AN IMMUNOLOGICAL STUDY OF LEISHMANIASIS IN THAI WORKERS RETURNING FROM THE MIDDLE EAST COUNTRIES

ระดับภูมิคุ้มกันโรค LEISHMANIASIS ในแรงงานไทย ที่กลับจากประเทศตะวันออกกลาง

Lt.Col. Tipprapa Piamjinda
พ.ท.หญิง ทิพย์ประภา เปี่ยมจินดา
Col. Sriwattana Chitchang
พ.อ.หญิง ศรีวัฒนา ชิตช่าง
Maj. Saovanee Leelayoova
พ.ต.หญิง เสาวณี ลีละยูวะ

Pramongkutklao College of Medicine
วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า

ABSTRACT

Many Thai workers returning from the Middle East countries are suffered from leishmaniasis which is one of the important epidemic diseases in the world. Suspicious cases are still in the native districts and may cause the spread of this disease in Thailand. A serological study of visceral and cutaneous leishmaniasis (VL, CL) was carried out among 570 Thai workers who had lived in the Middle East countries not less than 1 year. A developed immunofluorescent antibody test (IFAT) was applied to these suspected cases of VL, CL and healthy controls. From 11 (1.93%) serologically positive of VL, 2 was parasitologically confirmed either by staining or in vitro *cultivation. Leishmanial organisms were isolated from lesions of 2 patients suffering from CL whereas 18 (3.16%) cases of them were provided only IFAT positive of CL. Eight (1.4%) out of 29 suspected cases were positive to both VL and CL antigens. This is the first immunological leishmaniasis surveyed report in Thailand of the doubtful Thai workers returning from the Middle East countries. Further studies and prevention measures of this disease should be established since many Thais are still working in those countries.*

บทคัดย่อ

การศึกษาหาระดับภูมิคุ้มกันโรค leishmaniasis ในแรงงานไทยจากประเทศตะวันออกกลาง จำนวน 570 คน โดยวิธี immunofluorescent antibody test (IFAT) พบว่ามี 11 ราย (1.93%) ที่มีระดับภูมิคุ้มกันต่อโรค visceral leishmaniasis (VL) ซึ่งในจำนวนนี้มีอยู่ 2 ราย ที่สามารถแยกเชื้อ leishmania ได้จากน้ำกระดูกไขสันหลัง โดยวิธีย้อมสีและการเพาะเชื้อในห้องทดลอง และพบว่ามีจำนวน 18 ราย (3.16%) ที่มีระดับภูมิคุ้มกันต่อโรค cutaneous leishmaniasis (CL) ซึ่ง 2 ใน 18 รายนี้มีแผลเป็นผื่นขึ้นที่บริเวณผิวหนังของมือและเท้า จากแผลสามารถแยกเชื้อ leishmania ได้ นอกจากนี้ยังพบว่าใน 29 ราย ของผู้ที่มีระดับภูมิคุ้มกันโรค leishmaniasis มีอยู่ 8 ราย (1.4%) ที่มีระดับภูมิคุ้มกันโรคทั้ง 2 ชนิด การศึกษาครั้งนี้นับเป็นการศึกษาหาระดับภูมิคุ้มกันโรค leishmaniasis ในคนไทยที่เคยอยู่ในประเทศตะวันออกกลางเป็นครั้งแรก ซึ่งสามารถใช้เป็นข้อมูลในการศึกษาและป้องกันการระบาดของโรคนี้ในประเทศไทยในอนาคตได้

INTRODUCTION

Leishmaniasis is a widespread disease in Africa, Central America, South America, Europe, India and Middle East countries.[22] The disease is caused by *Leishmania* spp; the protozoa, which is found in an amastigote form in the mononuclear phagocytic cells of man and in promastigote form in sand flies of the *Phlebotomus* spp. which serve as intermediate host.[22] Biologic and biochemical studies suggest that the organism complex of *Leishmania tropica* cause cutaneous leishmaniasis (CL) in the Old World, the *Leishmania mexicana* and *Leishmania braziliensis* complexes cause cutaneous and mucosal disease in the New World, and the *Leishmania donovani* complex causes visceral leishmaniasis (VL)[3]. CL of the Old World occurs in two main forms : the urban, dry form is generally attributed to *L. tropica* and characteristically consists of a single, 1-3 cm ulcer that is said to heal itself in the course of 1 year. The rural wet form cause by *L. major* - consists of ulcers that are frequently multiple and that also are said to heal over 1 year[1]. Like Old World disease, CL of the New World characteristically progresses through the lesion, ulcerative and healing stages. The Old World CL affects man and dog in the big cities and towns of the Middle East, the Mediterranean area, North Africa, Northwest India and Pakistan, whereas the New World CL is found in Central and South America. Kala-azar or VL in the Old and New World is disseminated and infected the reticuloendothelial system. It is characterized by fever, hepatosplenomegaly, hypergammaglobulinemia and pancytopenia[22]. These widely distributed diseases are disfiguring and sometimes fatal[22].

In Thailand, the first report of leishmaniasis was the imported VL case found in a Pakistan female in 1960[15]. Seven years later the imported VL case was found in an Indian female and in 1984 in a Bangladesh boy[15]. The first VL in Thai patients has been

reported in 1985[4] and the other in 1986[6]. CL in Thai patients has been firstly reported in 1981 from many hospitals[16,17,19] At present, there are several patients who are suffering from lesion of CL come to the hospitals[3]. All of them went to the Middle East countries and were bitten by sand flies. Thailand is therefore now one of the countries in the epidemiological area of leishmaniasis[20], so it may be one of the serious problem in Thailand in the future.

In this study, the authors have developed the immunofluorescent antibody test (IFAT)[9, 11], using intact promastigotes as antigen to detect antibodies in the sera of 570 Thai workers both with and without clinical features, from the hospitals in and outside Bangkok.

MATERIALS AND METHODS

Case selection

The criterion for case selection was the Thai workers who lived in the Middle East countries not less than 1 year. They either had not been responding to antimalarial treatment or their blood smear was negative for *Plasmodium*. Previous history, general data (age, sex) and clinical characteristics (VL: fever, hepato-splenomegaly, enlargement of lymph nodes; CL: location, number and aspect of the lesions) were recorded on a standard observation form.

Sera

The sera used as negative controls were 430 sera from healthy Thai blood donors, who had the general data and residential area, province, similar to that of the study cases. These healthy Thai blood donor sera also responded negative results to HIV serological test. The leishmania sera were from 2VL and 2CL clinically and parasitologically confirmed in Kala-azar patients and cutaneous leishmaniasis patients, respectively, before the beginning of chemotherapy. Other controls for specificity included sera from 10 malaria Thai patients.

Parasite isolation and culture

Patients with suspected VL had aspiration of bone marrow performed, whereas that of CL, the small tissue samples were removed by punch-biopsy from the indurated edges of the cutaneous lesions. The aspirate and the fragment (after triturating in phosphate-buffered saline) were cultured directly in modified NNN medium[5]. The tubes which incubated at 23°C were examined every week and discarded if they were negative after 20 days. If organisms developed, cultures were routinely subcultured each week in modified Tobie's medium.

Antigen

Antigen was prepared isolately from the promastigotes of local strain of VL and CL grown in modified Tobie's medium. The growth was harvested on the 5th-7th post inoculation day and promastigotes were washed thrice with PBS pH 7.1. Before use in the IFAT test the parasites were adjusted to 10^6 promastigotes/ml in PBS pH 7.1.

Immunofluorescent antibody test (IFAT)

Ten μl washed promastigotes were dispensed regularly as thin smear of blood film, left uncovered on glass slide which coated with 0.01% BSA. After drying and fixation 15 min in 4°C octanol, small circles of the expected protozoan were determined and a determined serum dilution of 1:16 was used through out. As conjugate, sheep antihuman Ig G (H+L) labelled with FITC was used in dilution of 1:40. Fluorescence intensity was arbitrarily assessed as +, 2+, 3+, or 4+. Since a considerable number of individuals without leishmaniasis and malaria showed no fluorescence, ≥ + was considered IFAT seropositive. A quantitative standardization procedure of IFAT was performed by the unknown sera of Thai workers with 430 controls who had no known contact history of leishmaniasis.

RESULTS

Serological results of individuals from Thailand obtained with IFAT on the leishmanial antigens were shown in Table 1. Cross reactions between the 2 species (*L. donovani* and *L. tropica*) were present, but they were not found with the IFAT in sera of 10 patients with malaria and 430 healthy controls. The highest titres in VL and CL to homologous antigen are 1:1,024 and 1:256, respectively. In contrast, there was no positive result to leishmanial antigen in the healthy control and malarial sera diluted at 1:16. Thus a titre of 1:16 or greater in sera reacting with leishmanial antigen may be considered as diagnostic of leishmaniasis.

The results in IFAT of the 570 sera of Thai workers on both *L. donovani* and *L. tropica* antigens were shown in Tables 2 and 3. The prevalence rate of positive results with *L. donovani* and *L. tropica* antigens were 1.93% (11) and 3.16% (18), respectively. Of these positive results, 8 cases (1.4%) were positively responded with both antigens. Two had become positive cultures with clinically diagnosed VL and their IFAT titres with both antigens were 1:1,024. Other 2 patients had cutaneous lesions with confirmed parasitologically and their titres with both antigen were 1:256. The others who had positive results of IFAT with leishmania antigens, had negative parasitological results.

DISCUSSION

The first immunological survey of leishmaniasis in Thai workers returning from the Middle East countries was studied by using IFAT. The test was specific and considered reliable for diagnosis of leishmaniasis[7, 8]. Cross reactions between the 2 species of *Leishmania* were present, but they were not found with the IFAT in sera of healthy controls and patients with parasitic infections. Malaria sera, in particular, cross-reacted significantly with *L. donovani* antigen in IFAT[12]. In the results such a degree of cross-reactivity were not found in any of 10 malarious sera as agree with 36 malarious sera reported for the IFAT by Harith *et al.*[11] Quantitative standardization procedure of IFAT with 430 controls were performed to reassure that a titre of 1:16 was the minimum titre of IFAT positive results used for Thai people.

The highest titres to homologous antigen in VL were usually higher than that in CL. Edrissian & Darabian[8] also found that IFAT titres in Kala-azar patients were higher than the non-specific antibody titres in other diseases. The titres of antibody to VL antigen in this study were not as high as those reported by the others such as Edrissian & Darabian[8], ($\geq$ 1:256); El Amin *et al.*[9], ($\geq$ 1:50); Harith *et al.*[11],($\geq$ 1:100); whereas the titres to CL antigen were found the same ($\geq$ 1:16); as reported by Edrissian & Darabian[8]. Jahn and Diesfeld[13] found that cutaneous leishmaniasis and healthy people in Endao had the low titres of antibody to leishmania antigen. Thus, asymptomatic infection must be very common, but only in few of the infected individuals. The parasites managed to overcome immune defense mechanisms and to cause a generalized infection. At this stage of the disease, most of the affected persons showed typical symptoms and *L. donovani* could be demonstrated in splenic aspirate or bone marrow.

The negative result of the parasitological examination in Thai seropositive cases could be due to the low parasitic load and the methods of the isolation. The isolation of the parasite from human cases after direct inoculation of culture medium was not more successful than the inoculation into hamster[10]. This was especially true under field conditions where the chances of bacterial and fungal contamination were extremely high. Inoculation of hamsters was proposed as an easy method for isolation of strains from human cases, but there were still the problems of the maintenance of infected hamsters in the laboratory and the vectors of the diseases. *Phlebotomus squamipleusis, P. bailyi* var *campester* & *P. barraudi* var *siamese,* 3 species of sand fly found in Thailand[2, 21] were in doubt that they could act as the vectors of *Leishmania* or not.

The isozyme analysis of *Leishmania* isolates[14] leading to identification was not in progress in this study, so the strains of the parasites were concluded by the parasitological & clinical characteristics, the history of the patients, the countries where the patients came from and the sites of the isolated specimens only.

IFAT in this study was developed differently from the others. By using 4°C octanol, 15 min to fix the antigen slides, it was found that the background of the appearance was clearer than fixing with acetone[9, 11]. However further studies for the techniques in immunological assay (IFAT, ELISA, leishmania test) to detect the leishmaniasis should be developed, especially in cutaneous leishmaniasis which is epidemiologically carried by Thai workers returning from the Middle East countries. It is expected that there are many Thai workers suffering from this disease, still working in these countries and want to be back home soon. And from the prevalence rate of positive results with leishmania antigen in this study (1.93% VL and 3.16% CL), including the suspicious widespread vectors, sand fly, all might effect to the future of public health in Thailand. So, it should be aware of how to confine and solve this problem which might be one of the important epidemiological disease in Thailand.

SUMMARY

1. Of 570 Thai workers returning from Middle East countries, 11 (1.93%) and 18 (3.16%) were IFAT positive of VL and CL, respectively.
2. Of these serologically positive, 2 were parasitologically and clinically positive of VL whereas the other 2 were positive considerably of CL.
3. Eight (1.4%) sera were positive with both VL and CL antigens.
4. Four degrees Celsius octanol was used as a better fixation agent than acetone in developed IFAT for detecting leishmaniasis.

ACKNOWLEDGEMENT

This work was partially supported by grant from the National Research Council of Thailand. The authors are grateful to Dr. Somnate Boonpuckanavig, Head of the Department of Pathology, Faculty of Science, Mahidol University and her staffs for their assistance and suggestion; Dr. Sombat Tanprasertsiri for his valuable cooperation.

REFERENCES

1. Berman, J.D. Chemotherapy for Leishmaniasis : Biochemical Mechanisms, Clinical Efficacy, and Future Strategies. *Rev. Infect. Disease,* 1988, **10**, 560–586.
2. Causey, O.R. Phlebotomus of Siam with a Description of a New Variety. *Am. J. Hyg.,* 1938, **28**, 487–489.
3. Charoenlarp, P. Leishmaniasis. *J. Parasitol. Trop. Med. Assoc. Thailand,* 1986, **9**, 38, 43.
4. Chutabuddhi, A. Kala-azar in Thailand. *Folk Doctor Magazine,* 1985, **7**, 34–36.
5. Decker-Jackson, J.E. and Haniberg, B.M. Glycoprotein Released by *Leishmania donovani* : Immunologic Relationship with Host and Bacterial Antigens and Preliminary Biochemical Analysis. *J. Protozool.* 1978, **25**, 514–525.

IFAT in this study was developed differently from the others. By using 4°C octanol, 15 min to fix the antigen slides, it was found that the background of the appearance was clearer than fixing with acetone[9, 11]. However further studies for the techniques in immunological assay (IFAT, ELISA, leishmania test) to detect the leishmaniasis should be developed, especially in cutaneous leishmaniasis which is epidemiologically carried by Thai workers returning from the Middle East countries. It is expected that there are many Thai workers suffering from this disease, still working in these countries and want to be back home soon. And from the prevalence rate of positive results with leishmania antigen in this study (1.93% VL and 3.16% CL), including the suspicious widespread vectors, sand fly, all might effect to the future of public health in Thailand. So, it should be aware of how to confine and solve this problem which might be one of the important epidemiological disease in Thailand.

SUMMARY

1. Of 570 Thai workers returning from Middle East countries, 11 (1.93%) and 18 (3.16%) were IFAT positive of VL and CL, respectively.
2. Of these serologically positive, 2 were parasitologically and clinically positive of VL whereas the other 2 were positive considerably of CL.
3. Eight (1.4%) sera were positive with both VL and CL antigens.
4. Four degrees Celsius octanol was used as a better fixation agent than acetone in developed IFAT for detecting leishmaniasis.

ACKNOWLEDGEMENT

This work was partially supported by grant from the National Research Council of Thailand. The authors are grateful to Dr. Somnate Boonpuckanavig, Head of the Department of Pathology, Faculty of Science, Mahidol University and her staffs for their assistance and suggestion; Dr. Sombat Tanprasertsiri for his valuable cooperation.

REFERENCES

1. Berman, J.D. Chemotherapy for Leishmaniasis : Biochemical Mechanisms, Clinical Efficacy, and Future Strategies. *Rev. Infect. Disease,* 1988, **10**, 560–586.
2. Causey, O.R. Phlebotomus of Siam with a Description of a New Variety. *Am. J. Hyg.,* 1938, **28**, 487–489.
3. Charoenlarp, P. Leishmaniasis. *J. Parasitol. Trop. Med. Assoc. Thailand,* 1986, **9**, 38, 43.
4. Chutabuddhi, A. Kala-azar in Thailand. *Folk Doctor Magazine,* 1985, **7**, 34–36.
5. Decker-Jackson, J.E. and Haniberg, B.M. Glycoprotein Released by *Leishmania donovani* : Immunologic Relationship with Host and Bacterial Antigens and Preliminary Biochemical Analysis. *J. Protozool.* 1978, **25**, 514–525.

Table 1. Results of the developed IFAT on sera of patients with VL, CL, malaria and healthy control (HC) on leishmania antigen

Group	No.	Antigen	IFAT intensity of fluorescence	
			—	* ⩾ +
VL	2	L.d.	0	2**
		L.t.	0	2
CL	2	L.d.	0	2
		L.t.	0	2***
Malaria	10	L.d.	10	0
		L.t.	10	0
H.C.	430	L.d.	430	0
		L.t.	430	0

VL : visceral leishmaniasis with positive parasitic culture
CL : cutaneous leishmaniasis with positive parasitic culture
L.d. = *Leishmania donovani*
L.t. = *Leishmania tropica*
*Serum dilutions in IFAT was 1 : 16
**IFAT : VL titre = 1 : 1024
***IFAT : CL titre = 1 : 256

Table 2. Results of IFAT on 570 sera Thai workers on leishmania antigen (L.d. = *L. donovani* antigen, L.t. = *L. tropica* antigen)

Antigen	No.	IFAT intensity of fluorescence	
		−	$\geqslant$ + *
L.d.	570	559	11**(1.93%)***
L.t.	570	552	18**(3.16%)

*Serum dilutions in IFAT was 1:16

**Four patients were positive leishmania culture and clinically diagnosed as 2 VL and 2 CL

***% Prevalence rate

Table 3. IFAT titres in sera of Thai workers positive with *L. donovani* and *L. tropica* antigen

End-titre dilutions				No.of case	Antigen
1:16	1:64	1:256	1:1,024		
3	4	2(2)	2(2)	11	L.d.
2	1	3(2)	3(2)	8	L.t.
9	4	3(2)	2(2)	18	L.t.
2	2	2(2)	2(2)	8	L.d.

( ) Parasites + ves