Faculty of Resource Science and Technology

Assessment of *Cryptosporidium* and Helminth Contamination in Vegetables in Kuching, Sarawak

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Bachelor of Science with Honors
(Resource Biotechnology)
2017

ACKNOWLEDGEMENT

In the name of 'Allah', the most beneficent and merciful who gave me strength and knowledge to complete this project. This project has given me the most knowledge and experience in Biotechnology life. This has proved to be a greatexperience.

I would like to express my gratitude to my supervisor, Dr. Lesley Maurice Bilung and my co-supervisor Dr. Elexson Nillian who gave me this opportunity to fulfil this project. They gave me moral support and guided in different matters regarding the project. They had been supportive and patient throughout my progress in this project.

Truthfully thanks to my beloved family particularly to my parents, Tunku Syed Azhar bin Syed Mohamed and Nursyaza Lin binti Abdullah that have been fully support throughout this project and biotechnology course. They have been mentally, spiritually and financially support me, indirectly encourage me to move on in this life. My profound gratitude to my lab mate project members, Syatir bin Tahar and Nur Emyliana binti Yunos for the outmost encouragement for me to move along this project. I also would like to thank all my biotechnology friends, my best friends Nazreen, Ammar, Vjay, Munashamira, Nuranie, Raudah, Mohd Nasharuddin and Valarie Noormylisza for the mental support. Thank all of you.

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LIST OF ABREVIATION

μm	-	Micrometer
°C	-	Degree Celcius
mL	-	Millilitre
μL	-	Microlitre
mm	-	Millimeter

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Assessment of *Cryptosporidium* and Helminths Contamination in Vegetables in Kuching, Sarawak

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ABSTRACT

Vegetables are edible in both raw and cooked depending on vegetable types. It supplies good values of nutrition to human health and yet also can be the main vehicle of transmission for parasites by which can cause gastrointestinal illness. Thirty two vegetable samples taken from 10 different locations were washed in saline solution with physical rub and were let for 24 hours sedimentation. The top solution was discarded and only the bottom sediment was concentrated by centrifugation before the samples were brought to sucrose flotation technique. Modified Zeihl-Neelsen technique and Lugol's iodine solution were applied to *Cryptosporidium* and helminths detection, respectively. Lastly, the processed samples were observed under a compound microscope for confirmation of morphology, physical size and identification types of parasite. The parasite most encountered was *Ascaris* ova (29.4%) and followed by Hookworm ova (23.5%), *Toxocara* ova (11.8%), and *Strongyloides* ova (5.9%) from the vegetable samples in organic farms and retail markets in Kuching, Sarawak. *Ascaris* ova, *Toxocara* ova and hookworm ova were found positive to most ground type of vegetables. Further study are recommended on detection and enumeration of these parasites for future food security and health concerns.

Keywords: *Cryptosporidium*, Soil-transmitted Helminth, Organic Farms, modified Zeihl-Neelsen technique, Lugol's iodine solution

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ABSTRAK

Sayur-sayuran boleh dimakan sama ada secara mentah atau dimasak bergantung kepada jenis sayur-sayuran. Ia menyediakan nutrisi yang berkhasiat kepada manusia akan tetapi juga salah satu peredaran parasit yang mampu memberi penyakit gastrousus. Sebanyak 32 sampel sayur-sayuran telah dipungut dari 10 lokasi ladang yang berbeza untuk dibasuh dalam larutan garam disertai dengan gosokan fizikal dan dibiarkan untuk 24 jam pemendapan. Seterusnya, larutan atas telah dibuang dan hanya mendapan bawah telah dimampatkan melalui pengemparan sebelum sampel-sampel terbabit dibawa ke pengampungan sukrosa. Kemudian, teknik Zeihl-Neelsen terubahdan larutan Lugol's iodin telah digunakan untuk pengesahan Cryptosporidium dan helmin. Akhir sekali, sampel yang telah di proses telah dibawa ke mikroskop kompaun untuk pengesahan morfologi, saiz dan pengenalan jenis parasite. Parasit yang paling banyak dijumpai adalah telur Ascaris (29.4%) dan diikuti dengan telur Hookworm *(23.5%),* telur *Toxocara (11.8%),* dan telur *Strongyloides (5.9%) dalam sayur-sayuran ladang organik dan di pasaran Kuching, Sarawak. Telur-telur Ascaris, Toxocara, dan hookwormtelah dijumpai positif dalam sayur-sayuran yang jenis tanah. Maka dari itu, Cryptosporidium oocyst adalah susah dijumpai dalam sayur-sayuran tetapi helmin adalah mudah. Disarankan dalam aspek lanjutan pembelajaran untuk pengesahan dan penghitungan untuk parasite tersebut dalam tujuan masa depan keselamatan makanan dan masalah kesihatan*

Kata kunci: *Cryptosporidium,* Soil-transmitted Helminth, *Ladang organik, teknik Zeihl-Neelsen, larutan Lugol's iodine.*

INTRODUCTION

1.1 Background Study

Vegetables are an essential food for human to consume in respect of their best nutritional value. Some of vegetables are eaten raw to retain natural taste and preserve heat labile nutrients (Amoah, Drechsel, Abaidoo & Klutse, 2007). Global production of vegetables has increased to 9.4% in 2012 from 2003 which 1.74 billion tons of vegetables being recorded each year (Agriculture Market Information, 2014). Current concern around the globe is the disease that human can get by consuming vegetables, especially intestinal diseases.

Helminthic infection is closely related to consumption of contaminated vegetables and is endemic in many areas and it has been the principal to occur in poor environment sanitation country and inadequate personal hygiene (Hoek *et al.,* 2003). This problem has been increasingly concerned because of the expanding number of susceptible people, increase production of trade across international borders and change in national and international policies regarding food safety involvement (Akhlaghi & Oormazdi, 2000). Helminths basically populate in soil and have put up to high chance of transmission to human that gives no disregard aboüt it peculiarly on children (Uga *et al.,* 2005). Abougrain *et al.* (2010) reported foodborne illness linked to vegetables and fruits consumption that plays a major role in the transmission of parasitic foodborne diseases.

A protozoa of the genus *Cryptosporidium* can cause cryptosporidiosis had been a major source of human illness from 2001 to 2010 since the first outbreak *Milwaukee Cryptosporidiosis* in 1993 in Milwaukee, Wisconsin due to the parasite contamination in the public water supply (Hlavsa *et al.,* 2014). The treated water was contaminated by *Cryptosporidium* and the turbidity level was above normal during water treatment turbidity detection experimented by MacKenzie *et al.*, (1994). The previous study showed that the possible source of spreading the parasite is through water irrigation by which it is one of the sources that spreads *Cryptosporidium* oocyst throughout the vegetable farm, soil and eventually spread to vegetables (Hong *et al.*, 2014).

Based on the study by Maikai *et al.* (2012), 35% (70/200) of randomly picked vegetables were positive for *Cryptosporidium* oocyst and 40% of cooked foods were confirmed positive with *Cryptosporidium* oocyst. Natividad *et al.* (2015) reported on the significant increment of cryptosporidiosis case infected paediatric patients (2.9%) compared to adult patients (0.2%). Children have a higher risk of infection at the age of 5-9 year old group compared to adult group (Lim, 2008). On the other hand, the prevalence of intestinal helminths in raw vegetables commonly consumed in Khorramabad, Iran was positive with 52.7% and it went to the highest of 80% during spring (Ezatpour *et al.*, 2013). These parasites can lead to serious vegetable contamination that may lead to human infection. Presently, there is no information on parasitic contamination by *Cryptosporidium* and helminths on vegetables in Sarawak, despite of several studies have been conducted in Peninsular Malaysia. Thus, the main purpose of this study is to detect the presence of intestinal parasites (*Cryptosporidium* and helminths) on vegetables in organic farm and retail market level.

The main objectives of this study are:

i. To determine the occurrence of *Cryptosporidium* oocyst and helminth ova from vegetable samples using modified Ziehl-Neelson technique and Lugol's iodine solution respectively.

ii. To compare the distribution of the parasites in vegetables at organic farm and retail market.

LITERATURE REVIEW

2.1 Protozoa classification

Protozoa is a unicellular eukaryotic organism with animal-like behaviours that consist of six phyla Plasmodium, Sarcomastigophora, Retortamonada, Axostylata, Sarcomastigophora and Apicomplexa. Sarcomastigophora and Apicomplexa have the most significant species that can cause human disease. The categorization of these protozoans are based on their morphology, internal structure, criteria of geographic distribution, clinical manifestation, and the host that they infect. Determination of isoenzyme patterns and to identify nucleotide sequence in RNA, DNA or both (Yaeger, 1996).

2.2 Manifestation of *Cryptosporidium*

Based on Saneian *et al.* (2010), *Cryptosporidium parvum* is a well-known pathogenic protozoa of the phylum Apicomplexa as shown in **Figure 1** that can cause moderate to severe gastrointestinal illness, which also known as cryptosporidiosis. This disease was the fourth most frequent cause of diarrhoea during the period noted and it has been estimated that the cause of infections from 7% to 38% of immunocompromised patients, such as AIDS patients (Monroe, 1991). Nowadays, scientists have recognised 26 species as valid on the basis of biological, morphological and molecular data. *Cryptosporidium parvum* and *Cryptosporidium hominis* are the main causative agents of *Cryptosporidium* infection in human (Ryan *et al.* 2014). Borowski *et al.* (2009) have noted that *Cryptosporidium* life cycle completes within a week or two and the invasion starts at early of 48 hours, right after the oocysts have excysted, which is the release of sporozoites from oocyst, especially when they are getting mature within six days as they have microvilli for their mobility. Thus, they are quick in mobility due to its wide spread of surface area in the lining of ileum, that possibility to infect one whole gastrointestinal tract. In immunological aspect, through the invasion of *Cryptosporidium* will trigger the rapid expression of inflammatory chemokines, this response is important for the early development of inflammation (Chen, 2002).

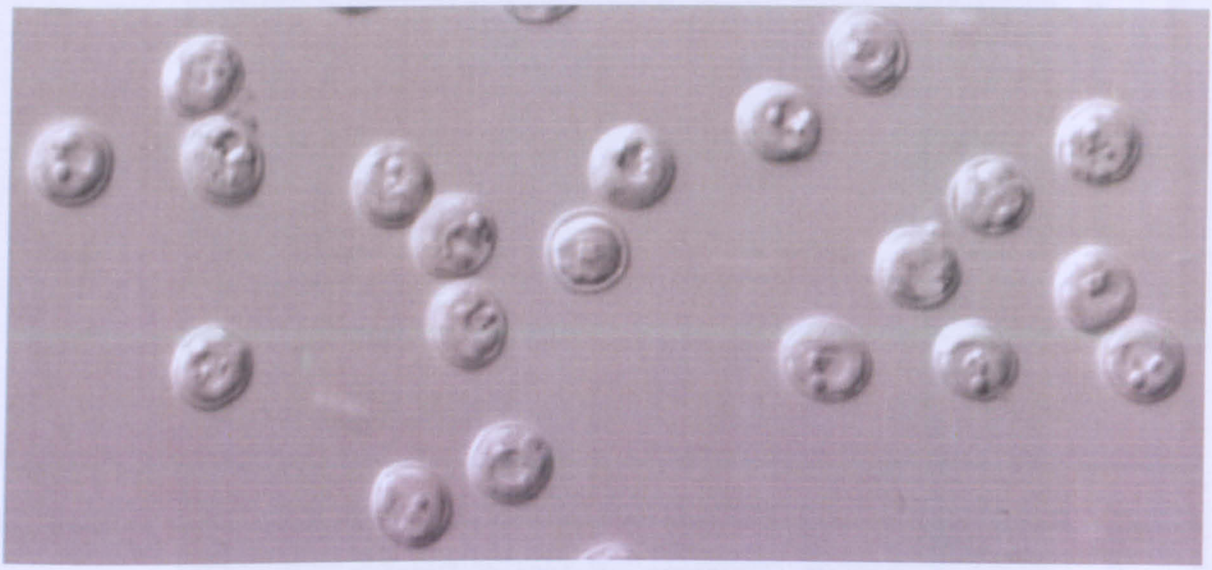

Figure 1: *Cryptosporidium parvum* oocyst (Stone, 2015)

2.3 The *Cryptosporidium* oocyst survivability

Cryptosporidium oocyst is resistant in prolong exposure of environment at any water sources and to many commonly used disinfecting agents such as chlorination, acid disinfectant and dilute bleach (Leitcha, 2011). The surface receptors at *Cryptosporidium* oocysts also play an important role to ensure that this parasite stage is match enough to the host target tissue in the small intestine, specifically at ileum (Smith, 2005). If it is in our gastrointestinal tract, observations by Upton *et al.* (1987), noted that bile salts and 37°C are particularly effective at stimulating excystation, while pancreatic enzymes are not at all. Koh *et al.,* (2014) had illustrated under electron microscope the changes of morphology of *Cryptosporidium* oocyst during excystation within *Cryptosporidium*-exposed biofilms as shown in **Figure 2.** Thus, they are triggered by the *in vitro* acidity environment. However, if taurocholic acid (involved in emulsification of fats) is present, it is particularly effective if the oocysts are to be exposed to initial acidified (Widmer *et al.,* 2007).

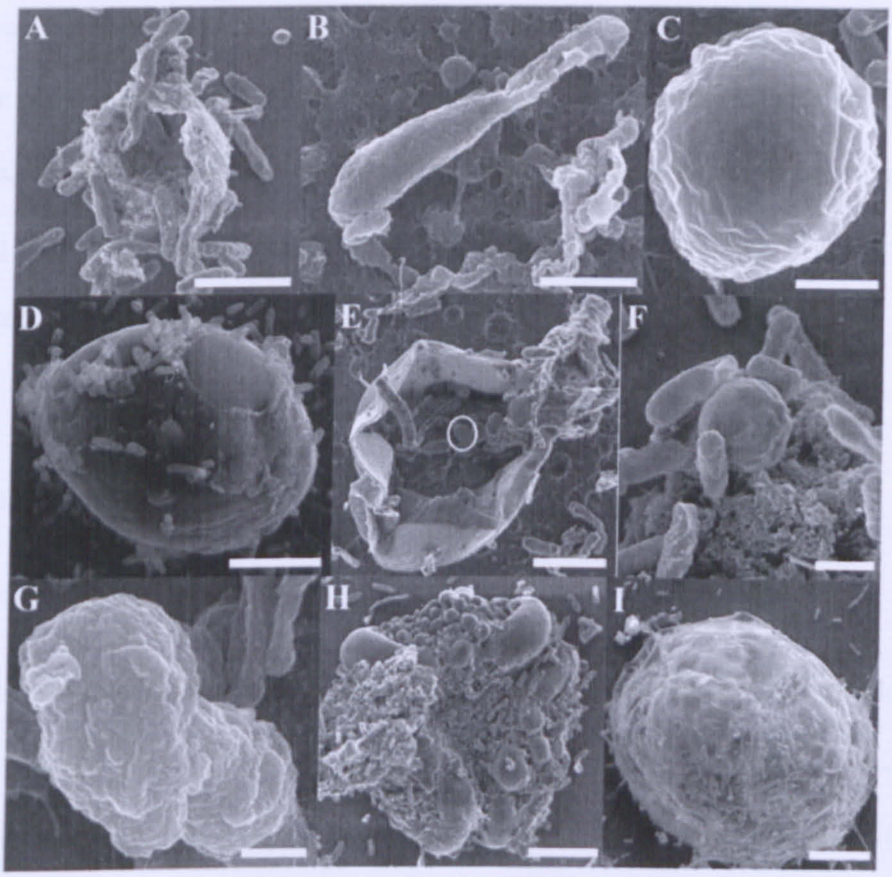

Figure 2: Scanning electron micrographs of *Cryptosporidium* within *Cryptosporidium*-exposed biofilms. (A) Empty oocysts with a rough membrane appearance; (B) Free sporozoite; (C) Trophozoite; (D) Large gamont cells (meronts) identified within 6 day-old biofilms; (E) type II meront containing type II merozoites within (circled); (F) Free type I merozoites; (G) Free type II merozoites; (H) Microgamont; (I) Extra-large gamont. Scale bars = A & B: 2.5 μm; C & F: 1 μm; D & E: 3 μm; G: 500 nm; H: 5 μm; I: 8 μm. (Koh *et al.*, 2014)

2.4 Ecology of *Cryptosporidium* in vegetables

To achieve high potential of farming productivity is something critical in terms of increasing vegetable production and reducing manpower needed. Most large and wide vegetable farms need a timely watering schedule to large number of plants. By doing that, it needs irrigation that spreads water like natural rain does. Irrigation with poor-quality water is a way for the vegetables at farms to be contaminated, normally, ground water, surface water and human waste water are water being used for irrigation (Steel *et al.*, 2004). Sometimes, slurry and animal wastes as fertiliser are sprayed on the land for supplying nutrients and maintaining soil topography, which has been practised even during the traditional time.

2.5 Parasitic Helminths

2.5.1 Classification of Helminths

Parasitic helminths are classified into three groups which are nematode, cestode and trematode *Ascaris* (large roundworm), *Strongyloides* (threadworm), *Trichuris* (whipworm) hookworms and *Taenia* (tapeworm) are examples of significant parasites in medical field.. Identification of these helminths are easily based on the morphology of their eggs (ova), confining to different organs or parts in adult worms and several organs or tissues regarding on their migration of larvae (Hansen *et al.*, 1998).

2.5.2 *Ascaris*

Ascaris is a parasitic nematode worm that is known to invade small intestinal tract. (Bogitsh, Carter & Oeltmann, 2012). *Ascaris lumbricoides* is a common parasite that infect human to cause ascariasis. It is the largest intestinal parasitic worm infecting human. The female (7.9 – 19 inch) has greater size than male (5.9 – 12 inch), whereby the fertilized eggs are oval round in shape (45 – 75 μm) while the unfertilised eggs are longitudinal oval shape (88–94 μm) (Roberts *et al.*, 2009) as shown in **Figure 3**. The disease is generally asymptomatic, that normally give symptoms to specific target locations such as lung, digestive track and gall bladder which are caused by larva and worm migration throughout different parts of human internal organs during maturation (Cano, Piñero, Tejero & Martínez, 2014).

The life cycle starts from larva that hatch in small intestine and penetrate through the wall and migrate to lungs, liver, trachea and flow back to small intestine through blood circulation andthis happens within three weeks. There is where the ova mature to an adult worm (Murray *et al.*, 2005). Tremendously, the female produces 200,000 eggs per day for a year that each egg persists in soil for more than 10 years (Murray *et al.*, 2005). The eggs have lipid layer that protects from the effect of alkalis, acids and other chemicals (Ross, 2007). It has its huge impact particularly in children (Albonico *et al.*, 2008). Ascariasis in children is often related to nutritional deficiency such as lactose and vitamin A, diminished in physical and cognitive development (Taren *et al.*, 1987).

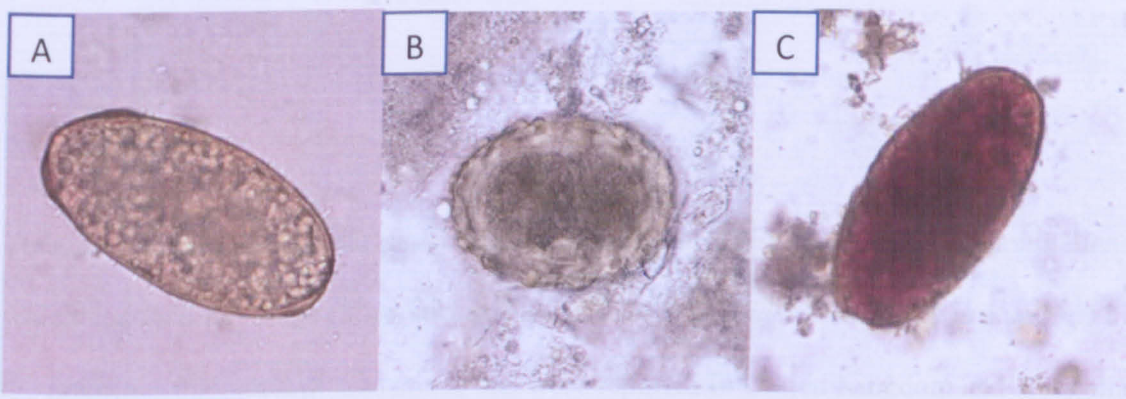

Figure 3: Figure above shows (A) unfertilized egg, (B) decorticated egg and (C) fertilized egg (Centers for Disease Control and Prevention, 2016)

2.5.3 Whipworms

Trichuris or whipworms are part of a genus of parasitic worms from the family of roundworm family Thrichuridae. *Trichuris trichiura* with specific size of male, 30-45 mm and female, 35-50 mm, is a type of whipworms that infect human to cause thricuriasis (Donkor *et al.,* 2016). Trichuriasis in children around 5-15 year old may cause non-specific abdominal discomfort and a dysenteriform picture, although it is generally asymptomatic (Hotez *et al.,* 2009). The eggs are covered with thick shell that stays in lining wall of intestines after ingestion and hatching.

First stage larvae migrates to the large intestine after they are released, then they develope into adult that burrow themselves into the mucosal lining of the intestinal wall and feed tissue fluids until they are mature, then to reproduce eggs (Diniz-Santos *et al.,* 2006). The most infected individuals are from immunocompromised adults due to weak immune system, while in children is due to deficiency in vitamin A. Minimal blood in loose stools may be present with the development of chronic anaemia. The whipworm has general life cycle which first ingested by the host, reach the duodenum in small intestine that eventually the eggs hatch and travel along the caecum of the large intestine for four weeks (Pedersen & Saeed, 2002).

2.5.4 Threadworm

Threadworm is a pathogenic parasitic roundworm that infects humans and mammalians which causes the disease called strongyloidiasis. *Strongyloides stercoralis* is a species of the notorious threadworm nematodes that were reported in United Kingdom and Australia (Vanderkooi, 2000). *Strongyloides fuelleborni* is the second species that can cause human strongyloidiasis but it is less common by which it is found in Africa and Papua New Guinea

(Siddiqui, 2001). The life cycle is much as other soil-transmitted disease. There are pathenogenetic female that capable of reproducing without males that stays in small intestine duodenal mucosa and lay embryonated eggs *in situ* that later will grow to rhabditiform larvae in the wall of intestines (Siddiqui, 2001). They can also form a free living generation with both males and females (Eberhardt *et al.,* 2007). **Figure 4** illustrates an egg known as ellipsoid with range of 40 – 85 μm in length coated with a thin wall containing a larva in the cavity of the egg (Viney & Lok, 2007)

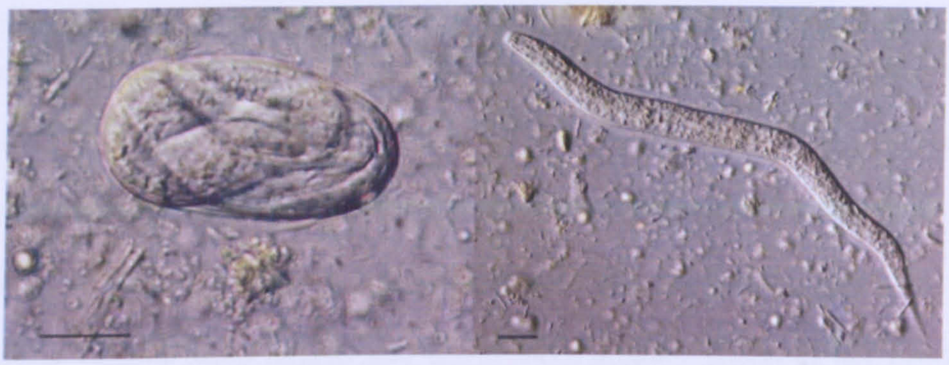

Figure 4: An egg and a first stage larva (left and right, respectively) of S. ratti in fresh faeces. Scale bars = 10μm (Viney & Lok, 2007)

2.5.5 Hookworm

Hookworm is named after the infection of hookworm disease that known to a parasitic bloodsucking roundworm. There are 2 species infecting human which are *Ancylostoma ceylanicum* and *Necator americanus*. Ancylostomiasis is a disease caused by *Ancylostoma ceylanicum* that it brings about iron deficiency anemia to the host by sucking blood in the small intestine (Hotez *et al.,* 2003). Whereas necatoriasis is caused by *Necator americanus* that gives apparent signs and symptoms such as abdominal pain, diarrhoea, cramps and weight loss that eventually leads to anorexia (Croese *et al.,* 2006). Infection starts when larvae penetrate the skin or ingested by the host. It migrates via blood circulatory system to the lungs as they erupt into the brinchi, travel up into trachea and are swallowed into the intestinal tract. They enter into the small intestine where they mould two times and grow to adult form, on that they attach themselves on the intestinal mucosa by their buccal capsule, feed on blood and reproduce (Anon, 2005). Almost half a billion of human population is infected by hookworms, leading to health problems in children and mothers in developing countries of the tropics and subtropics (Alan, 2012; Hotez *et al.,* 2005).

2.5.6 *Toxocara*

Toxocara is a parasite that cause toxocariasis in human. There are four important species which include *Toxocara canis, Toxocara cati, Toxocara leonine* and *Toxocara vitulorum.* They are specifically infect dogs and cats. Moreover, they are parasitic zoonotic nematodes that leads to different larval migrans syndromes related to toxocariasis includes covert toxocariasis, visceral larva migran and ocular larva migrans (Jones *et al.,* 2008). It is asymptotic whereby fatigue, anorexia, weight loss and abdominal pain muscle. *Toxocara* eggs are embryonated in 2 to 5 weeks and become infective. The larvae penetrates the bowel wall and migrate through vessels to the muscles, then liver, to lungs and eyes. *Toxocara spp.* are unable to reproduce in human host but exist in tissue in a state of arrested development which then later on will triggers inflammatory reaction. Hence, this can cause the symptoms of toxocariasis (Lee *et al.,* 2014).

2.6 Conventional vegetable farm

Based on the United States Department of Agriculture (2007), the term conventional farming is also known as industrial agriculture, it is a form of modern farming that refers to production that are industrialised of livestock, fish, poultry and crops by which they favour the inclusion of innovation in agricultural machinery and farming methods such as genetic technology, techniques to achieve economies of scale by productions, market evolutions and global trade. It is somehow for the global food demand projected for the next 50 years poses huge obstacles, challenges to sustain both food production and terrestrial, and aquatic ecosystem and services for the society (David *et al,. 2002*). On the other hand, it increases crop yields, makes crops more fruitful as it is genetically modified and helps farmers to be able to overcome food demand (Crystal, 2015). Speaking of farming yields, a study conducted by Verena *et al.* (2012), noted that conventional farming has 66% higher yields compared to organic farming depending on system and site characteristic, which is, this difference is highly contextual.

2.7 Organic vegetable farm

Basically, organic farming is a method of crop and livestock production that favours not to use pesticide, fertilizers, genetically modified organism (GMO), antibiotics and growth hormones (John & Benjamin, 2016). In this era of modern, the farmer's management toolkit combines three types of techniques, cultural, biological and mechanical, that minimizes the impact of other than organic farm system (Pamela, 2012). According to the study of Brandt (2001) stated that crops from organic farm are more likely to contain higher nutritional value such as vitamins, minerals, protein and carbohydrates compared to conventional crops, which determines that food from organic farm bring benefit to human health more than from conventional farm. In fact, a research done by Marcin *et al.* (2014), stated that, organic crops have higher concentrations of antioxidants and also lower concentrations to both cadmium (Cd) and incidence of pesticide residues that could prevent any pulmonary irritation, leukaemia and others related to the effects of these components.

2.8 Incidences and Outbreaks of *Cryptosporidium*

A research done by Latif *et al.* (2015) has proven that with direct wet mount, formalin-ether sedimentation, modified Zeihl-Neelsen and Direct Fluorescent Antibody (DFA) gave the same result (4.62%) to Sheather's sugar flotation (3.85%) that gives a confirmation that Malaysia has 4.6 of infection rate out of another two countries, Iraq and Jordan. Besides, currently breaking news in 2016, Canadian researchers have discovered the presence of intestinal parasite, *Cryptosporidium* for the first time in Arctic (CTVNews, 2016).

2.9 Detection technique

2.9.1 Modified Ziehl-Neelsen

Ziehl-Neelsen is also known as the acid-fast stain by which a special bacteriological stain used to identify acid-fast organism (Clarke, 1996). Acid fast organisms have large amount of lipid substances called mycolic acids that makes it to resist towards acid stains, thus makes methylene blue to form colour (Shui *et al.*, 2012). According to UK NEQAS Parasitology, standard concentration techniques do not concentrate well for acid-fast organism such as *Cryptosporidium.*

2.9.2 Lugol's Iodine Solution

Lugol's iodine solution is used for the detection of intestinal protozoa and helminth ova and larvae with wet mount preparation and technique of concentration of Iodine crystals (5.0 g) and Potassium Iodide (10.0 g) in 100 mL (Garcia & Bruckner, 1988). It is widely used in parasitology field to stain and making cell nuclei more visible under microscope.

It is known a rapid and non-specific contrast dye that apply to a wet mount (one drop is adequate on a 50 μL sample) to assist differentiation of parasitic cell from sediments. Dye brown colour will appear while other non-membrane sediments will remain clear (Forbes *et al.*, 1998).