DETECTION OF ANTI-GIARDIA IgG IN SELECTED ORANG ASLI SERUM SAMPLES

ABDOULIE M. SANYANG

UNIVERSITI SAINS MALAYSIA

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by

ABDOULIE M. SANYANG

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	Approximately
%	Percentage
>	More than
°C	Degree Celsius
µg	Microgram
µL	Microliter
CBB	Coomassie brilliant blue
cm	Centimetre
mm	Millimetre
CSA	Crude soluble antigen
dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay
et al.	*et alii* – 'and others'
× *g*	multiply by gravitational force
g	Gram
IgG	Immunoglobulin G
IHA	Indirect haemagglutination assay
kDa	Kilodalton
L	Litre
mA	Milliampere
mL	Millilitre
mM	Millimolar
NaCl	Sodium chloride

NaOH	Sodium hydroxide
OD_{450nm}	Optical density at wavelength of 450 nm
TBS	Tris-buffered saline
TBST	TBS-Tween 20
PBS	Phosphate buffered saline
PBST	PBS-Tween 20
s	Second
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
TMB	3,3’,5,5’-tetramethylbenzidine
rpm	Revolutions per minute
LB	Luria Bertani
NDI	Neglected diseases initiative
WHO	World Health Organization

PENGESANAN ANTIBODI ANTI-GIARDIA IgG DALAM SAMPEL SERUM TERPILIH ORANG ASLI

ABSTRAK

Giardiasis ialah penyakit parasit tularan air yang disebabkan oleh protozoa enterik, *Giardia lamblia*. Penyakit ini berleluasa di penempatan Orang Asli disebabkan faktor-faktor seperti sistem kumbahan yang kurang baik, amalan kebersihan yang kurang memuaskan dan bekalan air yang tidak bersih. Individu yang dijangkiti kebanyakannya kekal sebagai pembawa penyakit tanpa tanda klinikal, tetapi penyakit itu boleh menyebabkan tanda usus klinikal yang ringan, sederhana atau teruk. Untuk pengawalan penyakit giardiasis yang lebih baik, data epidemiologi yang mencerminkan populasi yang dijangkiti penyakit ini memainkan peranan penting. Data epidemiologi giardiasis dalam kalangan Orang Asli yang sedia ada hanya untuk jangkitan aktif yang dikaji pada suatu masa melalui reka bentuk kajian keratan rentas. Data yang dilaporkan mungkin lebih rendah daripada nilai sebenar populasi yang dalam risiko mendapat jangkitan ini. Oleh itu, kajian ini bertujuan untuk mengesan anti-*G. lamblia* IgG dalam sampel serum terpilih Orang Asli melalui CSA-IgG-ELISA buatan dalaman. Sejumlah 150 sampel serum arkib telah digunakan dalam kajian ini. Antigen larut kasar telah disediakan daripada *G. lamblia* yang dikultur dan digunakan untuk pembangunan CSA-IgG-ELISA buatan dalaman. ELISA buatan dalaman ini dijalankan dengan antigen salutan sebanyak 10 μg/mL, pencairan serum manusia pada 1:50 dan pencairan antibodi sekunder yang dikonjugasi HRP pada 1: 6000. Analisis kajian menunjukkan bahawa 68.7% sampel kajian menunjukkan bacaan ELISA OD_{450nm} sekurang-kurangnya 1.000; keputusan ini ialah sekurang-kurangnya tiga kali ganda daripada keputusan kajian-kajian lain yang dilaporkan

sebelum ini. Analisis juga menunjukkan bahawa tiada perhubungan antara pembacaan ELISA OD_{450nm} dan pembolehubah demografi subjek kajian iaitu umur dan jantina. Selain itu, tidak terdapat perbezaan yang ketara antara bacaan ELISA OD_{450nm} sampel serum positif dan negatif RT-PCR. Secara kesimpulan, seroprevalence anti-giardia IgG mungkin boleh menjadi petunjuk yang lebih baik untuk menganggarkan populasi yang terdedah kepada giardiasis.

DETECTION OF ANTI-GIARDIA IgG IN SELECTED ORANG ASLI SERUM SAMPLES

ABSTRACT

Giardiasis is a water-borne parasitic disease caused by the enteric protozoan, *Giardia lamblia*. The disease is prevalent in Orang Asli settlements due to the risk factors such as substandard sanitary system, poor hygiene practice and unclean water supply. Individuals harbouring the parasite mostly remain as asymptomatic carriers, but the disease may progress to mild, moderate or severe clinical intestinal symptoms. To better control the disease, epidemiological data reflecting on the range of affected population plays a pivotal role. The currently available epidemiological data of giardiasis in Orang Asli inferred only to active infection at a single timepoint via cross-sectional study design. The reported data might be underestimating the population at risk or exposed to giardiasis. Hence, the present study aimed to detect the presence of anti-*G. lamblia* IgG in selected Orang Asli serum samples via in-house CSA-IgG-ELISA. A total of 150 archived serum samples were used in the present study. Crude soluble antigen was prepared from axenically grown *G. lamblia* and used for development of in-house CSA-IgG-ELISA. The in-house ELISA was set up with coating antigen of 10 µg/mL, serum dilution of 1:50 and HRP-conjugated secondary antibody of 1:6000. From the analysis, 68.7% showed ELISA optical density (OD) at 450nm readings of at least 1.000; it was at least three-fold of previously reported cases by PCR or microscopy. There were no associations between ELISA OD_{450nm} readings and subjects' demographic variables i.e. age and genders. Similarly, there were no significant difference found between ELISA OD_{450nm} readings of RT-PCR positive and negative serum samples. The finding

suggested seroprevalence of anti-giardia IgG could be a better indicator for estimating population exposed to giardiasis.

CHAPTER 1

INTRODUCTION

1.1 *Giardia lamblia*

Giardia lamblia, also known as *Giardia intestinalis*, is a flagellate enteric protozoan that causes giardiasis. The disease affects over 280 million people each year (Choy et al., 2014). The use of advanced molecular techniques has revealed that *G. lamblia* has eight different "assemblages" assigned as A-H, which look similar morphologically but different phenotypically and genotypically (Ryan and Cacciò, 2013; Heyworth, 2016). According to a reviewed journal by Choy et al. (2014), assemblages C and D infect dogs, assemblage F infects cats, assemblage E infects hoofed livestock, assemblage G infects rats, and assemblage H infects marine animals. Only assemblages A and B are associated with human infection (Ryan and Cacciò, 2013; Choy, 2016). Feng and Xiao (2011), Ryan and Caccio (2013) and Choy et al. (2014) re-affirm that only assemblages A and B are known to be associated with human infection; and assemblage B is more prevalent than assemblage A worldwide. Moreover, a higher proportion of assemblage B was observed over A in developing countries with a slightly higher proportion in developed countries. Choy et al (2014) have reported a variety of *Giardia* species based on their morphology and structure in mammals, amphibians, and birds to be *G. duodenalis, G. agilis, G. muris, G. ardeae, G. microti and G. psittaci.*

1.1.1 Life Cycle of *Giardia lamblia*

The life cycle of *G. lamblia* includes the dormant cysts and the active multiplying trophozoites (Ortega and Adam, 1997). Both cysts and trophozoites can be found in faeces of the carrier. The cyst is the infective stage and it can survive in water up to

months. Humans get infected upon the ingestion of cysts from contaminated food or water. The cyst is resistant to environmental stress such as gastric acid (Farthing, 1996). The cysts pass through the stomach and undergo excystation to form trophozoites in the duodenum and jejunum of the gastro-intestinal tract (one cyst could lead to the production of two trophozoites). Trophozoites are motile and feed on glucose and nutrient in the intestine (Ortega and Adam, 1997). The trophozoites can stay silently in the intestine as asymptomatic infection, or cause invasive giardiasis such as diarrhoea, constipation, headache, nausea and abdominal pain. It hatches from the cyst and adheres to the small intestine where it induces epithelial inflammation, villous flattening and diarrheoa due to malabsorption (Farthing, 1996; Buret, 2008; Cotton et al., 2011). Trophozoite encystation takes place in the large intestine to form new cysts that are shed into faeces thereby contaminate the environment. Cysts are hardy, resistant and can remain viable in faeces in different environments such as chlorinated water and lower temperatures. Its variability can range from 28 to 84 days in lake or river water but reduce in soil and cattle slurry (Cole et al., 1989; Olson et al., 1999; Grit et al., 2012). Each cyst transforms into four trophozoites through a process called excystation in the small intestine. The trophozoites are only present in watery stools while the cysts are found in nondiarrheal stools. The trophozoites multiply by binary fission to form into cysts. The thick wall of the cyst serves as a protective cover for the parasite against adverse conditions when passed into the faeces and facilitate the mode of disease transmission. Notwithstanding, the lack of trophozoite walls renders them easily killed when excreted into the stool. Trophozoites would hardly survive the gastric environment in the stomach. For this reason, the trophozoites mostly remain restricted to the intestinal lumen. It is evidently documented that some patients may

not manifest any symptoms of giardiasis and hence become asymptomatic carriers who pass cysts in their stools (Choy et al., 2014). As indicated by several studies, WHO has established that transmission of giardiasis occurs via the faecal–oral route; by eating or drinking cysts-contaminated food or water or directly by person-to-person contact (Escobedo et al., 2014).

1.1.2 Morphology

The life cycle of *G. lamblia* has two phases; the infective stage called the cyst and the feeding and active dividing stage known as the trophozoites. The trophozoites are about 10 to 20 µm long, 5 to 8 µm wide and 2 to 4 µm thick (Figure 1.1). The anterior end of the trophozoites is pear-shaped and tapered posterior end with a convex dorsal surface and concave ventral surface. Microscopically, it is easy to be recognized owing to its external features. The organism is bilaterally symmetrical with a sucking disc on the ventral side (Figure 1.2). The trophozoite has four pairs of flagella namely, anterior, posterior, ventral and caudal. The cysts are usually ovoid to spheroidal measuring 8 to 10µm by 7 to 8µm. The cyst has 4 nuclei when mature with different remnants of the organelles of the trophozoite. It has been found that the sucking disc occupies the entire region of the ventral portion of the anterior. The ventral disc is known to be organelle of attachment. The movement of the parasite is seemly supported by all flagella but when it becomes attached, only the ventral flagella move while others remain inactive.

The cytoplasm of mature cysts of *G. lamblia* usually has irregularly shaped glycogen and when stained with Lugol's iodine stain dark or orange-brown. The visibility of these structures is enhanced by imparting iodine or Trichrome dyes

(Gömöri-Wheatley technique). Figure 1.3 shows the cyst of *G. lamblia* (Technology, 2015).

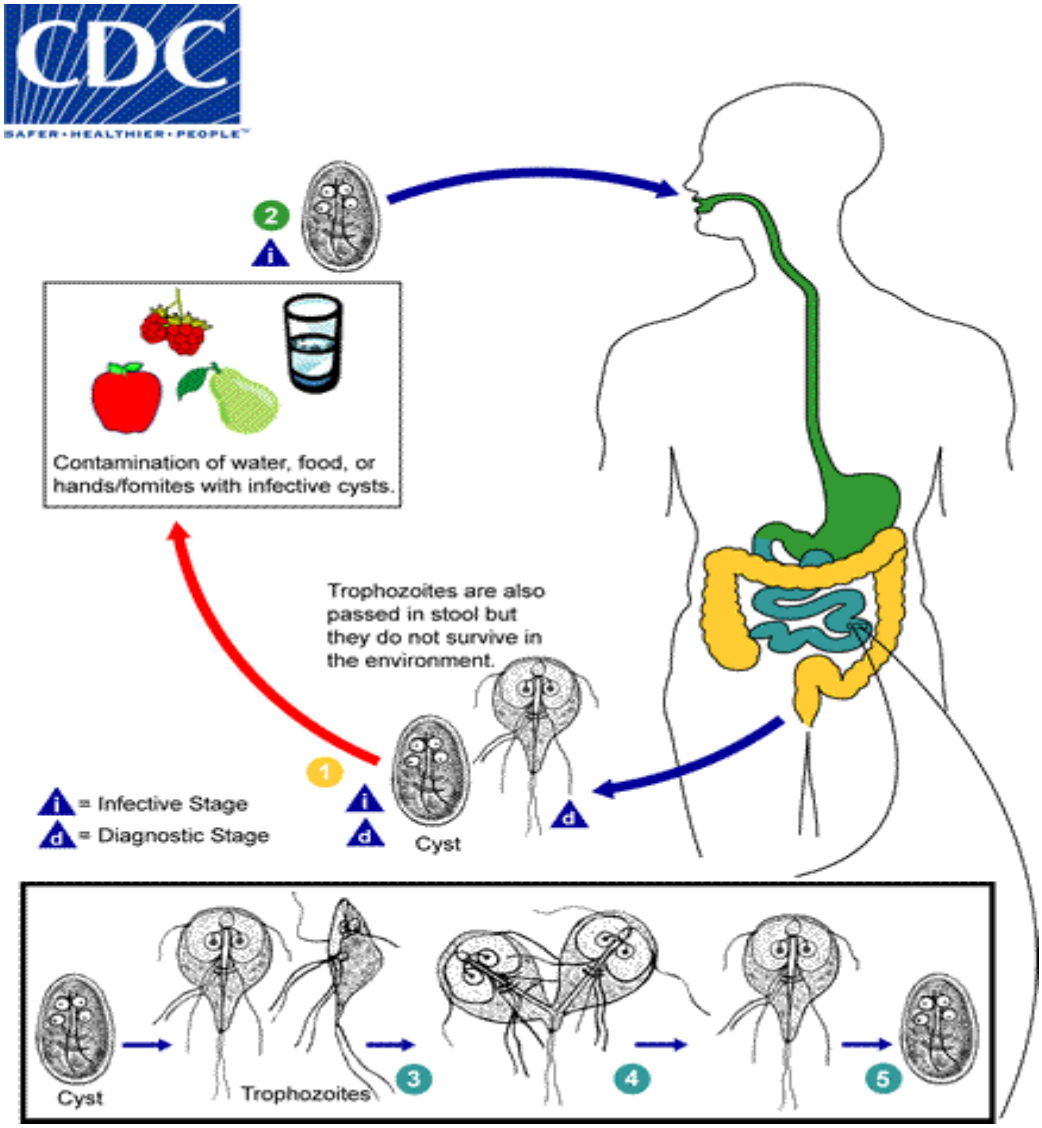
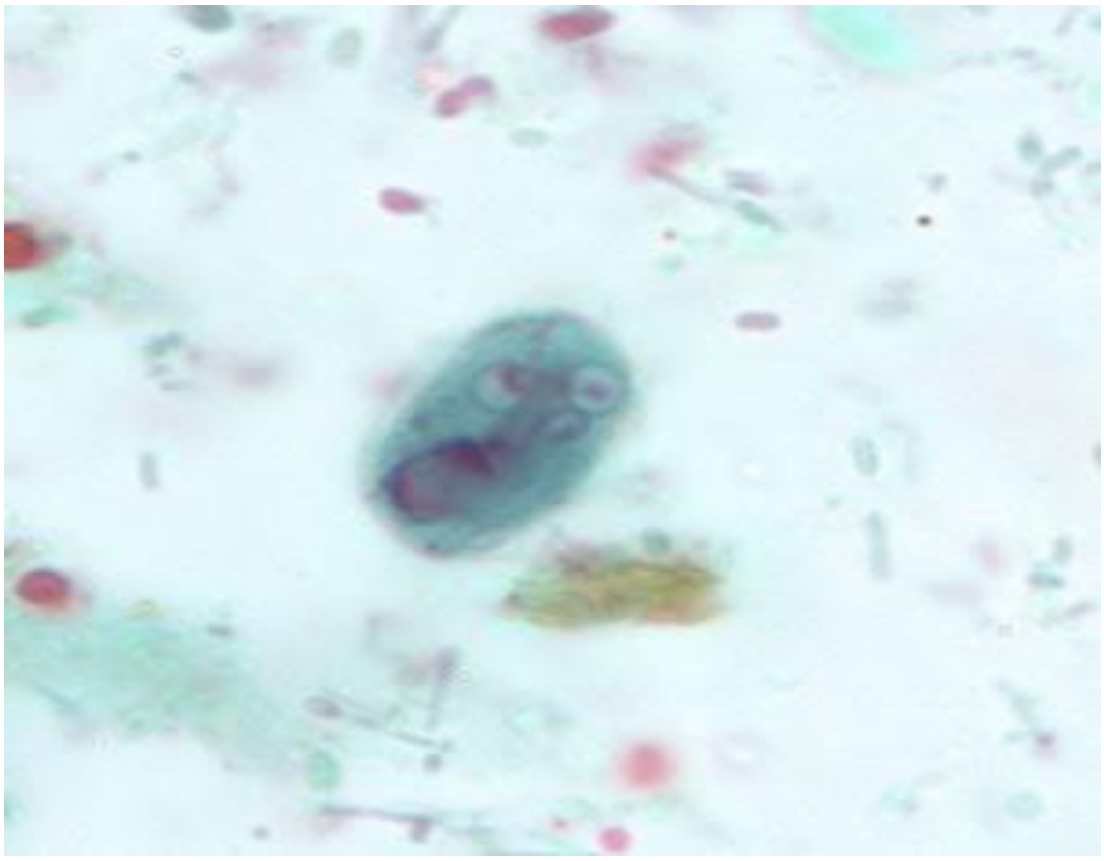
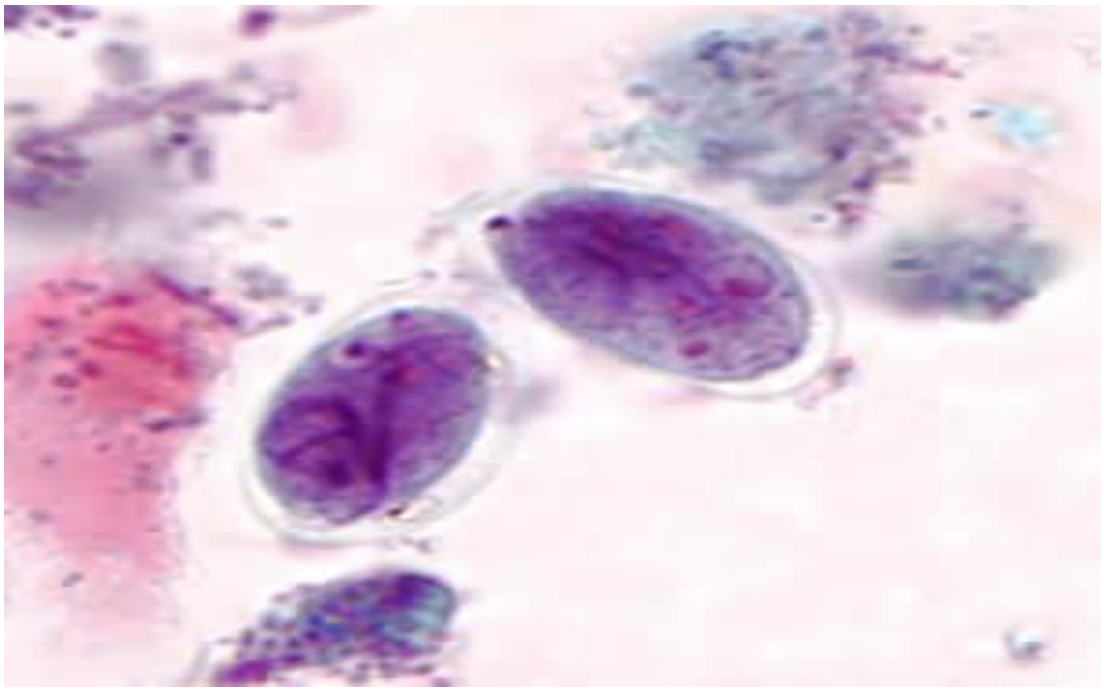


Figure 1.1 Life cycle of *G. lamblia* (CDC, 2017)



Source: Adopted from Technology (2015)

Figure 1.2 *G. lamblia* trophozoite in a trichrome-stained stool smear



Source: Adopted from Technology (2015)

Figure 1.3 Cyst stage of *G. lamblia* in a trichrome-stained stool smear

1.1.3 Axenic culture of *G. lamblia* trophozoites

G. lamblia was initially described by the Dutch Len-Maker Antonie Van Leeuwenhoek in 1681 from his own stool specimen but the establishment of the genus was later done in the 1990s (Ford, 2005). His description of the *Giardia* species was followed by many other researchers attempted to properly understand the species biology and its method of cultivation. Axenic culture went through phases of the evolutionary process. Karapetyan was the first to grow *Giardia in vitro* in 1960. Meyes was the first person to cultivate *Giardia* in an axenic medium. Later, Diamond noticed that the medium used for cultivation of *Entamoeba histolytica* (TYI-S-33) could also enhance the cultivation of *Giardia* trophozoites (Diamond et al., 1978; Davids and Gillin, 2011a). The modification of TYI-S-33 medium to better suit the cultivation of *Giardia* was done by Keister (Davids and Gillin, 2011b). The axenic cultivation technique was later adapted in the Hospital de Especialidades, Instituto Mexicano del Seguro Social, Mexico City.

1.2 Epidemiology of Giardiasis

1.2.1 Geographical Distribution of Giardiasis

Worldwide, giardiasis is known to be caused by a flagellated protozoan and it is reported to be the leading but treatable cause of infectious gastroenteritis. Its prevalence was reported to be 4-43% and 1-7% in high income and low-income countries respectively (Fletcher et al., 2012; Waldram et al., 2017). In 1981, the World Health Organization (WHO) added *Giardia* to its list of parasitic parasites (Committee, 1981). The WHO listed *Giardia* to the Neglected Diseases Initiative (NDIs) for surveillance because of the disease burden and association with poverty (Savioli et al., 2006). It has been found out to be a major cause of acute and chronic

diarrheoa, particularly among the children in underprivileged communities in developing countries with a prevalence between 10% and 50% (Savioli et al., 2006; Choy et al., 2014). Studies revealed that traveling to low-income settings was usually a risk factor, with countries in the Caribbean, North Africa, South Asia, and Southeast Asia were indicated the highest risk areas (Swaminathan et al., 2009; Ross et al., 2013). Hill et al. (2001) revealed that *Giardia* infection can be either asymptomatic or symptomatic with an estimated 5-15% among infected persons while known symptoms include diarrhoea, flatulence, abdominal pain and bloating. Globally, it is reported that *Giardia* infection greatly contributes to 2.5 million yearly deaths of diarrheal diseases (Adam, 2001). Travellers returning to the United Kingdom (UK) with gastrointestinal disorders, *Giardia* is the most frequently seen intestinal parasite (Swaminathan et al., 2009; Ross et al., 2013) ; and a high rate of giardiasis endemicity was reported in Guatemala, Peru, Nigeria and Bangladesh (Cedillo-Rivera et al., 2009).

1.2.2 Distribution of Giardiasis in Malaysia

The prevalence of *Giardia* infection among indigenous communities in rural Malaysia was found to be 11.6% (Choy et al., 2014) and age group ≤12 years old were most affected. Similar studies revealed the prevalence of *Giardia* infection among Orang Asli people living in Peninsular Malaysia (West Malaysia) was reported to be 29.2% (Bisseru and Ahmad, 1970; Anuar et al., 2012). According to Choy et al. (2016) several studies on the prevalence of *Giardia* infection among Orang Asli communities carried out between 1970s and 1990s revealed a prevalence rate from 4.8% - 25.0%. Additionally, the same review indicated the prevalence of *Giardia* infection among Orang Asli to be high at 4.0% - 29.2% for studies

conducted in a span of ten years period (2000 – 2010). Choy et at. (2014) revealed that the burden of the disease is higher in West Malaysia when compared to East Malaysia. This can be attributed to the poor sanitation and environmental conditions in Peninsular Malaysia (West Malaysia). Overall, the prevalence of *Giardia* infection rate among the general population is relatively low 3-9% (Choy et al., 2014). Furthermore, Choy et al. (2014) further revealed that from 1970s-1990s four study findings showed infection rate of >10% while two studies from the 2000s beyond indicated prevalence rates of 5.7% and 10.4%, respectively.

1.3 Pathogenesis of Giardiasis

It has been evidently proven that *G. lamblia* can be found mainly in places with poor sanitation and water systems contaminated with cysts of *G. lamblia* (World Health Organization 2016). Infection is initiated when the infective stage of the parasite, the cysts are ingested and subsequently excyst in response to the new environmental stimuli of the stomach**.** The low pH of the stomach is one such important signal. One cyst will give rise to two trophozoites during the process of excystation. These trophozoites have a concave ventral surface and a ventral disc to adhere to the epithelial surface of the small intestine. The trophozoites replicate by binary fission. For the trophozoites to evade the new hostile environment, they must transform into cysts (encystation) and exit the host to survive. These cysts can survive in unfavourable conditions for long periods of time if they do not dry out or freeze. Studies have revealed that the encysted parasites are resistant to chlorinated water (Painter et al., 2015). The trophozoites from the cysts attach to the small intestine and trigger epithelial inflammation, villous flattening and diarrhoea ensue due to malabsorption. Proceeding to the large intestine, the trophozoites change to new

cysts that are shed in faeces and can cause environmental contamination. The attachment of the parasite to the mucosal surface of the small intestine villi causes changes in the microvilli borders and affects its normal activity. This can lead to competition for essential nutrients, mechanical blockage and impairment of the mucosa, which will consequently lead to malabsorption (Brandborg et al., 1967; Morecki and Parker, 1967; Painter et al., 2015). Risk factors such as poverty, illiteracy, poor sanitation and water treatment systems (Faustini et al., 2006), have been identified as potential risk factors. These risk factors can negatively affect the cognitive development of children and contribute to iron deficiency anaemia, zinc deficiency, vitamin A and B deficiency, protein-energy malnutrition perhaps due to malabsorption of carbohydrate (Berkman et al., 2002; Al-Mekhlafi et al., 2010; Ignatius et al., 2012; Al-Mekhlafi et al., 2013; Halliez and Buret, 2013b)

1.4 Host Immunity

G. lamblia trophozoite is an anaerobic flagellated parasitic protozoan and the presence of high content of oxygen such as the reactive oxygen species (ROS) and nitric oxide (NO) can kill them in the host environment. NO has numerous and complex biological uses and it is synthesized from L-arginine by the enzyme NO synthase (NOS). It is an essential mediator of homeostasis and change in its actions can contribute to pathological states. Eosinophils are known to be specific to parasitic infections and are the first innate cellular immune response to giardiasis invasion across the small intestine. As a result of their interaction with *G. lamblia* trophozoites, they become activated and release ROS, which is toxic to the trophozoites thereby resulting in their killing. In addition to eosinophils, activated macrophages produce NO to kill trophozoites when they are stimulated by S-nitroso-

acetyl-penicillamine (SNAP) and sodium nitroprusside (SNP) in a dose-dependent manner (Fernandes and Assreuy, 1997). Macrophages have been shown to ingest and kill *Giardia* trophozoites *in vitro*. In innate immunity, the disease is naturally taking care of at the mucosal sites and the mediated immunity by the circulating antibodies in serum. *G. lamblia* trophozoites are also killed by the products of lipolysis present in human duodenal and upper jejunal (Halliez and Buret, 2013a).

1.5 Treatment of Giardiasis

The treatment of giardiasis can utilize four main classes of agents of the nitroimidazoles which includes metronidazole, tinidazole, paromomycin, and ornidazole (Table 1.1). Metronidazole (1-(hydroxyethyl)-2-methyl-5-nitroimidazole) effectiveness in treating giardiasis is well established (Darbon et al., 1962; Minetti et al., 2016). It remains to be the mainstay drug in treating giardiasis. The mechanism of killing of *Giardia* by metronidazole is that it utilizes the anaerobic metabolic pathways existing in *Giardia.* Once the drug is taken, it enters the cell wall of the dividing and motile trophozoites, then the electron transport of protein ferredoxins from the parasite donate electrons to the nitro group of the drug. Consequently, the drug becomes activated by reduction of this nitro group. The reduction reaction is established by the gradient supporting the intracellular transport of metronidazole. Reduced metronidazole serves as a terminal electron acceptor, which binds covalently to DNA macromolecules (Müller, 1983; Edwards, 1993). According to Gillis and Wiseman (1996), the reduction of products, which are toxic intermediates, exert their killing effects on the cell by reacting and damaging intracellular DNA macromolecules. This binding effect of the drug to the DNA damage molecule structure affects its roles leading to subsequent trophozoites death. Additionally, metronidazole inhibits trophozoite

Table 1.1 The recommended dosing and adverse effects of anti-Giardia drugs

Drug	Adult dose[f]	Pediatric dose	Adverse effects
Metronidazole[a]	250 mg t.i.d. × 5–7 days	5 mg/kg t.i.d. × 5–7 days	Headache, vertigo, nausea, metallic taste, urticaria Disulfiram-like reaction with alcohol ingestion Rare: pancreatitis, central nervous system toxicity, reversible neutropenia, peripheral neutropathy, T-wave flattening with prolonged use Mutagenic/carcinogenic?
Tinidazole[b]	2 g, single dose	50 mg/kg, single dose (max, 2 g)	As for metronidazole
Ornidazole[c]	2 g, single dose	40–50 mg/kg, single dose (max, 2 g)	As for metronidazole
Quinacrine[c]	100 mg t.i.d. × 5–7 days	2 mg/kg t.i.d. × 7 days	Nausea and vomiting, dizziness, headache Yellow/orange discoloration of skin and mucous membranes Rare: toxic psychosis
Furazolidone[d]	100 mg q.i.d. × 7–10 days	2 mg/kg q.i.d. × 10 days	Nausea, vomiting, diarrhea Brown discoloration of urine; disulfiram-like reaction with alcohol ingestion Reacts unfavorably with MAO inhibitors Mild hemolysis in G6PDH deficiency Carcinogenic?
Paromomycin[a]	500 mg t.i.d. × 5–10 days	30 mg/kg/day in 3 doses × 5–10 days	Ototoxicity and nephrotoxicity with systemic administration
Albendazole[a]	400 mg q.d. × 5 days	15 mg/kg/day × 5–7 days (max, 400 mg)	Anorexia, constipation Rare: reversible neutropenia and elevated liver function tests Teratogenic?
Bacitracin zinc[e]	120,000 U b.i.d. × 10 days	Not tested in children under 10 yr	Nausea, vomiting, abdominal discomfort Nephrotoxicity with systemic absorption

Notes: Adopted from Gardner & Hill (2001)

respiration thereby leading to parasite death. Also, the reductive activation of the drug may lead to toxic radicals, which react with essential cellular components leading to the extermination of trophozoites (Upcroft and Upcroft, 1998). One advantage of the metronidazole is that it is quickly and completely absorbed after oral administration by the body. The second most used and recommended drug of choice for the treatment of giardiasis is tinidazole. Like metronidazole, it is reported to have the same mechanism of action in treating giardiasis. It has also been evidently documented in many studies that a single-dose of tinidazole is more effective in treating giardiasis when compared to the same dose of metronidazole (Speelman, 1985; Gardner and Hill, 2001). The third antiparasitic drug used in treating giardiasis is known as paromomycin ; the blocking of the peptide synthesis at the level of the ribosome is its main mechanism of action (Murray et al., 2013). The fourth drug of choice is ornidazole whose therapeutic efficacy is similar to tinidazole mechanism of action against giardiasis but is not yet well documented (Gardner and Hill, 2001)

1.6 Prevention and Control of Giardiasis

Generally, in public health, the art of disease prevention is preferred for treatment. To eliminate the disease and reduce its health effects on the population, methods such as improved sanitation in resource-poor areas through maintaining enough water supplies and maintaining vigilance in personal hygiene are crucial. Besides, consistent hand washing, boiling water, thoroughly washing of fruits and vegetables and cooking food are all crucially important to break the chain of transmission (Masters, 2016). Although there is no known vaccine available for giardiasis yet

there exist prevention and control measures that if strictly followed can greatly help in eliminating the giardiasis scourge.

1.7 Diagnosis of Giardiasis

1.7.1 Laboratory Diagnosis

To diagnose giardiasis, the following laboratory tests are applicable currently such as microscopy, serology tests, culture, antigen detection, biopsy, ultrasound, and polymerase chain reaction. The trophozoites are usually seen in loose stool samples while the cysts are usually seen in formed samples. Usually, stool samples for three consecutive days are recommended for collection and sent to the laboratory for direct faecal smears examination using physiological saline and Lugol's or methylene blue is added to aid identification of trophozoites or cysts. It is recommended to collect three different samples on separate days because the cysts are usually shed intermittently. Therefore, collecting stool samples on different days will increase the chances of detecting the trophozoites or cysts commonly known as ova and parasites (OP) examination (Oberhuber et al., 1997). It has been reported that one stool sample will allow the detection of 60-80% of the infections, two samples 80-90% and three samples 90-100% (Goka et al., 1990; Hiatt et al., 1995). Polyvinyl alcohol (PVA) and 10% formalin should be used to preserve the samples to maintain their integrity and whenever it is to be tested at a later time, using methods such as trichrome and merthiolate-iodine-formalin concentrations (MIFC). However, direct wet mount smear preparation on fresh stool samples are commonly performed in many laboratories to save cost and time, but the sensitivity is reportedly low. Therefore, concentration techniques such as MIFC and permanent smears stained with Wheatley's trichrome methods can be employed on negative samples to minimise the likelihood of reporting the false negative result. Research has revealed that trichrome

and MIFC are the best identification methods for cysts of *G. lamblia*. However, trichrome is a superior method for identification of trophozoites. In numerous cases, live trophozoites could not be detected via concentration technique, as many will deteriorate during the process (Salleh et al., 2012).

1.7.2 Stool Microscopy

Slide microscopy remains to be the method of choice for diagnosing giardiasis in most laboratory settings, especially in developing countries to confirm or refute the clinician's suspicion. Because of this, there is a great need to have well-trained microscopist in the laboratory to perform this procedure. Poor correlation of patients infected with G*iardia* and the development of symptomatic giardiasis have been reported as one of the challenges with about 90% of infected persons present as asymptomatic carriers (Nazer, 2018). Owing to this challenge, under-diagnosis of the diseases is often reported by clinicians among patients and hence patients are rarely sent to the laboratory for confirmation in most cases, except if the person has an epidemiological link to the disease endemic or tropical areas. Another challenge is the poor sensitivity of laboratory methods, poorly-trained personnel, supervision and low laboratory proficiency (Flanagan, 1992; Cheesbrough, 2006). Despite the highlighted challenges above, slide microscopy of stool samples remains routinely practiced in numerous clinical parasitology laboratories across the globe to diagnose giardiasis. One advantage of this technique despite the availability of advanced diagnostic techniques is that it remains to be the only technique that provides the physical presentation of the parasite, which further indicates the active infection of the disease. It is a simple technique for an experienced microscopist to microscopically differentiate between trophozoite and cysts of *Giardia* especially

when staining methods are used such as methylene blue, Lugol's iodine, Wheatley's trichrome as seen in Figures 1.4, 1.5 and 1. 6. Trophozoites of *G. lamblia* has a pyriform shape; its cyst is ovoid (Table 1.2).

Slide microscopy of stool specimens can be performed directly by wet preparation or later after staining of the smeared slide. If the specimen(s) is/are to be examined later after the sample has been processing, then they need to be well-preserved to ensure the integrity of the material to remain intact to enhance easy detection, identification, and speciation. For direct smear microscopy, which looks for stool appearance (colour), consistency watery, semi-formed or formed; the presence of blood, mucus, or pus; the presence of an adult worm (*Ascaris lumbricoides*). Watery specimens should be processed and examined within 30 minutes of the collection because of the rapidly disintegrating nature of the trophozoites. The formed specimens should be processed and analysed within an hour following their collection and receipt in the laboratory for detection of the cysts. To increase the chances of detecting the parasite, the formed specimens should be processed and analysed within 24 hours. The trophozoites and cysts of *Giardia* can be easily identified but the challenge is to see the nucleus and karyosome. According to Thornton et al. (1983), trichrome and merthiolate-iodine-formalin concentrations (MIFC) are the best techniques for identification of *G. lamblia* cysts when compared to other techniques but trichrome supersedes MIFC method in identification of trophozoites. Similarly, it has been reported that both trichrome and methylene blue stains are both efficient in detecting and preserving the trophozoites of *G. lamblia* structure while the stained smears can be kept as a permanent record (Thornton et al., 1983; Rajurkar et al., 2012).

Table 1.2 Morphologic features and pathogenicity of intestinal *Giardia*

Organism	Size	Other Morphologic Features	Preferred Morphologic Diagnostic Test
Giardia duodenalis	Trophozoites, 10–20 µm long Cysts 8.0–10 µm long	Trophozoites: pyriform shape; sucking disk; 2 nuclei; 2 median bodies; 8 flagella (4 lateral, 2 ventral, 2 posterior) Cysts: ovoid shape; 2–4 nuclei; fibrils and median bodies; no flagella	Trophozoites: direct wet mount; trichrome stain Cysts: FEA concentration wet mount; trichrome stain

Note: Adopted from Cama and Mathison (2015)

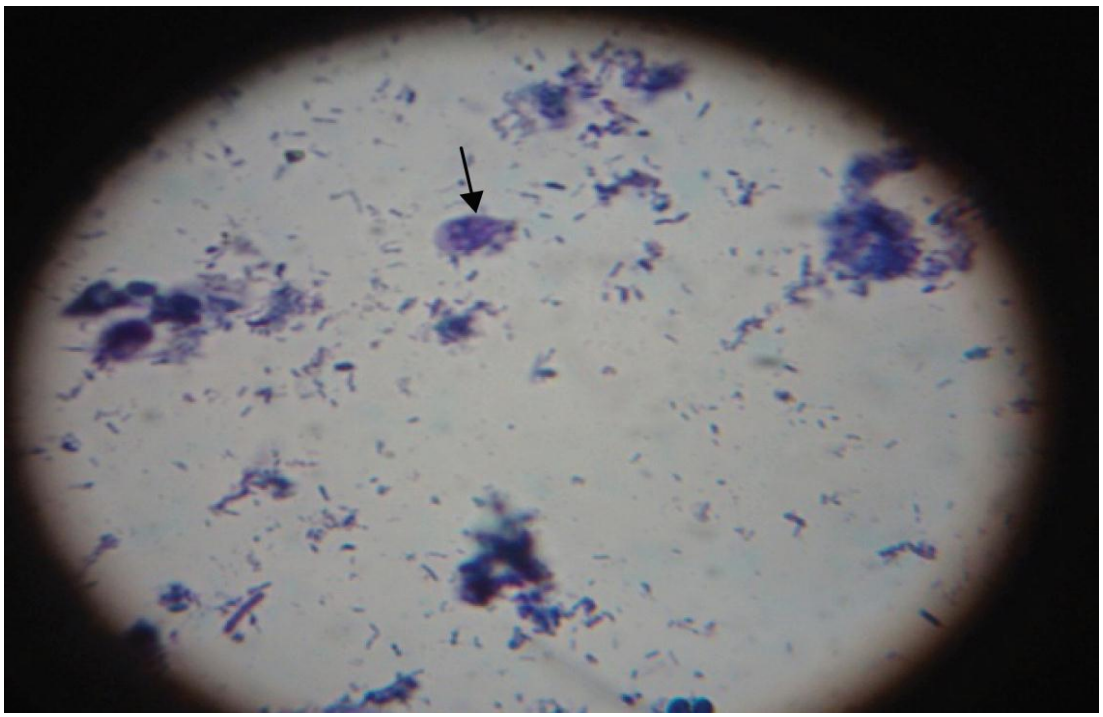


Figure 1.4 Methylene Blue staining of *Giardia* trophozoite

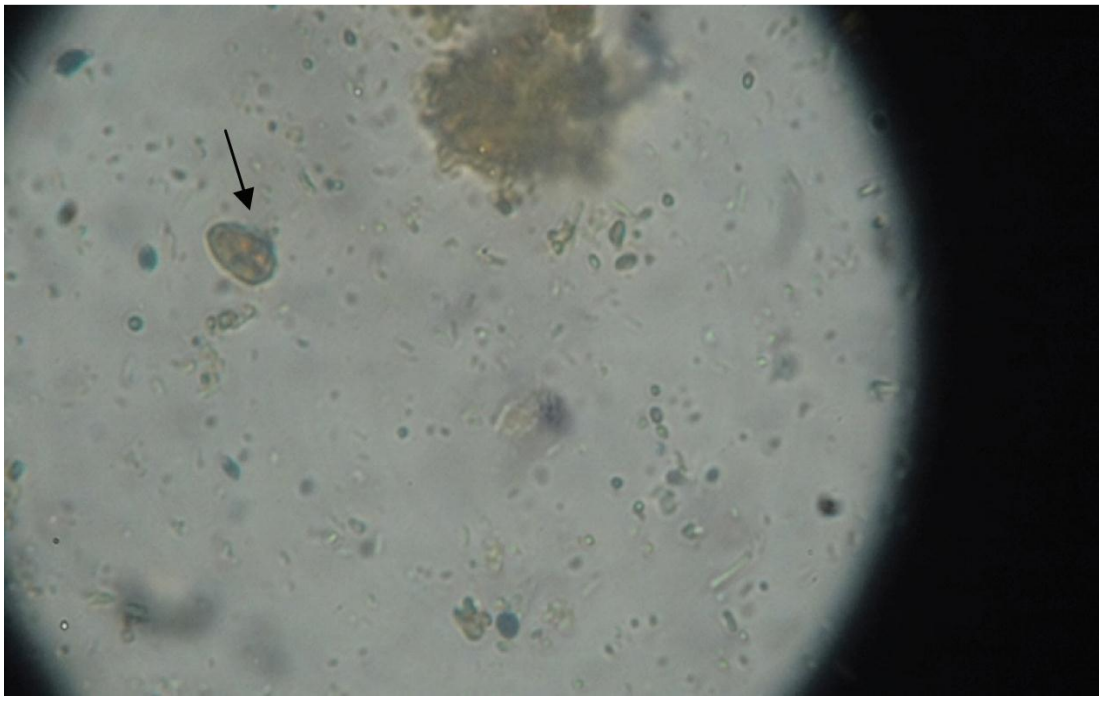


Figure 1.5 Iodine wet mount of *Giardia* cyst

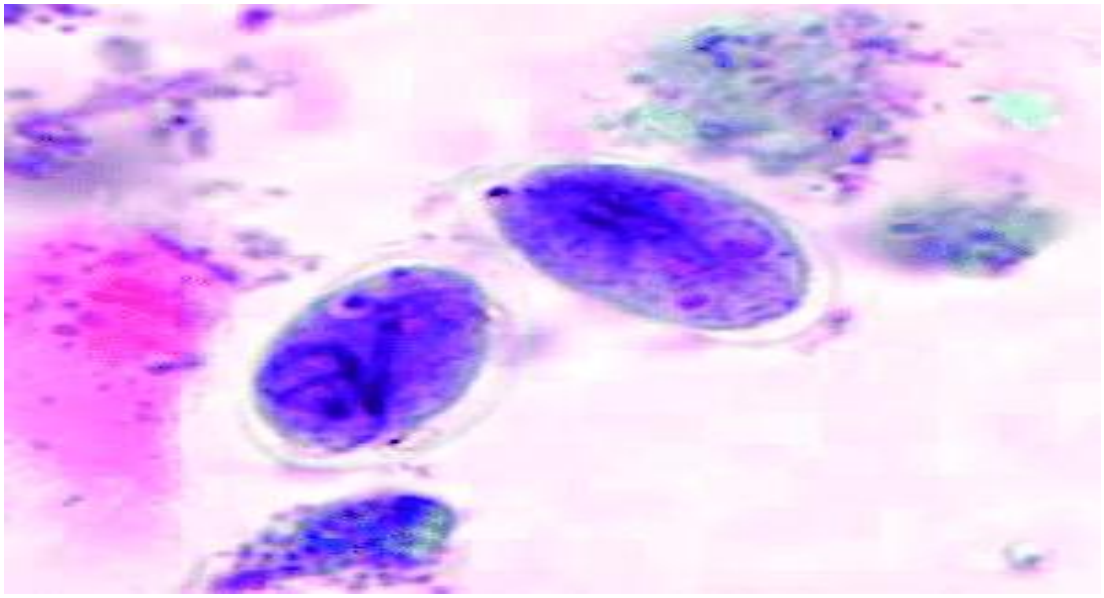


Figure 1.6 *Giardia* cysts are stained with trichrome stain

1.7.3 Stool Antigen Detection

Diagnosis of intestinal giardiasis using antigen detection test has several advantages. The test can rapidly detect the antigen present in stool and serum specimens. One of the methods often used is the SIMPLE-READ *Giardia* rapid assay (Medical Chemical Corporation). It is a qualitative test that detects *G. lamblia* in an aqueous extract of stool samples. This test kit demonstrated high sensitivity and specificity of 97.2% and 100% respectively with human stool specimens (Garcia and Garcia, 2006). Giardia-Strip is another rapid, simple to perform test. It is a strip test manufactured by Coris BioConcept, which aims to detect the membrane antigens of *G. lamblia* cysts in unformed fresh stool specimens and not on specimens preserved using PVA or MICF. Positive results are shown by clearly visible pink-red lines. The test strip has been compared with other methods such as the enzyme immunoassay and microscopy and showed an accuracy of 92.9%, sensitivity and specificity of 91.6% and 93.5% respectively (Cheesbrough, 2006). Another rapid test kit used to detect antigens of *G. lamblia* in stool samples are Ridascreen Giardia and Serazym Giardia. In one study both strips were tested, using microscopy as a reference standard, sensitivity and specificity by Ridascreen *Giardia* was 72.9% and 100% while Serazym *Giardia* had a sensitivity and specificity of 93.8% and 100% respectively. Clearly, the indications of the test outcomes of the rapid test strips showed that they are useful tools for parasite stool diagnosis. However, the commercially available test kits are usually costly when compared to the traditional parasitological methods such as the slide microscopy method (Jelinek and Neifer, 2013).

1.7.4 Stool Culture

There exist three types of cell culture methods for cultivation of *G. lamblia* namely xenic, monoxenic and axenic. In 1960, Karapetyan first cultivated *Giardia* parasite in

a mixed culture *Candida guilliermondi* and chick fibroblasts (Clark and Diamond, 2002). Monogenic culture is a medium with certain host example *E. coli*. Meyer was the first to report axenic cultivation of *Giardia* from small mammals in 1970 (Clark and Diamond, 2002). Axenic cultivation was first accomplished by Diamond in 1961 (Clark and Diamond, 2002). It is a medium without a host. It only needs a broth such as *G. lamblia* and *E. histolytica.* Although, the two main types of cell culture methods often used for axenic cultures are namely TP-S-1 and TYI-S-33 yet the organism can grow well similarly in a modified YI-S. For the two main cultures used, the use of TYI-S-33 supersedes the TP-S-1 in 1978. Axenic medium is used in the production of pure *Giardia* trophozoites (Clark and Diamond, 2002). Culture and isolation of *G. lamblia* can be done using stool specimens and rectal biopsy specimens. However, the success rate is reported to be between 44% and 55% with a significant false-negative rate (Yousefi, 2000). Worldwide, culture technique for *G. lamblia* from stool is done by few renowned research laboratory establishments. Moreover, culturing of the parasite is time-consuming, laborious, expensive and often unrewarding with a sensitivity of about 50% and further testing is usually necessary for differentiation (Yousefi, 2000; Fotedar et al., 2007).

1.7.5 Molecular Diagnosis

The advancement of research has evidently proven polymerase chain reaction (PCR) technique is more sensitive and specific than the traditional parasitological method (slide microscopy) as well as the antigen detection and the antigen-antibody binding techniques in the diagnosis of giardiasis. PCR method is sensitive as reported to detect as low as 2.4 pg rRNA gene of *Giardia*, and has the ability to detect as low as 10 trophozoites using conventional PCR procedure (Ghosh et al., 2000). Considering

the presence of PCR inhibitors in faeces (*e.g.* urobilnogens, bilirubins, bile salts) that may cause false-negative results, steps in DNA extraction are very important before conducting the test itself (Winiecka-Krusnell and Linder, 1995). Therefore, following the test procedure strictly can aid to reduce these inhibitors to obtain desired DNA amplification results (Winiecka-Krusnell and Linder, 1995). The sensitivity and specificity of PCR-based detection that multiplies the 552-bp intergenic spacer (IGS) region of multicopy rRNA gene of *G. lamblia* when compared with countercurrent immunoelectrophoresis (CIEF) and ELISA indicated a positivity of 20% while 7.14% and 12.85% for CIEF and ELISA respectively (Ghosh et al., 2000). Similarly, IgA antibody was detected in 75% of infected persons while 20% among non-infected persons with sensitivity and specificity of 75% and 90% respectively for both infected and non-infected groups (S.E. Mahmoud et al., 2010). *Giardia* DNA detection using PCR can be done on specimens such as stool, blood, and saliva (Ghosh et al., 2000; Rijsman et al., 2016). It has been scientifically documented that one of the demerits of antibody testing (ELISA) is that it cannot distinguish between present and past infections while PCR technique has the ability to detect the existence of the parasite in the clinical specimens (Rochelle et al., 1997).

1.7.6 Serology

Axenic cultivation was first accomplished by Louis Diamond in 1961. He successfully grew *G. lamblia* trophozoites using modified TYI-S-33 medium. This achievement by him and team had contributed significantly to the advancement of scientific knowledge about culturing the parasite. Besides the strides made by him and team, several other established research institutions have also conducted similar studies using *in vivo* and *in vitro* experiments and their findings have strongly

pointed to the evidence that the use of TYI-S-33 medium enhances the growth of pure Giardia trophozoites. The pure Giardia trophozoites obtained from the cell cultures can be used for diagnosis purposes and for seroprevalence study.

Recent studies on the use of human anti-giardia *lamblia* IgG ELISA test kits for the detection of human anti-*Giardia lamblia* IgG antibody has revealed 86% of infected patients develop serum antibody IgG against *G. lamblia* (Soliman et al., 1998). In a study of 147 samples serum, antibodies were detected in 93 (63.3%) and 100 (68%) using Indirect immunofluorescence (IIF) and ELISA techniques respectively. Moreover, the sensitivity of ELISA and IIF were 72% and 82%, respectively (Guimarães and Sogayar, 2002). Similarly, in another study, 34% of asymptomatic studied subjects showed a titre of 1:500 or less in anti-parasite response measured via Immunofluorescence (IFA), while more than 29% of symptomatic studied subjects showed a titre of 1:8000 or more analysed via ELISA (Soliman et al., 1998). The other biomarkers still in research includes, polypeptides, heat shock proteins, *Giardia* lectin antigen, Giardins, and tubulins.

1.7.7 Diagnosis of Extraintestinal Giardiasis

Diarrheoa is hardly found in case of extraintestinal giardiasis (Nazer, 2018). On the other hand, allergic presentations namely urticaria, erythema multiforme, bronchospasm, reactive arthritis, and biliary tract disease were associated with extraintestinal giardiasis. The origin of these presentations is likely a result of the host immune reaction (Homan and Mank, 2001). Evidently, symptoms for extraintestinal patients of intestinal giardiasis hardly exist, thus, the diagnostic stages of the parasite (cysts and trophozoites) can rarely be seen in stool specimens. In view

of this, medical practitioners have to draw a provisional diagnosis based on a combination of clinical manifestations, radiological imaging, and serological test. Besides, biopsy can also provide more insights into the diagnosis of both intestinal and extraintestinal disease.

1.8 The Rationale of the Study

Orang Asli who live in the remote part of West Malaysia with little access to safe water and poor sanitation are at great risk of giardiasis (Ngui et al., 2011). To control and prevent the disease, continuous surveillance of the distribution of anti-Giardia IgG is necessary among the Orang Asli dwellers (Choy et al., 2014). According to a study conducted in Peninsular Malaysia by Choy et al. (2014), the prevalence of *Giardia* infection was 11.6%. A similar study conducted by Al-Mekhlafi et al (2013), revealed 22.2% aboriginal school-going children were infected with *Giardia*. Routine diagnosis of giardiasis relies on microscopy, which is relatively impractical for large scale field work. Usually, the stool samples collection will require several visits and the management and analysis of samples are not automated. Serology techniques such as Enzyme-Linked Immunosorbent Assay (ELISA) could aid in identification of potential endemic areas. With ELISA technique as opposed to the microscopy, sample collection and analysis of samples can be performed on the same day because the former method is automated method. The sensitivity of serological method in the diagnosis of invasive intestinal disease was found to be 84% (Nath et al., 2013). Culture methods are time-consuming and tedious with a sensitivity of only about 50%, hence further diagnostic techniques such as speciation may be required (van Hal et al., 2007). High seroprevalences of anti-Giardia IgG were shown in endemic countries such as Mexico (55%) (Cedillo-Rivera et al., 2009) and Caribbean

countries (40%). In both studies, age and gender were recorded and reported. In Malaysia, Orang Asli settlements were endemic for intestinal parasitic diseases including giardiasis (Hotez, 2014). However, to date, there is limited seroprevalence data on anti-Giardia IgG among Orang Asli and its distribution which is useful for field surveillance of giardiasis and its targeted-area control measure. The determination of the distribution of anti-Giardia IgG will provide insightful information about the burden of the disease among certain Orang Asli settlements.

1.9 General Objective

The general objective of the study is to determine the presence of anti-Giardia IgG in selected serum samples of Orang Asli in Malaysia by using an in-house ELISA. The specific objectives of the study include:

1. To develop an in-house CSA-IgG-ELISA
2. To determine the distribution of ELISA OD at 450 nm readings among selected Orang Asli serum samples
3. To determine the association between the anti-Giardia IgG ELISA OD at 450nm readings and demographic variables (i.e. age and gender)
4. To compare the ELISA OD at 450 nm readings between real-time PCR positive and negative serum samples

1.10 Overview of the Study

The methodology flowchart of the study is shown below

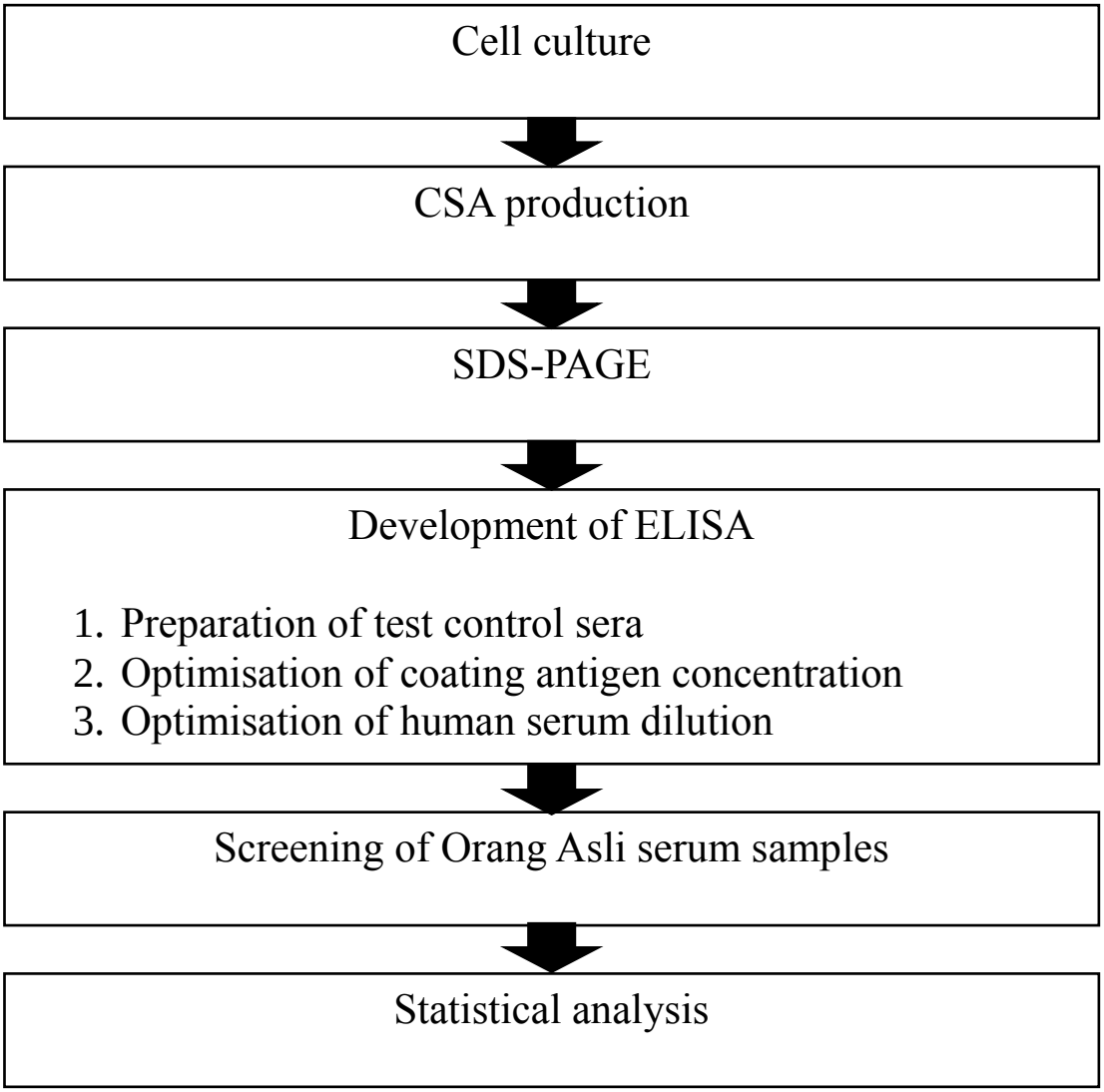


Figure 1.7 Flowchart of study

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Axenic Trophozoite Culture

The axenic strain of *G. lamblia* trophozoite was purchased from ATCC, USA. The cell was received in a cryopreserved vial. Upon receiving, the culture was revived and maintained in modified TYI-S-33 medium. Upon expansion of the culture, a portion of the culture was prepared for cryopreservation storage (Clark and Diamond, 2002).

2.1.2 Serum Samples

The current study utilized archived Orang Asli serum samples originally from a Tuberculosis (TB)-Parasite Correlational Study among Orang Asli. The samples were collected from Orang Asli settlements (i.e. located in Pahang, Perak, Kelantan, and Selangor) between year 2011 and 2014. The use of these serum samples was previously approved by Malaysian Department of Aborigine Affairs (Jabatan Kemajuan Orang Asli, JAKOA) and Universiti Sains Malaysia Human Ethical Committee (JEPeM USM Code: USM/JEPeM/[247.3(9)]), as shown in Appendices 1 and 2. For inclusion criteria: archived serum samples with known age and gender, and/or known real-time PCR analysis were used in the present study. The 150 serum samples used for evaluation of CSA were as follow:

Group I : Stool-PCR Positive (N=52)
Group II : Stool-PCR Negative (N=4)
Group Ⅲ : Stool-PCR Unknown (N=94)

2.1.3 List of Chemicals

Chemicals used in this study are listed in Table 2.1.

2.1.4 List of Kits and Consumables

Kits and consumables used in this study are listed in Table 2.2.

2.1.5 List of Equipment

Equipment used in this study is listed in Table 2.3.

Table 2.1 List of chemicals

Chemical / Reagent	**Manufacturer**
3,3,5,5-tetramethylbenzidine (TMB) solution	Sigma-Aldrich, USA
Acetic acid (glacial) 100%	Merck, Germany
Acrylamide solution 30%	Bio-Rad, USA
Ammonium iron (III) citrate	Sigma-Aldrich, USA
Ammonium persulfate (APS)	Bio Basic, Canada
Ascorbic acid	Sigma-Aldrich, USA
Beta-mercaptoethanol (β-ME)	Amresco, USA
Bio-Rad protein assay reagent	Bio-Rad, USA
Biosate peptone	BD, USA
Block One	Nacalai, Japan
Bovine Serum	Gibco, New Zealand
Bromophenol blue	Amresco, USA
cOmplete, Mini Protease Inhibitor Tablets, without EDTA	Roche, Germany
CBB R250	Sigma, USA
Dextrose	Sigma-Aldrich, USA
Diamond Vitamin Tween80	Sigma-Aldrich, USA
Ethanol absolute	HmbG, USA
Glycine	Vivantis, Malaysia
Hydrochloric acid (HCl) fuming 37%	Merck, Germany
Methanol	Merck, Germany
Potassium phosphate dibasic	Sigma-Aldrich, USA
Potassium phosphate monobasic	Sigma-Aldrich, USA
Sodium bicarbonate ($NaHCO_3$)	Merck, Germany
Sodium carbonate (Na_2CO_3)	Merck, Germany
Sodium chloride (NaCl)	Merck, Germany
Sodium dodecyl sulphate (SDS)	Vivantis, Malaysia
Sodium hydroxide granulated pellet	Merck, Germany
Sodium phosphate dibasic	Merck, Germany
Sodium phosphate monobasic	Merck, Germany
Spectra ^{TM} Multicolor Broad Range Protein Ladder	Thermo Scientific, USA
Tetramethylethylenediamine (TEMED)	Vivantis, Malaysia
Tris Base	Merck, Germany
Typtone	Sigma, USA
Tween 20	Amresco, USA

Table 2.2 List of kits and consumables

Consumable	**Manufacturer**
96-well maxisorp microplate	Nunc, Denmark
Centrifuge tube, 1.5 mL, 2.0 mL, 15 mL, 50 mL	Axygen, USA
Cuvette	Greiner Bio-One, Germany
Laboratory bottle	DURAN, Germany
Pipette tips	Axygen, USA

Table 2.3 List of equipment

Equipment	**Manufacturer**
-20°C freezer	SNOW, Malaysia
-80°C deep freezer	ilShin, Korea
Biophotometer Plus	Eppendorf, Germany
Bio-Rad Mini Protean 3 Electrophoresis System	Bio-Rad, USA
Centrifuge mikro 22R	Hettich, Germany
Centrifuge universal 32R	Hettich, Germany
Hot plate & magnetic stirrer	ERLA Technologies, Malaysia
Image analyzer	Vilber Lourmat, France
Thermo Scientific™ Varioskan™ LUX multimode microplate reader	Thermo Scientific, USA
Inverted microscope	Leica, Germany
PowerPac™ HC High-Current Power Supply	Bio-Rad, USA
Refrigerator	Samsung, Malaysia

2.1.6 List of Buffers and Reagents

Buffers and reagents used in the present study are listed in Appendix 2.

2.2 Methods

2.2.1 Cell Culture

The trophozoites were revived from liquid nitrogen as illustrated in Figure 2.1. The cryovial was revived from liquid nitrogen and incubated in prewarmed water bath at 37°C for 5 minutes without agitation. The cell suspension was then transferred to a culture tube prefilled with warm medium (Figure 2.2). The revived culture was then incubated in an incubator at 37°C. The viability and motility of the trophozoites were examined after 10 minutes of incubation via inverted microscope (Figure 2.3). *G. lamblia* trophozoites were hermetically maintained in polystyrene slant-end culture tubes (Nunc Nunclon, Denmark) containing about 80% filled modified TYI-S-33 medium. The change of medium procedure was done aseptically to minimize contamination as much as possible. Cultures were incubated at 37^{o}C and medium changed every 48-72 hours.

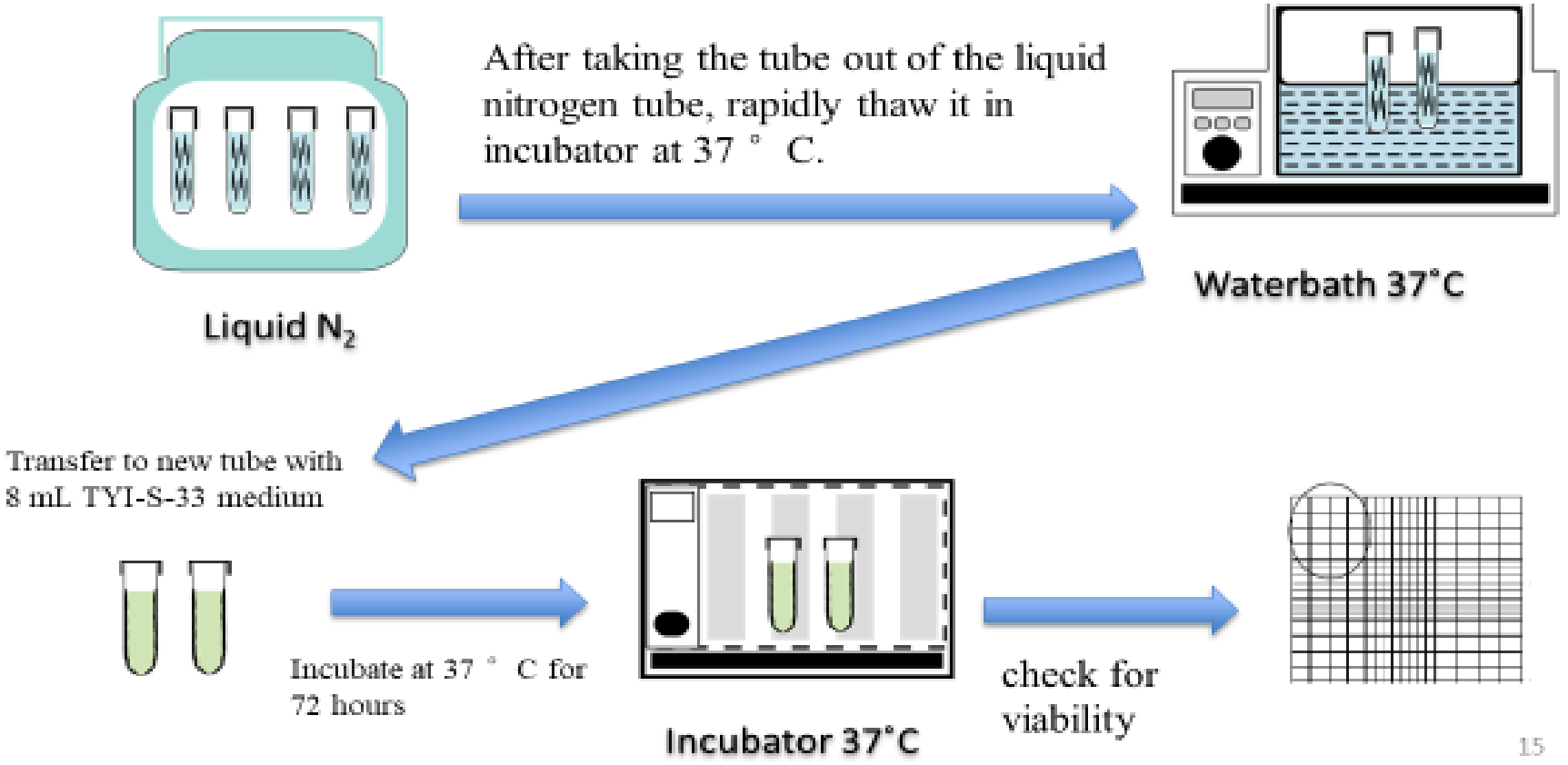


Figure 2.1 Diagram for the revival of cryopreserved trophozoites

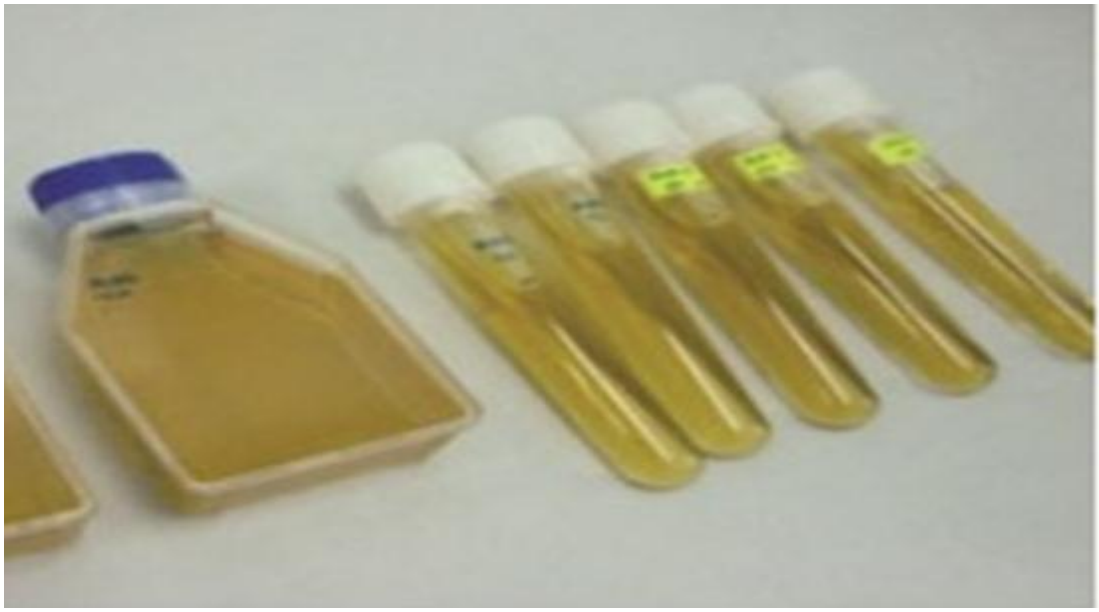


Figure 2.2 Culture of trophozoites maintained in culture tubes and flasks

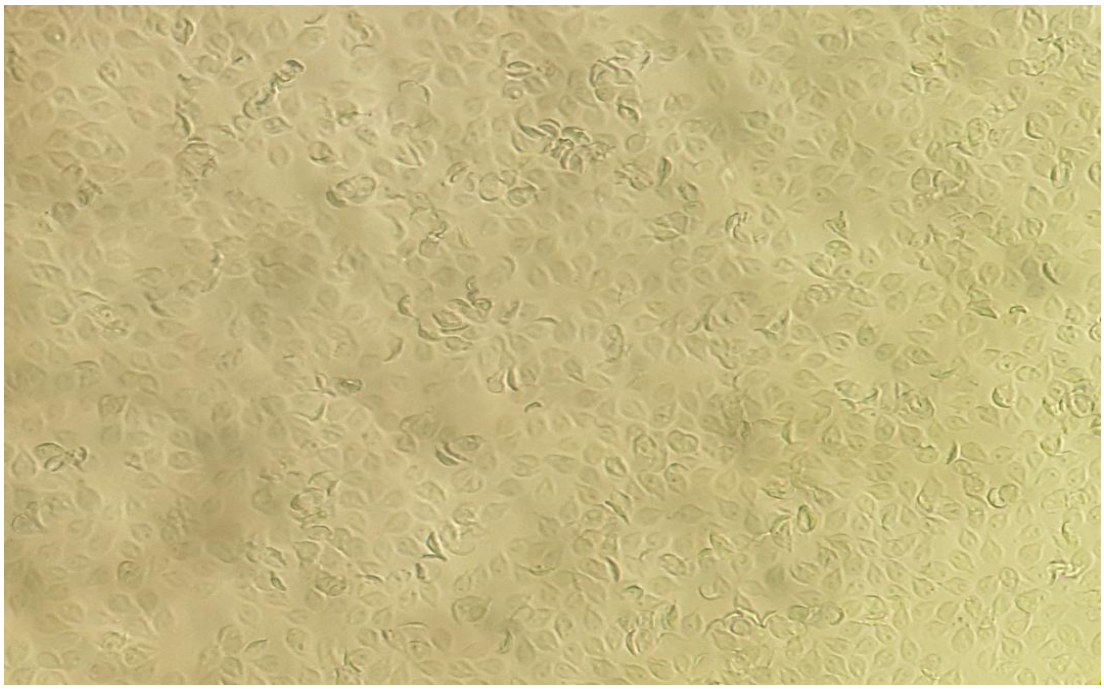


Figure 2.3 Morphology of axenic culture of *G. lamblia* trophozoites

2.2.2 Cell Harvest

The culture tubes were chilled in crushed ice for 15 minutes to detach the cells from the tube wall. The tubes were inverted repeatedly but diligently to ensure a thorough mixture of the cells. The cell suspensions were collected in a sterile 50 mL centrifuge tube and spun at 1000 × g for 10 minutes. After the centrifugation, the the cell pellet was obtained by discarding the supernatant. The cell pellet was transferred into a 14 mL microcentrifuge tube and 5 mL of sterile phosphate-buffered saline (PBS) was added. The solution was thoroughly mixed using a pipette with a tip before initiating centrifugation at 1000 × g for 5 minutes. This cell washing procedure was repeated twice. After the second cell washing step, the cell pellet was transferred into a 2 mL microcentrifuge tube and resuspended with 2 mL of PBS. The cell density of the suspension was then determined. The tube was then centrifuged at 1000 × g for 5 minutes. The supernatant was discarded, and the cell pellet was stored at -80^0 C until used.

2.2.3 Cell Counting

In this study, the cell counting was performed using Trypan blue exclusion method using a Neubauer's chamber. The dead cells and the background were stained blue, while the viable cells remained unstained. Upon collection of cell suspension in a centrifuge tube, the tube was chilled on ice for 2 min to prevent attachment of cells onto the tube wall. The cell suspension was resuspended, and then 50 µL of the suspension was mixed with 50 µL of 0.4% Trypan blue. Ten µL of the mixture were pipetted into the Neubauer's chamber. Duplicate counting was performed with the aid of the tally counter. Figure 2.2 shows the schematic diagram of the cell counting

area and the formula to deduce the cell density of the cell suspension (Yap et al., 1970).

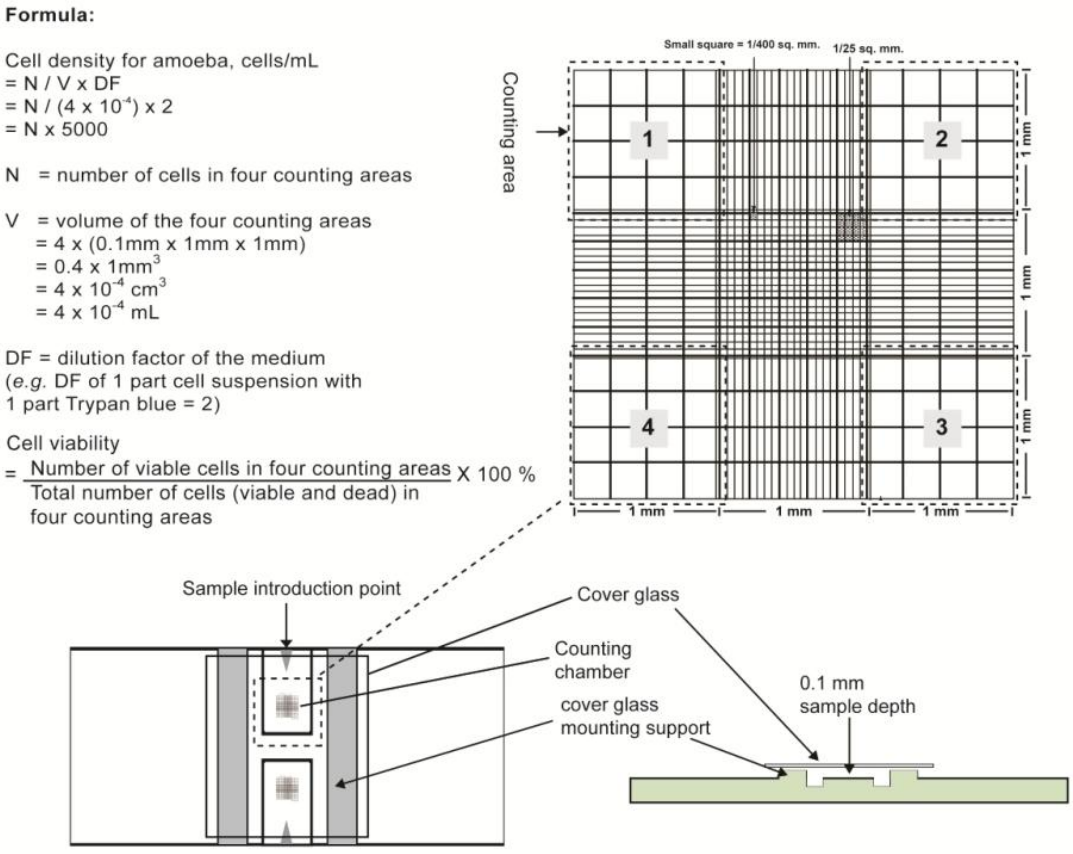


Figure 2.4 Schematic diagrams of cells counting area in Neubauer's chamber

2.2.4 Preparation of Crude Soluble Antigen (CSA)

Approximately 10 million trophozoites were harvested and resuspended in 500 µL of lysis buffer and 20 µL of 0.5 M iodoacetamide. This was followed by two cycles of sonication, a process of rupturing the cell membrane to release its content, for 1 minute at 10% amplitude with 0.5 sec vibration-on and 0.5 sec vibration-off, on crushed ice cubes. The cell lysate (soluble & insoluble proteins) was spun at 10,000 × *g* for 10 minutes at 4°C, and the supernatant used as CSA. Bradford Protein Assay was used to determine the protein concentration (Bio-Rad, USA). The CSA was stored at -80°C until used.

2.2.5 Protein Concentration Determination by Bradford Protein Assay

The Bradford Protein Assay was used to determine the concentration of protein sample *G. lamblia* (CSA) (Bradford, 1976). The working solution was prepared by diluting four parts of distilled water with one part of the Bradford protein assay dye (Bio-Rad, USA). For the construction of protein concentration-standard curve in Biophotometer Plus (Eppendorf, Germany), one blank tube and four different tubes of Bovine Serum Albumin (BSA) protein standards containing 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL were prepared. Twenty µL of each protein standard was mixed with 1 mL of Bradford protein assay reagent. The readings of the mixtures were read at OD_{595nm} via Biophotometer. A standard curve was plotted based on the readings and the known BSA concentrations. For determination of protein sample concentration, 1 mL of Bradford prepared reagent was mixed with 20 µL of samples. The protein samples were serially diluted 1:1, 1:2, 1:4 and 1:8 with dH_2O before mixing with the protein assay reagent. The mixture of protein sample and protein assay reagent was subjected to OD_{595nm} measurement using

Biophotometer. This procedure was repeated thrice for each sample allowing three measurements to be made for each sample or protein standard.

2.2.6 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine albumin protein standards and *G. lamblia* CSA. The protein samples were separated according to the respective molecular weight. It was performed in accordance with Laemmli (1970) description, however with some necessary adjustments. Acrylamide is an inert material and does not interact with the protein to form a matrix. The SDS Gel composed of two layers, namely the stacking and resolving gels. The SDS-PAGE gels were cast, and the electrophoresis set was assembled according to the manufactural instruction manual. For sample preparation, one part of the protein sample was mixed with one part of 2X sample buffer. Serially diluted protein samples were analysed to ensure the analysis covered a range of protein amount. The mixture of protein sample and sample buffer was then heated at 95°C for 5 minutes via heat block. The mixture was then cooled on ice and spun at 3000 × *g* for 5 minutes. The mixture supernatant was loaded into the stacking well. A protein molecular weight standard was loaded for an indication of protein band molecular weight subsequently. The electrophoresis was run at a constant current with 25 mA per gel until the sample tracking dye i.e. bromophenol blue was approximately 0.5 cm from the bottom of the glass plate. After the electrophoresis was completed, the gel was diligently removed from the glass plate and incubated in tap water for 10 minutes with two changes of water. The gel was then stained with RAMA stain for 30 minutes for colour development. The protein profile of the gel was captured using image analyser.

2.2.7 Development of *G. lamblia* CSA-IgG-ELISA

An in-house ELISA was developed for the detection of anti-*G. lamblia* IgG in Orang Asli serum samples. Prior to ELISA optimisation, test high and low OD_{450nm} sera were identified and pooled. A preliminary ELISA was performed using 96 serum samples based on standardised parameters i.e. coating antigen concentration of 10 µg/mL, serum dilution of 1:50 in PBS and HRP-conjugated antibody of 1:6000 (according to manufacturer recommendation). The ELISA was performed according to general ELISA procedure in section 2.2.8. Six serum samples presented the highest ELISA OD_{450nm} readings were identified; about 50 µL of the serum samples were pooled together and used as pooled positive sera. Similarly, six serum samples presented the lowest ELISA OD_{450nm} readings were identified; about 50 µL of the serum samples were pooled together and used as pooled negative sera.

2.2.7 (a) Optimisation Coating Antigen Concentration

In order to capture maximum amount of targeted antibody, it is important to ensure wells of microtiter plate were saturated with enough quantity of coating antigen. In a normal procedure, wells of microtiter plate were coated with 100 µL of coating antigen and incubated for overnight at 4°C in a humid box. The excess coating antigen was washed away with PBS-T. This was followed by a blocking step with 200 µL blocking reagent for 1 hour at room temperature to ensure saturation of any unbound areas in the wells. To determine the best protein concentration for coating the wells, two-fold diluted coating antigen with concentrations ranging from 80 µg/mL to 0.625 µg/mL were used to sensitise the wells. The wells were then tested with PBS control, pooled positive and negative sera, respectively. Wells saturated with coating antigen will show constant signals. Hence, the minimum coating antigen

concentration giving constant ELISA OD_{450nm} readings was selected for the development of in-house *G. lamblia* CSA-IgG-ELISA.

2.2.7 (b) Optimisation Human Sera Dilution

The present in-house ELISA aimed to detect anti- *G. lamblia* antibody in the human serum. Appropriate dilution of human sera in PBS is important to ensure the ELISA able to capture maximum targeted antibody over background antibody, which is present in non-infected human. Hence, wells saturated with the sensitised antigen were tested with a range of diluted pooled positive and negative human sera ranging from 1:25 to 1:3200, respectively. At optimum dilution, the OD_{450nm} readings of pooled positive and negative human sera will show maximum ratio. The minimum dilution of human sera giving maximum ratio between OD_{450nm} readings of pooled positive and negative human sera was selected for the development of in-house *G. lamblia* CSA-IgG-ELISA.

2.2.7 (c) Optimisation HRP-Conjugated Antibody Dilution

Secondary antibody plays a pivotal role in the detection of the targeted antibody and amplification of the signal. For optimisation of secondary antibody, wells saturated with sensitised antigen and fully bound with antibodies reactive to the coated antigen were probed with a range of two-fold serial diluted secondary antibody i.e. from 1:750 to 1:96000 in PBS. The minimum secondary antibody dilutions showed a maximum ratio between OD_{450nm} readings of pooled positive and negative human sera was selected for the development of in-house *G. lamblia* CSA-IgG-ELISA.

2.2.8 General ELISA Procedure

One hundred microliters (100 µL) of coating antigen were added to each well of the microtiter plate made from polystyrene at known protein concentration diluted in ELISA coating buffer and incubated at 4°C for overnight. On the next day, the excess coating antigen was discarded, and the wells were washed three times with 200 µL of PBS-T, ELISA washing buffer. Block One, ELISA blocking reagent, was then added to each well at room temperature for 1 hour with 200 rpm rotary shaking. The wells were washed three times with 200 µL PBS-T after completion of blocking. Hundred (100 µL) of a human serum sample or PBS control was added to each well accordingly and the microtiter plate was incubated for 1 hour at room temperature. After the incubation, the wells were washed 3 times with 200 µL of PBS-T to remove unbound antibodies. Then each well was loaded with 100 µL of HRP-conjugated antibody at dilution of 1:6000 in PBS and incubated at room temperature for 1 hour. Finally, each well was washed 3 times with 200 µL of PBS-T with 200 rpm rotary shaking. For signal development, each well was added with 100 µL of TMB substrate solution and incubated in dark at room temperature for 15 minutes. The signal development was ended by adding 100 µL of ELISA stop solution to each well. Optical density (OD_{450nm}) reading of each well was measured by Thermo ScientificTM Varioskan^{TM} LUX multimode microplate reader.

2.2.9 Statistical Analysis

The demographic data and ELISA OD_{450nm} readings were recorded using Mocrosoft Excel and analysed using Statistical Product and Service Solutions (SPSS) for Window. Upon checking of the normality distribution of the data, the association between ELISA OD_{450nm} readings and subjects age was analysed via Pearson's Chi-

square. The mean ELISA OD_{450nm} readings between genders were compared via independent t-test. While the mean ELISA OD_{450nm} readings between positive and negative real-time PCR subjects were compared via the Mann-Whitney test. A P value of less than 0.05 indicates a statistically significant difference.

CHAPTER 3

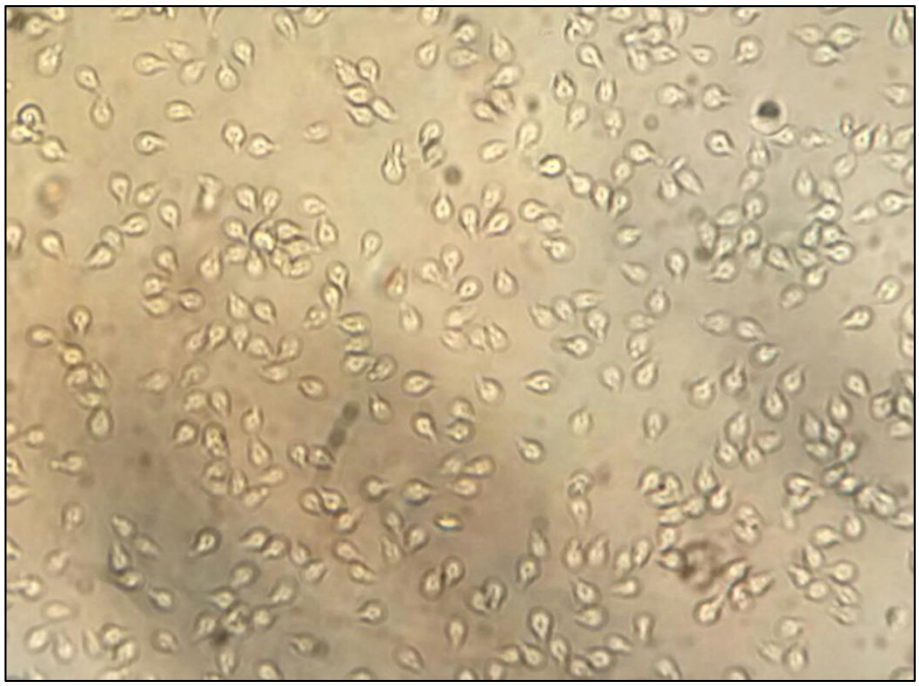
RESULTS

3.1 Culture of *G. lamblia* Trophozoites

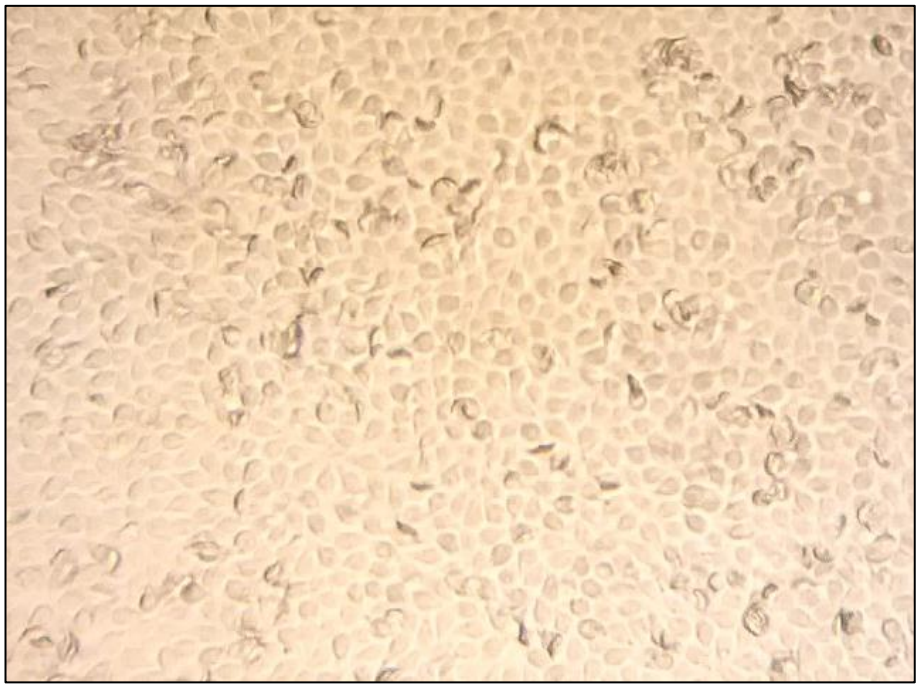
*G. lambli*a trophozoites were revived from liquid nitrogen and maintained in culture tubes. The medium was changed every 48 hours. The culture was harvested when the growth was confluence, as shown in Figure 3.1.

3.2 Preparation of *G. lamblia* CSA

Trophozoites CSA was prepared in preparation buffer containing protease inhibitors. The protein concentration was determined by Bradford method. The BSA protein standards were prepared and the quality was analysed by SDS-PAGE prior to use in Bradford protein assay. The protein band thickness and intensity reduced along with the two-fold dilution from Lane 1 to Lane 6 (Figure 3.2). The BSA standard curve for Bradford protein assay showed high r^2 value i.e. 0.9418, which mean at least 90% of the points fit the trendline (Figure 3.3). CSA was produced from *G. lamblia* trophozoites and the determined protein concentration was about 7 mg/mL. The protein profile of CSA was analysed by SDS-PAGE (Figure 3.2 & 3.4). The profile showed many distinct protein bands ranged from 25-260 kDa.



A
(Pre-confluence)



B
(Confluence)

Figure 3.1 *G. lamblia* trophozoite morphology at 200x magnification

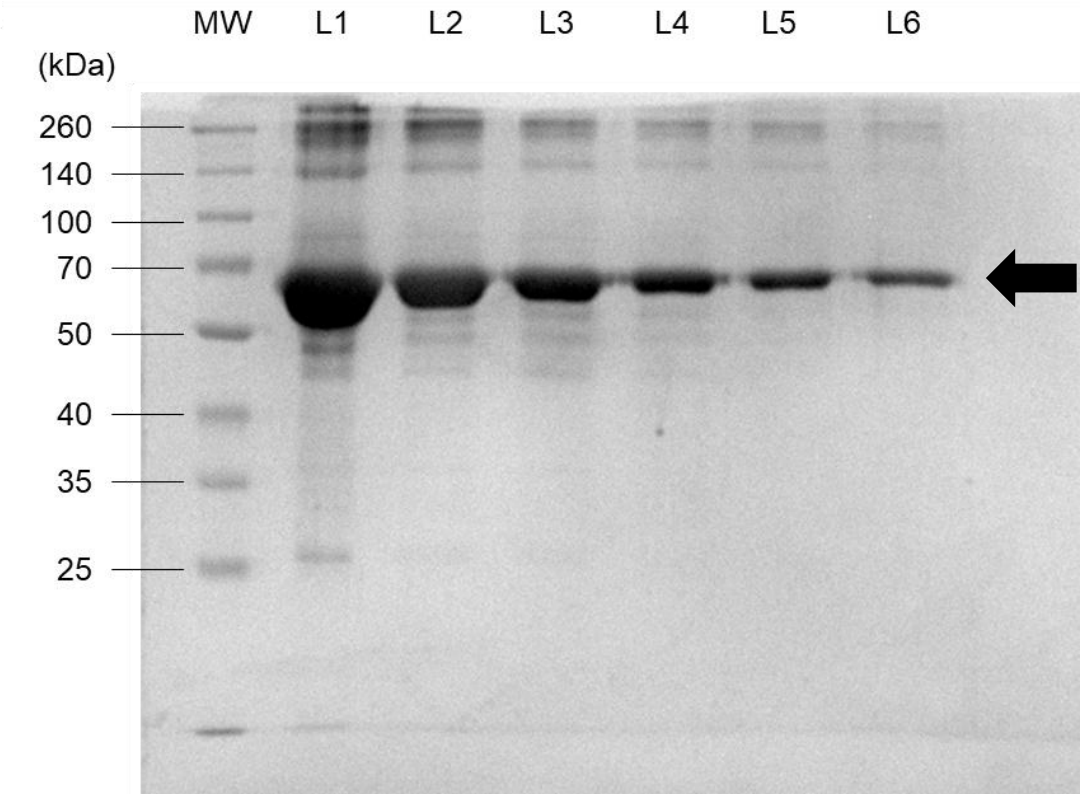


Figure 3.2 SDS-PAGE protein profiling of BSA protein standards

Note

MW : Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, USA)

Lane 1-6 : BSA protein standards of 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL

The arrow indicates protein bands of BSA.

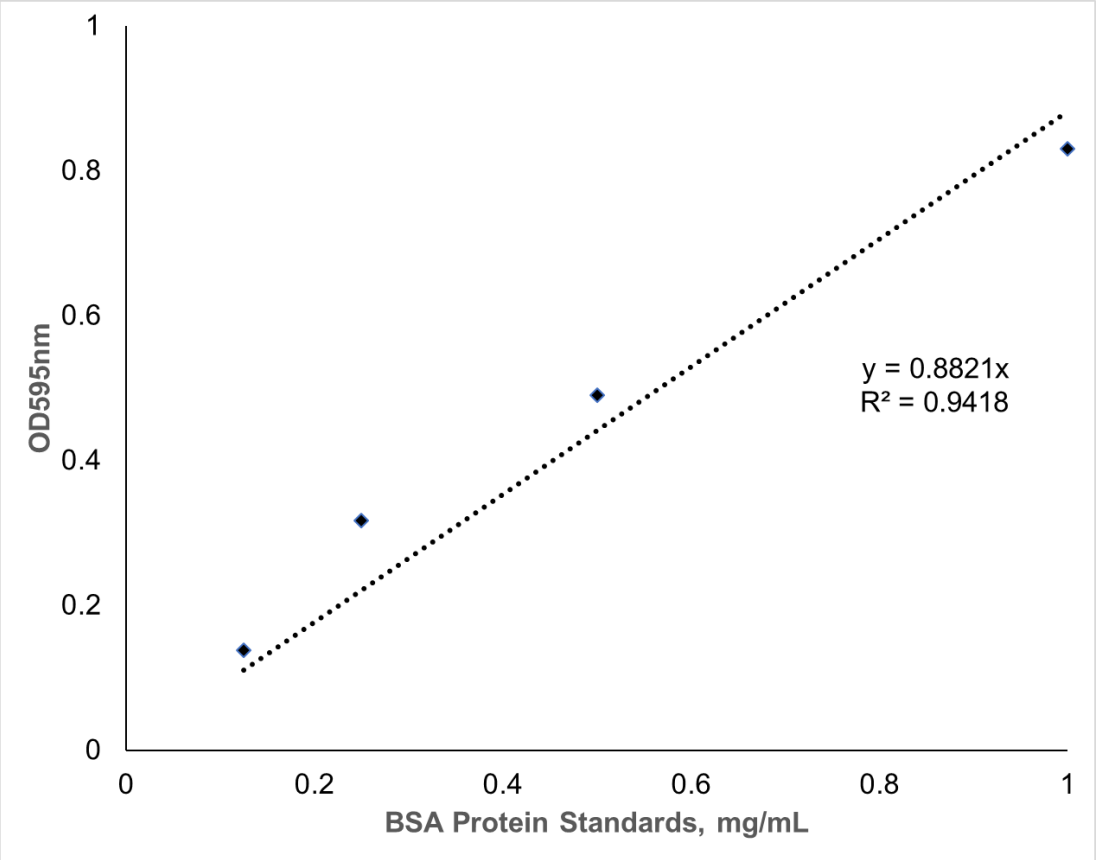


Figure 3.3 BSA standard curve for Bradford protein assay

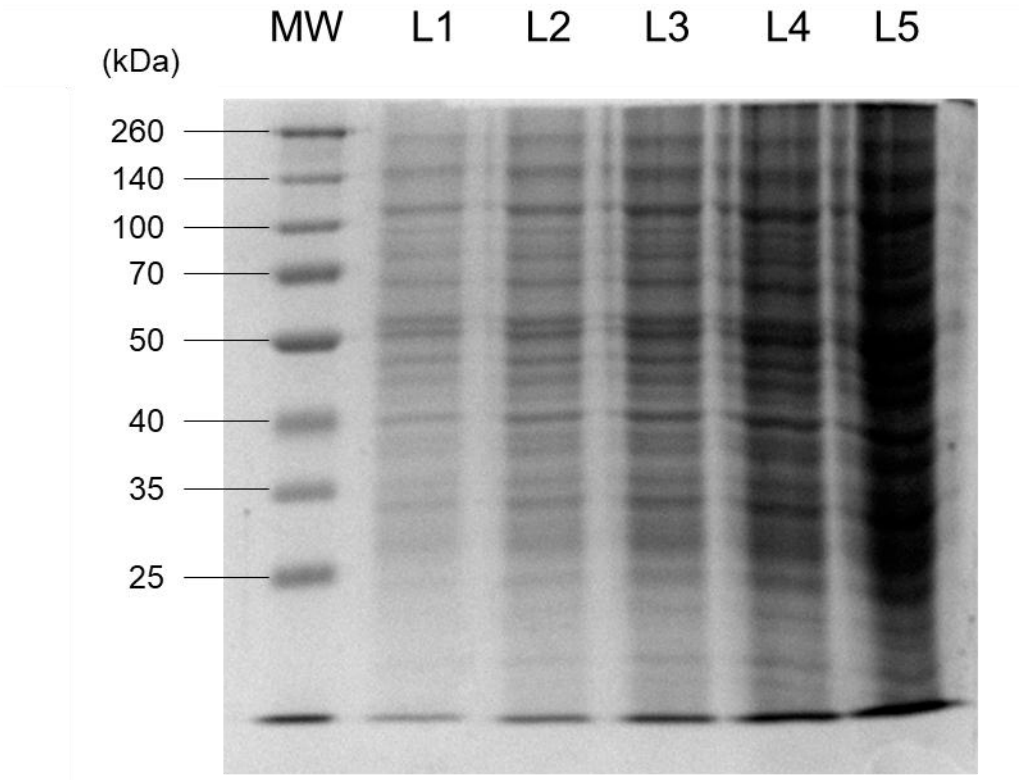


Figure 3.4 SDS-PAGE profiling of *G. lamblia* CSA

Note

MW : Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, USA)

Lane 1-5 : *G. lamblia* CSA of 2.5 µg, 5 µg, 10 µg, 20 µg, and 40 µg, respectively

3.3 Development of CSA-ELISA

An in-house ELISA was developed to detect the presence of anti-*G. lamblia* IgG in the Orang Asli serum samples. The antibodies were detected using coating antigen was prepared from axenically cultured *G. lamblia* trophozoites.

3.3.1 Preliminary Screening

A total of 96 serum samples were screened with ELISA based on standardised parameters i.e. coating antigen concentration of 10 µg/mL, primary and secondary serum dilution of 1:50 and 1:6000. Six serum samples with the highest OD_{450nm} were pooled together and used as a pooled positive control; while six serum samples with the lowest OD_{450nm} were pooled together and used as a pooled negative control. Tabulation of serum OD was presented in Figure 3.5.

3.3.2 Optimisation of Coating Antigen Concentration

To ensure the ELISA wells were saturated with *G. lamblia* CSA, the concentration of the coating antigen was determined. The wells were coated with the antigen of different concentrations ranging from 80 µg/mL to 0.625 µg/mL. Each range of coating antigen dilutions was tested with PBS control, pooled positive and pooled negative serum samples. The trend of positive OD_{450nm} readings remained plateau as the coating antigen concentrations decrease (Figure 3.6). For pooled negative and PBS control the OD_{450nm} readings showed reducing trend as the coating antigen concentrations were lower than 5 µg/mL, possibly due to the reduce in coating antigen in the well. Hence, coating antigen concentration of 10 µg/mL was chosen for subsequent optimisation.

Figure 3.5 Distribution of IgG-ELISA OD_{450nm} reading of Orang Asli serum samples (n=94)

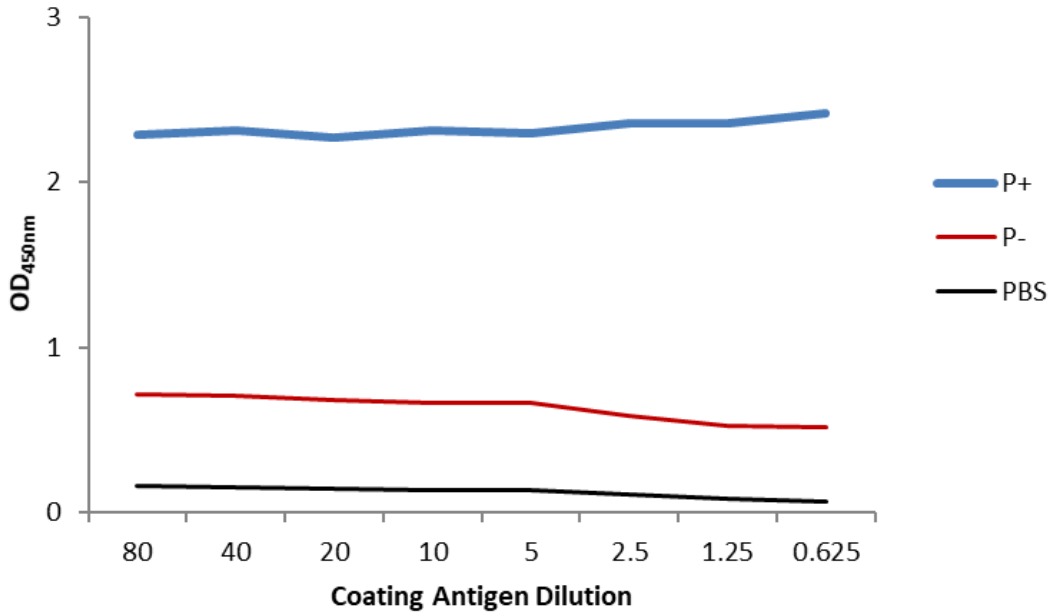


Figure 3.6 ELISA OD_{450nm} readings versus coating antigen concentrations

Note: P+: Pooled positive serum sample; P-: Pooled negative serum sample; PBS: Phosphate buffered saline

3.3.3 Optimisation of Serum Sample Dilution

The lowest serum dilution with the biggest ratio between OD_{450nm} reading of pooled positive and pooled negative serum samples was determined. ELISA wells coated with 10 µg/mL of *G. lamblia* CSA were tested with range of 2-fold serial diluted pooled positive and negative serum samples i.e. ranging from 1:25 to 1:3200 (Figure 3.7). From the graph, serum dilutions from 1:50 to 1:200 showed a ratio of pooled positive to pooled negative OD_{450nm} readings of about 3. The OD_{450nm} readings of PBS control remained plateau. Hence, the dilution factor of 1:50 was chosen for serum sample dilution.

3.3.4 Optimisation of Secondary Antibody Dilution

The lowest secondary antibody dilution with the biggest ratio between OD_{450nm} reading of pooled positive and pooled negative serum samples was determined. ELISA wells coated with 10 µg/mL of *G. lamblia* CSA were tested with range of 2-fold serial diluted pooled positive and negative serum samples i.e. ranging from 1:750 to 1:96000 (Figure 3.8). From the graph, serum dilutions of 1:6000 and 1:12000 showed a ratio of pooled positive to pooled negative OD_{450nm} readings of about 3. The OD_{450nm} readings of PBS control showed a slight increase trend as the secondary antibody dilution was below 1:6000. Hence, instead of 1:12000, a lower dilution factor of 1:6000 with a similar ratio of pooled positive to pooled negative OD_{450nm} readings was chosen for secondary antibody dilution.

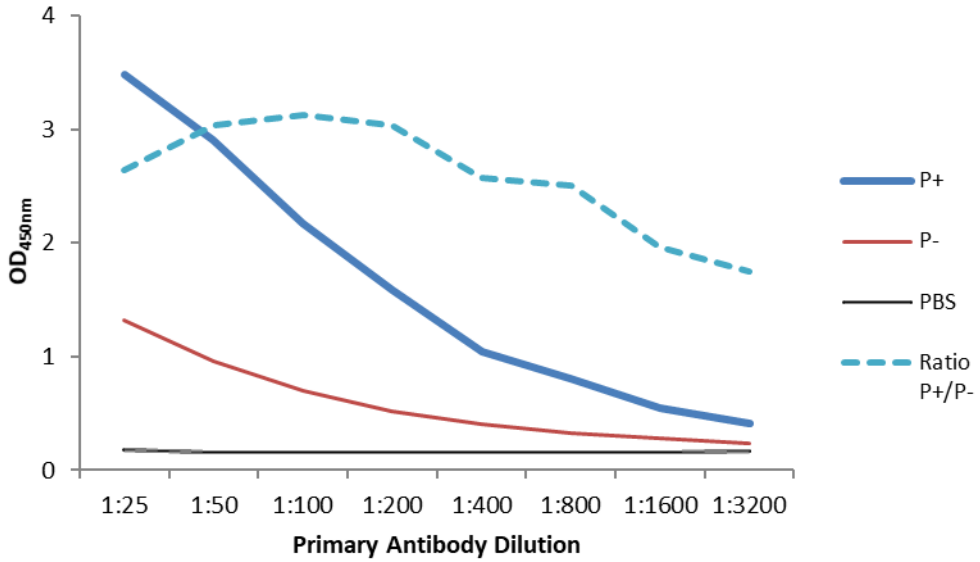


Figure 3.7 ELISA OD_{450nm} readings versus serum dilutions

Note: P+: Pooled positive serum sample; P-: Pooled negative serum sample; PBS: Phosphate buffered saline; ratio P+/P-: the ratio of positive OD_{450nm} reading to negative OD_{450nm} reading

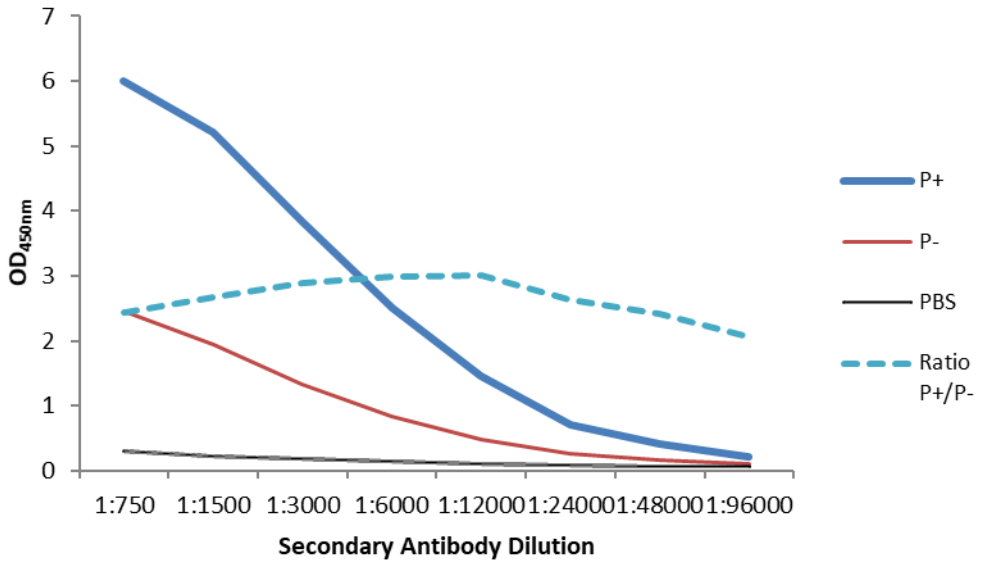


Figure 3.8 ELISA OD_{450nm} readings versus secondary antibody dilutions

Note: P+: Pooled positive serum sample; P-: Pooled negative serum sample; PBS: Phosphate buffered saline; ratio P+/P-: the ratio of positive OD_{450nm} reading to negative OD_{450nm} reading

3.3.5 Optimised Parameters for *G. lamblia* CSA-IgG-ELISA

The optimised parameters for the ELISA are presented in Table 3.1. Each well of the microtiter plate was coated with 10 µg/mL of *G. lamblia* CSA to ensure saturation of the antigen to the well surface. The dilution for serum sample was 1:50 i.e. the lowest dilution with the maximum ratio between high and low ELISA OD_{450nm} readings. The dilution for HRP-conjugated secondary antibody was 1:6000 as recommended by the manufacturer and it also showed a high ratio of pooled positive to pooled negative OD_{450nm} readings.

3.4 Screening of Selected Orang Asli Serum Samples

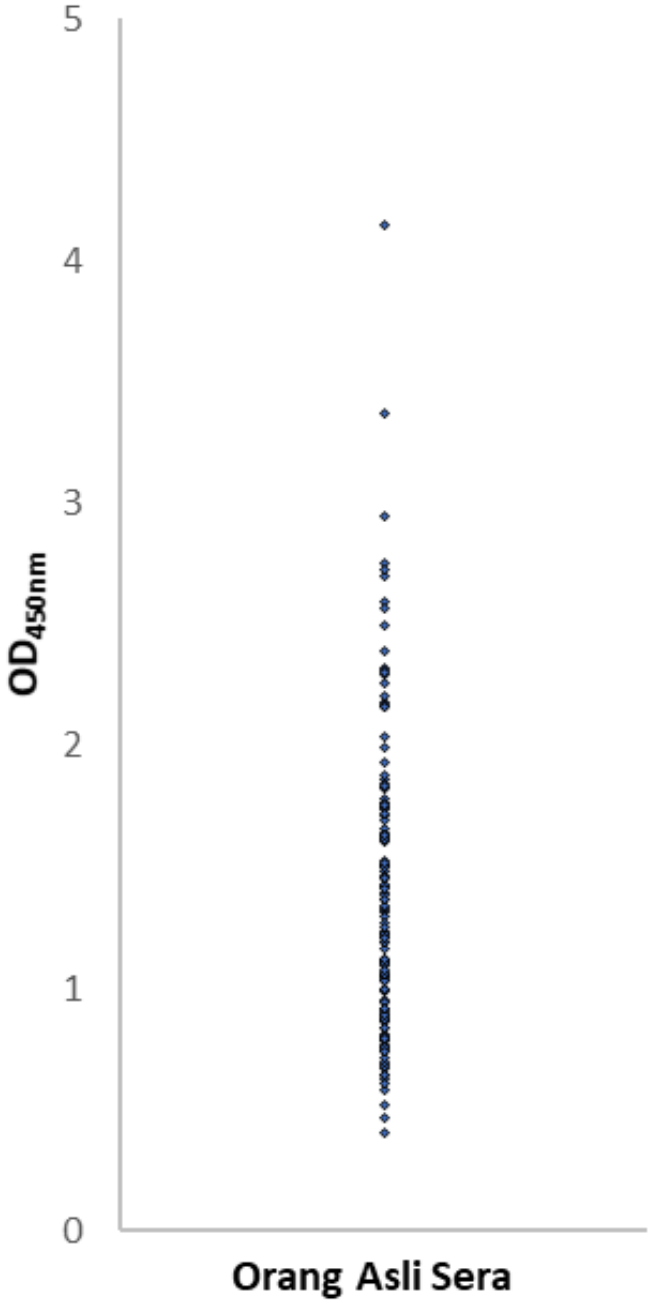
A total of 150 Orang Asli serum samples were screened with the developed *G. lamblia* CSA IgG-ELISA. The scattered plot of the OD_{450nm} readings is presented in Figure 3.9. From the plot, 31.3% of the readings were below 1; 54.7% of the readings were between 1 and 2, and 14.0% of the readings were above 2.

3.5 Subjects' Demographics and ELISA OD_{450nm} readings

The association between subjects' age and ELISA OD_{450nm} readings is as shown in Table 3.2. There was no significant association shown between the two variables. The ELISA OD_{450nm} readings between genders were compared via independent t-test (Table 3.3). Similarly, there was no significant difference in ELISA OD_{450nm} readings between genders.

Table 3.1 Optimized parameters for CSA-IgG-ELISA

Variables	**Parameters**
Coating Antigen Concentration	10 µg/mL
Serum Dilution in PBS	1:50
HRP-conjugated Secondary Ab Dilution in PBS	1:6000



OD_{450nm}	N	%
0.00-0.99	47	31.3
1.00-1.99	82	54.7
2.00-2.99	19	12.7
>3.00	2	1.3

Figure 3.9 IgG-ELISA OD_{450nm} readings of Orang Asli serum samples (n=150)

Table 3.2 Correlation between subjects' age and ELISA OD_{450nm}

Variable	OD_{450nm}		
	r	**Strength**	**P value**
Age (25-64 years)	0.014	Poor	0.868

Note: Pearson's correlation; Statistically significant if $p<0.05$.

Table 3.3 Comparison of ELISA OD_{450nm} between genders

Variable	N	OD_{450nm}		
		Mean±SD	Mean Difference	P value
Gender				
Male	68	1.3961±0.6023	0.3523	0.728
Female	82	1.3609±0.6289		

Note: Independent t-test; Statistically significant if $p<0.05$.

3.6 Association between Real-Time PCR and ELISA OD_{450nm} Readings

The ELISA OD_{450nm} readings between positive and negative RT-PCR samples were compared via the Mann-Whitney Test (Table 3.4). Similarly, there was no significant difference in ELISA OD_{450nm} readings between the two groups of samples.

Table 3.4 Comparison of ELISA OD_{450nm} between RT-PCR positive and negative samples

Variable	N	OD_{450nm}		
		Mean±SD	**Mean Difference**	**P value**
RT-PCR				
Positive	52	1.3870±0.6020	0.3839	0.227
Negative	4	1.0030±0.2581		

Note: Mann-Whitney Test; Statistically significant if $p<0.05$.

CHAPTER 4

DISCUSSION

Giardiasis remains an unresolved public health problem in Orang Asli communities. Infected individuals may stay as asymptomatic carriers or experience a severe disease with problems of fat malabsorption and lactose intolerance (Buret, 2008). Orang Asli children harbouring the disease are associated with protein-energy malnutrition, vitamin A deficiency, iron deficiency anaemia, zinc deficiency and poor cognitive and educational performance (Berkman et al., 2002; Gendrel et al., 2003; Al-Mekhlafi et al., 2010; Quihui et al., 2010; Al-Mekhlafi et al., 2013; Wong et al., 2016).

To better control the disease, epidemiological data of the disease plays a pivotal role; identification of high-risk group, source of pathogen and highly endemic areas could aid in ceasing the disease spreading (Choy et al., 2014). Data pertinent to associations of the disease with age and genders could help in educating the high risks group and provide insight into better disease control measures (Cedillo-Rivera et al., 2009; Soares and Tasca, 2016). Choy et al. (2014) indicated that giardiasis is more common in children below 12, but others showed that adults have the equal odds of harbouring the infection (Anuar et al., 2012; Painter et al., 2015). In the present study, no significant difference in anti-*G. lamblia* antibody between the two genders. No association between antibody titre and age of study subjects as well. The results suggested that both genders and Orang Asli of different age having the same odds of getting giardiasis. Several risk factors associated with these findings include ingestion of food and/or water contaminated with *Giardia* cysts, lack of proper

sanitary system, poor hygiene practice and low socioeconomic status (Anuar et al., 2012; Al-Mekhlafi et al., 2013; Choy et al., 2014; Lee et al., 2014; Choy, 2016). Furthermore, there was no significant difference in the ELISA OD_{450nm} reading between RT-PCR positive and negative samples. This finding might indicate that both groups were exposed to giardiasis in the past, but RT-PCR only illustrated subjects with active infections.

In Malaysia, current epidemiological data relies on conventional or routine detection methods such as faecal concentration, permanent stained smears, and modified acid-fast staining for the detection of the presence of *G. lamblia* trophozoites in the faecal samples (Al-Mekhlafi et al., 2010; Ngui et al., 2011; Anuar et al., 2012). To obtained results of higher accuracy, advanced molecular methods such as indirect fluorescent assay, faecal antigen detection assay and nucleic acid detection method are being used for the detection of either trophozoites specific DNA or antigen (Garcia and Garcia, 2006; Choy et al., 2014; Lee et al., 2014; Choy, 2016; Soares and Tasca, 2016). These data provide information on individuals harbouring the infection as the parasite's markers such as trophozoites, cysts, antigen, and/or nucleic could be found in the specimens. However, the specimen of epidemiological study often limited to one-off sample examination in a cross-sectional study, as it is challenging in terms of time and the cost incurred to collect follow-up specimens in consecutive days or weeks to improve the accuracy of the research data (Dryden et al., 2006). These data inferred to only the population who were exposed to the disease at a specific time point but not a range of time duration. On the other hand, serological data pertinent to the parasitic disease, particularly IgG, could help in detecting both present and past infection, as the titre of anti-parasite

IgG remains high in the infected individual. There were 68.7% study subjects presented OD_{450nm} reading of at least 1, suggesting a high seroprevalence of anti-*G. lamblia* IgG, as compared to the highest report prevalence of 29.2% determined by microscopy.

In the present finding, many protein bands were shown in the SDS-PAGE protein profile of CSA. The present CSA protein profile was different in terms of band intensity for the proteins ranging from 25 kDa to 260 kDa, from the one published by Guimarães et al. (2002), Reiner and Gillin (1992) and Soliman et al. (1998). For instance, protein bands of 100 kDa and 40 kDa were not observed in the protein profile reported by Guimarães et al. (2002). Besides, previous studies have shown that only certain proteins were antigenic based on Western blot analysis (Reiner and Gillin, 1992; Soliman et al., 1998; Guimarães et al., 2002). Using such a complex protein in ELISA for detection of antibody might lead to varying degrees of assay affinities toward anti-*G. lamblia* antibody. The variations can be contributed by different CSA preparation protocols or even CSA of different batches. An effort on the identification of *Giardia* specific protein markers specific to human giardiasis might be able to improve the enzyme immunoassay. Utilisation of such marker in serological assay would help in standardisation of serological findings and limiting batch-to-batch variations (Flores et al., 2016).

The present study experienced several limitations. First, the present study required a control group from non-endemic areas for the determination of cut-off value for discriminating seropositive and seronegative groups. Second, detection of anti-*G. lamblia* IgG using CSA is highly sensitive due to the use of highly

heterogeneous antigen. However, the effect of batch to batch variation is yet to be examined. Third, a bigger sample size is required to acquire an adequate statistical power in the analysis.

CONCLUSION

In the present study, CSA was prepared from axenically grown *G. lamblia* culture. The CSA was used for the development of an in-house ELISA for detection of anti-*G. lamblia* IgG. The in-house ELISA was used for screening of 150 archived Orang Asli serum samples; there were 68 male samples and 82 female samples. There were 56 samples analysed with RT-PCR; in which 52 were positive and 4 were negative. From the analysis, 68.7% showed ELISA OD_{450nm} readings of at least 1.000. There were no associations between ELISA OD_{450nm} readings and subjects' demographic variables i.e. age and genders. Similarly, there was no significant difference found between ELISA OD_{450nm} readings of RT-PCR positive and negative serum samples. The findings suggested that seroprevalence of anti-giardia IgG could be a better indicator for estimating population exposed to giardiasis, recently and in the past. Analysis of serum samples from a prospective follow-up study can be conducted in the future on selected Orang Asli population who lived in a highly endemic area in order to assess the time duration required for seroconversion post-treatment. This will provide insight into the usefulness of serological assay on the control of giardiasis.

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APPENDICES

APPENDIX 1HUMAN ETHICAL APPROVAL

PEJABAT DEKAN
DITERIMA
12 SEP 2018

UNIVERSITI SAINS MALAYSIA — USM APEX — JEPeM JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian, Kelantan, Malaysia
T : (6)09-767 3000/2354/2362
F : (6)09-767 2351
E : jepem@usm.my
L : www.jepem.kk.usm.my
www.usm.my

4th September 2018

Prof. Dr. Norazmi Mohd Noor
Dean
School of Health Sciences
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan

JEPeM USM Code: USM/JEPeM/[247.3(9)]

Study Protocol Title: Host Immunogenetics and Susceptibility to M. tuberculosis – Towards a Rational Design of Vaccines against M. tuberculosis.

Dear Prof:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) acknowledged receipt of Continuing Review Application dated 5th August 2018.

Upon review of JEPeM-USM Form 3(B) 2017: Continuing Review Application Form, the committee's decision for the **EXTENSION OF APPROVAL IS APPROVED (start from 1st September 2018 till 31st August 2019)**. The report is noted and has been included in the protocol file.

Thank you for your continuing compliance with the requirements of the JEPeM-USM.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

(ASSOC. PROF. DR. AZLAN HUSIN)
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

Ams (12A).

17/9/18

PROFESOR DR. NORAZMI MOHD NOR
Dekan
Pusat Pengajian Sains Kesihatan
Kampus Kesihatan
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

CERTIFIED BY: NPRA MALAYSIA — National Pharmaceutical Regulatory Agency (NPRA) | FERCAP — Forum for Ethical Review Committees in Asia & Western Pacific Region

UNIVERSITI SAINS MALAYSIA

JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

DITERIMA
12 SEP 2018

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian, Kelantan, Malaysia
T : (6)09-767 3000/2354/2362
F : (6)09-767 2351
E : jepem@usm.my
L : www.jepem.kk.usm.my
www.usm.my

4th September 2018

Prof. Dr. Norazmi Mohd Noor
Dean
School of Health Sciences
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan.

JEPeM USM Code: USM/JEPeM/[247.3(9)]

Study Protocol Title: Host Immunogenetics and Susceptibility to *M. tuberculosis* – Towards a Rational Design of Vaccines against *M. tuberculosis*.

Dear Prof:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) approved the proposed amendments in your study entitled, **"Host Immunogenetics and Susceptibility to *M. tuberculosis* – Towards a Rational Design of Vaccines against *M. tuberculosis*" [USM/JEPeM/[247.3(9)]** during its meeting on 16th August 2018.

Upon review of JEPeM-USM FORM 3(A) 2017: Study Protocol Amendment Submission Form, the following amendments have been approved:

1. Added co-investigator –
   (i) Dr. Wong Weng Kin – who works on TB-Parasite co-infection
   (ii) Nur Hidayati Mohd Balia, a new research assistant of the project
   (iii) Nurul Syahidah Shaffee, a new research assistant of the project.

Thank you.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

(ASSOC. PROF. DR. AZLAN HUSIN)
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

Asma (CRA)
17/9/18
PROFESOR DR. NORAZMI MOHD NOR
Dekan
Pusat Pengajian Sains Kesihatan
Kampus Kesihatan
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

CERTIFIED BY: National Pharmaceutical Regulatory Agency (NPRA) | Forum for Ethical Review Committees in Asia & Western Pacific Region

BAHAGIAN PERANCANGAN DAN PENYELIDIKAN
JABATAN KEMAJUAN ORANG ASLI MALAYSIA
(KEMENTERIAN KEMAJUAN LUAR BANDAR DAN WILAYAH)
TINGKAT 3, WEST BLOK
WISMA SELANGOR DREDGING
142-C, JALAN AMPANG
50548 KUALA LUMPUR
TELEFON : 03-2161 0577 / FAKSIMILI : 03-2164 8920

KIRIMAN FAKSIMILI

NO. FAKSIMILI PENERIMA	:		TINDAKAN: BIASA [ ] SEGERA [ ] SERTA MERTA [√]
TARIKH DIHANTAR	:	17/2/2012	
KEPADA	:	Prof Dr Norazmi Mohd Nor	
DARIPADA	:	*Rogayah Bhg. Perancangan& Penyelidikan Ibupejabat*	
BIL. MUKA SURAT	:	5	
PERKARA / TAJUK	:	*Kelulusan menjalankan kajian/program/ lawatan keperkampungan Orang Asli*	
RUJUKAN SURAT	:	*Seperti surat yang disertakan*	
PESANAN	:	*Sila akui penerimaan fax ini seperti talian di atas ext:328*	

TANDATANGAN

JABATAN KEMAJUAN ORANG ASLI MALAYSIA
(KEMENTERIAN KEMAJUAN LUAR BANDAR DAN WILAYAH)
TINGKAT 3,4,10 & 20, WEST BLOCK,
WISMA SELANGOR DREDGING,
142 – C, JALAN AMPANG,
50548 KUALA LUMPUR.

JAKOA

Telefon : 03-21610577
03-21610994-8(5 talian)
Gomsak : 03-61892122
Fax : 03-21621470 (IP)
03-61863160 (GBK)
Laman Web : www.jakoa.gov.my

Ruj. Kami : JAKOA.PP.30.052 Jld. 5 (73)
Tarikh : 23 R. Awal 1433H
16 Februari 2012

Ketua Program LRGS Perubatan Tropikal
Pusat Pengajian Sains Kesihatan
Kampus Kesihatan
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.
(u/p:Prof. Dr.Norazmi Mohd Nor)

Tuan

KEBENARAN MENJALANKAN PROJEK PENYELIDIKAN PENYAKIT TIBI DAN CACING TULARAN TANAH DI KALANGAN ORANG ASLI DI MALAYSIA

Dengan hormatnya saya diarah merujuk kepada perkara tersebut di atas.

2. Sukacita dimaklumkan bahawa Jabatan ini tiada halangan untuk memberi kebenaran kepada pihak tuan bagi menjalankan kajian bertajuk **"Tropical Tuberculosis: Delineating Host-Environment-Pathogen Interactions"**, bagi mengenalpasti imunogenetik seseorang yang menghadapi infeksi cacing/protoza di perkampungan Orang Asli.

3. Untuk makluman pihak tuan, JAKOA telahpun menyerahkan Hospital Orang Asli kepada Kementerian Kesihatan Malaysia (KKM) yang berkuat kuasa 1 Januari 2012. Oleh itu pihak tuan perlulah mendapat persetujuan daripada Kementerian Kesihatan Malaysia. Kelulusan ini juga hanya tertakluk di penempatan Orang Asli sahaja. Sebarang pemasalahan yang melibatkan pentadbiran Hospital perlulah tuan merujuk kepada pihak yang berkenaan. Pihak tuan dibenarkan menjalankan kajian tersebut pada tarikh dan tempat yang telah ditetapkan seperti berikut:-

Ruj. Kami : JAKOA.PP.30.052 Jld. 5 (

Tempat	:	i. Hospital Orang Asli, Kementerian Kesihatan Malaysia (KKM) ii. Semua Transit Orang Asli, KKM semua negeri iii. Perkampungan Orang Asli di Semenanjung Malaysia
Tarikh	:	Februari 2012 - Februari 2013
Penyelidik	:	Di lampiran A

3. Untuk makluman pihak tuan juga, kebenaran hanya diberikan dalam tempoh setahun sahaja sebagaimana prosedur Jabatan sedia ada. Sehubungan dengan itu, pihak tuan adalah dipohon untuk mengemukakan notis permohonan perlanjutan tempoh kajian kepada pihak Jabatan sebulan sebelum tempoh kajian tamat.

5 Pihak tuan juga diminta supaya capat mematuhi syarat-syarat seperti mana terkandung dalam borang permohonan seperti dilampiran Appendix 1. Di samping itu, pihak tuan juga diminta mengemukakan 2 salinan *hard copy* dan 1 salinan *soft copy* hasil penyelidikan kepada JAKOA Ibu Pejabat **(Bahagian Perancangan dan Penyelidikan)**.

6 Sehubungan itu, pihak tuan boleh menghubungi Pegawai Jabatan Kemajuan Orang Asli Negeri dan Daerah yang berkaitan untuk mendapatkan maklumat lanjut mengenai perkara di atas. Kerjasama pihak tuan berhubung perkara di atas amat dihargai dan diucapkan terima kasih.

Sekian,

" BERKHIDMAT UNTUK NEGARA "

" KOMUNITI BERDAYA DESA BERJAYA "

Saya yang menurut perintah,

(SARGI BIN BAKAM)

Bahagian Perancangan dan Penyelidikan
b.p Ketua Pengarah
Jabatan Kemajuan Orang Asli Malaysia

Ruj. Kami : JAKOA.PP.30.052 Jld. 5 (93)

s.k - *Pengarah Hospital Orang Asli, Gombak (No.Tel: 03-61892669)*

- *Semua Transit Orang Asli di semua Negeri*

- *Pengarah JAKOA semua negeri* - *Selangor/W.Persekutuan*
Kelantan/Terengganu
Perak/Kedah
N/Sembilan/Melaka
Pahang
Johor

#7034 P.005/005

Ruj. Kami : JAKOA.PP.30.052 Jld. 5 (93)

Lampiran A

Nama Penyelidik

1. Prof. Dr. Norazmi Mohd Nor
2. Dr. Lim Boon Huat
3. En. Wong Weng Kin
4. Dr. Siti Suraiya Md Noor
5. Dr. Vasantha Kumari Neela
6. Dr. Aziah Ismail
7. Prof. Rahmah Noordin
8. Dr. Zary Shariman Yahaya
9. Dr. Syafinaz Amin Nordin

APPENDIX 2 BUFFER AND REAGENT PREPARATIONS

<table>
<tr><th>Buffer/Reagent/Glassware</th><th>Description</th></tr>
<tr><td>Ammonium Persulfate, APS, 20%</td><td>0.02 g of APS salt was freshly mixed with 1 mL of dH_2O prior to being used.</td></tr>
<tr><td>Electrode Buffer, 10X</td><td>30 g of Tris base, 144 g glycine and 10 g of SDS were mixed in 800 mL of dH_2O. After that, the buffer was made up to the final volume of 1 L and stored at 4 °C. The 1X electrode buffer was freshly made by mixing 1 part of 10X electrode buffer with 9 part of dH_2O.</td></tr>
<tr><td>ELISA Coating Buffer (0.05 M Sodium Carbonate, 0.02% NaN_3)</td><td>1.465 g $NaHCO_3$, 0.795 g Na_2CO_3 and 0.1 g of sodium azide were dissolved in 300 mL of dH_2O. The pH was adjusted to 9.6 and made up to final volume of 500 mL.</td></tr>
<tr><td>ELISA Stop Solution (0.5 M H_2SO_4)</td><td>2.788 mL H_2SO_4 was diluted to a final volume of 100 mL with dH_2O.</td></tr>
<tr><td>Ethanol, 70%</td><td>A volume of 700 mL absolute ethanol was mixed with 300 mL of dH_2O.</td></tr>
<tr><td>Gel mixtures for SDS-PAGE</td><td>
<table>
<tr><th>Ingredient</th><th>Resolving Gel
10%</th><th>Stacking Gel
3%</th></tr>
<tr><td>Resolving buffer, 4X</td><td>2.5 mL</td><td>-</td></tr>
<tr><td>Stacking buffer, 4X</td><td>-</td><td>1.25 mL</td></tr>
<tr><td>dH_2O</td><td>3.96 mL</td><td>3.27 mL</td></tr>
<tr><td>Acrylamide (29:1), 30 %</td><td>3.33 mL</td><td>0.375 mL</td></tr>
<tr><td>10 % SDS</td><td>0.1 mL</td><td>0.05 mL</td></tr>
<tr><td>10 % APS</td><td>0.1 mL</td><td>0.05 mL</td></tr>
<tr><td>TEMED</td><td>0.01 mL</td><td>0.005 mL</td></tr>
<tr><td>Total</td><td>10 mL</td><td>5 mL</td></tr>
</table>
</td></tr>
<tr><td>Heat-Inactivated Bovine Serum</td><td>Frozen bovine serum was thawed at 37°C and then incubated at 56°C for 30 min. The heat-inactivated serum was then aliquoted and stored at -20°C until used.</td></tr>
<tr><td>Iodoacetamide, 0.5 M</td><td>0.185 g of iodoacetamide was dissolved in 2 mL of dH_2O and stored at 4°C until used.</td></tr>
</table>

Buffer/Reagent/Glassware	Description
Luria-Bertani, LB Broth	An amount of 12.5 g LB powder (Merck, Germany) was dissolved in 500 mL of dH_2O and adjusted to pH 7.0. The solution was autoclaved at 121°C for 15 min and kept at 4°C. Antibiotic was added accordingly if needed after the medium was cooled to 55°C.
Phosphate Buffered Saline, PBS (10X)	A total of 2 g KCl, 80 g NaCl, 11.375 g Na_2HPO4, and 2 g KH2PO4 was dissolved in 800 mL of dH_2O. pH was adjusted to 7.2 and the final volume was made up to 1 L with dH_2O. 1X PBS working solution was made by mixing one part of 10X stock solution with nine part of dH_2O.
Phosphate Buffered Saline-Tween 20, PBS-T	PBS-T, 1X was made by diluting 100 mL of PBS, 10X in 800 mL of dH_2O. 500 µL of Tween-20 solution was added into the solution and made up to a final volume of 1 L with dH_2O.
RAMA Stain	**a. CBB 250/Methanol Stain, 4X** 1 g CBB R250 was dissolved in 300 mL of methanol and made up to a final volume of 500 mL with dH_2O. **b. 30% Ammonium Sulfate, 10X** 15 g ammonium sulphate was dissolved in 30 mL of dH_2O, and made up to a final volume of 50 mL with dH_2O. **c. RAMA Stain Working Solution** 25 mL of CBB 250/methanol stain (4X), 10 mL of 30% ammonium sulphate (10X) and 10 mL of acetic acid were mixed together and made up to the final volume of 100 mL.
Roche Complete Lysis-M buffer, Without EDTA	One tablet of cocktail protease inhibitor (Roche, Germany) was dissolved in 10 mL of lysis-M buffer (Roche, Germany) and stored at 4 °C until used.
Sample Buffer, 2X	Sample buffer (2X) was prepared by mixing 3.2 mL of dH_2O, 1.0 mL of stacking buffer, 1.6 mL of glycerol, 1.6 mL of 10% SDS and 0.2 mL of 0.05% Bromophenol blue in a 15 mL centrifuge tube. The mixture was then aliquoted into eight microcentrifuge tubes in which each tube was filled with 0.95 mL sample buffer and stored at –20°C. Fifty µL of *β-mercaptoethanol was freshly added into each tube prior to use.*

Buffer/Reagent/Glassware	Description
SDS-PAGE Resolving Buffer	54.45 g Tris base was dissolved in 150 mL of dH_2O. The pH was adjusted to 8.8 with HCl and made up to the final volume of 300 mL with dH_2O. The buffer was autoclaved and stored at 4°C until used.
SDS-PAGE Stacking Buffer	6 g Tris base was dissolved in 60 mL of dH_2O. The pH was adjusted to 6.8 with HCl and made up to the final volume of 100 mL with dH_2O. The buffer was autoclaved and stored at 4°C until used.
Sodium Dodecyl Sulfate, SDS, 10 %	10 g of SDS powder was dissolved in 80 mL of dH_2O and made up to the final volume of 100 mL with dH_2O.
Sodium Hydroxide Solution, 0.5 M	2 g of sodium hydroxide pellet was dissolved in 50 mL of dH_2O and the solution was made up to the final volume of 100 mL with dH_2O. The solution was filter-sterilized and stored at 4°C until used.
Modified TYI-S-33	Solution A and solution B were made to prepare incomplete TYI-S-33. Solution A was prepared by dissolving 30 g of Biosate peptone in 500 mL of distilled water (dH_2O). Solution B was prepared by dissolving 0.75g bile salt, 1.0 g of potassium phosphate dibasic, 0.6 g of potassium phosphate monobasic, 2 g of sodium chloride, 1 g of L-cysteine and 0.2 g of ascorbic acid in 370 mL of dH_2O. Both solution A and B were mixed together, and 22.5 mg of ammonium iron (III) citrate were added. The pH was then adjusted to 6.8. The mixture was filtered sterilised with 22 µm membrane. Complete modified TYI-S-33 was made by adding 125 mL of heat inactivated bovine serum and 25 mL of Diamond vitamin Tween 80 (40X). The complete medium was then aliquoted and stored at -20°C until used.

APPENDIX 3EXPERIMENTAL DATA

Experiment 1: Screening of 96 Orang Asli Serum Samples

ELISA Parameters:

Coating antigen concentration : 10 µg/mL in coating buffer
Serum dilution : 1:50 in PBS
HRP-Conjugated antibody : 1:6000 in PBS

Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
C	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
D	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
E	S49	S50	S51	S52	S53	S54	S55	S56	S57	S58	S59	S60
F	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70	S71	S72
G	S73	S74	S75	S76	S77	S78	S79	S80	S81	S82	S83	S84
H	S85	S86	S87	S88	S89	S90	S91	S92	S93	S94	S95	S96

Readings

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8633	0.6711	1.1683	1.1227	0.6415	1.4568	1.4170	2.5982	1.2311	1.3650	1.5021	0.9425
B	1.8269	0.8074	1.8585	0.8700	1.7516	1.6370	1.5062	2.1815	0.9952	1.7383	0.7906	0.8940
C	0.5155	2.3893	1.2276	0.4046	0.8763	1.1237	0.4639	1.4207	1.1072	1.0498	2.2634	2.7029
D	0.7625	1.7512	0.8011	0.8370	0.7839	0.9845	0.6270	1.1196	1.7109	1.9328	1.1867	0.5822
E	1.0372	0.9084	0.7820	1.8478	0.6063	1.6230	1.0004	1.1091	2.3236	2.7536	0.9940	1.1032
F	0.9515	0.6845	1.0334	0.7536	0.6766	1.0954	1.2143	0.6954	0.7400	0.6452	0.7527	0.8808
G	0.8048	1.5232	1.3135	1.7662	1.6072	1.3218	1.1871	0.7154	0.7905	1.2951	0.8605	0.6404
H	0.9014	1.6561	1.3977	2.7285	1.9954	1.3870	1.2241	0.8718	1.4257	0.7611	1.4133	1.2660

Experiment 2: Optimization of coating antigen concentration

ELISA Parameters

Coating antigen concentration : Varying concentrations in coating buffer (80-0.625 µg/mL)
Serum dilution : 1:50 in PBS
HRP-Conjugated antibody : 1:6000 in PBS

Layout

	Pooled Positive		Pooled Negative		PBS Control							
	1	2	3	4	5	6	7	8	9	10	11	12
A	80 µg/mL	80 µg/mL	80 µg/mL	80 µg/mL	80 µg/mL	80 µg/mL						
B	40 µg/mL	40 µg/mL	40 µg/mL	40 µg/mL	40 µg/mL	40 µg/mL						
C	20 µg/mL	20 µg/mL	20 µg/mL	20 µg/mL	20 µg/mL	20 µg/mL						
D	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL						
E	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL						
F	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL						
G	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL						
H	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL						

Readings

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.3074	2.2709	0.7114	0.7256	0.1618	0.1607						
B	2.1877	2.4367	0.7092	0.6985	0.1484	0.1557						
C	2.2476	2.2989	0.6727	0.6860	0.1443	0.1477						
D	2.2638	2.3538	0.6499	0.6766	0.1362	0.1415						
E	2.2708	2.3116	0.6688	0.6563	0.1371	0.1352						
F	2.3391	2.3691	0.6074	0.5734	0.1107	0.1116						
G	2.3342	2.3762	0.5422	0.5033	0.0790	0.0823						
H	2.3269	2.5010	0.5357	0.4978	0.0652	0.0677						

Experiment 3: Optimization of human sera dilutions

ELISA Parameters

Coating antigen concentration : 10 µg/mL
Serum dilution : Varying dilutions in PBS (1:25 to 1:3200)
HRP-Conjugated antibody : 1:6000 in PBS

Layout

	Pooled Positive		Pooled Negative		PBS Control							
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:25	1:25	1:25	1:25	-	-						
B	1:50	1:50	1:50	1:50	-	-						
C	1:100	1:100	1:100	1:100	-	-						
D	1:200	1:200	1:200	1:200	-	-						
E	1:400	1:400	1:400	1:400	-	-						
F	1:800	1:800	1:800	1:800	-	-						
G	1:1600	1:1600	1:1600	1:1600	-	-						
H	1:3200	1:3200	1:3200	1:3200	-	-						

Readings

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.3646	3.5919	1.2913	1.3410	0.1708	0.1792						
B	2.9999	2.8226	0.9567	0.9578	0.1494	0.1641						
C	2.1378	2.1979	0.6609	0.7280	0.1585	0.1579						
D	1.5902	1.5745	0.5022	0.5413	0.1577	0.1591						
E	1.0282	1.0593	0.3921	0.4205	0.1610	0.1519						
F	0.8097	0.7978	0.3188	0.3225	0.1493	0.1602						
G	0.5422	0.5497	0.2805	0.2751	0.1639	0.1567						
H	0.4051	0.4063	0.2267	0.2381	0.1652	0.1696						

Experiment 4: Optimization of HRP-conjugated secondary antibody dilutions

ELISA Parameters

Coating antigen concentration : 10 µg/mL in coating buffer
Serum dilution : Dilution of 1:50 in PBS
HRP-Conjugated antibody : Varying dilutions in PBS (1:750 to 1:96000)

Layout

	Pooled Positive		Pooled Negative		PBS Control							
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:750	1:750	1:750	1:750	1:750	1:750						
B	1:1500	1:1500	1:1500	1:1500	1:1500	1:1500						
C	1:3000	1:3000	1:3000	1:3000	1:3000	1:3000						
D	1:6000	1:6000	1:6000	1:6000	1:6000	1:6000						
E	1:12000	1:12000	1:12000	1:12000	1:12000	1:12000						
F	1:24000	1:24000	1:24000	1:24000	1:24000	1:24000						
G	1:48000	1:48000	1:48000	1:48000	1:48000	1:48000						
H	1:96000	1:96000	1:96000	1:96000	1:96000	1:96000						

Readings

	1	2	3	4	5	6	7	8	9	10	11	12
A	6.0000	6.0000	2.4468	2.4629	0.2871	0.3094						
B	5.1972	5.2282	1.8819	2.0100	0.2172	0.2469						
C	3.8240	3.8493	1.3367	1.3249	0.1860	0.1878						
D	2.4450	2.5667	0.8334	0.8409	0.1464	0.1467						
E	1.4304	1.4754	0.4798	0.4856	0.1133	0.1127						
F	0.6813	0.7186	0.2694	0.2638	0.0854	0.0876						
G	0.3959	0.4084	0.1666	0.1651	0.0695	0.0703						
H	0.2119	0.2182	0.1050	0.1038	0.0588	0.0633						

Experiment 5: Screening of 150 Orang Asli Serum Samples

ELISA Parameters:

Coating antigen concentration : 10 µg/mL in coating buffer
Serum dilution : 1:50 in PBS
HRP-Conjugated antibody : 1:6000 in PBS

Layout

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
C	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
D	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
E	S49	S50	S51	S52	S53	S54	S55	S56	S57	S58	S59	S60
F	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70	S71	S72
G	S73	S74	S75	S76	S77	S78	S79	S80	S81	S82	S83	S84
H	S85	S86	S87	S88	S89	S90	S91	S92	S93	S94		

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	P+	P-	S95	S96	S97	S98	S99	S100	S101	S102	S103	S104
B	S105	S106	S107	S108	S109	S110	S111	S112	S113	S114	S115	S116
C	S117	S118	S119	S120	S121	S122	S123	S124	S125	S126	S127	S128
D	S129	S130	S131	S132	S133	S134	S135	S136	S137	S138	S139	S140
E	S141	S142	S143	S144	S145	S146	S147	S148	S149	S150	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Readings

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8633	0.6711	1.1683	1.1227	0.6415	1.4568	1.4170	2.5982	1.2311	1.3650	1.5021	0.9425
B	1.8269	0.8074	1.8585	0.8700	1.7516	1.6370	1.5062	2.1815	0.9952	1.7383	0.7906	0.8940
C	0.5155	2.3893	1.2276	0.4046	0.8763	1.1237	0.4639	1.4207	1.1072	1.0498	2.2634	2.7029
D	0.7625	1.7512	0.8011	0.8370	0.7839	0.9845	0.6270	1.1196	1.7109	1.9328	1.1867	0.5822
E	1.0372	0.9084	0.7820	1.8478	0.6063	1.6230	1.0004	1.1091	2.3236	2.7536	0.9940	1.1032
F	0.9515	0.6845	1.0334	0.7536	0.6766	1.0954	1.2143	0.6954	0.7400	0.6452	0.7527	0.8808
G	0.8048	1.5232	1.3135	1.7662	1.6072	1.3218	1.1871	0.7154	0.7905	1.2951	0.8605	0.6404
H	0.9014	1.6561	1.3977	2.7285	1.9954	1.3870	1.2241	0.8718	1.4257	0.7611	-	-

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.3198	1.2276	1.8765	1.6313	2.1716	1.1057	0.8869	1.0515	1.0630	3.3752	1.6173	2.2964
B	0.9468	1.4841	1.3366	1.2710	4.1511	1.5101	1.0597	2.3116	1.2060	1.0332	1.0586	1.3404
C	2.2087	1.7846	0.9159	0.9189	1.2063	2.1660	1.6355	0.7384	2.1623	2.9530	1.6106	1.6307
D	1.5055	1.4105	1.3677	1.7691	1.5023	2.4988	1.4690	0.8366	2.0366	1.7589	1.6965	2.5730
E	1.7167	1.8275	1.4517	1.0719	1.1188	1.2512	1.4532	2.3022	1.8322	1.5147	0.1331	0.1311
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-