DEVELOPMENT OF AN IN-HOUSE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SERODIAGNOSIS OF HUMAN FASCIOLIASIS

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by

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LIST OF SYMBOLS

%	Percentage
<	Lower than
>	Greater than
x *g*	Gravitational force
mM	Millimolar
µm	Micrometer
mL	Milliliter
µL	Microliter
mg	Milligram
µg	Microgram
°C	Degree celsius

LIST OF ABBREVIATIONS

AUC	Area Under Curve
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CIAS	Computer Image Analysis System
CL1	Cathepsin L1
CSA	Crude Soluble Antigen
CT	Computer Topography scan
ELISA	Enzyme-Linked Immunosorbent Assay
epg	Eggs per Gram
ES	Excretory/Secretory
et al	And Others
HRP	Horseradish Peroxidase
i.e.	Id est (That is)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Inc.	Incorporation
INFORMM	Institute for Research in Molecular Medicine
JEPeM	Jawatankuasa Etika Penyelidikan Manusia
kDa	Kilo Dalton
MRI	Magnetic Resonance Imaging
NaCl	Sodium Chloride
NPA	Negative Percent Agreement
NPV	Negative Predictive Value
NTD	Neglected Tropical Disease
OD	Optical Density
p.i.	Post-infection
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PPA	Positive Percent Agreement

PPV	Positive Predictive Value
ROC	Receiver Operating Characteristic
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SGD	Sustainable Development Goals
spp.	Species (plural form)
SPSS	Statistical Package for Social Sciences
USA	United States of America
USM	Universiti Sains Malaysia
WHO	World Health Organization

PEMBANGUNAN ELISA DALAMAN BAGI SERODIAGNOSIS FASIOLIASIS MANUSIA

ABSTRAK

Fasioliasis ialah sejenis jangkitan parasit tularan air dan makanan oleh *Fasciola* spp. yang menjangkiti haiwan dan manusia. Kaedah diagnostik standard bagi jangkitan fasioliasis manusia adalah bergantung pada pengesanan telur fasciolid secara koprologi yang kurang sensitf dan hanya sesuai untuk diagnosis jangkitan kronik. Kaedah serodiagnosis boleh digunakan untuk menambah baik kapasiti diagnosis fasioliasis bagi tujuan klinikal dan penyelidikan. Tujuan kajian ini adalah untuk membangunkan dan mengoptimum ELISA dalaman tidak langsung berdasarkan antigen kasar *F. gigantica* untuk tujuan serodiagnosis dalam kalangan penduduk tempatan. Dalam kajian ini, antigen larut kasar (CSA) disediakan daripada cacing dewasa dan diguna dalam pengoptimunan ELISA dalaman. Sembilan puluh sampel arkib serum manusia yang diperoleh dari kajian keratan rentas terdahulu (60 seropositif, 30 seronegatif) disaring menggunakan ELISA yang dibangunkan. Nilai pemotong esai ini ditentukan dengan plot analisis lengkung ciri operasi penerima (ROC) dan peratusan persetujuan ini dibandingkan dengan kit ELISA komersial (Diagnostics Automation/Cortez Diagnostics, USA). Kepekatan salutan antigen, pencairan serum manusia dan pencairan antibody sekundar dikonjugasi HRP dioptimumkan masing-masing pada 20 µg/mL, 1:100 dan 1:6000. Esai ini menunjukkan prestasi yang hampir setara dengan kit ELISA komersial pada nilai pemotongan 0.65 (nilai kappa=0.910), dengan peratusan persetujuan positif dan peratusan persetujuan negatif masing-masing pada 100% dan 96.67%.. Kesimpulannya, ELISA dalaman yang dibangunkan adalah setanding dengan ELISA

komersial dalam kajian ini dan berpotensi untuk diaplikasikan dalam serodiagnosis fasioliasis manusia dalam komuniti.

DEVELOPMENT OF AN IN-HOUSE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SERODIAGNOSIS OF HUMAN FASCIOLIASIS

ABSTRACT

Fascioliasis is waterborne and foodborne parasitic disease caused by *Fasciola* spp. which infect animals and humans. Current gold standard of human diagnosis relies on coprological detection of fasciolid eggs which lacks sensitivity and only applicable in chronic infections. Serodiagnosis can be used to improve diagnostic capacity of fascioliasis for both clinical and research purposes. The aim of this study was to develop and optimize an in-house indirect ELISA based on *F. gigantica* crude antigen for serodiagnosis of human fascioliasis in local population. In the study, a crude soluble antigen (CSA) was prepared from adult flukes and was used in the optimization of in-house ELISA. Ninety archived human sera from a previous cross-sectional study (60 seropositive, 30 seronegative) were screened using the developed ELISA. The cut-off value of the assay was determined by plotting the receiver-operating characteristic (ROC) curve analysis and the percentage agreements were calculated in comparison to a commercially available ELISA kit (Diagnostics Automation/Cortez Diagnostics, USA). The coating antigen concentration, serum dilution and HRP-conjugated secondary antibody dilution were optimized at 20 µg/mL, 1:100, and 1:6000 respectively. The assay exhibited near perfect agreements with the commercial ELISA kit at a cut-off value of 0.65 (kappa value=0.910) in which the positive percent agreement and negative percent agreement were 100% and 96.7 % respectively. In conclusion, the developed in-house ELISA is comparable to that of commercial ELISA

in this study and is potentially applicable for the serodiagnosis of human fascioliasis in local community.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Fascioliasis is a human and animal parasitic disease caused by *Fasciola* spp. The main infective species are *Fasciola hepatica* (which has a wider and more cosmopolitan distribution across Europe and Americas) and *Fasciola gigantica,* (more common for countries in Africa and Asia, including Malaysia). The foodborne trematodiasis is recognized by World Health Organization (WHO) as one of the important global neglected tropical disease (NTD) due to the morbidity as well as economic losses caused by the decrease in milk and meat production, particularly in rural and farming communities (Mas-Coma, 2004).

Human fascioliasis was initially seen as a disease secondary to its animal counterpart, with reported sporadic cases amounted to only 2,000 between 1970s until 1990s. The situation changed when WHO launched a worldwide investigation which led to its recognition as an emerging or re-emerging tropical disease and requires global attention (Mas-Coma, 2009). Human fascioliasis is estimated to affect 2.4 million to 17 million people worldwide, with the highest incidence in Peru's and Northern Bolivian Altiplano, while the number of people at risk of infection is estimated at 180 million (Mas-Coma, 2005; Figueroa et al, 2006). The global human fascioliasis distribution stretches extensively across regions in Americas, Europe, Africa and Asia. Vietnam, Thailand and Philippines are known as the major endemic countries of this disease in Southeast Asia (Mas-Coma et al, 2005a). Malaysia has only one human case of fascioliasis reported, and no prevalence analysis has been documented thus far (Naresh et al, 2006; Najib et al, 2020b).

The major source of infection for both ruminants and human is the consumption of aquatic vegetables such as lettuce and water cress; and edible plants that are washed with water contaminated with the cystic stage metacercaria (Walker et al., 2008). Studies carried out in Ethopia found signficant risk association with sheep and/or cattle ownership, besides raw vegetable consumption, use of unsafe drinking water sources, and irrigation practices (Fentie et al., 2013). Diagnosis of fascioliasis is accomplished by the identification of eggs in the coprological microscopic examination, which is considered as a "gold standard", or by the immunological testing of serum and/or stool samples (Mas-Coma et al, 2014; Sarkari et al, 2017). Chemotherapy with triclabendazole is the mainstay of fascioliasis treatment, although resistance to this drug has been reported (Cabada et al, 2006; Diaz et al, 2010).

1.2 Problem Statement

The epidemiological status of human fascioliasis has been well-documented across the globe, particularly in the endemic regions that have suffered significant morbidity such as Peru, Iran, Egypt and Vietnam. Numerous prevalence studies on ruminant fascioliasis have been carried out in Malaysia, many of which focus on cattle (Khadijah et al, 2019; Masrin et al, 2015; Rita et al, 2017; Zainalabidin et al, 2015). A study conducted by Diyana et al revealed the highest prevalence of bovine fascioliasis in the Bukit Tengah Regional Veterinary Laboratory (5.55%), (Diyana et al, 2019). Terengganu is the most endemic state for ruminant fascioliasis, with 82% seroprevalence in cattle, and 89% seroprevalence in sheep and goat (Khadijah et al, 2017; Rita et al, 2017). To date, data regarding the seroprevalence and distribution of human fascioliasis in Malaysia is fragmented or not well documented. The previous

study has demonstrated 37.3% and 67% seroprevalence of fascioliasis among farmed cattle and cattle breeders in Kelantan respectively (Najib, 2020a).

Currently, fascioliasis is diagnosed based on egg or *F. hepatica*-specific antigen detection in faeces or by antibody detection in serum or milk. All these assays have some limitations; for example, egg shedding by adult flukes only occurs 3 – 4 months post-infection (p.i.); hence, coprological examination is only feasible in chronic infections, during which severe liver damage and other complications may have already occur (Chen and Mott, 1990; Mas-Coma et al 2014; Sarkari et al, 2017). The eggs may also still be undetectable in this period due to low infection burden, irregular egg-shedding, or the intermittent nature of egg production. Thus necessitating repeated examinations over several days which is time-consuming (Mas-Coma et al, 2014). Delay in obtaining results often leads to delay in the commencement of treatment in the absence of definitive diagnosis. Availability of an effective, simple and cost-effective diagnostic tool will enable infected individuals to be treated in a targeted manner, and will help the community to move away from the traditional repeated blanket treatment regime. Human immune response to *Fasciola* antigens occurs early in infection. Antibody detection using an immunological method such as enzyme-linked immunosorbent assay (ELISA) may be regarded as a more reliable, easier and cheaper mean in evaluating the prevalence rate and disease exposure of human fascioliasis than coprological analysis especially among high risk individuals such as farm workers and animal breeders. An in-house ELISA for human fascioliasis is not yet available and such testing would be equally as reliable and accurate, as well as more cost effective if used for local screening or diagnostic evaluation in the community.

1.3 Rationale of Study

ELISA is a serodiagnostic tool routinely used for immunodiagnosis in acute infection and its excellent specificity and sensitivity have been proven in multiple past studies (O'Neill et al, 1999; Espenoza et al, 2007; Valero et al, 2012a Khan et al, 2017;). Indirect ELISA is the most frequently used test in serodiagnosis of human fascioliasis because of the relative simplicity and early detection of infection (usually 1–2 weeks) (Sanchez-Andrade et al, 2001). Its high level of specificity has been proven in studies conducted in developed countries (Mas-Coma et al, 2014; Sarkari and Khabisi, 2017). It is also capable of diagnosing asymptomatic or subclinical fascioliasis and ectopic fascioliasis where no eggs are shed in stool for detection by coprological examination (Mas-Coma et al, 2014). The aforementioned advantages as well as its ease of application and relatively rapid result makes it a preferred diagnostic tool for use in human fascioliasis seroprevalence studies in Malaysia, of which the present data is unavailable.

Currently, the commercial ELISA kit available for use in our country is *Fasciola* IgG ELISA manufactured by Diagnostic Automation/Cortez Diagnostics, Inc. (USA). Conducting a surveillance study involving a large sample size using imported kit would incur high cost to the research. Moreover, the commercial kit is constructed based on *F. hepatica* antigen and could potentially produce less accurate result for diagnosis of human fascioliasis in Malaysia, which is endemic for *F. gigantica* species. Furthermore, the result interpretation may be misleading when tested in the local population as the cut-off values of anti-Fasciola titre are different compared to the manufacturer's value at different geographical areas. Developing an in-house ELISA test that corresponds to the local settings would be extremely valuable in terms of study cost-effectiveness as well as ensuring improved accuracy in

serodiagnosis of fascioliasis. Serodiagnosis remains an important approach in routine diagnosis (in addition to thorough clinical assessment of the infected patient) due to its many advantages over the conventional method. In the long run, determining and establishing the current seroprevalence and epidemiology of human fascioliasis in Malaysia will certainly be useful in designing appropriate control measures and prevention strategies to improve the economic status of endemic areas, such as those of farming and cattle breeding communities in Perak, Terengganu and Kelantan.

Therefore, this research aims to develop an in-house ELISA serodiagnostic test and to compare its performance with a currently available commercial fascioliasis serodiagnosis kit for human fascioliasis. The research findings are essential to the health service providers in the state to assist cattle breeders in monitoring and/or controlling of the zoonotic disease to reduce the risk of ecomonic loss and improve the economic wellbeing and health of the cattle breeders.

1.4 Research Question

Is anti-Fasciola IgG antibody detection by the developed in-house ELISA in good agreement with that of commercial ELISA kit?

1.5 Study Objectives

1.5.1 General Objective

The general objective of this study was to develop an in-house ELISA for serodiagnosis of human fascioliasis and evaluate its diagnostic performance.

1.5.2 Specific Objectives

1) To develop and optimize an in-house ELISA for detection of anti-*Fasciola* IgG antibodies in human sera.

2) To compare the positive and negative percentage agreements between the in-house ELISA and the commercial ELISA kit.

1.6 Flowchart of Study

The overview of study methodology is shown in Figure 1.1.

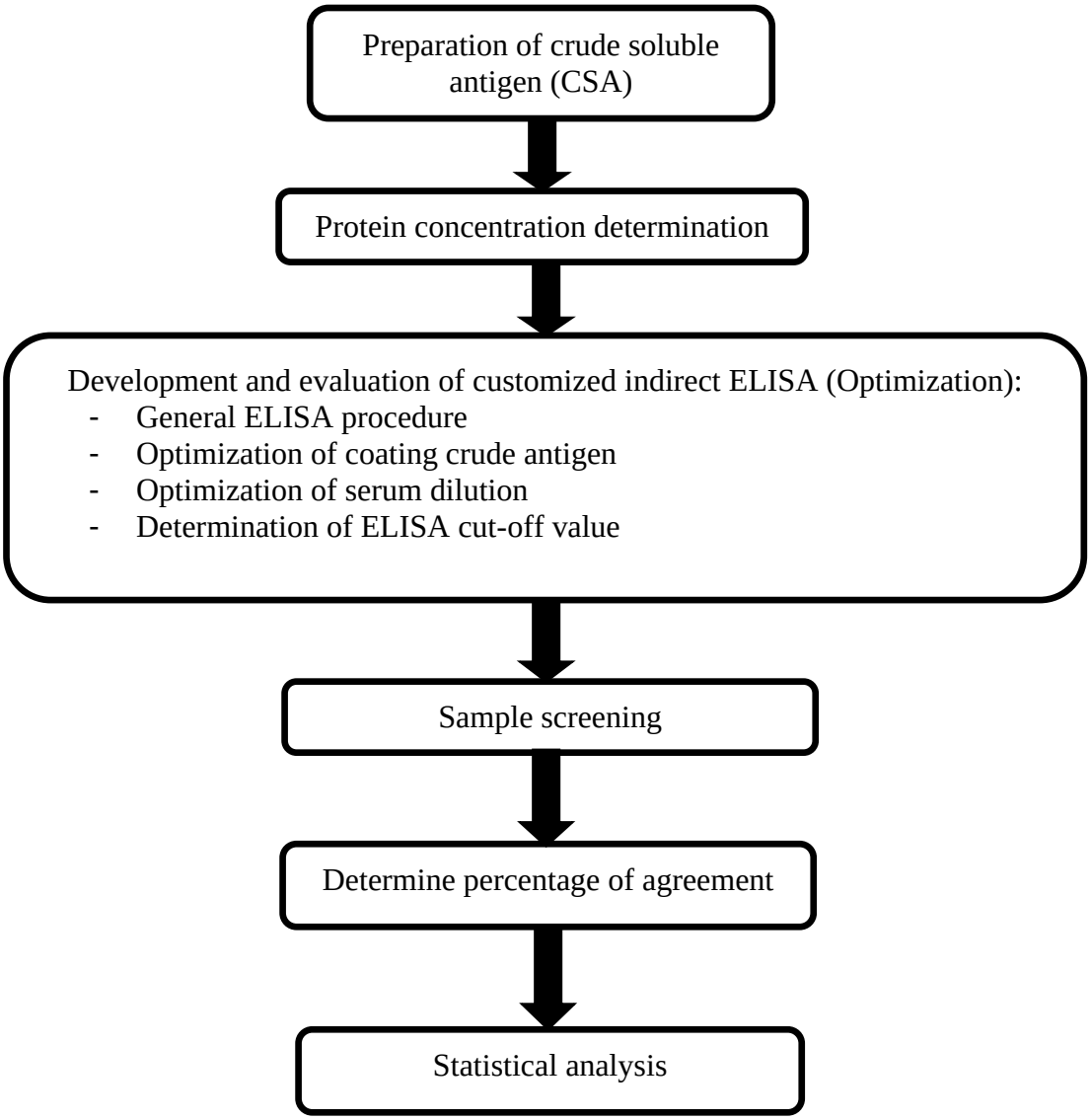


Figure 1.1 The flowchart of study

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Fascioliasis is a waterborne and foodborne zoonotic disease caused by two parasites of genus *Fasciola.* According to animal taxonomy, *Fasciola* spp. belong to class Trematoda under phylum Platyhelminthes within the kingdom Animalia. Beneath the class Trematoda, it is classified under order Protostome, suborder Echinostomata, superfamily Fasciolidiae, and genus *Fasciola* (Figure 2.1). The main *Fasciola* species commonly related to the human parasitic disease are *Fasciolia hepatica* and *Fasciola gigantica* (Keiser and Utzinger, 2009).

F. hepatica is commonly found in countries with temperate weather such as Europe, the Americas, the Oceania, and some parts of Africa and Asia. Meanwhile, *F. gigantica* has a more restricted global distribution which is mainly located in tropical regions of Africa and Asia (Valero et al, 2009). Both species have been found to co-exist or overlap in Egypt, Asia Japan, Korea and Philippines (Mas-Coma, 2005a; Valero et al, 2009). The most recent esteimated number of human fascioliasis cases is around 17 million globally with *F. hepatica* being the dominant infective strain due to its higher colonizing capacity (Mas-Coma, 2005a).

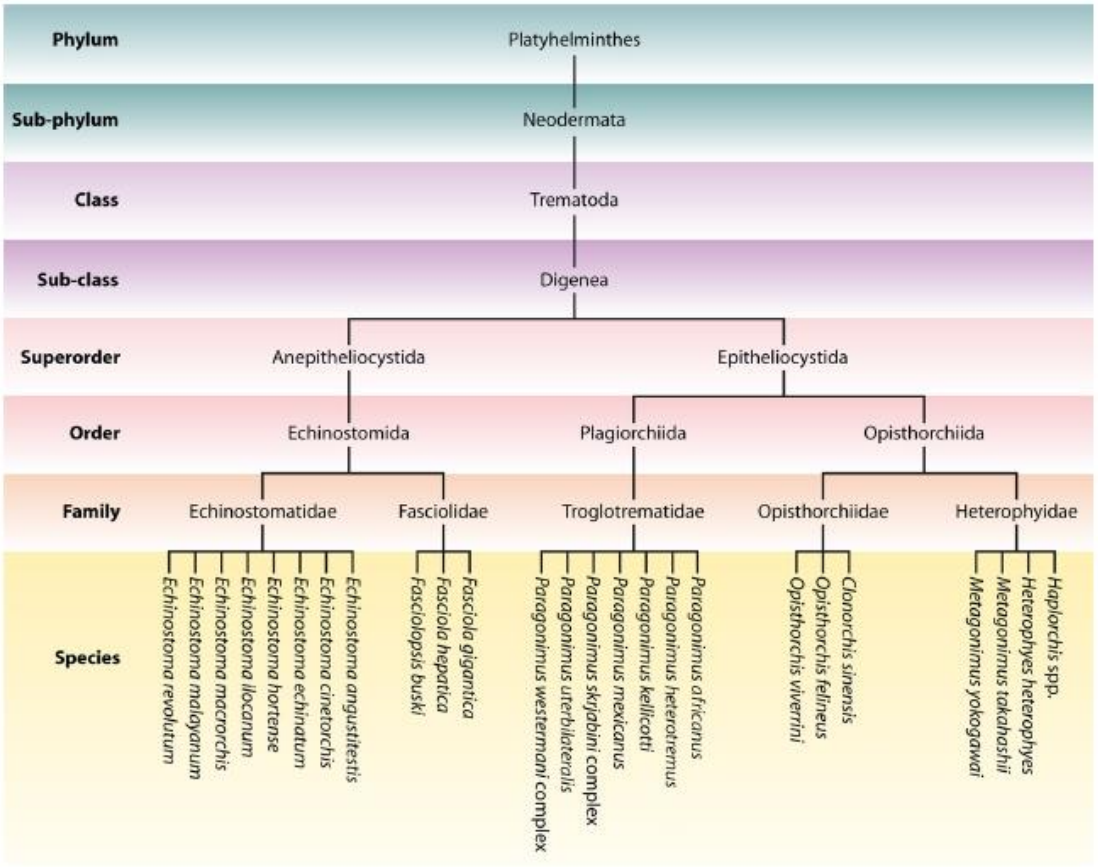


Figure 2.1 Taxonomy of *Fasciola* spp. (Keiser and Utzinger, 2009)

Human fascioliasis was regarded as a secondary disease that had resulted from its ruminant counterpart in areas endemic of the animal disease. However, as the trend in the number of human cases continued to increase even in areas with sporadic animal fascioliasis in the 1980s and 1990s, human infection was beginning to be treated with its own importance (Ashrafi *et al.*, 2014), so much so that WHO has included human fascioliasis as one of the neglected tropical infections which required control and prevention measure on a global level (Valero *et al.*, 2009; WHO, 2017). This importance is further strengthened by the evidence of its impact on the economic losses in livestock as well as meat and dairy production which is vital to the livelihood of many rural farming communities in developing countries (Mas-Coma *et al.*, 2005b; Mehmood *et al.*, 2017).

In line with Sustainable Development Goals (SGD), WHO had implemented a series of global measures dubbed as "The NTD Roadmap to 2020" in a bid to reduce the worldwide prevalence and burden of tropical diseases, of which human fascioliasis is included, by means of elimination and eradication particularly in poor and developing countries beginning in 2013 with a set of goals to achieve by 2020. The WHO Strategic and Technical Advisory Group for Neglected Tropical Diseases has addressed the challenges of the roadmap and proposed its extension and further development for the next decade (WHO, 2020).

2.2 Life Cycle and Transmission of *Fasciola* spp.

Fascioliasis is a zoonotic disease that infects both animals and humans, also referred to as the definitive or reservoir hosts. The animals that host this parasite are common livestock ruminants such as cattle, sheep, buffaloes and goats, and wild animals such as deer, rodents, horses, wild pigs, mules and camels (Ashrafi et al., 2014; Mas-Coma,

2004; Sripa et al, 2010). Fasciolids' two-host lifecycle occurs in four main phases and takes place in approximately 14 to 23 weeks (Figure 2.2) (Mas-Coma, 2004);

(i) Definitive hosts are infected through ingestion of vegetation contaminated with metacercariae of the parasites. The parasitic cysts then excyst within the duodenum of these hosts, penetrate the intestinal walls and migrate through the liver to mature in the biliary tree into adult flukes. The sexual maturation and oviposition of flukes in humans occur around three to four months, while their lifespan lasts between 9 to 13.5 years

(ii) The unembryonated eggs are expelled with the faeces into the external environment, become embryonated in freshwater within two weeks, and hatch to release miracidia under certain physicochemical properties, particularly that of water temperature ranging between 15 – 25 ^{o}C (Mas-Coma, 2004). The transit between definitive mammal host and intermediate snail vector consists of the long resistance phase of the egg and the short active phase of miracidium.

(iii) The miracidia penetrate into the snail vector and develop into sporocysts, rediae and cercariae. The cercariae are then shed into the water with temperature 9 – 26 °C regardless in daylight or night time. The duration of prepatent period (i.e. period between ingestion and appearance of eggs) is 38 – 86 days, depending on temperature as higher temperatures will reduce the period.

(iv) Transit between intermediate snail host and definitive mammal host includes the short swimming phase of cercaria and the long resistance phase of metacercaria. The released cercariae swim until they encounter solid

support, such as the leaves of nearby aquatic vegetation, and encyst to form metacercariae above or below the waterline. The metacercariae will become infective within 24 hours

2.3 Morphology of Adult Flukes of *Fasciola* spp.

The classical method of discriminating *F. hepatica* and *F. gigantica* is by observing and comparing the morphological characteristics of both species under microscope. However, the extensive phenotypic variation between the two species as well as the identification of hybrid or intermediate forms of *Fasciola* spp. in some regions has made this task difficult (Mas-Coma et al, 2005b). Generally, the adult worms of *F. hepatica* are shorter in length, broader and conical, whereas adult worms of *F. gigantica* are narrower, larger in size and more elongated (Figure 2.3) In the past two decades, precise measurements of linear lengths, areas and ratios of the adult flukes are achieved by computer image analysis system (CIAS), such as shown in Figure 2.4 (i.e. BL = Body length, BW = maximum body width, BWOv = body width at ovary level, CL = cone length, CW = cone width, OS max = maximum diameter of oral sucker, OS min = minimum diameter of oral sucker, VS max = maximum diameter of ventral sucker, VS min = minimum diameter of ventral sucker, PhL = pharynx length, PhW = pharynx width, TL = testicular space length, TW = testicular space width; Periago et al, 2006). A recent comparative morphometrical analysis was carried out by Shafiei et al (2014) between adult flukes of *F. hepatica* and *F. gigantica* isolated from common livestock ruminants in southwest Iran. The study demonstrated morphometric measurements differences between the two species, bandut also between *F. hepatica* flukes isolated from different mammalian host species (Shafiei et al, 2014).

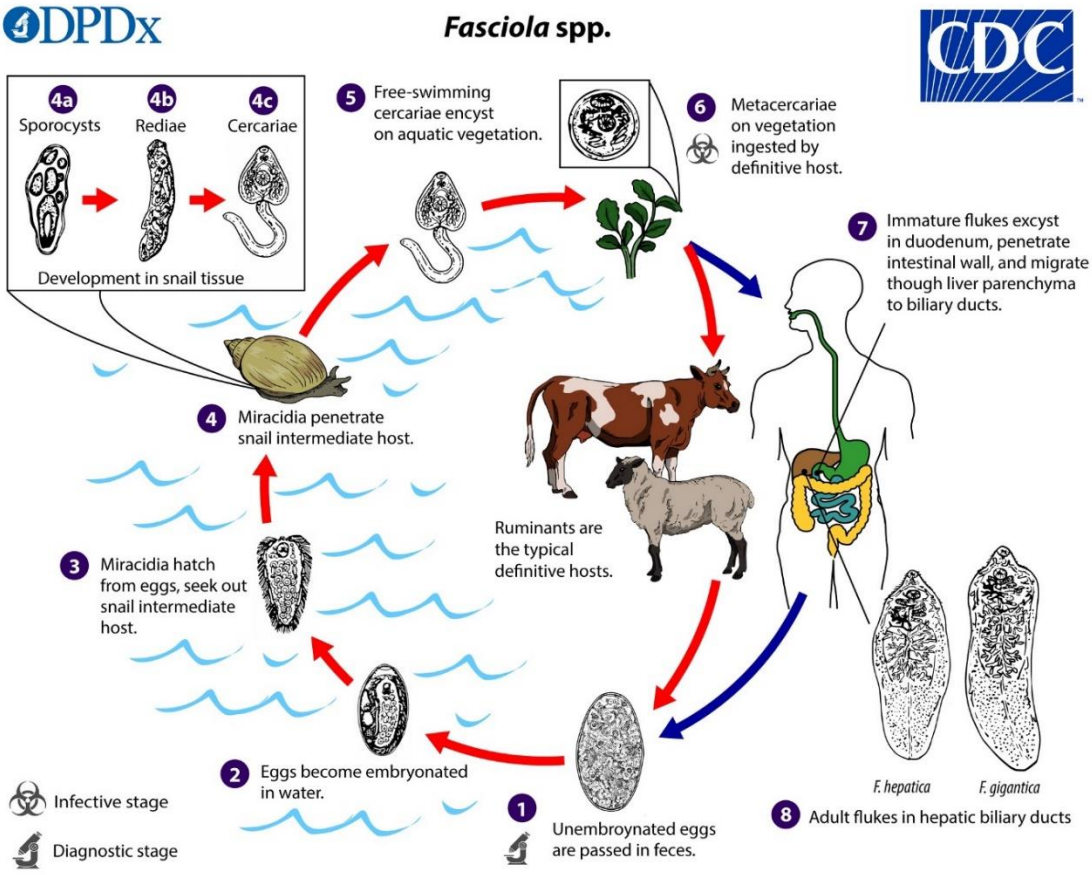
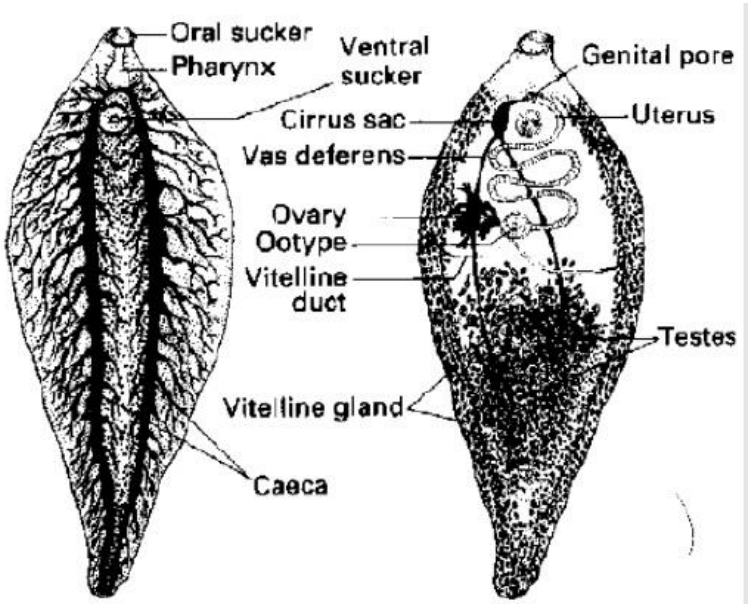


Figure 2.2 The two-host life cycle of *Fasciola* spp. in intermediate hosts (i.e.snail) and definitive host (i.e. ruminants and humans). (CDC, 2018)

(a) (b)



(c)

Figure 2.3 (a) Morphology of adult fluke of *F.hepatica* isolated from sheep. (b) Morphology of adult fluke of *F. gigantica* isolated from cattle. (Shafiei et al, 2014) (c) Structures of an adult *F. hepatica* fluke (Alemneh et al, 2019).

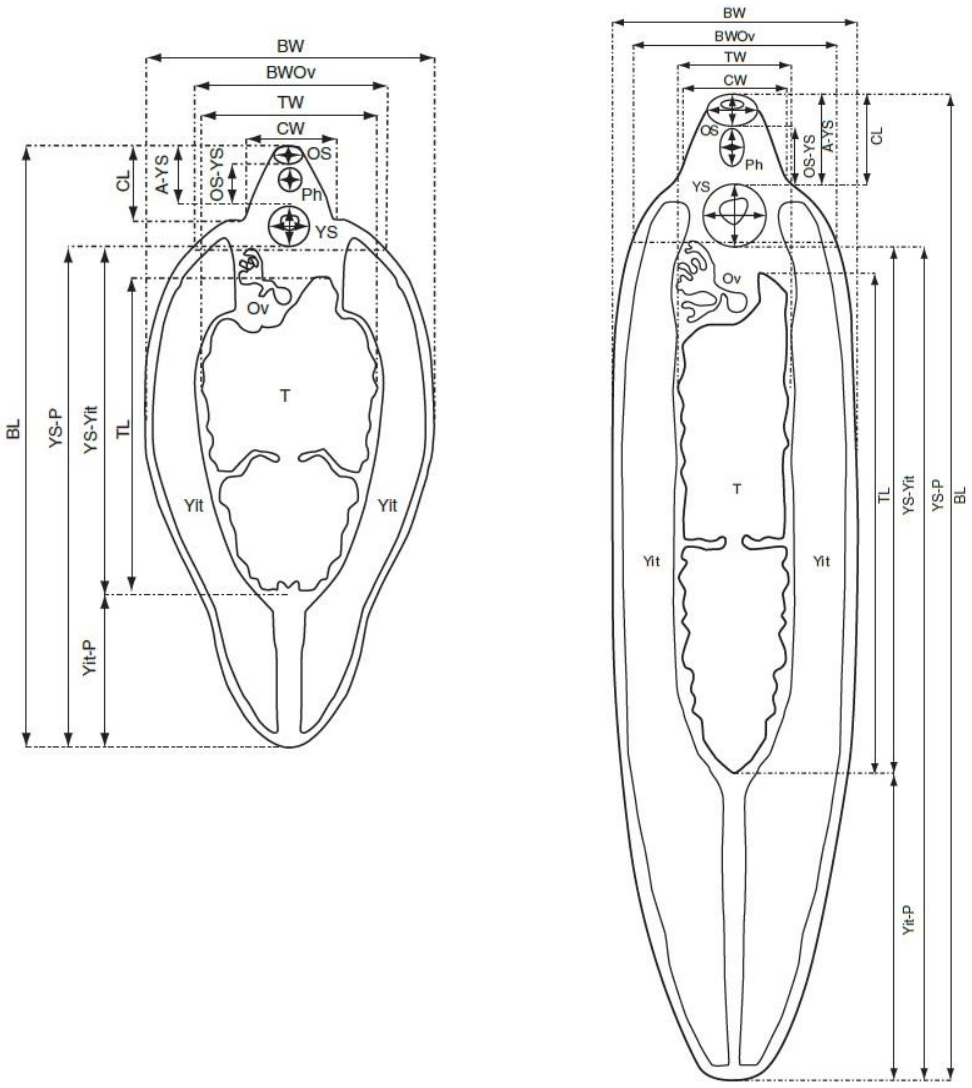


Figure 2.4 Standardized measurements obtained under microscope of gravid *F. hepatica* adult fluke (left) and *F. gigantica* adult fluke (right). (Periago et al, 2006)

2.4 Epidemiology

The epidemiological classification for human fascioliasis proposed by Mas-Coma et al in 1999 is still useful in describing the prevalence and disease intensity in different regions around the world (Mas-Coma et al, 1999; Mas-Coma et al, 2018). The classification includes; (i) imported cases, (ii) autochthonous, isolated cases, (iii) epidemic situation, and (iv) endemic situations which are further classified into hypoendemic, mesoendemic and hyperendemic scenarios, as described in Table 2.1. To date, the most well-known hyperendemic region recorded for human fascioliasis lies in the Northern Bolivian Altiplano with prevalence of 72% and 100% for coprological and serological surveys respectively, with infection intensity reaching up to 5000 eggs per gram (epg) (Mas-Coma, 2005a).

Interestingly, fascioliasis is recognized as the food-borne parasitic disease with broadest distribution (Figure 2.3) because its human endemic regions are discovered in areas ranging below sea-level along the Caspian, to the communities living in mountainous ranges (Mas-Coma et al, 2005b). *F. gigantica* is the sole species of fasciolid found in Malaysia (Khadijah et al, 2019; Rajamanickam et al, 1996). The most recent global estimation of human fascioliasis is 2.4 million to 17 million cases (Mas-Coma et al, 2018). The number of cases affected by the liver fluke in the whole of Asia, or even in Southeast Asia alone, is yet undetermined. However, its incidence is notably increasing in Vietnam, which the most endemic country in this region. According to an official report by WHO in 2008, there has been an estimated 5000 cases throughout Vietnam, with more cases being reported from 2004 onwards in response to treatment availability. Climate change is also credited as one of the important attributes to the increasing incidence of human fascioliasis cases (Mas-Coma et al., 2009; Sripa et al., 2010; WHO, 2008)

Table 2.1 Epidemiological classification of human fascioliasis prevalence and infection intensities.

Classification	Description
Imported cases	Diagnosed in a zone lacking *F. hepatica* (even among animals) but were infected in an area where *F. hepatica* transmission occurs
Autochthonous cases	Acquired the infection in the area where they reside and animal fascioliasis is also present; these cases appear non-constant and sporadical
Endemic cases	**Hypoendemic** <1% prevalence, mean intensity <50 eggs per gram (epg) **Mesoendemic** 1 – 10% prevalence, mean intensity 50 – 300 epg **Hyperendemic** >10% prevalence, mean intensity >300 epg
Epidemic cases	Outbreaks appearing in animal-endemic zones where previous human reports have always been isolated and sporadic; or Outbreaks in zones human-endemic in humans but involving a greater number of individuals than usual

Footnote: Adapted from Mas-Coma, 1999

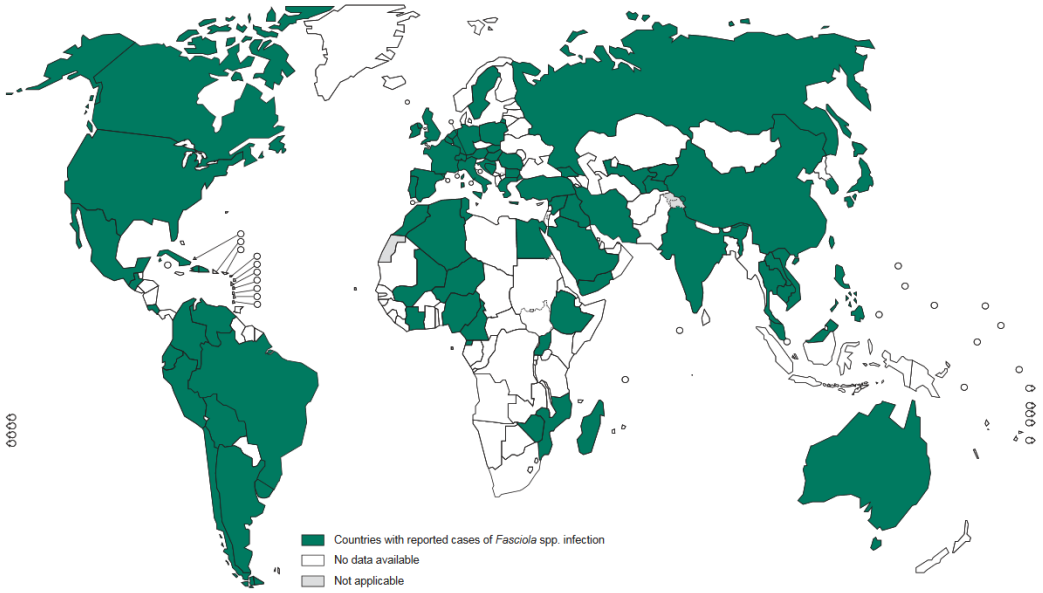


Figure 2.5 Worldwide distribution of *Fasciola* spp. infection, based on data of the most recent year available. (Gandhi et al, 2019.)

In Malaysia, the epidemiological studies of fascioliasis have focused mainly on animal disease, and published data are only limited to the states of Terengganu and Perak (Khadijah et al., 2017, 2019; Rita et al., 2017; Zainalabidin et al., 2015). To date, there has only been one published case of human fascioliasis in Malaysia in 2005 (Naresh et al, 2006). Startlingly, a recent study conducted in cattle farms throughout Kelantan has demonstrated 67% seropositive prevalence among the cattle breeders **(**Najib et al, 2020c).

2.5 Pathogenesis and Clinical Manifestations

Fascioliasis is a zoonotic disease typically transmitted from livestock to humans through ingestion of aquatic vegetables contaminated by metacerceriae. The parasitosis commonly presents as liver disease as it is caused by hepatic flukes that deposit and mature into adult worms in the large biliary passages. Untreated, the disease may complicate into chronic granulomatous inflammation.

The course of this parasitic disease consists of four distinct clinical phases; (i) incubation phase, (ii) acute or invasive phase, (iii) latent phase and (iv) chronic or biliary phase. Patients are commonly diagnosed in acute or chronic phases, during which symptoms are most apparent, prompting the patients to seek medical attention. Incubation phase, which ranges from several days to two or three months, refers to the period beginning from the ingestion of metacercariae to the manifestations of the initial symptoms. The acute or invasive phase occurs during the migration of juvenile flukes along the passages of biliary tree and liver where maturation takes place, giving rise to symptoms such as fever, general malaise, loss of appetite, abdominal pain, nausea and diarrhoea. The duration of this period ranges from two to four months. In the latent phase, patients are asymptomatic. Here, the parasites mature into adult flukes in the

liver and begin to lay eggs, the entire process ranging from months to several years. The chronic phase, otherwise known as biliary or obstructive phase, occur months or years after infection. The symptoms that present in this phase are characteristic of hepatic disease due to chronic inflammation and fibrosis developed in response to long months of infection in the liver, potentially causing obstruction in the biliary passages. Those symptoms include jaundice, nausea, epigastric or right upper quadrant pain, pruritis, intolerance to fatty food and even cholelithiasis. (CDC, n.d.; Ashrafi et al, 2014).

However, there are instances where the immature flukes deviate from the typical path along the biliary tree during their migration, entering other organs and causing ectopic fascioliasis. The most frequent organs affected by this deviation are those along the gastrointestinal tract. The lesions have also been reported to be found in lymph nodes, heart and blood vessels, muscles, lungs, pancreas, spleen, subcutaneous tissues, epididymis, brain and spine (Mas-Coma et al, 2014). Intracranial disease due to fluke migration into the brain is known as neurofascioliasis and is manifested by neurological, meningeal or psychiatric symptoms, whereas ocular symptoms indicate presence of infection in the orbits, otherwise known as ophthalmofascioliasis. Both instances of migration are rare, though they may be underestimated due to misdiagnosis (Ashrafi et al, 2014).

2.6 Clinical Approach to Diagnosis and Treatment

The process of confirming diagnosis of human fascioliasis requires examination of clinical manifestations as well as indicative results from parasitological or serological investigations (Hillyer, 1999; Tran et al, 2019). Prior to 1990s, diagnosis of human fascioliasis had been individual-based, guided by presence of eosinophilia (increased eosinophil count in the blood) had been sufficient without the need to resort to stool analysis, as co-infections were rare in autochthonous people hence there was less risk of misdiagnosis. Diagnosis by serology was done through passive detection of cases when patients come to seek treatment during the invasive phase of infection. Information obtained through history-taking which pinpoints to the source of a well-known aquatic vegetation associated with human fascioliasis, is helpful in reaching a diagnosis. Starting in the mid-1990s, field studies conducted demonstrate heterogenous transmission patterns and epidemiological scenarios that had been previously neglected, thus calling for the need of community surveys (Mas-coma, et al 2014).

Stool examination to recover fasciolid eggs is only useful 3 – 4 months post infection (p.i.). Serology is thus usually utilized for earlier detection of infection (i.e. invasive or acute phase); as early as 2 weeks p.i., for anti-Fasiola abs detection, or 8 weeks for antigen detection. Furthermore, flukes may not have achieved maturity as humans are "*generally believed to be poor fasciolia hosts*" (Mas-Coma et al, 2014), resulting in only small percentage of patients with eggs in their stool sample in non-endemic regions. Analysis of infective burden by quantifying egg number per faecal gram (epg), however is only useful in human endemic areas with very high intensity infections. Despite that, epg was proven to not necessarily reflect adult fluke number in animal fascioliasis, it is nevertheless the only method available to indirectly assess infective burden, intensity-related pathogenicity and post-treatment colic risk. For

patients in the biliary or chronic phase who are shedding eggs, the disappearance of eggs is the most frequent criterion used to establish successful treatment in post-treatment surveillance using coprological examination. For patient in the invasive phase, resolution of clinical symptoms, eosinophilia and hepatic function, indicate successful treatment. Invasive and imaging techniques such as laparoscopy and ultrasound are also be helpful for this scenario (Mas-Coma et al, 2014).

Chemotherapy is the mainstay of treatment for helminthiases, and triclabendazole, a derivative of benzimidazole, is the drug of choice for treating fascioliasis. This drug was initially developed for treatment of veterinary fascioliasis in livestock and its use in humans began in 1990s after the reduced manufacturing of bithionol, the traditional medication for fascioliasis in humans (Gandhi et al, 2019). Triclabendazole is administered as a single post-prandial dose of 10 mg/kg, and a repeated dose is recommended after 12 or 24 hours for patients with severe infection (Keiser and Utzinger, 2009). In the event of treatment failure from this initial administration, a doubled dose (20 mg/kg) is recommended and should be administered as split doses 12 to 24 hours apart. Resistance to triclabendazole in human fascioliasis has been reported (Diaz et al, 2010; Cabada et al, 2016), however such cases appear to be rare and sporadic compared with livestock where triclabendazole use has been widespread and its resistance well-established. Triclabendazole still remains the WHO's current recommended drug of choice for human fascioliasis (WHO, n.d.).

2.7 Laboratory Diagnostic Approaches

There are generally three main modalities of laboratory investigation to diagnose human fascioliasis, which include parasitological testing, serological or immuno-diagnosis, and molecular diagnosis. Although complementary testing using imaging techniques

such as ultrasound, computer topography (CT) scan and magnetic resonance imaging (MRI) have been utilized to aid in diagnosis during infections in chronic or obstructive phase to visualize the biliary tree (Keiser and Utzinger, 2009):

2.7.1 Parasitological Diagnosis

This technique involves recovery of fasciolid eggs in stool samples or samples obtained from gastric, duodenal or biliary lavage. Parasitological test has long been the gold standard to reach definitive diagnosis of human fascioliasis despite its many disadvantages. Fasciolid eggs are only detectable after 3 – 4 months of prepatent period (Chen and Mott, 1990; Valero et al, 2012b), hence stool examination is unreliable in early diagnosis of human fascioliasis. In chronic fascioliasis, fasciolid eggs may be detected in stool samples but the inherently insensitive faecal smear microscopy technique often escapes detection of infected individuals with light infections. Complete absence of eggs in a stool sample even in biliary phase of disease during which oviposition should occur is not uncommon due to poor quantity or irregularly intermittent nature of eggs shedding (Attallah et al, 2013). Humans are also recognized as poor hosts for *Fasciola* spp. and the liver flukes often do not achieve full maturation thus failing to produce eggs, which could lead to negative stool samples despite repeated examinations. Eggs may also be absent in ectopic fascioliasis. (Mas-Coma et al, 2014; Sarkari and Khabisi, 2017; Tran et al, 2019).

Sedimentation techniques, such as formalin-ether or formalin-ethyl-acetate techniques, are favoured over flotation techniques due to their superior accuracy and sensitivity (Chen and Mott, 1990; Mas-Coma et al, 1999; Mas-Coma et al, 2014a). They are also well established in routine diagnostic laboratories, and methods such as

FLOTAC (Cringoli, et al, 2010) and Flukefinder (Flukefinder Diagnostic System®, USA) are available. The cellophane faecal thick-smear technique, or Kato-Katz, is also commonly used in both qualitative analysis and egg quantification due to its simplicity, rapidity, low cost and reproducibility – advantages that allow this method to be applicable under field conditions (Keiser and Utzinger, 2009; Mas-Coma et al, 2014a).

2.7.2 Immunodiagnosis

Immunodiagnostic tests are currently the best method for diagnosis of *Fasciola* spp. infection. These tests based on detection of specific antibodies or antigens in an infected person. It is a favoured approach that overcomes the issue of low sensitivity associated with parasitological diagnosis, particularly in the invasive phase of infection or in situations where egg production is poor due to low infective burden. Anti-*Fasciola* antibodies are detectable as early as 2 weeks post-infection, allowing for much earlier diagnosis, faster initiation of appropriate treatment and ultimately preventing serious complications like cirrhosis (Mas-Coma et al, 2014). Antigens are also detectable in serological or stool samples at an earlier period than fasciolid eggs (i.e. at 8 weeks post-infection).

There are numerous immunological tests employed in the past, most of which are no longer used, including complement fixation, indirect haemagglutination, immunofluorescence assay, precipitation tests, skin tests, and radioisotope tests. Nowadays, the most frequently used tests in immunodiagnosis are based on indirect enzyme-linked immunosorbent assay or ELISA due to its ease of performance (Chen and Mott, 1990; Keiser and Utzinger, 2009; Mas-Coma et al, 2014). However, antibody detection tests do not distinguish active infection from past or resolving one. Antibodies