung einer Narbe spontan abheilen. Falls es sich um eine Infektion mit einer weniger virulenten Form des Parasiten gehandelt hat, ist der Patient geheilt und für den Rest des Lebens resistent gegen diese Form. Andere Stämme des Parasiten lösen aber die mucocutane Form der Krankheit aus, die zu schmerzhaften destruktiven Läsionen der Lippen- und Nasenschleimhäute führt, oder die viszerale Form der Leishmaniose, die innere Organe angreift und unbehandelt tödlich verläuft. In der Republik Jemen sind die cutane und die viszerale Leishmaniose endemisch, werden aber aus Mangel an Geld in der Bevölkerung weder hinreichend diagnostiziert noch behandelt.

Aufzeichnungen über epidemiologische Daten gibt es nur für wenige staatliche Krankenhäuser, die Zahl der unregistrierten Fälle ist wahrscheinlich sehr hoch. Um in Zukunft zu Kontrollmaßnahmen gegen die Krankheit beitragen zu können, wurde der Versuch unternommen, neue molekulare Diagnoseverfahren zu entwickeln, die einerseits sehr empfindlich und spezifisch sind, andererseits aber nicht zu kompliziert und teuer, so dass sie unter den limitierten Voraussetzungen des Landes realisiert werden können.

Um einen Einblick in das wahre Ausmaß der Krankheit im Jemen zu erhalten, wurden die in den staatlichen Krankenhäusern in den letzten Jahren gesammelten Daten ausgewertet. Außerdem wurden in verschiedenen Teilen des Landes mehr als 200 Proben von Patienten mit dem Verdacht auf cutane oder viscerale Leishmaniose gesammelt. Diese Proben wurden zunächst im Jemen mikroskopisch auf cutane Leishmaniose und mit Hilfe einer sehr unspezifischen Formol-Präzipitation-Methode auf viscerale Leishmaniose untersucht. Für einige Proben stand ein kommerzieller ELISA zur Verfügung. Von allen gesammelten Blutproben wurde die Plasmafraktion und aus angereicherten Leukozyten die DNA gewonnen und für nähere Analysen zur Universität Giessen gebracht.

Von den mittlerweile verfügbaren Sequenzdaten für die *Leishmania*-Stämme *L. infantum* (viszerale Leishmaniose) und *L. major* (cutane Leishmaniose) wurden

spezifische PCR-Primer für die Entwicklung neuer Diagnoseverfahren abgeleitet. Die neuen Verfahren wurden anhand von gereinigter DNA dieser beiden im Labor kultivierten Stämme getestet. Zur Entwicklung neuer PCR-Verfahren wurden fünf verschiedene Zielsequenzen von hoch-repetitiven Regionen der Genome ausgewählt. Zwei Genus-spezifische Tests und ein Test, der die Unterscheidung von cutaner und viszeraler Leishmaniose zulässt, wurden erfolgreich entwickelt.

Die Anwendung dieser Tests für die im Jemen gesammelten Patientenproben ergab, dass die PCR-Diagnose wesentlich empfindlicher und spezifischer ist, als die traditionell eingesetzten Methoden. Freilich konnte im Rahmen dieser Arbeit weder ein vollständiges Bild über die Epidemiologie der Leishmaniose im Jemen noch ein endgültig perfektes Diagnoseprotokoll entwickelt werden. Dies wird Aufgabe für die folgenden Jahre sein. Immerhin weisen die ermittelten Daten einerseits darauf hin, dass die Lage der Leishmaniose im Jemen sehr viel schwerwiegender ist, als offiziell zugegeben. Andererseits wurden diagnostische Verfahren entwickelt, die ausreichend einfach und so kostengünstig sind, dass sie im Land selbst eingeführt werden können. In optimierter Form könnten diese Verfahren zu einem besseren Stand der Kenntnis über diese Krankheit im Land führen und auf Dauer zu einer Verbesserung der gesundheitlichen Situation der Bevölkerung beitragen

SUMMARY

Leishmaniasis is an infectious disease caused by different species of unicellular *Leishmania* parasites which are transmitted by the bite of sandflies. As first clinical symptom, a non-healing ulcerous wound of up to several centimeters diameter forms around the bite of the insect. After several months, the wound may heal spontaneously leaving a scar. If the infection was due to a less virulent species of the parasite, the patient is healed and resistant against this form for the rest of life. However, other strains of the parasite lead to mucocutaneous leishmaniasis which lead to painful destructions of the mucosa of lips and nose, or to visceral leishmaniasis which affects inner organs and is lethal without treatment. Cutaneous as well as visceral leishmaniasis are endemic in the Republic of Yemen, but due to limiting financial resources, the disease is neither appropriately diagnosed nor treated in the population.

Recorded epidemiological data exist only for some governmental hospitals, but the number of unregistered cases may be huge. In order to shed more light in the real extent of the disease in Yemen and to contribute to future control measures, it was attempted to design new molecular procedures for the diagnosis, which are, at the one hand, highly sensitive and specific, at the other hand not too complicated and inexpensive, so that they could be performed under the limiting conditions of the country.

To obtain some insight in the situation of leishmaniasis, the data registered in governmental hospitals during the past years were collected and evaluated. In addition, samples from more than 200 patients with suspected cutaneous and visceral leishmaniasis were collected in different parts of the country. These samples were analysed first in Yemen by microscopy for cutaneous leishmaniasis and by a highly unspecific procedure called formol gel precipitation for visceral leishmaniasis. Some of the samples were also analysed by a commercial ELISA. From all collected blood samples the plasma fraction and DNA from enriched leucocytes were prepared and transported to the University of Giessen for further analysis.

By using the available sequence data of the *Leishmania* strains *L. infantum* (visceral leishmaniasis) and *L. major* (cutaneous leishmaniasis), specific PCR primers were developed for the design of new diagnostic procedures. The assays were tested by means of purified DNA from these two strains which had been

cultivated in the laboratory. Five different target sites from highly repeated sequences of the genomes were selected to develop new diagnostic PCR assays. Two assays specific for the genus *Leishmania*, and one assay allowing differentiation of the two strains were constructed allowing to discriminate between cutaneous and visceral leishmaniasis.

Applying these tests on the patient samples collected in Yemen revealed that PCR diagnosis is more sensitive and specific than the traditional methods used. Of course, neither a comprehensive epidemiology of Yemen nor a definitive perfect diagnostic protocol could be obtained during this work. This has to be established during the following years. However, the results reveal at the one hand that the situation of leishmaniasis in Yemen is more severe than officially stated. At the other hand, diagnostic procedures were developed which are simple and inexpensive enough to be introduced in the country. When optimized, these techniques may lead to more awareness of this disease in the country and to an improvement of the health condition of the suffering population.

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1. INTRODUCTION

1.1 Epidemiology of leishmaniasis

Leishmaniasis is a protozoan parasitic disease which is prevalent in many parts of the tropics, subtropics, and Southern Europe. The disease is caused by infection with different species of *Leishmania*, which are transmitted by the bite of phlebotomine sand flies. The most common forms of leishmaniasis in the Old World are *cutaneous leishmaniasis* (CL), causing skin sores (review: Reithinger et al., 2007), and *visceral leishmaniasis* (VL), affecting internal organs of the body such as spleen, liver, and bone marrow (review: Berman, 2006). A third form of the disease, *mucocutaneous leishmaniasis* (MCL) leading to severe destruction of mucosal areas of the mouth, nose and pharynx occurs exclusively in Latin America (reviewed by Myler and Fasel, 2008). There is an estimated prevalence of approximately 12 million infected people worldwide with 1.0 - 1.5 million new cases of cutaneous leishmania and 400,000 - 600,000 new cases of visceral leishmania per year. Leishmaniasis is endemic in 88 countries and It has been estimated that 350 million people are at threat. Confirmed cases of VL have been reported from 66 countries, 90% of the world's VL occurs in five countries (India, Bangladesh, Nepal, Sudan and Brazil), and 90% of all CL cases are concentrated in 7 countries (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria) showing that Middle East is a focus for cutaneous leishmania (Kumar et al., 2007). At present 90% of all MCL occurs in Bolivia, Peru and Brazil. It is the most feared form of leishmaniasis because of its disfiguring lesions of the face.

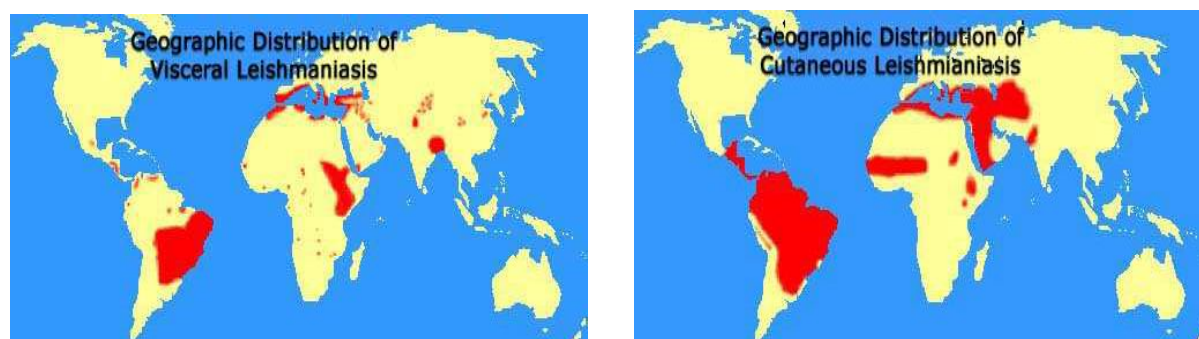


Figure 1: World distribution of visceral and cutaneous leishmaniasis (source: WHO)

4.1 Epidemiology of leishmaniasis

There are no solid data on the incidence of *Leishmania* infections in Yemen, but the disease is certainly under-reported, especially in women and children, and may exceed 10.000 cases per year. Most cases correspond to cutaneous leishmaniasis due to infections with *L. major* and *L. tropica* which may not necessarily need treatment, but up to 10 % of the infections may be due to *L. infantum* leading to visceral leishmaniasis which is associated with up to 90% mortality when untreated.

There are only few reports on leishmaniasis in Yemen in the literature (Sarnelli 1933; Rioux 1986; Pralong et al. 1995; Khatri et al., 2006) which can be used to predict the parasite strains circulating in the country (*L. major* and *L. tropica* for cutaneous leishmaniasis and *L. infantum* for visceral leishmaniasis), but these publications do not indicate the frequency and severity of the disease in the population. In order to get more information on the occurrence of leishmaniasis, data collected during the years 2005-2008 by the the National Center of Surveillance of Disease from all governmental hospitals in the 20 governorates were analysed (Table 1). However, these data can only be used in a qualitative way, but they are by no means quantitative. Data obtained during a visit of the (private) Alolfi Hospital in the Alhodeidah governorate indicated 105 cases during the same period of time (2005-2008) in this single place which is twofold the number of cases reported by the government for the whole governate (53 cases).

The closest countries with similar kinds of *Leishmania* strains, phlebotomine vectors and comparable geographic conditions are Oman in the East, Saudi Arabia in the North-West and, in a distance of some 2000 km North-West from Yemen, Jordan and Palestine. Some useful epidemiological data exist from Saudi Arabia (al-Zahrani et al., 1989 and Ibrahim et al., 1992), whereas most informative data have been published for Jordan and Israel (Nimri et al., 2002; Wasserberg et al., 2002; Al-Jawabreh et al., 2003; Al-Jawabreh, 2005; Singer et al., 2009; Amro et al., 2009; Hamarsheh et al., 2009). These data could form a basis for the understanding of leishmaniasis in Yemen. As in Yemen, two forms of transmission of the disease exist: in urban areas the parasites are transmitted from infected individuals by the bite of phlebotomine sand flies to naïve persons (anthroponotic cutaneous leishmaniasis), whereas in rural areas animals are thought to be the reservoir (zoonotic cutaneous leishmaniasis). The anthroponotic form of disease is frequent in large cities where

many people live close together under poor hygienic conditions. The vectors can multiply in small water reservoirs close to the dwellings as illustrated by the following pictures (Figure 25).



Figure 25: Conditions for anthroponotic cutaneous leishmaniasis. In many cities of Yemen, leishmaniasis is not dependent on animal hosts, but is transmitted by sand flies directly from infected individuals to naïve persons. In such locations the infection rate can be very high. The larvae of the sand flies can perfectly thrive in nearby water pools.

Hosts involved in the transmission of the zoonotic form of the disease include primarily dogs and small rodents living in arid regions. In the neighbouring countries Iran, Jordan and Israel host species such as *Psammomys obesus*, *Rattus rattus*, *Gerbillus dasyurus*, *Nesokia indica*, *Rhombomys opimus* and *Meriones spp.*

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Giessen im Juli 2009

Abdullatif Ali