MORPHO-HISTOPATHOLOGICAL CHARACTERISTICS AND MOLECULAR IDENTIFICATION OF *FASCIOLA* SPECIES FROM INFECTED CATTLE LIVER

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by

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

MORPHO-HISTOPATHOLOGICAL CHARACTERISTICS AND

^0C	Degree Celsius
µg	Microgram
µL	Microliter
BW	Body width
Cm	Centimetre
TAE	Tris-acetate-EDTA
dH_2O	Distilled water
BL	Body length
et al.	*et alii* – 'and others'
CL	Cephalic length
CW	Cephalic width
F	Fasciola
ITS1	Internal Transcribed Spacer 1
RFLD	Restriction Fragment Length Polymorphism
PCR	Polymerase chain reaction
bp	Base pair
ORF	Open reading fram (ORF)Magnisumchloride 2
mL	Mililitter
PBS	Phosphate Buffered Saline
H&E	Haematoxylin and Eosin

CIRI MORFO-HITOPATOLOGI DAN PENGENALAN MOLEKUL SPESIES *FASCIOLA* DARIPADA HATI LEMBU YANG DIJANGKITKAN

ABSTRAK

Fasciola spesis cacing trematoda parasit kepada mamalia dan manusia, membawa risiko kesihatan awam dan -akibat sosio-ekonomi yang besar. Kajian mengenai spesis *Fasciola* di Kelantan masih sedikit dan kajian ini memberi tumpuan kepada ciri-ciri morfo-histologi serta pengenalpastian molekul mereka. Hati lembu yang dijangkiti telah dikumpulkan dari beberapa ladang di Kelantan. Parameter morfometrik untuk cacing muda, sederhana, dan dewasa seperti lebar badan (BW), panjang badan (BL), lebar kon kepala (CW), dan panjang kon kepala (CL) telah ditentukan menggunakan stereomikroskop yang dikalibrasi. DNA genom cacing telah diekstrak menggunakan kit Mini QIAGEN, dan pemotongan panjang fragmen polimorfisme restriksi rantai polimerase (PCR-RFLP) telah dilakukan pada fragmen DNA 463 bp di kawasan spacer transkripsi dalaman 1 (ITS1) dengan menggunakan enzim restriksi Tas1. Produk PCR-RFLP telah dipisahkan pada gel agarosa dan difotograf. Sampel hati telah diperiksa secara kasar untuk tanda jangkitan *Fasciola* dan perubahan pada morfologi. Sampel tisu telah dikumpulkan untuk pemeriksaan histopatologi menggunakan pewarnaan H & E dan perubahan makroskopik dan mikroskopik patologi hati dan kandung empedu telah diperhatikan. Analisis morfometrik cacing dewasa menunjukkan ukuran BL 4.260 ± 0.191 cm, BW 0.61 ± 0.180 cm, CW 0.37 ± 0.057 cm, dan CL 0.28 ± 0.058 cm. Nisbah BL/BW dan BW/CW adalah masing-masing 2.93-5.97 cm dan 1.55-2.72 cm. PCR-RFLP menghasilkan 3 jalur sepanjang 93 bp, 151 bp, dan 220 bp yang ditunjukkan sebagai *F. gigantica*. Parasit ditemui dalam hati dan kandung hempedu, dan penebalan lendir saluran

empedu diperhatikan dalam pemeriksaan makroskopik. Walau bagaimanapun, dalam pemeriksaan mikroskopik, pemerhatian menunjukkan infiltrasi sel-sel radang, fibrosis, nekrosis, dan degenerasi hepatosit. Di dalam kandung hempedu, nekrosis ditemui di dalam epitel mukosa saluran hempedu, infiltrasi serat kolagen, sel-sel radang, hipertrofi, dan hiperplasia epitelium kandung hempedu. Kajian ini mendapati bahawa gabungan kedua-dua kaedah dapat mengesahkan dengan tepat kehadiran *F. gigantica* dalam hati yang dijangkiti. Dalam pencirian histopatologi, ia dapat menunjukkan ciri-ciri morfologi yang berbeza pada hati lembu yang dijangkiti.

MORPHO-HISTOPATHOLOGICAL CHARACTERISTICS AND MOLECULAR IDENTIFICATION OF *FASCIOLA* SPECIES FROM INFECTED CATTLE LIVER

ABSTRACT

Fasciola species, parasitic trematodes of mammals and humans, pose a significant public health risk and carry substantial socio-economic consequences. There have been few studies in Kelantan to characterise species, and this study focuses on their morpho-histological characteristics and molecular identification. Infected cattle livers were collected from several farms in Kelantan. Morphometric parameters for juvenile, medium, and adult flukes such as body width (BW), body length (BL), cone width (CW), and cone length (CL) were determined using a calibrated stereomicroscope. Fluke genomic DNA was extracted using a QIAGEN Mini kit, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed on the 463 bp DNA fragment in the region of internal transcribed spacer 1 (ITS1) using Tas1 restriction enzyme. The PCR-RFLP product was separated on agarose gel and photographed, the livers were grossly investigated for *Fasciola* infection and morphological changes. Tissue samples were collected from histopathological examinations using H & E stain and the pathological macroscopic and microscopic changes of the liver and gall bladder were observed. The morphometric analysis of adult flukes revealed a BL of 4.260 ± 0.191cm, BW of 0.61 ± 0.180 cm, cephalic CW of 0.37 ± 0.057 cm, and CL of 0.28 ± 0.058 cm. The BL/BW and BW/CW ratios were 2.93-5.97 cm and 1.55-2.72 cm respectively. The PCR-RFLP produced 3 bands of 93 bp, 151 bp, and 220 bp, which indicated as *F. gigantica*. The parasite was found in the liver and gall bladder, and thickening of bile duct mucous was observed on macroscopic examination. Microscopic observation found

infiltration of inflammatory cells, fibrosis, necrosis, and degeneration of hepatocytes. In the gall bladder, necrosis was found in the epithelial mucosal bile duct, infiltration of collagen fibers, inflammatory cells, hypertrophy, and hyperplasia of the bladder epithelium. The study found that the combination of both morphometric analysis and molecular methods can accurately confirm the presence of *F. gigantica* in the infected liver. Histopathological characterisation has demonstrated different morphological features of infected cattle liver.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Fasciola species are parasitic trematodes that infect various mammalian hosts, including humans and livestock. Fascioliasis, a parasitic disease, has a substantial impact on both the global economy and public health (Tidman *et al.*, 2023). *Fasciola hepatica* and *Fasciola gigantica* are the two primary species responsible for causing fascioliasis in cattle and humans (Fanke *et al.*, 2017). Fascioliasis leads to significant financial losses for the livestock industries due to the decrease in body weight, liver condemnation, decline in milk production, increase in the cost of anthelmintic treatment, and a reduction in animal fertility (Kuerpick *et al*., 2019., Ahmad-Najib *et al.*, 2021). Humans can also acquire parasites through the consumption of contaminated water or ingestion of infected plants, leading to a range of clinical symptoms (Mas-Coma et al., 2018, Tidman *et al.*, 2023). Fascioliasis is widely acknowledged as a global issue within the realm of animal health, as it has been extensively documented in both developed and developing countries.

The identification and characterisation of *Fasciola* species are crucial in disease management and control strategies. Classical detection of fascioliasis requires either microscopic identification of *Fasciola* eggs in faeces by using sedimentation or flotation techniques, or recovery of the flukes from liver necropsy (Kelly *et al.*, 2019). Faecal analysis has been known to exhibit varying sensitivities, to the extent of displaying false-negative findings in the presence of chronic or lighter infection. Liver

necropsy, on the other hand, has been shown to have near-perfect sensitivity in the detection of the flukes but did not help much in species identification.

The identification of species has traditionally been centred around morphological and morphometric characteristics, including factors such as size, shape, presence or absence of shoulder, cephalic cone, and other specific anatomical components (Strydom *et al.*, 2023). These physical traits may vary and be subjective, which can lead to misidentification and inaccurate species classification. Furthermore, over the course of the previous decade, the intermediate forms of *Fasciola* have been documented in several Asian and African countries where both *F. hepatica* and *F. gigantica* coexist. It is widely believed that hybrid *Fasciola* flukes have originated from interspecific hybridizations that have taken place in various countries across East, Southeast, and South Asia (Sharbatkhori *et a*l., 2023).

In recent years, molecular approaches have developed into an effective tool for identifying *Fasciola* species. Polymerase chain reaction (PCR) and DNA sequencing have offered a reliable technique for distinguishing between *F. hepatica* and *F. gigantica*, as well as detecting mixed infections and probable hybridization between the two species (Shafiei *et al.*, 2014). Molecular identification not only improves our understanding of fascioliasis epidemiology and transmission dynamics, but it also aids in disease management.

Previous research has predominantly concentrated on either morphological or molecular methodologies in isolation, with a limited combination of the two approaches (Fairweather *et al.,* 2011).

Therefore, it is necessary to conduct a research study that integrates morphological observations with molecular approaches and histopathological observation to obtain a more comprehensive and accurate identification of *Fasciola* species in cattle.

1.2 Statement of Problems

At present, particularly in Kelantan, the knowledge of fascioliasis is inadequate. No preliminary studies have been done in Kelantan to describe and classify *Fasciola* species. Recent data from coprological and serological studies in Kelantan showed a high prevalence of fascioliasis in ruminants and among cattle farmers (Najib *et al.*, 2020). Most cattle in Kelantan were brought in from Thailand and Cambodia, countries where fascioliasis is highly prevalent and *Fasciola* hybrids were found causing misunderstanding in identification (Amor *et al.*, 2011). Diagnostic tests for *Fasciola* species on infected cattle livers can generate some challenges in differentiating between *F. hepatica* and *F. gigantica* (Periago *et al.*, 2008). Variability in morphological seen among *Fasciola* species, particularly across developmental stages and geographic regions might be challenging to identify and categorize different species or strains using simple morphological criteria (Valero, 2019). The pathogenic implications of *Fasciola* species infections in cattle, notably the degree of liver damage, have not yet been thoroughly examined (Martínez-Sernández *et al.*, 2016).

1.3 Rationale of the Study

Species identification of the *Fasciola* species infecting the livers of cattle is essential for comprehending the epidemiology, transmission, and appropriate control measures. It can be difficult to distinguish between *F. hepatica* and *F. gigantica*, the two most prevalent species that infect cattle, based basically on morphological characteristics alone. Therefore, molecular characterization can provide more reliable species

identification. Various diagnostic methods, such as molecular methods, in the identification of *Fasciola* species in cattle liver infections are essential for enhancing diagnostic accuracy. This knowledge can aid in early and accurate detection, resulting in timely and effective treatment and control measures. Lastly, understanding the pathological effects of *Fasciola* species. infections in the livers of cattle are essential for determining the impact on animal health. Characterizations at the morpho-histopathological and molecular levels can help correlate specific species or strains with variations in the severity of liver injury, associated clinical symptoms, and disease severity.

This study aims to address current knowledge gaps and contribute to a better understanding of the morphology and pathological characteristics and genetic diversity of *Fasciola* parasites in cattle populations in Kelantan, Malaysia by performing morpho-histopathological characterization and molecular identification methods.

1.4 Research Objectives

1.4.1 General Objective

To determine the morpho-histopathological characteristics and molecular identification of Fasciola species from infected cattle liver.

1.4.2 Specific objectives

1. To determine the morphological characteristics of *Fasciola* spp. from infected cattle liver via morphometric parameters**.**
2. To identify the *Fasciola* species from infected cattle liver by molecular methods.

3. To describe the pathological changes of infected cattle liver by histopathological examination

1.5 Research Questions

The purpose of this study is to answer the following research concerns about the morphological characterisation and molecular identification of *Fasciola* species in cattle:

1. What morphological characteristics and how do these characteristics differ amongst *Fasciola* species in cattle?
2. Can specific genetic markers, such as the internal transcribed spacer (ITS) region, accurately differentiate between *F. hepatica* and *F. gigantica* in cattle?
3. What is the concordance between morphological characterisation and molecular identification methods in identifying *Fasciola* species in cattle?
4. What are the histopathological features of infected cattle liver, and can this be correlated to the stages and the extent of the infection?

These research questions will guide the investigation and provide a comprehensive understanding of the morphological characteristics, molecular identification, genetic diversity, and epidemiology of *Fasciola species* in cattle. By addressing these questions, this research will contribute to the advancement of knowledge in the field and facilitate evidence-based practices for disease control and management.

1.6 Research Hypotheses

The hypotheses of the study were based on the objectives of the study and the research questions. Therefore, the hypotheses of this study were:

1. Null Hypothesis (H0): There are no significant differences in key morphological characteristics between *Fasciola hepatica* and *Fasciola gigantica*.
2. Alternative Hypothesis (H1): Key morphological characteristics show significant differences between *Fasciola hepatica* and *Fasciola gigantica*.
3. Null Hypothesis (H0): The internal transcribed spacer (ITS) region cannot accurately differentiate between *Fasciola hepatica* and *Fasciola gigantica* in cattle.
4. Alternative Hypothesis (H1): The internal transcribed spacer (ITS) region can accurately differentiate between *Fasciola hepatica* and *Fasciola gigantica* in cattle.
5. Null Hypothesis (H0): There is no significant concordance between morphological characterization and molecular identification methods in identifying *Fasciola* species in cattle.
6. Alternative Hypothesis (H1): There is significant concordance between morphological characterization and molecular identification methods in identifying *Fasciola* species in cattle.

1.7 Research Scope and Thesis Organization

The research scope covers the study on the morpho-histopathological characterisation and molecular identification of *Fasciola* species from infected cattle liver. The research study hopes to integrate morphological analysis, histopathological observation and molecular approaches to obtain a more comprehensive and accurate identification of *Fasciola* species in cattle.

This study involved a necroscopy of the infected liver obtained from the local butcher. Gross observation and histopathological examination of the liver were done using a haematoxylin-eosin staining procedure. Liver flukes of varying developmental stages were isolated for morphological characterization by morphometric methods and

identification by molecular methods. All these studies are described following the thesis organisation presented in the study chapters below:

Chapter 1: This chapter is an introductory part of the thesis. The introduction section deals with the study background, problem statement, rationale and justification of the study, research scope and thesis organization and the research objectives. This section sets a stage and provides the necessary context for readers to understand the purpose and significance of the study.

Chapter 2: This chapter is the literature review section of the thesis. The literature review section presents an overview of *Fasciola* species, current studies on the aspects of morpho-histopathological characterization and molecular identification of *Fasciola* species from infected cattle liver and discusses the gaps in the current knowledge as well as the shortcomings and advantages of various diagnostic methods used in this study. Discussion on research questions and the direction of the study based on the above aspects are emphasized.

Chapter 3: This chapter contains the description of all materials and methods used in the study. It described the gross morphology of the infected liver, morphological characterization by morphometric methods, histopathological examination using H&E staining methods and identification by molecular methods. This section also provides a detailed description of morphological and histopathological analysis and molecular identification methods used in the study.

Chapter 4: This chapter deals with the results obtained from the study. The results on the morphological characterization, histopathological examination, and identification of *Fasciola* species. by molecular methods are explained.

Chapter 5: This chapter contains a clear, constructive, and creative discussion of the research findings. It will discuss the relationship between the literature review, the methods used and the findings and their significance.

The scope of study described above is intended to provide clear and orderly information to facilitate the implementation of the research and to set forth for the smooth reading of the thesis. This study is expected to deliver current information on the morpho-histopathological characterization and molecular identification of *Fasciola* species and helped us understand the distribution and genetic diversity of *Fasciola* parasites, as well as the adoption of more precise diagnostic procedures.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *Fasciola* Species

Fasciola species are large parasitic trematodes that are generally referred to as liver flukes. *F. hepatica* and *F. gigantica* are two important species of veterinary and medicinal importance globally. Understanding these species' features and life cycles are critical for disease management and control.

Both *Fasciola* species in adult stages have a leaf-like body with a broad tip at the back (Figure 2.1). In a cone-shaped area on the front, the two suckers are small and close together.The pharynx can be seen clearly. There are numerous side branches and a long intestinal caecum that extends to the back of the body. The second and third quarters of the body are divided in half by the two testes, each of which has a branch. Large and situated close to the acetabulum, the circular bag splits open into a genital pore. It has a protruding twisted cord. The branching ovary is dextral and before the testes. The ovaries reach up to the back of the body on both sides. Between the ovary and the location where the caecum divides in half is the short uterus. The deposited eggs are ovoid, yellow, and empty inside when they are laid. The picture below indicated the species of *Fasciola* species (Valero *et al.*, 2009)

Figure 2.1 Adult stage and eggs of *Fasciola* species.

There are differences according to the size, width, intestinal, and ovaries in both species. In adults and eggs of *Fasciola* species: (a) adult stage of *F. hepatica*; (b) adult stage of *F. gigantic*a; (c) egg of *F. hepatica* found in stools of a human patient; (d) *egg of F. gigantica* found in a fecal sample of a bovine, understanding the life cycle of the parasite can help develop control techniques that target either the mammalian host or the vector (Figure 2.2). Immature eggs of parasite are expelled in the biliary ducts and pass through the stool. Embryonated eggs release miracidia, which enter a suitable snail (intermediate host). Lymnaeid snails from the *Galba* and *Radix* genera, notably *Galba truncatula*, are the most widely transmitted snail genera of *Fasciola* species. worldwide (Caravedo and Cabada, 2020). The figure below shows the life cycle of Fasciola species (Mas-Coma *et al.*, 2020)

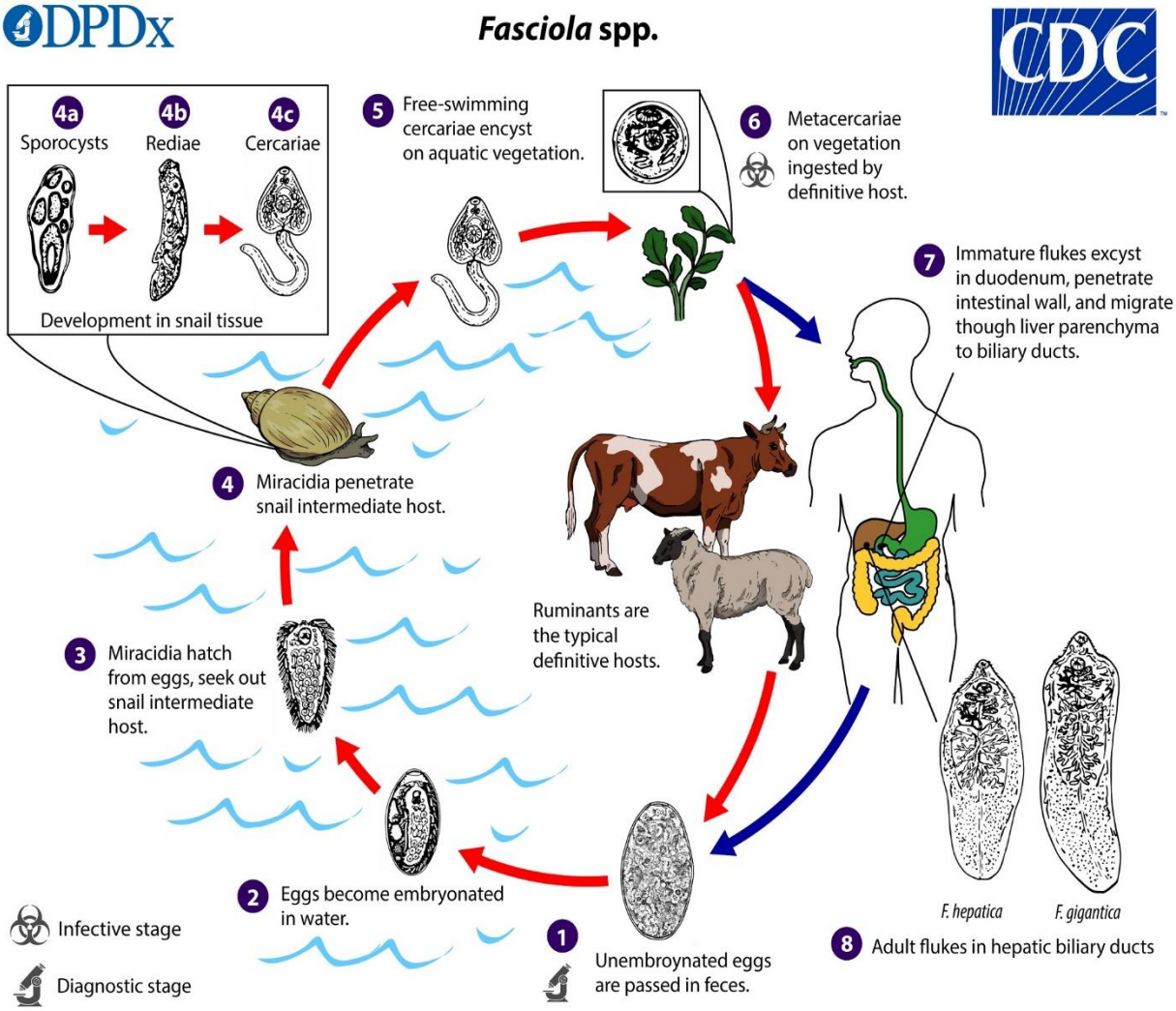

Figure 2.2 Life cycle of *Fasciola* species.

In the snail, the parasites undergo several developmental stages (sporocysts, radiate, and cercariae). The cercariae are released from the snail and encyst as metacercaria on aquatic vegetation or other substrates. Humans and other mammals become infected by ingesting metacercaria-contaminated vegetation (e.g., watercress). After ingestion, the metacercaria excysts in the duodenum penetrates through the intestinal wall into the peritoneal cavity. The immature flukes then migrate through the liver parenchyma into biliary ducts, where they mature into adult flukes and produce eggs (Sumruayphol *et al.*, 2020).

The migratory phase can last more than 12 weeks and causes severe symptoms like fever and pain in the upper right quadrant, as well as high eosinophil counts and lesions on the liver that look like tracks. The chronic phase of the infection is caused by sexually mature parasites that have established themselves in the bile ducts. This phase, which can last more than 10 years, is characterised by biliary tree inflammation and obstructive symptoms like sporadic abdominal pain and jaundice. In humans, maturation from metacercaria into adult flukes usually takes about 3–4 months (Caravedo and Cabada, 2020).

F. hepatica is widespread and infects a wide variety of animal hosts, including cattle, sheep, goats, and humans. It is found across the world but is most common in clement and subtropical areas. Adult *F. hepatica* parasites live in the livers of their hosts and produce fascioliasis, which causes liver damage, weight loss, and decreased production (Lalor *et al.*, 2021). Its life cycle includes intermediate hosts, most notably freshwater snails, where the parasites reproduce asexually and grow into infective larvae (cercariae). Infection can occur when animals or humans consume contaminated water or plants with metacercariae (Mas-Coma, Bargues and Valero, 2014).

F. gigantica can be found in tropical and subtropical regions of Africa, Asia, and parts of the Americas. It affects a wide variety of herbivores, including cattle, buffaloes, and sheep. *F. gigantica*, like *F. hepatica*, causes fascioliasis and generates major economic losses in animal production. *F. gigantica's* life cycle is identical to that of *F. hepatica*, with freshwater snails serving as intermediate hosts (Fanke *et al.*, 2017). There is a possibility of hybridisation between *F. hepatica* and *F. gigantica* in

places where both species coexist, leading to genetic heterogeneity and potential issues in proper species identification (Sharbatkhori *et al.*, 2023).

Accurate identification and differentiation between *Fasciola* species are essential for understanding their epidemiology, transmission dynamics, and control strategies. Traditional morphological identification based on size, shape, and anatomical features can be challenging due to overlapping characteristics and variations within and between species. Therefore, molecular methods, including PCR and DNA sequencing, have become valuable tools for species differentiation and detection of mixed infections.

2.2 Overview of *Fasciola* species studies in cattle

Several prior investigations on the morphological characterisation and molecular identification of *Fasciola* species in cattle have been conducted. These studies have helped us understand the distribution, prevalence, and genetic diversity of *Fasciola* parasites, as well as the creation of more precise diagnostic procedures.

2.3 Morphological characterisation of *Fasciola* species

Detailed morphological observations have been used in studies to identify *Fasciola* species in cattle. Size, shape, and the presence of anatomical components have all been investigated to find distinguishing characteristics between *F. hepatica* and *F. gigantica*. Adult fluke size and shape, as well as the morphology of oral and ventral suckers, reproductive organs, and tegumental spines, have all been reported in research (Beesley *et al.*, 2018).These morphological investigations have significantly contributed to the identification of *Fasciola* species in cattle.

Morphological characterization of *Fasciola* species entails examining the parasites' surface and internal properties, such as size, shape, colour, and other physical characteristics. These data can then be used to distinguish between the various *Fasciola* species. The parasites, *F. hepatica and F. gigantica*, have oral and ventral feeding adaptations, which allow them to attach to the host's tissues while eating. This is a key morphological feature for differentiating *Fasciola* species (Shafiei *et al.*, 2014). For example, *F. hepatica* has a circular oral sucker and a crescent-shaped ventral sucker, but *F. gigantica* has a larger and longer oral sucker and a circular ventral sucker (Amor *et al.*, 2011).

Additional morphological criteria used to differentiate *Fasciola* species include body size and form, the presence or lack of spines on the body surface, and the anatomy of the reproductive organs. *F. hepatica*, for example, has a shorter, broader body with fewer spines than *F. gigantica*, and the two species' reproductive organs differ greatly in structure and size. Morphological characterisation of *Fasciola* species has aided in the identification and classification of these parasites. In recent years, molecular techniques such as DNA sequencing have become more crucial for reliable identification of *Fasciola* species (Heydarian, Jajarmi, Spotin, Ashrafi, Mohebali, Aryaeipour, Bozorgomid, Hajialilo, Afshar, *et al.*, 2022).These procedures are more accurate and reproducible than morphological techniques, and they can be used to identify species even when the morphology of the parasites is confusing.

Both species feature a leaf-like flat body with a different appearance but a comparable pointed posterior end, as well as two relatively small suckers near the cephalic front of their bodies. The pharynx, which is part of the digestive system, is visible, and the flatworms have two branching testes that are arranged in tandem within

the second and third halves of the body. The cirrus pouch, which includes a protruding spined cirrus (a reproductive organ), is in front of the suckers and opens in a postbifurcal genital orifice. The ovary is pretesticular and dextral, which means it is in front of the testicles and on the right side of the body.

The vitellaria, which makes the egg yolk, extend bilaterally (on both sides) up to the hard body. The eggs are operculated (they have a lid-like covering), ovoid (egg-shaped), yellow, and non-embryonated when laid (meaning they have not yet developed into embryos) (Valero, 2019). The adult stage of *F. hepatica* has a maximum length and width of 29 mm and 14.1 mm, respectively, while *F. gigantica* has a maximum length and width of 52.3 mm and 11.8 mm, respectively.

As a result, *F. gigantica* is longer and slimmer, with parallel lateral walls and absent or diminished cephalic cone shoulders. Furthermore, *F. gigantica's* caecae are more branching, particularly along the midline of the body, and its ovary branches are more numerous and longer. All measurements in "pure form" of *F. hepatica* and *F. gigantica* specimens overlap except for the maximum body length, maximum body width, body length-body width ratio, body roundness, and distance between the ventral sucker and the posterior end of the body. These phenotypic differences allow the two species to be identified (Imani Baran, Cheraghi Saray and Katiraee, 2017).

This latter remark on *Fasciola* species identification was also reported in a South African study that demonstrates length, width, and the ratio of length to width may be used to measure body shape reliably (Haridwal *et al*., 2021). According to the findings, the average length-to-width ratio of the two species was substantially different. Their body lengths differed statistically significantly, with *F. gigantica*

having the longest and *F. hepatica* having the shortest. However, there was no significant difference in body breadth across the groups, according to the study.

2.4 Molecular Identification of *Fasciola* Species Using PCR and DNA Sequencing Techniques

PCR (Polymerase Chain Reaction) and DNA sequencing are powerful molecular techniques used in the identification of *Fasciola* species. PCR allows for the amplification of specific DNA sections, whereas DNA sequencing enables a complete examination of the genetic information, allowing for accurate *Fasciola* species differentiation. PCR is used in *Fasciola* species identification to amplify certain DNA sections or genetic markers that are unique to each *Fasciola* species. The diversity and discriminatory capacity of the target gene in distinguishing between various species are important considerations. The internal transcribed spacer (ITS) region, COX1 gene, and the ND1 gene are all widely targeted genes (Wu *et al.*, 2021).

The PCR process involves several steps. DNA is taken from *Fasciola* samples acquired from infected hosts, such as animals or humans. This is followed by designing the species-specific or genus-specific primers, which is to target the specified gene areas. Then, the PCR machine is used to amplify the targeted DNA regions of the designed primers, suitable PCR-restriction enzyme approach based on species - specific variation in ITS1, that would accurately differentiate between *F. hepatica* and *F. gigantica.* Lastly, the PCR results are sorted by size using gel electrophoresis, allowing the amplified fragments to be visualized.

Following PCR amplification, the DNA fragments can be further analysed by DNA sequencing to determine the exact nucleotide sequence. The specific order of

nucleotides in the amplified gene area is provided by DNA sequencing, enabling the precise identification and distinction of *Fasciola* species. Depending on the size of the study and the available resources, DNA sequencing can be performed using a variety of techniques, such as Sanger sequencing or Next-Generation Sequencing (NGS). The acquired DNA sequences are compared to existing reference sequences in genetic databases to precisely identify the *Fasciola* species.

Molecular techniques such as PCR and DNA sequencing have been utilised to identify *Fasciola* species infecting cattle and detect genetic alterations. Specific genetic markers, such as the internal transcribed spacer (ITS) region, cytochrome oxidase (COX) genes and NADH dehydrogenase subunit 1 (ND1) gene, have been targeted for PCR amplification and subsequent sequencing ((Ai *et al.*, 2011.,Ahmad *et al.*, 2021). These investigations were able to identify *F. hepatica* from *F. gigantica* and discover potential hybridization events between the two species.

PCR amplification of the mitochondrial gene COX1 has been used to differentiate *Fasciola* species. COX1-based PCR tests can distinguish *F. hepatica* from *F. gigantica* (Heydarian et al., 2022). The mitochondrial gene ND1 has also been amplified by PCR for the molecular identification of *Fasciola* species. *F. hepatica* and *F. gigantica* can be distinguished using species-specific primers targeting ND1 (Nazari *et al.*, 2022). To detect genetic variation between *Fasciola* species, researchers used Random Amplified Polymorphic DNA (RAPD) analysis. This method amplifies random sections of the genome, resulting in species-specific banding patterns that can be used to differentiate across species ((Nuchprayoon, Junpee and Poovorawan, 2007).

Restriction Fragment Length Polymorphism (RFLP) is a technique that identifies genetic variation by digesting amplified DNA with specific restriction enzymes. The resulting fragment sizes are then compared to distinguish between different species. By using different restriction enzymes, unique fragment patterns can be generated for each *Fasciola* species (Othman *et al.*, 2023). ITS region of ribosomal DNA (rDNA) has been targeted for PCR amplification to differentiate *Fasciola* species. Primers specific to the ITS region, such as ITS1 and ITS2, have been used to amplify *Fasciola* DNA, followed by sequencing or restriction enzyme analysis (Shafiei et al., 2013).

PCR and DNA sequencing are indispensable tools in *Fasciola* species identification. They provide a reliable and accurate approach for differentiating between *Fasciola* species, aiding in the understanding of their epidemiology, pathogenicity, and control strategies. Some research has compared morphological observations with molecular identification methods to determine concordance and reliability. These investigations emphasized the advantages and disadvantages of each strategy, as well as the significance of combining morphological and molecular techniques for reliable species identification (Saboor *et al*., 2020; Cwiklinski *et al*., 2021). The combination of morphological and genetic data has resulted in a better understanding of *Fasciola* species in cattle populations.

In summary, past research on the morphological characterization and molecular identification of *Fasciola* species in cattle has provided useful insights into the parasites' features, distribution, prevalence, and genetic diversity. The combination of morphological and genetic approaches has improved species identification and our understanding of fascioliasis epidemiology in cattle populations.

2.5 Histopathological of *Fasciola* species infected cattle liver

Histopathological changes of infected *Fasciola* species the infection exhibited mild swelling and a pale type along the rounded edges. On the other hand, additional livers demonstrated significant swelling, accompanied by a few small irregular whitish regions that suggested the presence of fibrosis on the outer surface. In certain instances, the capsule exhibited a notable thickness and coarse texture, accompanied by a whitish or reddish discoloration. Additionally, the parenchyma displayed hardness attributed to the presence of fibrous tissue. The presence of fibrosis in the bile ducts, characterized by the occurrence of multiple small and large patches distributed throughout the parietal surface, as well as the liver exhibiting a pipe stem appearance, was observed. Under microscopic examination, it was observed that the liver structures of *Fasciola* -infected cattle displayed notable alterations compared to the typical structures observed in non-infected livers. The extent and severity of these changes varied depending on the duration and intensity of the infection. the instances of necrosis and fibrosis within the hepatic parenchyma cells(Nduka,.. 2015). The migration of juvenile flukes within the liver tissues and parenchyma cells has resulted in significant damage to the hepatocytes. The cellular walls have undergone degeneration, resulting in the deformation of the nuclei. The cytoplasmic contents were discharged into the sinusoids. Macrophages and lymphocytes have infiltrated the infection site and are observed to aggregate around antigenic substances, specifically the fluke eggs. This statement suggests the presence of either the acute phase or the parenchymal phase of the infection, along with the resulting pathological alterations, The degradation of the tissues ensued after the migration of the flukes (Adam *et al.*, 2022).

On gross pathology and histopathology of the gall bladder and liver by the presence of eosinophils, fibroblasts, and lymphocytes infiltrating the regions that were previously traversed by juvenile flukes. Additionally, the presence of eosinophil infiltration, a distinctive characteristic of helminthic infection in animals, has been recognized as a significant diagnostic indication for this condition. The observed occurrence of biliary duct hyperplasia accompanied by areas of fibrosis in this study may be attributed to an immunological response involving infiltration of mononuclear cells. Hepatic enlargement may arise as a consequence of red blood cell hemoglobin destruction, resulting in hypoproteinemia, which is caused by a parasitic infection and characterized by the accumulation of non-hepatic cells and increased cellular content, such as hemosiderin deposition (Adam *et al.*, 2022)**.** The Infection can result in changes in gross pathology changes, such as hepatomegaly and liver damage, is possible. The infection of fasciolosis has been observed to be associated with gall duct obstruction and gall hardening. The histopathological analysis demonstrated the presence of linear lesions in varying stages, ranging from acute haemorrhagic necrosis to active granulomas characterized by the formation of organized fibrotic regions caused by the presence of eggs and worms from the parasite within the liver parenchyma. Many of the chronic lesions exhibited the presence of lymphocytes and macrophages. The aetiology of epithelial hyperplasia is attributed to the persistent irritation resulting from the presence of parasitic worms. The presence of *Fasciola* species. within the gall, duct is suggestive of chronic trauma, as the continuous presence of these parasites results in the persistent extraction of blood from the same location. Dystrophic calcification arises due to the presence of calcium deposits within regions that have previously undergone degeneration or necrosis (Kardena *et al.*, 2017).

2.6 Current Knowledge and Research Gaps in Morphological Characterization and Molecular Identification of *Fasciola* Species

While significant progress has been made in the study of morphological characterization and molecular identification of *Fasciola* species in cattle, significant gaps in this knowledge area still remain. These gaps highlight areas that require additional research to better our understanding of *Fasciola* parasites and improve diagnostic and control strategies. Some key knowledge gaps are described below.

Many studies have focused on specific regions or nations, resulting in a limited representation of *Fasciola* species in cattle. More research is needed to close these regional gaps and gain a better understanding of the prevalence, distribution, and genetic diversity of *Fasciola* species around the world.

While previous research has recorded the morphological properties of *Fasciola* species, more detailed and standardized morphological characterization methodologies are still required. Standardization would enhance consistency and comparability among studies, allowing for more accurate species identification and reducing subjectivity in morphological observations.

Although many genetic markers, such as the ITS region, COX and ND genes, have been used to identify *Fasciola* species in cattle, additional research and development are required. Additional markers would enable a stronger and exact species distinction, especially in situations where hybridization or co-infection with multiple species is likely.

Fasciola species' genetic diversity in cattle populations is still unknown. More research is needed to understand the extent of genetic variation within and between

Fasciola species, including the identification of specific genetic markers associated with pathogenicity, host specialization, and drug resistance. Understanding genetic variation can aid in the development of targeted management strategies as well as the identification of potential emergent strains or genotypes. *Fasciola* species have zoonotic potential, and understanding their transmission patterns between animals and humans is crucial for public health. More research is needed to investigate the zoonotic risk associated with *Fasciola* species in cattle, such as identifying the prevalence of human illnesses linked to livestock reservoirs and looking into the genetic relationship between human and bovine isolates.

Filling these knowledge gaps will lead to a fuller understanding of *Fasciola* species morphology, genetic diversity, and epidemiology. It will also help to create improved diagnostic tools, effective control methods, and therapies to lessen the impact of fascioliasis on both animal and human health.

CHAPTER 3

MATERIALS AND METHODS

This chapter describes the materials and methods used to accomplish the objectives of the study. The study involved obtaining healthy and infected cattle liver from a local butcher between April and June 2023.

3.1 Materials

3.1.1 List of Chemicals

Chemicals used in this study are listed in APPENDIX A.

3.1.2 List of Kits and Consumables

Kits and consumables used in this study are listed in APPENDIX B.

3.1.3 List of Equipment

Equipment used in this study are listed in APPENDIX C.

3.2 Research Design

This is an experimental study designed to examine, characterize, and identify *Fasciola* species from anfected cattle liver. The study is laboratory-based research. The study design is illustrated in Figure 3.1. Cattle livers were obtained from a local butcher in Pengkalan Chepa and Kota Bharu, Kelantan. Gross and histopathological examinations were done on the healthy and infected liver. *Fasciola* species. morphological characterization was done using the morphometric methods. Identification of species was carried out using molecular methods.**,** Liver fluke was extracted using QIAGEN Mini kit, 463 bp DNA fragments in the region on internal transcribed spacer 1 (ITS1) of Fasciola spp. will be amplified using conventional polymerase chain reaction (PCR).

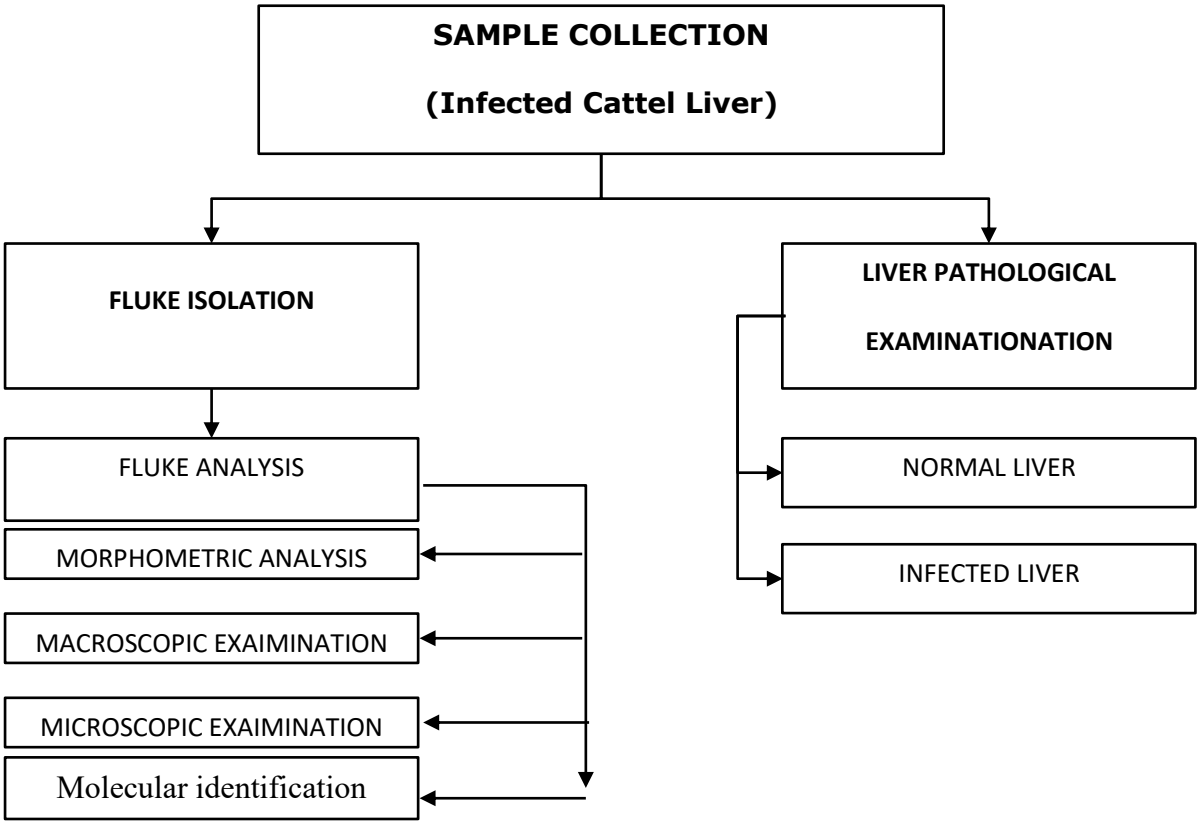

Figure 3.1 Flow chart of the study