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Detection of human Sarcocystosis using dried blood on filter papers: An Immunofluorescent Antibody Test

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Abstract. Sarcocystosis, a parasitic infection caused by a protozoa belonging to the genus *Sarcocystis*, is found worldwide in both and animals. *Sarcocystis* spp., require two animal hosts to complete their life cycle. The infection has gathered more global attention after recent outbreaks, especially amongst wester travellers to Malaysia. Other than sporadic cases and the current outbreaks, little information is available regarding human *Sarcocystis* infection in Malaysia. The present study aims to determine the prevalence of sarcocystosis among humans using an immunofluorescent antibody (IFA) test applied to dried blood on filter papers. A total of 200 blood samples were collected on filter papers from autopsy cases at two Malaysian hospitals: Sungai Buloh Hospital (peninsular Malaysia) and Queen Elizabeth Hospital (Malaysian Borneo). Antigens were prepared from bradyzoites harvested from positive goats' muscle samples. Of the 200 samples, 32 (16%) had *Sarcocystis* antibodies that showed positive fluorescence reactions on filter papers. There was no significant difference (t-test, p value > 0.05) in prevalence rates between samples collected from autopsies at peninsular Malaysia and Malaysian Borneo. The results demonstrated that the filter paper technique can be used as one of the alternative serological tests in the diagnostic of human sarcocystosis.

INTRODUCTION

Sarcocystosis is a parasitic infection of men and animals with worldwide distribution. It is caused by a protozoan parasite belonging to the genus *Sarcocystis* and requires two different hosts to complete its life cycle. In humans, the infection is recognized in two forms, muscular and intestinal (Fayer *et al.*, 2015). The parasite utilizes humans as the definitive host and various species of animals as intermediate hosts (Dubey, 2015; Fayer *et al.*, 2015).

In Malaysia, human muscular sarcocystosis has been reported since 1975, with most cases found incidentally during autopsy or biopsy (Kutty & Dissanaike, 1975; Tappe *et al.*, 2013). Most medical practitioners do not perform invasive procedures (e.g., muscle biopsies) on patients complaining muscle ache during regular visits. Hence, the true prevalence of the infection of sarcocystosis could be underreported. In addition to that, the symptoms of muscular sarcocystosis are broad, ranging from

asymptomatic to chronic forms of myalgia and myositis (Fayer *et al.*, 2015).

It is believed that sarcocystosis is endemic in Malaysia. Despite some sporadic case reports, little information is available regarding human *Sarcocystis* infection in Malaysia (Wong & Pathmanathan, 1992; Abu Bakar, 2012). Nevertheless, research on animal sarcocystosis focusing on meat producing livestock has been conducted extensively in recent years and showed a high prevalence in goat, cattle, and buffalo (Latif *et al.*, 2013; Kutty *et al.*, 2015; Latif *et al.*, 2015; Ng *et al.*, 2015). As such, transmission of sarcocystosis to human hosts is possible through ingestion of under cooked meat or contaminated water (Fayer *et al.*, 2015).

In 1978, Thomas & Dissanaike conducted the first immunofluorescent antibody (IFA) test to determine the prevalence of sarcocystosis in Malaysia. The study used human serum samples collected from Aborigines, Malays, Indians and Chinese and found that the prevalence rate varied from 3.6% to 39.7% between ethnic groups. To the best of our knowledge, this study has been the only seroprevalence research carried out in Malaysia. However, antibody detection using serum samples is not always feasible under field conditions. However, the use of blood collected on filter papers for later laboratory-based clinical diagnosis in epidemiological studies of other protozoal diseases such as malaria (Corran *et al.*, 2008), leishmaniasis (Al-Alousi *et al.*, 1980) and toxoplasmosis (Bartova *et al.*, 2006) suggests that this approach may be useful in studying sarcocystosis. Furthermore, according to More *et al.* (2008) and Poulsen *et al.* (2014), there is no cross-reactivity between *Sarcocystis* and *Toxoplasma* in IFA test. Therefore, it is reasonable to extrapolate that the likelihood of cross reaction of other parasite antigens with those against *Sarcocystis* spp. is minimal. The aim of this preliminary study is to determine the feasibility of IFA technique relying on dried blood samples collected to filter paper in the detection of *Sarcocystis* spp., and secondly, to use this approach for determining the prevalence of human sarcocystosis from autopsy cases at Sungai Buloh Hospital, Selangor and Queen Elizabeth Hospital, Kota Kinabalu, Sabah.

MATERIALS AND METHODS

Study sites

Samplings were conducted in two sites: Sungai Buloh Hospital, Selangor (located approximately 24 km from the Capital of Malaysia, Kuala Lumpur) and Queen Elizabeth Hospital, Kota Kinabalu, Sabah (Figure 1). These sites were selected to represent both peninsular Malaysia and Malaysian Borneo.

Data collection

Human blood samples were collected on filter papers (No. 4; Whatman, USA) between September 2015 to March 2016 (six-month duration) during post-mortem examinations. Causes of death varied, but automobile accidents and heart-related diseases were predominant. Blood samples were allowed to dry, placed in self-sealing bags containing silica gels, and stored at -20°C until further processing. Blood samples from a healthy person were collected and served as negative control. All samples were transported to and stored at the Institute of Medical Molecular Biotechnology (IMMB), Universiti Teknologi MARA for laboratory analysis.

Antigen preparation

Antigens were prepared from *Sarcocystis* bradyzoites collected from the muscles of infected goats (*Capra aegragrushircus* (L.)) slaughtered at Shah Alam, Selangor (Figure 1). All positive samples (those containing a microsarcocyst) were crushed/homogenized to extract the bradyzoites (Al-Alousi *et al.*, 1980). This homogenate was centrifuged (Haraeus Biofuge Pico, UK) at 1886xg for 10 minutes and the resulting supernatant discarded. The remains were mixed and 1.0 µl aliquots of bradyzoites transferred to circle slides (Thermomenzel, Germany). After an hour of air drying, bradyzoite samples were fixed with absolute methanol and washed twice in phosphate buffered saline (PBS) for

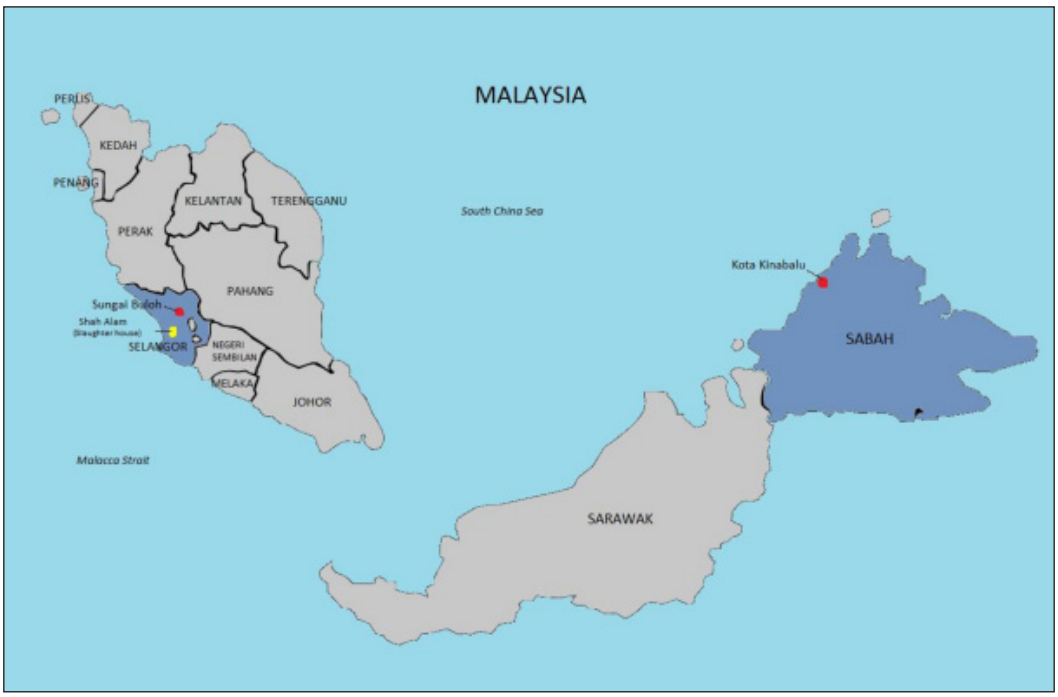

Figure 1. Map showed the locations (red dots) where dried blood samples from autopsy cases were collected. Sungai Buloh Hospital (Left) is located in Selangor, peninsular Malaysia and Queen Elizabeth Hospital (Right) is located in Sabah, Malaysian Borneo. Yellow dot denotes the slaughter house location (Shah Alam, Selangor) where parasite specimens were obtained from infected goats for antigen preparation.

five minutes each. The antigen slides were allowed to air-dry, then wrapped with paper towel and aluminium foil, placed in a self-sealing plastic bag containing silica gel, and stored at -20°C until further use.

Immunofluorescent Antibody Test

The immunofluorescent antibody test was performed following the method used in a previous study on leishmaniasis (Al-Alousi *et al.*, 1980). A 6 mm of the blood spot was punched out of the filter paper. Preliminary work showed that a 6 mm disc in 50 µl PBS solution was equivalent to approximately 1:50 diluted serum (Al-Alousi *et al.*, 1980). A total of 15µl of PBS solution and the discs were transferred to the antigen-containing slide and incubated under moist conditions for one hour at room temperature. The filter paper discs were removed with a jet of PBS and the slides washed twice five minutes each, with PBS solution. Flourescein-isothiocyanate (FITC) conjugated rabbit antihuman immunoglobulin (Abcam, USA) diluted 1:10 in PBS was used as a conjugate and incubated for one hour under high-humidity conditions at room temperature. The slides were mounted in Pro-Long Gold anti-fade reagent (Life Technologies, USA) and examined under a fluorescent microscope (Olympus BX61, Japan) using an FITC filter (excitation 490 nm, emission 525 nm) at x100 magnification. Fluorescent results at 1:40 or greater dilutions were considered positive as baseline titre.

Statistical analysis

Microsoft Excel (2007) was used as a statistical tool in data analysis. Student t-test was employed to compare the results between prevalence of human sarcocystosis from autopsy cases at the Sungai Buloh Hospital, Selangor and Queen Elizabeth Hospital, Kota Kinabalu, Sabah.

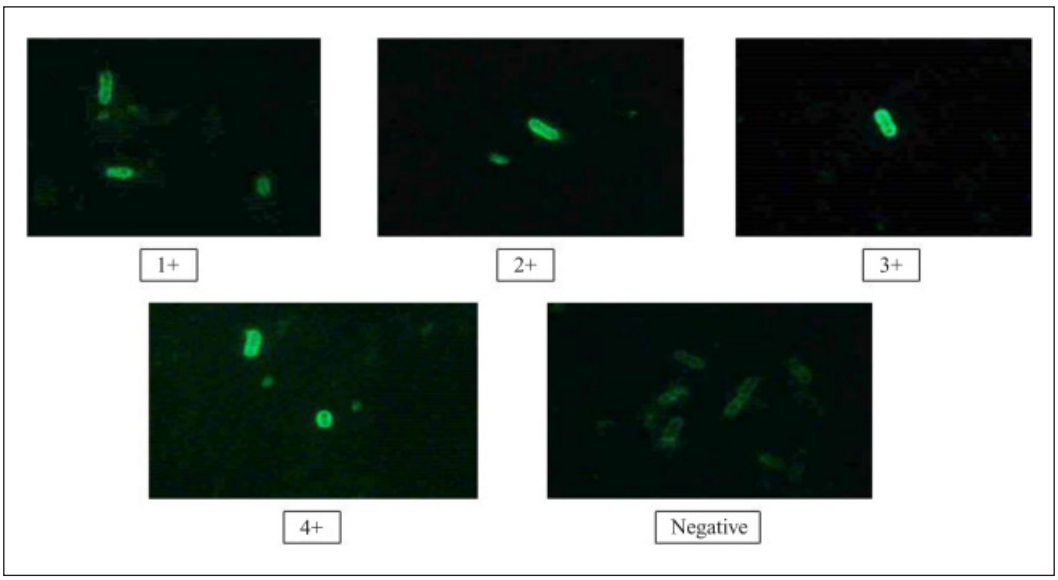

Figure 2. Degree of fluorescence reaction.

Table 1. Results of IFA tests on the prevalence of *Sarcocystis* spp. antibody using dried blood samples on filter papers collected from autopsy cases according to hospitals, nationalities, and ethnicity

	Sg. Buloh Hospital		Queen Elizabeth Hospital		Total no. of positive samples (%)
	No. tested	No. positive (%)	No. tested	No. positive (%)	
Malaysian					
Malay	22	7 (31.8)	72	9 (12.5)	16 (17.0)
Chinese	13	1(7.7)	17	4 (23.5)	5 (16.7)
Indian	25	3 (12.0)	1	0 (0.0)	3 (11.5)
Subtotal	60	11 (18.3)	90	13 (14.4)	24 (16.0)
Foreign Nationals					
Indonesian	15	2 (13.3)	9	1 (14.3)	3 (12.5)
Bangladeshi	13	2 (15.4)	–	–	2 (15.4)
Myanmar	4	2 (50.0)	–	–	2 (50.0)
Nepalese	4	1 (25.0)	–	–	1(25.0)
Other	4	0 (0.0)	1	0 (0.0)	0 (0.0)
Subtotal	40	7 (17.5)	10	1 (10.0)	8 (16.0)
Total	100	18(18.0)	100	14 (14.0)	32 (16.0)

RESULTS

A total of 200 blood spots on filter papers: 100 from Sungai Buloh Hospital and 100 from Queen Elizabeth Hospital, were collected respectively for this study. Amongst these samples, 32 (16%) had *Sarcocystis* antibodies and showed positive fluorescent reactions (Figure 2). In the present work, there was no significant difference (t-test, p value > 0.05) in prevalence rates between samples collected from autopsies at peninsular Malaysia and Malaysian Borneo. Based on the highest prevalence rate of *Sarcocystis* infections among Malaysians were 17% (Malay) followed by 16.7% (Chinese) and 11.5% (Indian) (Table 1).

DISCUSSION

The present study demonstrated that 16% of the Malaysian population from the two hospitals were putatively positive for *Sarcocystis*' antibodies regardless of race. The prevalence is comparable between peninsular Malaysia and Malaysian Borneo as represented by the Sungai Buloh and Queen Elizabeth hospitals, with 18% and 14% respectively. Furthermore, prevalence rates appear to be equivalent between Malaysian citizens and foreign nationals from other Asian countries. The last known seroprevalence study of *Sarcocystis* antibodies in Malaysian citizens was conducted in 1978 (Thomas & Dissanaike, 1978). The present work is also the first antibody test using dried blood on filter paper as an alternative detection tool for sarcocystosis.

This study showed that dried blood on filter paper for indirect fluorescent antibody testing is practically feasible for the diagnosis of sarcocystosis. The technique is relatively simple and would prove useful for epidemiological studies which usually involve a huge number of samples. It is easy to perform through collection of a drop of blood from a patient on filter paper and without the difficulties of sera collection by venepuncture.

Additionally, these types of samples (dried blood on filter paper) can be kept at different temperatures without affecting the quality of the immunoglobulin. Latif (1972) stated that globulins in dried blood on filter papers retained their original activity for 30 days at room temperature (18-20°C) and 60 days at -20°C.

CONCLUSION

Sarcocystosis is a neglected parasitic disease that has only recently begun to receive attention globally. In this study, we demonstrated that an IFA test using dried blood on filter paper is possible and could be used for screening or epidemiological studies of human sarcocystosis. We have also shown that this approach can determine the prevalence of human sarcocystosis from autopsy cases at the Sungai Buloh Hospital, Selangor and Queen Elizabeth Hospital, Kota Kinabalu, Sabah.

Abbreviations

IFA: immunofluorescent antibody; IMMB: Institute of Medical Molecular Biotechnology; MARA: Majlis Amanah Rakyat; PBS: phosphate buffered saline; FITC: Flourescein-isothiocyanate.

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Competing Interests

The authors declare that they have no competing interests.

Ethics and consent to participate

The study was approved by the Ethical Committee of the Institute of Research Management & Innovation (iRMI), Universiti Teknologi MARA on 22 June 2015 (reference number: 600-RMI (5/1/6). Consent from the family was obtained prior to blood collection from all autopsy cases.

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