Tropical Biomedicine 33(4): 739–745 (2016)

Prevalence and Distribution of Anti-Amoebic IgG Antibody among Orang Asli (Aborigines) in Peninsular Malaysia

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Received 12 December 2015; received in revised form 14 June 2016; accepted 16 June 2016

Abstract. *Entamoeba* species are commonly detected in stool samples of Orang Asli due to their substandard living conditions and poor hygiene. Among the *Entamoeba* spp., *Entamoeba histolytica* is the only known primary pathogenic species. This study determined the prevalence and distribution of anti-amoebic IgG antibody among Orang Asli in Peninsular Malaysia. The results would reflect the prevalence of amoebiasis in the population. This study analysed a total of 375 serum samples from archives of two Orang Asli projects conducted between 2011 and 2014. They were from six different states in Malaysia, namely Johor, Kedah, Kelantan, Pahang, Perak, and Selangor. Anti-amoebic IgG antibody was detected using an enzyme-linked immunosorbent assay (ELISA) with crude soluble antigen produced from axenically grown *E. histolytica* trophozoites. From the analysis, the overall seropositivity was approximately 71% (266/375), while the seropositivity rates for each of the three Orang Asli tribes *i.e.* Senoi, Negrito and Proto-Malay, were 66% (137/208), 92% (103/112), and 43% (17/41) respectively. Orang Asli from Kedah [95% (52/55)] showed the highest seropositivity, followed by Kelantan [79% (54/68)], Perak [73% (78/107)], Pahang [60% (57/95)], Selangor [56% (14/25)], and Johor [48% (10/21)]. Orang Asli from rural [76% (192/254)] and peripheral urban [65% (69/106)] areas showed significantly higher seropositivity (p=0.002) than those from urban areas [36% (4/11)]. The high prevalences of anti-amoebic IgG antibody in these Orang Asli populations comprised both active and past infections. This study provides current insights of amoebiasis in selected Orang Asli settlements in Peninsular Malaysia. The high seropositivity of anti-amoebic IgG antibody suggests that the settlements are endemic for amoebiasis and there is a high risk of acquiring *E. histolytica* infection among the dwellers.

INTRODUCTION

Amoebiasis is a parasitic disease caused by the enteric protozoan, *Entamoeba histolytica*. This disease is prevalent in tropical and sub-tropical areas associated with substandard living conditions and poor hygiene. It is estimated that 10% of the world population are affected by this disease (Samie *et al.*, 2012), and ~10% of those infected progress to symptomatic amoebic disease. The majority of asymptomatic cases are due to *E. dispar* and *E. moshkovskii* which are microscopically indistinguishable from *E. histolytica* (ElBakri *et al.*, 2013). *E. histolytica* is the primary pathogenic species and can cause invasive amoebiasis such as amoebic colitis and amoebic dysentery. The disease may progress to extraintestinal infection through haematogenously spread of trophozoites to other organs causing potentially fatal abscesses (Mortimer & Chadee, 2010).

The estimated 150,000 Orang Asli population in the Peninsular Malaysia comprises three main tribes namely Senoi,

Negrito and Proto-Malay. These tribes are further sub-divided into 18 sub-tribes (JAKOA, 2011). There were reports which showed high prevalences of *Entamoeba* spp. among Orang Asli stool samples (Shahrul-Anuar & Norhayati, 2011; Hotez, 2014). In a recent study, the prevalence of intestinal *Entamoeba* spp. among Orang Asli based on formalin-ether concentration and trichrome staining techniques was found to be 18.6% (93/500) (Shahrul-Anuar *et al.*, 2012b). Subsequently analysis by polymerase chain reaction (PCR) on the samples showed 18% (16/88) of the positive cases were due to *E. histolytica*; whereas the rest were caused by *E. dispar* and/or *E. moshkovskii* (Shahrul-Anuar *et al.*, 2012a).

Serodiagnosis based on *E. histolytica* antigen is a useful tool to determine the prevalence of amoebiasis. Only *E. histolytica* causes symptomatic disease which stimulates humoral immune response upon infection of the intestinal mucosa (Mortimer & Chadee, 2010; Shahrul-Anuar & Norhayati, 2011; Yang *et al.*, 2012). Furthermore serodiagnosis can detect infection (present and past) in a broader timeline because IgG antibody titer does not drop dramatically after recovery. Currently, there is no new published report on sero-prevalence of amoebiasis among Orang Asli in Malaysia. Back in 1976, a study among Orang Asli children and adults in Western Peninsular Malaysian reported anti-amoebic IgG seropositivities of 79% and 87%, respectively (Gilman *et al.*, 1976; Farhana *et al.*, 2009). Ten years later, another sero-prevalence study at Hospital Orang Asli Gombak in state of Selangor revealed 9.7% (16/165) of the patients were positive for anti-amoebic IgG (Thomas & Leng, 1986).

The present study investigated the prevalence and distribution of anti-amoebic IgG antibodies of Orang Asli from six states in Peninsular Malaysia using enzyme-linked immunosorbent assay (ELISA). The optimized immunoassay utilized soluble antigen produced from *E. histolytica* trophozoites that were axenically cultured at the School of Health Sciences, Universiti Sains Malaysia.

MATERIALS AND METHODS

Sample Collection and Processing

Archived Orang Asli blood samples collected between the year 2011 and 2014 from two previous projects were used in the present study. Samples from the first project consisted of 188 serum samples from a 'Tuberculosis (TB)-Parasite Correlational Study' among Orang Asli. The serum samples were categorized into three groups: Notified TB cases who are under treatment; healthy contact (HC)-close contacts of TB patients, and others (OT) who were admitted into the hospitals for other ailments as the control group. The second project comprised 187 serum samples from 'Orang Asli Genomic Evolutional and Anthropometric Study', which focused on six diminishing Orang Asli sub-tribes, namely Bateq, Che Wong, Kanaq, Lanoh, Semai, and Kensiu. Purposive sampling method was applied in both studies.

Orang Asli participants were recruited after they consented to the aim and potential future benefits of the project findings. Blood samples were collected into blood collection tubes containing either clot activator or heparin from the field sites. The samples were processed to obtain serum or plasma, and then stored at -20°C.

Preparation of Crude Soluble Antigen (CSA)

CSA was prepared as previously described by Tan *et al.* (2013). Briefly, 10 million *E. histolytica* trophozoites were mixed with 500 µL of Complete Lysis-M buffer (Roche Diagnostic, Germany) added with a cocktail of protease inhibitors and 20 µL of 0.5 M iodoacetamide (Sigma, USA). The cells were then disrupted by sonication at 10% amplitude for three cycles of 1 min each (Branson, USA). The lysate was centrifuged at 10 000 × *g* for 10 min at 4°C, and the supernatant was used as CSA. The protein concentration of CSA was determined using Bradford method and kept in -20°C until used.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed as described by Tan *et al.* (2013). Wells of microtiter plate (Nunc, Denmark) were coated overnight with 4 µg/well of CSA in 100 µL of 0.1 M carbonate coating buffer, pH 9.6, at 4ºC. Next day, the wells were washed 3×5 min with 200 µL of phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), then blocked with blocking reagent (Roche, Germany) for 1 h at room temperature (RT). The wells were again washed 3×5 min with 200 µL of PBS-T. A volume of 100 µL primary antibody (serum samples) diluted 1:200 with PBS was introduced into the each well, and incubated at RT for 1 h. After another washing step, a volume of 100 µL secondary antibody [horseradish peroxidase conjugated monoclonal mouse anti-human IgG (Invitrogen, USA)] diluted 1:500 with PBS was added into each well, and incubated at RT for 1 h. After a final washing step, 100 µL of tetramethylbenzidine (TMB) substrate solution was added into each well and incubated for 15 min at RT in the dark. A volume of 100 µL/well of 1 N H_2SO_4 was added to stop the substrate reaction. The optical densities (OD) of the wells were read at 450 nm using Multiskan™ FC Microplate Photometer (Thermo Scientific, USA). The result was expressed as mean OD of the duplicate wells for each sample. The cut-off value of the ELISA was determined from mean OD + 2 standard deviations (SD) of 30 blood donor serum samples from Hospital Universiti Sains Malaysia which were negative for anti-amoebic IgG by IHA Cellognost® Amoebiasis Kit (Dade Behring Marburg GmbH, Germany). Pooled samples from 30 patients with amoebic liver abscess (ALA) was used as the positive control; while 30 pooled IHA-negative blood donor serum sample was used as the negative control.

Data Analysis

The demographic data were recorded using Microsoft Excel 2013. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22. All categorical variables were analyzed using chi-square test for homogeneity. The distribution of the cases was mapped with the aid of Google Map application (Google Inc.), based on the addresses of the participants. The classification of urbanization of Orang Asli settlements was based on the criteria determined by Department of Orang Asli Development (JAKOA), Malaysia: (i) rural area – can be reached through ground or water route; without clean water supply, 24-hour electricity, other basic facilities and stable economic resources (ii) near urban – nearby to Malay villages, reachable through premix road, equipped with clean water, 24-hour electricity, has land development project and stable economic resources; and (iii) urban – equipped with most basic facilities and there is no undergoing land development project (JAKOA, 2011).

Ethical Considerations

The methodology of this study was approved by Universiti Sains Malaysia Human Research Ethics Committee (JEPeM) [ref. no. USMKK/PPP/JEPeM[247.3(9)]], National Medical Research Register (NMRR) [ref. no. (2) dlm.KKM/NIHSEC/08/0804/P11-567], Malaysia and Department of Orang Asli Development (JAKOA) [ref. no. JAKOA.PP.30.052 Jld.5(96)]. Participants were recruited after providing written informed consents.

RESULTS

Sociodemographic Characteristics

A total of 375 serum and plasma samples were examined. The samples were collected from 206 (55%) females and 169 (45%) males whose ages ranged from 13 to 79 years old with mean age ± SD of 34.3 ± 13.0 and 37.8 ± 15.6, respectively.

Ethnic Groups, Sub-Tribes and anti-Amoebic IgG Antibody

Among the three ethnic groups, Negrito (92%) showed the highest seropositivity ($p<0.001$), followed by Senoi (66%) and Proto-Malay (41%). The seropositivity of anti-amoebic IgG among each Negrito sub-tribe was at least 77%. Among the Senoi tribe, only Semai

Table 1. Prevalence of anti-amoebic IgG antibody among Orang Asli in Peninsular Malaysia

Variable	No.	Seropositivity, N (%)	*P*
Overall	375	266 (71)	
Sex			0.454
Female	206	143 (69)	
Male	169	123 (73)	
Age			0.321
10-19	43	29 (67)	
20-29	118	80 (68)	
30-39	80	59 (74)	
40-49	57	47 (82)	
50-59	41	30 (73)	
60-69	22	12 (55)	
70-79	5	3 (60)	
Unknown	9	6 (67)	
Ethnic Group			<0.001*
Senoi	208	137 (66)	
Che Wong	25	5 (20)	
Semai	62	49 (79)	
Temiar	119	83 (70)	
Jah Hut	1	0 (0)	
Mah Meri	1	0 (0)	
Negrito	113	103 (92)	
Kensiu	53	50 (94)	
Lanoh	22	17 (77)	
Bateq	28	28 (100)	
Lanoh+Kintak	1	1 (100)	
Mendriq	8	7 (88)	
Proto-Malay	41	17 (41)	
Kanaq	11	3 (27)	
Jakun	8	5 (63)	
Temuan	16	7 (44)	
Semelai	6	2 (33)	
Orang Asli	14	9 (64)	
BMI			0.370
Underweight	70	55 (79)	
Normal	217	152 (70)	
Overweight	73	50 (68)	
Unknown	15	9 (60)	
State			<0.001*
Johor	21	10 (48)	
Kedah	55	52 (95)	
Kelantan	68	54 (79)	
Pahang	95	57 (60)	
Perak	107	78 (73)	
Selangor	25	14 (56)	
Unknown	4	1 (25)	
Location of Settlements			0.002*
Rural	254	192 (76)	
Near Urban	106	69 (65)	
Urban	11	4 (36)	
Unknown	4	1 (25)	

*Significant based on chi-square test for homogeneity

Table 2. Prevalence of anti-amoebic IgG antibody among TB patient, Healthy Contact and Others

Variable	No.	Seropositivity, N (%)	*P*
Overall	188	125 (67)	
Health Status			
TB	90	65 (72)	0.098
HC	65	43 (66)	
OT	33	17 (52)	
TB+HC	155	108 (70)	0.045*
OT	33	17 (52)	

*Significant based on chi-square test for homogeneity.

and Temiar sub-tribes showed seropositivity of at least 70%. Among the Proto-Malay tribe, Jakun (63%) showed the highest seropositivity; while Kanaq, Temuan and Semelai showed seropositivity of less than 50% (Table 1).

TB and Anti-Amoebic IgG Antibody

The descriptive analysis revealed relatively higher ($p < 0.05$) seropositivity among the TB and HC groups, as compared to OT (control group). TB group presented the highest seropositivity (72%) as compared to HC (66%) and OT (52%) (Table 2).

Distribution of Anti-Amoebic IgG Antibody

The distribution of seropositivity is shown in Figure 1. Participants from rural areas showed the highest seropositivity rate (76%), followed by near urban (65%) and urban areas (36%). Orang Asli settlements in Kedah state showed the highest seropositivity (95%), followed by Kelantan (79%), Perak (73%), Pahang (60%), Selangor (56%), and Johor (48%) (Table 1).

DISCUSSION

Amoebiasis is still a public health problem in rural and remote areas of Malaysia. The infection spreads through faecal-oral route and may cause intestinal and/or invasive amoebiasis. It may lead to potentially fatal extraintestinal amoebiasis involving organs

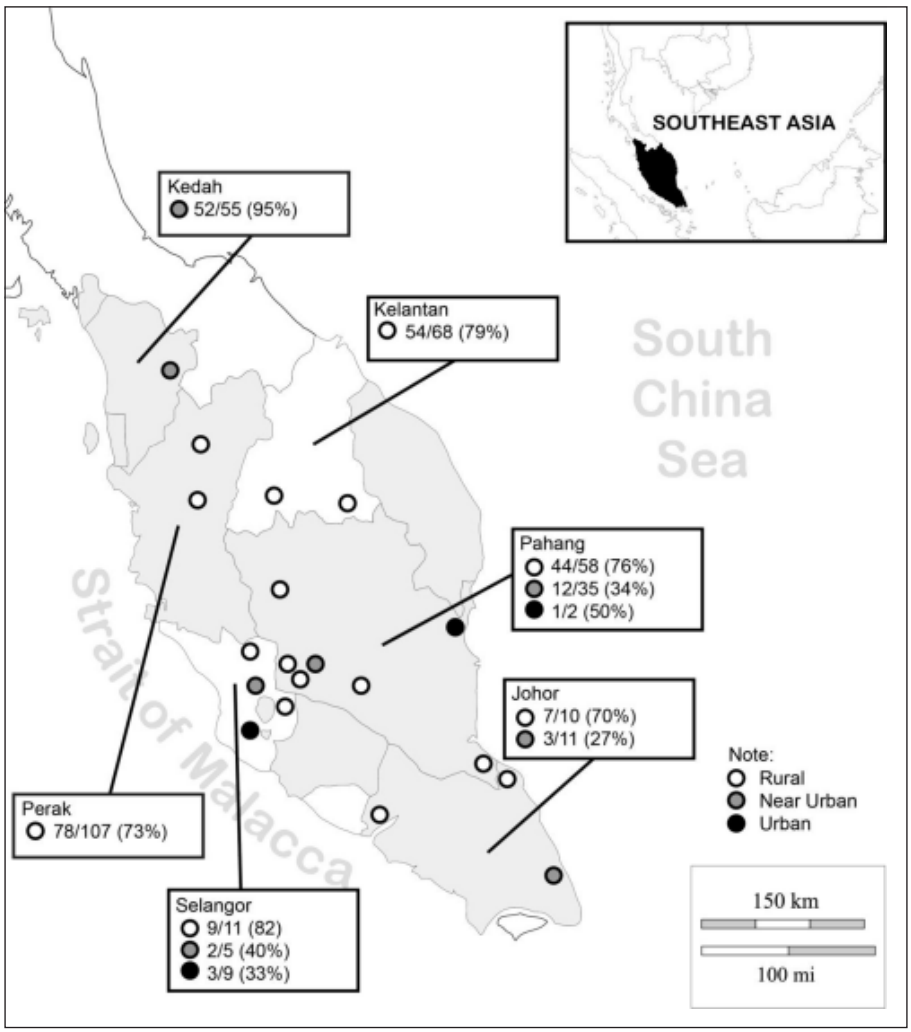

Figure 1. Prevalence of anti-amoebic IgG antibody among Orang Asli in Peninsular Malaysia (2011-2014).

like liver, lung, and brain with amoebic liver abscess as the most common manifestation (Shahrul-Anuar & Norhayati, 2011). Early diagnosis and treatment are the two key factors to prevent the exacerbation of the disease.

Most Orang Asli in Peninsular Malaysia lives in rural and remote areas. This population is known to be at risk of parasitic diseases like soil-transmitted helminthiases, amoebiasis, giardiasis, cryptosporidiosis, malaria and toxoplamosis (Hotez, 2014). The economy of this population depends mainly on agriculture, and hand washing is not a common practice after their daily planting or gardening activities. This unhygienic practice might be partly due to inaccessibility of clean water, inadequate water piping system, and lack of protective equipment like gloves and boots. In addition, consumption of raw vegetables and close contacts with domestic animals like cats, dogs, and poultry, which are part of Orang Asli lifestyle were reported to be associated with the parasites transmissions (Shahrul-Anuar & Norhayati, 2011; Shahrul-Anuar *et al.*, 2012a; Shahrul-Anuar *et al.*, 2012b). This study determined the prevalence and distribution of anti-amoebic IgG antibody among the Orang Asli in Peninsular Malaysia.

The findings indicate the presence of amoebiasis in the population although amoebic seropositivity do not differentiate between present and past infections (Tanyuksel & Petri, 2003).

The results showed that the seropositivity of anti-amoebic IgG antibody among the Orang Asli adults was 71%. The highest seropositivity was found in rural areas, followed by near urban to urban areas. Compared to the 87% seropositivity among Orang Asli in 1976 as reported by Gilman *et al.* (1976), there is a decrease in prevalence of ~16%. The reduction in the prevalence is probably due to the effective modernization programme by the government. However, it takes continuous and sustained efforts to eliminate the disease. Besides improving the socio-economic welfare of Orang Asli, education and health services need to be further upgraded to better control amoebiasis and other parasitic diseases with almost similar risk factors.

A study by Shahrul-Anuar *et al.* (2012b) revealed a high prevalence of intestinal *Entamoeba* spp. infection among the three Orang Asli tribes. Interestingly, the present study also showed a high overall potential invasive amoebiasis among this indigenous population based on seropositivity of anti-amoebic IgG antibody. Hence effective intervention programmes should be carried out to reduce the amoebiasis threat. In congruence with the microscopic findings of Shahrul-Anuar *et al.* (2012b), the highest seroprevalence was also found among the Negrito tribe, followed by Senoi and Proto-Malay. This phenomenon is probably due to the different means of subsistence among the Negrito who mainly survive on semi-nomadic hunting, gathering and fishing (HGF), as compared to Senoi and Proto-Malay who depend on shifting cultivation, plantation and HGF (Dunn, 1972).

Seropositivity of amoebiasis was significantly higher ($p<0.05$) among TB patients and HC, as compared to the OT control group. Among them, TB patients showed the highest seropositivity of amoebiasis ($p>0.05$). This suggested the potential correlation between TB and amoebiasis. In this regard, there are previous reports which postulated that harboring of intestinal parasites might increase the probability of developing TB due to the skewing of protective immune response (Resende Co *et al.*, 2007; Li & Zhou, 2013).

One limitation of the present study was the selection of the participants, since they were based on serum samples collected previously by purposive sampling. Hence, the seroprevalence results of anti-amoebic IgG antibody can only be taken as an indication of possible invasive amoebiasis prevalence among the three main Orang Asli tribes identified from the 6 states in Peninsular Malaysia.

In conclusion, the seroprevalence of invasive anti-amoebic IgG antibody among the Orang Asli was still high. The highest seroprevalence was observed among the Negrito ethnic group; and the states of Kedah, Kelantan and Perak showed high seropositivity of at least 70% with higher seropositivity observed in rural and near urban areas than the urban areas. Future efforts should focus on implementing intervention programmes at Orang Asli settlements with high anti-amoebic IgG antibody prevalence to reduce the seemingly unceasing problems.

Acknowledgements. The authors wish to acknowledge the cooperation and support from the participating State Health Directors and the respective TB and Leprosy Departments; Dr. Mohd Akmal Dato' Dahlan, doctors, medical assistants and nurses of Hospital Orang Asli Gombak; and Director and staff of Department of Orang Asli Development (JAKOA). We would also like to thank the Director General of Ministry of Health, Malaysia for permission to publish this paper; and Dean of School of Health Sciences, Universiti Sains Malaysia for the laboratory facilities. This study was funded by the Malaysia Ministry of Education Long Term Research Grant Schemes (LRGS), No. 203/PSK/6722002 and No. 304/PPSK/6150116/U132.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Dunn, F.L. (1972). Intestinal parasitism in Malayan aborigines (Orang Asli). *Bulletin of the World Health Organization*, **46**(1): 99.

ElBakri, A., Samie, A., Ezzedine, S. & Odeh, R.A. (2013). Differential detection of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* in fecal samples by nested PCR in the United Arab Emirates (UAE). *Acta Parasitologica*, **58**(2): 185-190.

Farhana, F., Jamaiah, I., Rohela, M., Abdul-Aziz, N. & Nissapatorn, V. (2009). A ten year (1999-2008) retrospective study of amoebiasis in University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. *Tropical Biomedicine*, **26**: 262-266.

Gilman, R., Davis, C., Gan, E. & Bolton, M. (1976). Seroepidemiology of amebiasis in the Orang Asli (Western Malaysian aborigine) and other Malaysians. *The American Journal of Tropical Medicine and Hygiene*, **25**(5): 663-666.

Hotez, P.J. (2014). Aboriginal populations and their neglected tropical diseases. *PLoS Neglected Tropical Diseases*, **8**(1): e2286.

JAKOA (2011). Department of Orang Asli Development: Strategic Plan 2011-2015.

Li, X.-X. & Zhou, X.-N. (2013). Co-infection of tuberculosis and parasitic diseases in humans: A systematic review. *Parasites and Vectors*, **6**(1): 79.

Mortimer, L. & Chadee, K. (2010). The immunopathogenesis of *Entamoeba histolytica*. *Experimental Parasitology*, **126**(3): 366-380.

Resende Co, T., Hirsch, C.S., Toossi, Z., Dietze, R. & RibeiroRodrigues, R. (2007). Intestinal helminth coinfection has a negative impact on both anti-Mycobacterium tuberculosis immunity and clinical response to tuberculosis therapy. *Clinical & Experimental Immunology*, **147**(1): 45-52.

Samie, A., ElBakri, A. & AbuOdeh, R.E. (2012). Amoebiasis in the tropics: Epidemiology and pathogenesis. In, *Current Topics in Tropical Medicine*: InTech, pp 201-226.

Shahrul-Anuar, T., Al-Mekhlafi, H.M., Abdul Ghani, M.K., Abu Bakar, E., Azreen, S.N., Salleh, F.M., Ghazali, N., Bernadus, M. & Moktar, N. (2012a). Molecular epidemiology of amoebiasis in Malaysia: Highlighting the different risk factors of *Entamoeba histolytica* and *Entamoeba dispar* infections among Orang Asli communities. *International Journal for Parasitology*, **42**(13): 1165-1175.

Shahrul-Anuar, T., Al-Mekhlafi, H.M., Ghani, M.K.A., Osman, E., Yasin, A.M., Nordin, A., Azreen, S.N., Salleh, F.M., Ghazali, N. & Bernadus, M. (2012b). Prevalence and risk factors associated with *Entamoeba histolytica/dispar/moshkovskii* infection among three Orang Asli ethnic groups in Malaysia. *PloS One*, **7**(10): e48165.

Shahrul-Anuar, T. & Norhayati, M. (2011). Public health and clinical importance of amoebiasis in Malaysia: A review. *Tropical Biomedicine*, **28**(2): 194-222.

Tan, Z., Wong, W., Noordin, R., Zeehaida, M., Olivos, G. & Lim, B. (2013). Efficacies of two in-house indirect ELISAs for diagnosis of amoebic liver abscess. *Tropical Biomedicine*, **30**(2): 250-256.

Tanyuksel, M. & Petri, W.A. (2003). Laboratory diagnosis of amebiasis. *Clinical microbiology reviews*, **16**(4): 713-729.

Thomas, V. & Leng, Y.P. (1986). Immunodiagnosis and seroepidemiology of amoebiasis in Selangor, Malaysia. *Philippine Journal of Microbiology and Infectious Diseases*, **15**: 48-52.

Yang, B., Chen, Y., Wu, L., Xu, L., Tachibana, H. & Cheng, X. (2012). Seroprevalence of *Entamoeba histolytica* infection in China. *The American Journal of Tropical Medicine and Hygiene*, **87**(1): 97-103.