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Molecular Prevalence of *Setaria digitata* and its Associated Risk Factors Among Horses in Kelantan

By

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MOLECULAR PREVALENCE OF *SETARIA DIGITATA* AND ITS ASSOCIATED RISK FACTORS AMONG HORSES IN KELANTAN

ABSTRACT

Setaria digitata is a filarial nematode primarily infecting cattle but capable of causing ocular and neurological diseases in horses through aberrant migration. Despite Kelantan being a major equine hub, molecular data on *Setaria digitata* in horses are limited in literature. This study investigated its molecular prevalence and associated risk factors in 31 horses sampled across three districts, using conventional PCR targeting mitochondrial 12S rDNA and COI genes. The 12S assay produced faint or multiple bands, with three samples showing the expected 450 bp band leading to a suspected prevalence of 9.7%, while COI amplification yielded no positive results. Several samples showed low A260/A230 ratios, indicating contamination that may have impaired PCR outcomes. Fisher's Exact Test showed no significant associations between PCR results and sex, age, breed, water source, or cattle proximity. Small sample size, limited site coverage, and DNA purity issues likely reduced detection sensitivity. These findings indicate a low but detectable presence of Setaria DNA in horses in Kelantan, highlighting the need for larger studies, improved DNA quality, and sequencing for definitive species confirmation.

Keywords: 12S rDNA, COI gene, Horses, Kelantan, Molecular detection, PCR, Prevalence, *Setaria digitata*

ABSTRAK

Setaria digitata ialah nematoda filaria yang lazimnya menjangkiti lembu tetapi juga mampu menyebabkan penyakit okular dan neurologi pada kuda melalui migrasi aberrant. Walaupun Kelantan merupakan hab ekuestrian utama, data molekul mengenai S. digitata dalam populasi kuda masih terhad. Kajian ini menilai prevalens molekul dan faktor risiko berkaitan dalam 31 ekor kuda dari tiga jajahan, menggunakan PCR konvensional yang menyasarkan gen mitokondria 12S rDNA dan COI. Ujian 12S menghasilkan jalur samar atau berganda, dengan tiga sampel menunjukkan jalur 450 bp seperti kawalan positif, memberikan prevalens disyaki sebanyak 9.7%, manakala amplifikasi COI tidak menghasilkan keputusan positif. Beberapa sampel menunjukkan nisbah A260/A230 yang rendah, menandakan pencemaran yang mungkin menjejaskan prestasi PCR. Ujian *Fisher's Exact* menunjukkan tiada hubungan signifikan antara keputusan PCR dengan jantina, umur, baka, sumber air atau jarak dengan ladang lembu. Saiz sampel yang kecil, liputan kawasan yang terhad, serta isu ketulenan DNA berkemungkinan mengurangkan sensitiviti pengesanan. Dapatan ini menunjukkan kewujudan DNA Setaria pada tahap rendah dalam kuda di Kelantan, dan menekankan keperluan kajian berskala lebih besar, peningkatan kualiti DNA, serta penjujukan untuk pengesahan spesies yang lebih tepat.

Kata kunci: 12S rDNA, gen COI, kuda, Kelantan, PCR, pengesanan molekul, prevalens, *Setaria digitata*

CERTIFICATION

This is to certify that we have read this research paper entitled 'Molecular Prevalence of *Setaria digitata* and its Associated Risk Factors Among Horses in Kelantan' ' by Isabel Sio Hwee Ling and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirements for the course DVT 55204 - Research Project.

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LIST OF ABBREVIATIONS

PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
COI / cox1	Cytochrome c Oxidase Subunit I
12S	12S Ribosomal RNA Gene (mitochondrial)
bp	Base Pair
µL / µl	Microlitre
GS Column	Geneaid Spin Column (from gSYNC Kit)
NFW	Nuclease-Free Water
TAE	Tris-Acetate-EDTA Buffer
EDTA	Ethylenediaminetetraacetic Acid
CNS	Central Nervous System
FPV	Faculty of Veterinary Medicine
UMK	Universiti Malaysia Kelantan
FYP	Final Year Project
BLAST	Basic Local Alignment Search Tool

CHAPTER 1

1.0 INTRODUCTION

Vector-borne parasitic infections remain a concern in tropical regions, especially those involving filarial nematodes like *Setaria* spp., which commonly infect ruminants with minimal clinical impact (Deplazes *et al.*, 2016). However, in aberrant hosts such as horses, *Setaria digitata* can migrate to the central nervous system or eyes, causing neurological or ocular disease (Gilger, 2022; Hafiz *et al.*, 2011).

Kelantan, a northeastern Malaysian state with a strong equine culture, holds nearly 20% of the national horse population in 2019. Horses in the state are often kept near cattle and mosquito breeding grounds, increasing the risk of *Setaria* transmission (Syamsul *et al.*, 2020; Hornok *et al.*, 2007). These horses are mainly raised for riding or equestrian sports which require good vision of the horse. However, the corneal opacity, decreased vision, or blindness due to ocular setariasis can be detrimental and even fatal to the horse and rider (Shin *et al.*, 2017).

While morphological identification of *Setaria* is challenging due to species similarity, molecular techniques such as PCR targeting the cox1 and 12S rDNA genes have proven reliable for accurate species identification (Chamuah *et al.*, 2015; Yatawara *et al*., 2010). These methods have successfully confirmed *Setaria digitata* in various hosts across Asia (Helmi *et al.*, 2025). The use of molecular diagnostics can significantly improve the detection rates of *Setaria* spp., allowing for more accurate epidemiological assessments and the establishment of effective control measures.

1. 1 RESEARCH STATEMENT

Setaria digitata infections in horses are often underdiagnosed due to their subclinical presentation and the limitations of conventional diagnostic methods such as blood smear microscopy and post-mortem examination. These traditional techniques are time-consuming and may fail to detect low-level or early-stage infections. Despite Kelantan's strong equestrian culture and presence of an indigenous horse breed, there is a lack of data on the molecular prevalence of *Setaria* spp. in the region's equine population. This gap in knowledge hinders effective surveillance and control measures. Given the advantages of molecular techniques like PCR in providing accurate and sensitive detection, this study seeks to determine the occurrence of *Setaria digitata* among horses in Kelantan using molecular diagnostic methods.

1.2 RESEARCH QUESTIONS

1. What is the molecular prevalence rate of *Setaria digitata* infection among horses in Kelantan?
2. What are the risk factors associated with *Setaria digitata* infection in horses?

1.3 RESEARCH HYPOTHESIS

1. There is a low (<16.9%) molecular prevalence rate of *Setaria digitata* infection among the sampled horse population in Kelantan.
2. The proximity of stable to still waters and cattle farms are significant risk factors associated with *Setaria digitata* infection in horses compared to host-related risk factors such as age, sex, and breed.

1.4 RESEARCH OBJECTIVES

1. To determine the molecular prevalence rate of *Setaria digitata* among horses in Kelantan.
2. To determine the risk factors associated with *Setaria digitata* infection in horses.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 HORSE POPULATION IN KELANTAN

In 2019, the horse population in Kelantan across 10 districts, namely Kota Bharu, Pasir Mas, Pasir Puteh, Bachok, Tumpat, Machang, Kuala Krai, Tanah Merah, Jeli and Gua Musang, was 951 horses (DVS, 2021). In the same year, 4904 horses were recorded in Malaysia (Quaza, 2019). Thus, Kelantan comprises 19.4% of the total horse population in Malaysia out of 14 states in 2019. The rearing or breeding of horses is considered to be part of the Kelantanese tradition (Bernama, 2019).

In Kelantan, horses are often kept as pets, for recreation or reared for racing (Nurul, 2023). To some, these horses offer emotional therapy from the tiredness of daily life. Pet horses are common among Kelantanese with injured racehorses to be sold as pets to non-racers. This naturally led to use of horses in recreational activities by owners (Razis, 2023). Horses are also involved in Kelantan's agrotourism with the addition of an animal farm in the Jeram Mengaji Agro Resort in the previous year (Rizal, 2024). In 2024, the Kelantan Youth and Sports Secretariat (U-BeS) aimed to popularise Mounted Archery as a leading sport in Malaysia as it is a well-known sport in Kelantan and Terengganu (Bernama, 2024). A training centre for this sport was set up in Persada Stud Farm at Jeram Mengaji Agro Resort 3 years prior (Bernama, 2021).

According to KudaKelate.blogspot.com (2012), the Kelantan mixed ponies (Kuda Kacukan) are considered to be Kelantan's indigenous breed of horses derived from the crossbreeding of Thoroughbred horses and Arabian horses introduced by ancient traders with Wild Asiatic horses from Mongolia descended from neighbouring countries centuries ago. These horses are hardy, resistant to diseases and unpredictable weather changes, able to eat local grass, smart, good temperament with great suitability for endurance racing. This breed is subdivided into three classes which are KKA (height between 51 to 55 inch) and KKB (height between 47 to 51 inch).

2.2 *SETARIA SPP.*

Species within this genus are typically non-pathogenic worms transmitted by mosquitoes and reside in the peritoneal or pleural cavities. They are slender white worms that grow up to 12 cm long with a spirally coiled posterior end. Their characteristic appearance include a mouth surrounded by a cuticular ring with dorsal, ventral and lateral prominences (Otranto *et al.*, 2024). Male worms possess four pairs of precloacal and generally four pairs of postcloacal papillae, with spicules that are unequal in size and shape. Female tails often exhibit several large, conical spines. The parasite's location and gross morphological features are usually sufficient for identification at the genus level. (Otranto *et al.*, 2024).

Multiple *Setaria* species, including *Setaria labiatopapillosa*, *Setaria digitata*, *Setaria cervi*, *Setaria marshalli*, *Setaria equina*, and *Setaria tundra*, have been reported globally. Among these, *Setaria digitata*, *Setaria marshalli*, and *Setaria labiatopapillosa*, are considered prevalent in cattle across Asian regions (Mrifag *et al.*, 2020).

According to Otranto *et al.* (2024), *Setaria equina* typically resides in the peritoneal cavity of equids and may occasionally be found in the scrotal area. *Setaria multipapillosa* inhabits the peritoneal cavity of cattle and various wild ruminants, including roe deer, red deer, antelopes, and giraffes. *Setaria digitata* is commonly located in the peritoneal cavities of cattle and buffaloes. Other species, such as *Setaria cervi* and *Setaria marshalli*, infect specific ruminants and are distributed across various geographical regions. Generally, adult *Setaria* species are considered to have low pathogenicity. However, in aberrant hosts like horses and goats, the larval stages, particularly of *Setaria digitata,* may migrate to the central nervous system, leading to localized encephalomyelitis, neurological impairment, and in severe cases, death (Deplazes *et al.*, 2016).

As mentioned, the parasite does not cause clinical manifestation and does not pose any harm when they are residing in their normal site and natural host, causing only mild fibrinous peritonitis that can be found during post-mortem. However, when these larvae migrate aberrantly away from their predilection site of peritoneal cavity and pleural cavity, they may enter the CNS or the eyes causing cerebrospinal or ocular setariasis (Gilger, 2022).

2.3 EQUINE SETARIASIS

Setaria spp. are known to cause microfilaraemia in their natural host. They typically inhabit the peritoneal cavity of animals world wide without causing significant clinical effects and are typically incidental findings during necropsy. In horses, clinical manifestations typically occur when the larval stage of these parasites migrate aberrantly to organs such as the central nervous system or the eyes. While *Setaria equina* is considered to be the species infecting equine, other *Setaria spp.* that are known to infect other species such as *Setaria digitata* and *Setaria labiatapapillosa*, can be transmitted to horses, causing clinical manifestation to these aberrant hosts (Hafiz *et al.*, 2011).

Migration to the central nervous system is associated with cerebrospinal nematodiasis in horses, whereby the parasite, particularly *Setaria digitata*, migrates to the brain or spinal cord, though spinal cord involvement is more frequently observed. This leads to clinical manifestations of paralytic signs which may include tail and bladder atony, ataxia, and deficits in conscious proprioception, depending on the region of affected spinal cord (Gilger, 2022). They also suffer from acute focal encephalomyelomalacia as a result of the cerebrospinal infiltration by the parasite. Clinical pathologically, the condition results in a decrease in total white blood cells and red blood cells, while causing an increase in eosinophils (Hafiz *et al.*, 2011).

Abnormal migration of the parasite into the eye causes ocular setariasis which is the most common form of setariasis in horses. This form manifests as corneal opacity, photophobia, lacrimations, blepharospasms as well as blindness (Gilger, 2022). In this form of setariasis, a white, thread-like worm can be seen moving in a swirling pattern in the aqueous humor. It may not be visible initially due to the corneal opacity and may require topical eye ointment such as Terramycin, to reduce the corneal opacity and allow the visualization of the worm within the aqueous humor.

2.4 *SETARIA DIGITATA*

Setaria digitata has distinctive physical characteristics. Males are typically 40 to 60 mm long, while females are larger, ranging from 60 to 120 mm. The species is identified by noticeable dorsal and ventral thickenings and a well-defined peribuccal ring. The tail of the female ends in a prominent button-like structure made up of several papillae. Male worms have two spicules of unequal length, with the left spicule about 2.5 times longer than the right. The microfilariae are enclosed in a sheath and measure between 240 and 260 micrometers. The parasite's life cycle begins with adult worms in the body cavity releasing larvae into the bloodstream. These microfilariae are taken up by mosquitoes, particularly from the Aedes and Culex genera, during feeding. Inside the mosquito, the larvae mature into the infective stage within 12 to 16 days and are transmitted to a new host during the mosquito's next blood meal.

The time from infection to the appearance of microfilariae in the host is approximately 8 to 10 months (Otranto *et al.*, 2024).

This species was specifically chosen as it is reportedly the most common cause of ocular setariasis in horses and most reported in tropical Asian countries where its vector thrives (Helmi *et al.*, 2025). Moreover, the first report of equine ocular setariasis and its molecular identification of *Setaria digitata* was done in Kelantan, showing that there is already some level of prevalence of this *Setaria* species in this state.

2.5 DIAGNOSIS

Generally, diagnosis of *Setaria* infection often are due to incidental findings of parasitic worms in peritoneal cavity during necropsy, findings of microfilariae during routine blood smear screenings or findings of these worms in the anterior chamber of the eyes during ocular examination.

As for cerebrospinal nematodiasis, confirmation of this parasite can only be done by observation of larval form in the microscopic examination of the spinal cord. In post-mortem, brown foci or streaks can be seen grossly due to the migration of larvae to the central nervous system. Microscopically, microcavitation and varying degree of haemorrhage can be seen. Loss of myelin and fragmentation of axons locally can be observed with the presence of inflammatory cells such as eosinophils, neutrophils, and macrophages, showing mild meningitis and vascular cuffing (Otranto *et al.*, 2024).

2.6 MOLECULAR DETECTION

The morphological identification has traditionally been used to identify *Setaria digitata*, its similarity to other *Setaria* species such as *S. equina* and *S. labiatopapillosa* makes accurate differentiation difficult (Maharana *et al.*, 2020). As a result, molecular techniques have become essential for precise species confirmation. The most widely used molecular markers are the mitochondrial cox1 (cytochrome c oxidase subunit I) gene and the 12S rDNA gene. The cox1 gene provides high resolution at the species level due to its variability, while 12S rDNA is more conserved and supports genus-level classification (Chamuah *et al*., 2015; Yatawara *et al.*, 2010). In a recent study on Aceh cattle, PCR amplification of these genes confirmed the identity of *Setaria digitata*, with BLAST results showing up to 99% sequence similarity with isolates from Sri Lanka, China, and India (Helmi *et al.*, 2025). Phylogenetic analysis using MEGA software further validated the grouping of the isolates within the *Setaria digitata* clade. These findings underscore the reliability of cox1 and 12S rDNA as molecular markers and highlight the importance of molecular detection in accurately assessing prevalence and guiding disease control strategies in endemic regions like Malaysia.

2.7 RISK FACTORS

The proximity of the stable with a cattle farm is closely related to the transmission of *Setaria spp.* from cattle to horse, leading to an increase in risk of aberrant setarial migration related to *Setaria digitata* (Hwang *et al.*, 2022). This leads to an increase in risk of abnormal forms of setariasis such as ocular and cerebrospinal setariasis. In a study by Hwang *et al.* (2022) on the epidemiological investigation of environmental risk factors for outbreak of nematode-related equine hindlimb ataxia, 85.4% of cases occur within 2km of a cattle ranch.

Located in the northeastern region of Peninsular Malaysia, Kelantan is primarily an agricultural state. The rearing of ruminants is commonly practiced on a small scale, often through backyard farming and smallholder systems (Syamsul *et al.*, 2020). This increases the risk of setarial transmission in this state.

Moreover, a close proximity to still water is a risk factor closely related to *Setaria spp.* transmission as it is a favoured habitat for the larval development of mosquitoes (Hornok *et al.*, 2007). Presence of still waters near to the stable or within the stable provides a breeding ground for mosquitoes increasing the population of vectors for Setaria infection. The parasite is spread through the bites of mosquitoes from several genera, including Anopheles, Culex, Aedes, Hyrcanus and Armigeres (Tung *et al.*, 2004). In the same study by Hwang *et al.* (2022) as mentioned previously, 75.6% of cases occur within 2 km from paddy fields with 5 times more likely to occur in horses near more than 2 paddy fields, whereas 61% occur within 0.5 km from rivers with 6 times more likely to occur compared to those located more than 0.5 km away.

Favorable temperature for the population growth and activity of mosquitoes is also a risk factor for the transmission of Setaria spp. (Hwang *et al.*, 2022). Due to the tropical climate of Malaysia, transmission of this disease is expected to be high due to the hot weather all year round leading to high mosquito activity throughout the year.

CHAPTER 3

3.0 RESEARCH METHODOLOGY

3.1 ETHICAL APPROVAL

Ethical approvals for using animals in the current study was obtained from the Animal Ethics Committee, Faculty of Veterinary Medicine of Universiti Malaysia Kelantan under the approval code of UMK/FPV/ACUE/FYP/012/2025 (Appendix A).

3.2 STUDY AREA AND TARGET POPULATION

The study was conducted in Kelantan, Malaysia, which comprises eleven districts. A total of 31 horses were sampled from five horse stables located in three districts, namely Kota Bharu, Bachok, and Tanah Merah. Of these samples, two were collected during clinical rotations at the Universiti Malaysia Kelantan (UMK) Veterinary Teaching Hospital during routine physical examinations. One horse originated from the teaching hospital in Bachok, while the other originated from Tanah Merah.

3.3 SAMPLE COLLECTION AND PROCEDURE

A total of 31 blood samples were collected from horses in Kelantan throughout July to October. The blood was aseptically withdrawn from the jugular vein with each horse providing approximately 3 to 5 ml of blood stored in an Ethylenediaminetetraacetic acid (EDTA) tube. The samples were collected using a 1 1⁄2 inch 21G vacutainer needle and each blood tube was labelled accordingly. They were kept chilled and wrapped with a layer of tissue paper in a polystyrene box with ice packs during transportation to the laboratory. At the laboratory, the samples will be stored in a chiller at 2 to 8 °C and processed within seven days.

Table 3.1 : Number of samples collected according to districts.

No.	District	No. of samples collected
1	**Bachok**	22
2	**Kota Bharu**	8
3	**Tanah Merah**	1
	Total	31

Whenever possible, information of the horses were obtained via verbal interview with the caretakers. Data such as the name, breed, and age of the horses were collected and recorded. Initially, information on the last deworming was planned to be collected. However, this information was unable to be collected as some caretakers were not aware of some of the horses' deworming status. Moreover, information on the risk factors such as the presence of cattle farms and water bodies within 2 km of the stable was collected. This was evaluated by observation during the journey to the stable as well as using Google Maps to search for nearby cattle farms and water bodies.

3.4 DNA EXTRACTION

Using the gSYNC™ DNA Extraction Kit (Geneaid, Taiwan), DNA was extracted from whole blood samples according to the manual provided by the manufacturer. Blood was left to warm to room temperature prior to beginning the DNA extraction process.

The blood sample was prepared by transferring 200 µL of whole blood to a 1.5 mL microcentrifuge tube. 20 µL of Proteinase K was added and mixed by pipetting. Then, the mixture was incubated at 60°C for 5 minutes. For cell lysis, 200 µL of GSB buffer was added and then mixed by shaking the mixture vigorously before incubating at 60°C for 5 minutes. The tube was inverted every 2 minutes. Moving on to DNA binding, 200 µL of absolute ethanol was added to the sample lysate and mixed immediately by shaking it vigorously for 10 seconds. Any precipitate was broken up as much as possible using a pipette. A GS Column was placed in a 2 mL collection tube. All of the mixture, including any insoluble precipitate, was transferred to the GS Column and centrifuged at 14,000 to 16,000 x g for 1 minute. The 2 mL collection tube containing the flow-through was discarded and the GS column was transferred to a new 2 mL collection tube.

For the next step, 400 µL of W1 buffer was added to the GS column and centrifuged at 14,000 to 16,000 x g for 30 seconds. The flow-through was discarded and the GS column was placed back into the 2 mL collection tube. 600 µL of Wash Buffer was added to the GS column and centrifuged at 14,000 to 16,000 x g for 30 seconds before discarding the flow-through. The GS column was placed back in the 2 mL collection tube and centrifuged for 3 mins at 14,000 to 16,000 x g to dry the column matrix.

For elution of the DNA from the column matrix, the dried GS column was transferred to a clean 1.5 mL microcentrifuge tube. 100 µL of pre-heated Elution Buffer was added to the centre of the column matrix and let stand for at least 3 minutes to allow the Elution Buffer to be completely absorbed. It was centrifuged at 14,000 to 16,000 x g for 30 seconds to elute the purified DNA. The extracted DNA was stored at -20°C until used in a PCR assay to detect the presence of setarial DNA.

3.5 DNA CONCENTRATION AND PURITY ASSESSMENT

DNA concentration and purity was assessed by using the NanoPhotometer® P-Class® (Implen, Germany). The machine was switched on and set up to use the dsDNA settings. The NanoPhotometer® P-Class Submicroliter Cell was inserted into the cell holder with the cell windows facing the light beam. Then, 1 µl of elution buffer was pipetted onto the centre of the measuring window while ensuring that there is no bubble and the droplet forms a dome-shape in the window. Then, lid 10 was put on and the blank button was pressed to obtain a blank reference range. After that, the steps were repeated using sample PCR products. It is important to repeat blank reference ranges using the same elution buffer regularly to ensure accuracy of readings. In this research, the blank reference range was repeated every three samples and samples with readings that are too low were repeated after the blank sample to confirm the low readings. All readings were recorded.

3.6 POLYMERASE CHAIN REACTION (PCR)

The molecular detection of *Setaria digitata* was performed using conventional PCR targeting the 12S rRNA gene of *Setaria digitata* and COI gene of Filaria. The 12S rDNA gene had the expected product size of 450 base pairs (bp). The forward and reverse primer sequence used was 5'- GTT CCA GAA TAA TCG GCT A -3' and 5'- ATT GAC GGA TCR TTT GTA CC -3' respectively. The COI gene had the expected product size of 680 bp using the forward and reverse primer sequence of 5'- TGA TTG GTG GTT TTG GTA A -3' and 5'- ATA AGT ACG AGT ATC AAT ATC -3', respectively.

Table 3.2 : Primer sequences used and the expected product sizes.

Target Gene	DNA Sequence	Product Size	Reference
12S rDNA	Forward: 5'- GTT CCA GAA TAA TCG GCT A -3' Reverse: 5'- ATT GAC GGA TCR TTT GTA CC -3'	450 bp	Junsiri *et al.* (2023), Helmi *et al.* (2025)
COI	Forward: 5'- TGA TTG GTG GTT TTG GTA A -3' Reverse: 5'- ATA AGT ACG AGT ATC AAT ATC -3'	680 bp	

A 20 µl PCR reaction mixture was prepared, comprising 5.5 µl of Nuclease Free Water (NFW), 12.5 µl of Go Taq® Green Master Mix (Promega, USA) , 1 µl of 10 µM forward primer, and 1 µl of 10 µM reverse primer. This mixture was scaled by the factor of 36 while taking into account an addition of four units for one positive control, one negative control and potential pipetting errors.

Table 3.3 : PCR master mix composition

PCR Components	1 unit	36 units
Nuclease Free Water (NFW)	5.5 µl	198.0 µl
Go Taq Green Master Mix	12.5 µl	450.0 µl
Forward Primer	1.0 µl	36.0 µl
Reverse Primer	1.0 µl	36.0 µl
DNA Template	5.0 µl	-
Total	25 µl	

The PCR amplification was conducted using the Mastercycler Nexus Gradient (Eppendorf, Germany). The PCR was done using two different techniques which are the standard PCR and the touchdown PCR, where by the standard technique was used for the 12S rDNA target and the touchdown PCR was used for the COI gene as per the protocol suggested by the Molecular Biology laboratory of the Faculty of Veterinary Medicine (FPV).

3.5.1 12S rRNA Using Standard PCR Protocol

As the standard PCR technique was applied, it uses a single, static annealing temperature for all cycles. The PCR conditions includes an initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 54°C for 45 seconds, and extension at 72 °C for 90 seconds, with a final extension step at 72 °C for 5 minutes.

Table 3.4 : PCR conditions for 12S rDNA

Step	Temperature / Time	Cycle
Initial Denaturation	94℃ / 3 min	1
Denaturation	94℃ / 45 sec	40
Annealing	54℃ / 45 sec	
Extension	72℃ / 90 sec	
Final Extension	72℃ / 5 min	1

3.5.2 COI Using Touchdown PCR

Touchdown PCR was applied for the primers targeting the COI gene which involved a starting high annealing temperature that gradually decreases over the first 8 cycles followed by 25 cycles of PCR conditions of denaturation at 94 °C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72 °C for 65 seconds, with a final extension step at 72 °C for 7 minutes.

Table 3.5 : PCR Conditions for COI (Touchdown PCR)

Step	Temperature / Time	Cycle
Initial Denaturation	94℃ / 2 min	1
Touchdown	94℃ / 45 sec 51℃ / 45 sec (-0.5℃ / cycle) 72℃ / 65 sec	8
Denaturation	94℃ / 45 sec	25
Annealing	45℃ / 45 sec	
Extension	72℃ / 65 sec	
Final Extension	72℃ / 7 min	1

3.7 GEL ELECTROPHORESIS

The resulting amplified PCR products were analysed using agarose gel electrophoresis. As the expected products were 450 bp for the 12S rDNA gene and 680 bp for the COI gene, 2% agarose and 1.5% agarose were used, respectively. To prepare the gel, 1.2g agarose powder was weighed and 80 ml of 1× TAE buffer in a Schott bottle was measured. 1.2g agarose powder and 60ml of buffer was added to the Schott bottle before swirling gently to dissolve the powder. The mixture was microwaved for 1 minute burst twice before adding the remaining 20ml and microwaved for another 1 minute. The addition of the buffer in two portions was done to prevent drying of the agar mixture. Once dissolved, 1 µl of Midori Green dye was added to the agarose solution and swirled immediately. To produce the 2% agarose gel, the same procedure was used with the change of 1.6 g of agarose was used instead.

Prior to the preparation of the agarose gels, a large dual slot gel tray was placed onto a surface and levelled using a circular bubble leveler to evaluate. Then, two 20-well combs were placed into the allocated slots. After the agarose gel was done, the gel was quickly and carefully poured onto the tray before the agarose gel solidified. Any bubbles observed are removed or pulled to the sides using a pipette tip. The gel tray was then left to solidify for a minimum of 30 minutes.

After the gel had set, the comb was gently removed and the gel tray was placed into an electrophoresis tank filled with a 1× TAE buffer ensuring the gel to be completely submerged. 6 µl of 100 bp DNA ladder was loaded into the first well of each row while 10 µl of PCR products was added into the remaining wells with 16 samples on the top row and 15 samples on the bottom row. 10 µl of positive control and negative control were added to the wells at the end of each row. Electrophoresis was performed at 100 V and 400 mA for 45 minutes. After that, the gel was visualised and the results were captured using Gel Doc (Biorad, USA). Positive results for the 12S rDNA primer should show 450 bp whereby the bands will be located between the 400 bp and 500 bp ladders, whereas the COI primer should show 680 bp whereby the bands will be located close to the 700 bp ladders.

3.8 STATISTICAL ANALYSIS

Data were analyzed using IBM SPSS Statistics (Version 29). Descriptive statistics were used to summarize frequencies and percentages of categorical variables, including sex, age category, breed category, presence of water source, and cattle farm status. Due to the variety of age and breed, they were grouped into two categories each. Ages were separated into the below and above 5 years old group as horses are considered to be adults at ages of 5 years old and above. The breeds were categorised into arabian line and non-arabian line to reduce the number of breeds. Anglo breeds are included in the Arabian line as it originated from a crossbreed between Thoroughbred and Arabian breed.

As several variables contained small cell counts (<5), the assumptions of the Chi-square test were not met. Therefore, the Fisher's Exact Test was selected as the primary statistical method to assess the association between categorical variables and PCR results. Fisher's Exact Test is appropriate for 2×2 contingency tables with low expected frequencies and provides an exact probability of observing the distribution by chance. A significance level of $p < 0.05$ was used for all analyses. Results were reported using two-sided p-values. Variables that were constant such as presence of cattle farms within 2 km, where all cases belonged to the same category, were excluded from inferential analysis.

CHAPTER 4

4.0 FINDINGS

4.1 DEMOGRAPHIC DATA OF HORSE SAMPLED

A total of 31 horses were sampled in this study. The majority of the horses were female (58.1%), while males made up 41.9% of the sample. In terms of age distribution, most horses were adults aged 5 years and above (77.4%), whereas only 22.6% were younger than 5 years. The horses represented several breeds. The largest group consisted of Anglo-Arab crosses (54.8%), followed by Warmbloods (19.4%). KKB/Pony and Thoroughbred horses each represented 9.7% of the sample. Polo and Arabian horses were the least represented, each making up 3.2% of the total sample.

This shows that the sample was mainly composed of adult horses and was dominated by the Anglo-Arab cross breed. There is a discrepancy in the number of horses in the age groups and breed is due to the type of sampling chosen, which is convenience sampling whereby samples were taken from stables randomly and these stables rears horses of different breeds and ages. Below is the summary of the total number of horses and its percentages of different groups.

Table 4.1: Demographics data of horses sampled

	No. of horse samples	Percentage (%)
Sex		
● Male	13	41.9
● Female	18	58.1
Age		
● < 5years old (young)	7	22.6
● ≥ 5 years old (adult)	24	77.4
Breed		
● Anglo-Arab cross	17	54.8
● KKB / Pony	3	9.7
● Warmblood	6	19.4
● Thoroughbred	3	9.7
● Polo	1	3.2
● Arabian	1	3.2

4.2 PRESENCE OF RISK FACTORS WITHIN 2 KM OF HORSES

The potential risk factors were identified during the literature review whereby journal articles mention that the presence of cattle farms and water bodies within 2 km to be significant in an increased risk of setaria infection. Thus, the presence of these factors within 2 km was identified. All stables except Stable 5 reported the presence of cattle farms within a 2 km radius. Large water bodies, such as rivers or lakes, were present near most stables as well. Only Stable 3 reported the absence of large water bodies, while Stable 5 had unknown data for both cattle farms and large water bodies.

Overall, this suggests that most horses were housed in areas with nearby cattle farms and water sources, which may potentially influence exposure to transmission of *Setaria digitata* microfilaria from cattle and increased breeding grounds for mosquitoes as the intermediate host.

However, due to the lack of variation in some variables, such as the fact that nearly all stables sampled had cattle farms nearby, statistical testing for these risk factors was limited.

Table 4.2: Presence of risk factors within 2 km of stables

	No. of horse samples	**Cattle farm**	**Water bodies**
Stable 1	1	Present	Present
Stable 2	6	Present	Present
Stable 3	8	Present	Absent
Stable 4	15	Present	Present
Stable 5	1	Present	Present

4.3 PCR RESULTS

4.3.1 12S rDNA

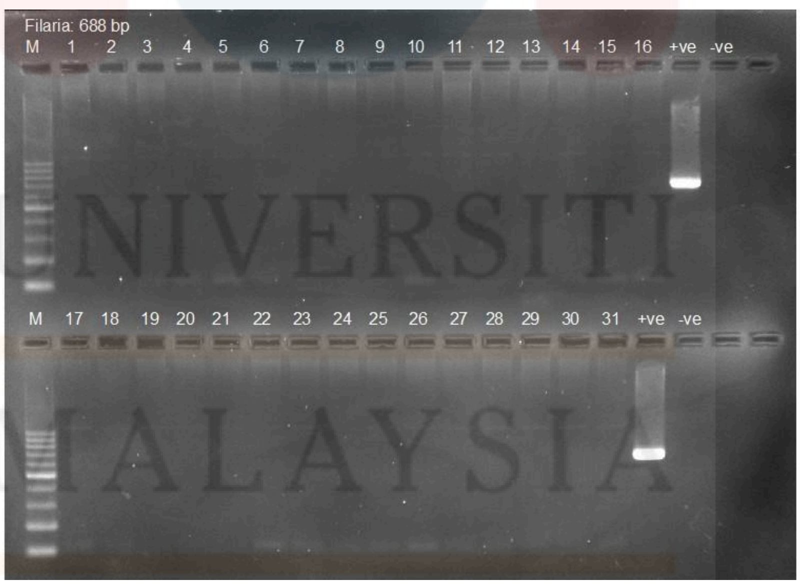
The amplification of the 12S rRNA gene for *Setaria digitata* revealed faint bands across 18 samples with 10 of them showing multiple bands. It was determined that there is no contamination of the sample as the negative sample showed an absence of bands. However, sample 1, 14 and 17 showed a faint band on the expected size despite having multiple bands. This raised a suspicion of *Setaria digitata* in these samples. The presence of a strong, bright single band at 450 bp in the positive control lane, validates the procedure.

Figure 4.1: Gel electrophoresis of 12S rDNA PCR (450bp)

4.3.2 COI

The amplification of the COI gene for Filaria revealed that all samples are negative by PCR. No bands were observed at the sample lanes at the expected position corresponding to the 680 bp for setarial DNA, as seen in the positive control. The presence of a band in the positive control lane validates the PCR procedure.

Figure 4.2: Gel electrophoresis of COI PCR (680 bp)



4.3.3 INTERPRETATION

The PCR analysis for detecting *Setaria digitata* in horse blood samples from Kelantan yielded conflicting results using two different targeting genes. Using the 12S rDNA primers, it yielded multiple bands in 18 samples with 3 samples showing bands with the expected product size of 450 bp, whereas the results using the COI gene primers, it yielded zero positive results. Both PCRs had an absence of bands on the negative control lane and a strong band on their respective expected product size on the positive control lane. This validates the results of the PCR done and confirms that there is no contamination of the PCR product. Thus, the sample 1, 14 and 17 was suspected to have detected presence of setarial DNA but unfortunately, it could not be confirmed as the sample was not sent for nucleotide sequencing.

Table 4.3: PCR interpretation of samples based on 12S results

Results	No. of horse samples	Percentage (%)
Positive	3	9.7
Negative	28	90.3

4.4 DNA CONCENTRATION AND PURITY

The assessment of DNA concentration and purity using the NanoPhotometer® P-Class® (Implen, Germany) yielded a result with a wide spectrum on DNA concentration ranging from as low as 12.5 ng/µl to 52 ng/µl. The DNA purity is represented by the A260/A280 ratio and A260/A230 ratio. The A260/A280 ratio assesses the protein contamination and should optimally lie within the range of 1.7 to 2.0 but the reading of 1.6 does not render DNA unsuitable but rather, lower ratios indicate more contaminants are present (Promega, 2025). The results obtained from this research were found to be 1.7 to 2.0 with only one sample falling below the optimal range at 1.6.

A260/A230 ratio indicates the contamination from organic compounds and salt used during extraction whereby Promega suggests that it should be more than 1.5. However, the ideal A260/A230 ratio should be within the range of 2.0 to 2.2 (Thermo Fisher Scientific, 2014). The results obtained from this research ranged from as low as 0.7 to as high as 2.7 with 11 samples yielding less than 1.5. This suggests that there is a high level of contamination from organic compounds and salt used during extraction, such as proteins, EDTA, phenol or guanidine HCL. across those 11 samples (Lucena-Aguilar *et al.*, 2016).

4.5 RISK FACTOR

4.5.1 DATA OF POSITIVE SAMPLES

The three samples suspected to be positive for *Setaria digitata* originated from two districts in Kelantan, namely Bachok (Samples 1 and 17) and Kota Bharu (Sample 14). Samples 1 and 14 were obtained from female horses aged 5 years and above, whereas Sample 17 was collected from a male horse below 5 years of age. The breeds represented among these suspected positive cases were Anglo for Sample 1, Arabian for Sample 14, and Arabian cross for Sample 17. All three horses were housed within 2 km of a cattle farm, which aligns with previous findings suggesting that proximity to cattle may increase exposure to *Setaria* species. In terms of water sources, Samples 1 and 17 were collected from locations with water bodies within a 2 km radius, while Sample 14 originated from an area without nearby water bodies. These findings indicate variability in demographic and environmental characteristics among the suspected positive horses, although the small number of cases limits definitive interpretation.

Table 4.4: Information of Positive samples

Variables	Sample 1	Sample 14	Sample 17
District	Bachok	Kota Bharu	Bachok
Sex	Female	Female	Male
Age group	≥ 5 years old	≥ 5 years old	< 5 years old
Breed	Anglo	Arabian	Arabian cross
Presence of cattle farm within 2 km	Yes	Yes	Yes
Presence of water bodies within 2 km	Yes	No	Yes

4.5.2 SEX

Fisher's Exact Test showed no significant association between sex and PCR status. Female horses had an 11.1% positivity rate, while males had 7.7%. The difference was not statistically significant ($p = 1.000$) (Table 1).

Table 4.5: Associations between sex and PCR results.

Sex	Negative (n)	Positive (n)	Total	Fisher's Exact p
Female	16	2	18	
Male	12	1	13	
Total	**28**	**3**	**31**	**1.000**

4.5.3 AGE GROUP

Age group (<5 years vs. >5 years) was not significantly associated with PCR results. Horses <5 years had a 14.3% positivity rate, compared to 8.3% in older horses. This difference was not statistically significant ($p = 0.550$) (Table 2).

Table 4.6: Associations between age groups and PCR results.

Sex	Negative (n)	Positive (n)	Total	Fisher's Exact p
Female	16	2	18	
Male	12	1	13	
Total	**28**	**3**	**31**	**1.000**

4.5.4 BREED

Arabian-line horses had a higher positivity rate (16.7%) compared to non-Arabian horses (0%). However, the association was not statistically significant (p = 0.245) (Table 4). The apparent difference may reflect the small sample size rather than a true biological effect.

Table 4.7: Associations between breed category and PCR results.

Breed Category	Negative (n)	Positive (n)	Total	Fisher's Exact p
Arabian-line	15	3	18	
Non-Arabian	13	0	13	
Total	**28**	**3**	**31**	**0.245**

4.5.5 PRESENCE OF CATTLE FARM WITHIN 2 KM

The variable Cattlefarm could not be analyzed because all horses belonged to stables with a cattle farm within 2 km, making the variable constant and non-informative for statistical comparison.

4.5.6 PRESENCE OF WATER BODY WITHIN 2 KM

The presence of a water source showed no association with PCR results. Positivity rates were similar for horses with (8.7%) and without (12.5%) a water source, with p = 1.000 (Table 3).

Table 4.8: Associations between water bodies within 2 km and PCR results.

Water Body	Negative (n)	Positive (n)	Total	Fisher's Exact p
Absent	7	1	8	
Present	21	2	23	
Total	**27**	**3**	**31**	**1.000**

CHAPTER 5

5.0 DISCUSSION

In this study, molecular detection of *Setaria digitata* using two genetic markers, 12S rDNA and COI, produced conflicting outcomes. While the 12S rDNA PCR successfully amplified multiple bands in several samples, with three samples showing a band consistent with the positive control, the COI PCR failed to yield detectable amplification in all samples. This contradicting results are likely due to inherent differences in biology marker, primer specificity and amplification dynamics.

Firstly, 12S rDNA genes are fragments of gene that encodes for the mitochondrial ribosome. It is a conserved gene as the sequence changes slowly over evolutionary time. It is frequently used for molecular detection due to its high sensitivity. Studies have demonstrated that 12S primers can robustly amplify helminth DNA, and are especially useful when parasite DNA is present in low quantity. It also has a broad species detection including across invertebrates such as nematodes, trematodes and cestodes whereby they were successfully identified to species level which are usually more difficult with other markers like 18S and COI (Chan *et al.*, 2022). 12S rDNA exists in a very high copy number as each cell contains many mitochondria and each mitochondrion has multiple copies of mitochondrial DNA.

Thus, the 12S rDNA is present in hundreds and thousands of copies per cell, making it easy to amplify even from degraded or small amounts of parasite DNA. However, this broad species detection comes at the cost of reduced specificity. A study aimed to detect filarial DNA in faecal samples found that the amplification results of 12S amplifies broadly but cannot differentiate species well (Gaillard *et al.*, 2020). This is consistent with the 12S rDNA PCR results of multiple bands found in 18 samples from this research.

In contrast, COI gene, also known as Cytochrome Oxidase 1 or COX1 gene is the main subunit of the cytochrome c oxidase complex which exists in multiple copies per cell but fewer than 12S rDNA. It is commonly used for DNA barcoding and offers higher inter-species resolution as it is more variable and less sensitive than the 12S gene. The higher variability and longer amplicons at 600 to 700 bp allows for a higher specificity leading to less likely result of multiple bands due to non-specific amplifications. In the same study of the detection of filarial DNA in fecal sample mentioned previously, the results show that COI gene generated a larger proportion of high-quality sequences usable for phylogenetic analysis which indicates that COI amplifies well as well as its variable sequence yields clearer and more reliable phylogenetic grouping (Gaillard *et al.*, 2020).

While its higher variability allows a more accurate species identification, it also pose an important challenge that is exhibited in this research findings. The COI primers may fail to bind in field isolates with sequence divergence, and PCR success can be compromised in samples with low parasite load or degraded DNA (PubMed, 2011). This is due to the fewer copies present as well as the longer amplicons at 600 to 700 bp, causing it to become harder to amplify. The longer amplicons means that it has more variable primer-binding sites, less tolerant to degraded DNA and is more affected by inhibitors. This explains why COI amplification failed in our samples, even though 12S rDNA indicated the presence of *Setaria* DNA. Importantly, COI failure in PCR does not necessarily indicate absence of infection, but rather may reflect suboptimal primer binding, low template concentration, or PCR inhibitors.

To evaluate the quality of DNA extracted, the DNA concentration and purity was assessed using a nanophotometer which yielded satisfactory DNA concentration and A260/A280 ratio. This indicates that there is sufficient DNA concentration within the sample with minimal protein contamination. However, the A260/A230 ratio is significantly lower than the ideal range at 2.0 to 2.2 value, whereas 11 samples did not reach the minimum value recommended by Promega at values lower than 1.5. This indicates that the extracted DNA sample contains contaminants of organic compounds such as phenol, guanidine or complex carbohydrates (Srirunguang *et al.*, 2022). The DNA extraction kit used contains guanidine hydrochloride in the GB buffer and does not contain phenols.. The presence of such components in the extracted DNA are the result of improper technique when carrying out the DNA extraction leading to the chemical contamination of the extracted DNA sample.

Guanidine is classified as a mixed mode inhibitor which inhibits both polymerase and DNA-binding (McCord *et al.*, 2015). According to the study done by McCord *et al.* (2015), guanidine partially denatures or inactivates Taq polymerase, leading to reduced PCR efficiency. It also affects DNA stability especially at the single-stranded regions. This causes the reduced availability of templates and ultimately, causes reduction in amplicons, especially in longer amplicons. Applying this information to this research, it is consistent with the absence of bands in the amplification of COI gene as compared to the shorter 12S rDNA gene. The presence of guanidine in the extracted DNA coupled with the lower sensitivity of COI gene, lower copies per cell and possibly too low level of setarial DNA in the sample, together these limitations significantly compromise amplification efficiency, supporting the observation that the COI PCR showed no detectable bands.

In short, one plausible interpretation of our results is that the 12S assay is sensitive enough to detect *Setaria* DNA in a subset of samples, but it also amplifies non-target DNA which causes the extra bands. Meanwhile, the COI assay may be failing not because *Setaria* is absent, but because of suboptimal binding or low template, leading to false-negative results. This suspicion can be overcome by assessing the DNA concentration and purity before performing the PCR. If the DNA purity is unsatisfactory, the extracted DNA samples should be purified using a purification kit before conducting the PCR.

The sample size in this study was limited to 31 horses which is insufficient for producing representative results as Kelantan has an estimated horse population of 951 (DVS, 2021). Using an appropriate sample size calculation, a sample size of 278 would be required for statistically significant findings. The small sample size in this study may not have adequately captured the diversity and prevalence of *Setaria digitata* in the population which leads to the lack of positive detection. A larger sample size is necessary to increase the probability of identifying infected individuals and obtaining a more accurate detection rate.

Furthermore, the horses were sampled from only two different districts in Kelantan which may not reflect the broader horse population across this state. The limited geographic representation may have contributed to the low detection rate as different farms or districts may have varying environmental conditions and risk factors for *Setaria digitata*. This study relied exclusively on blood samples for PCR analysis which may not be sufficient for detecting *Setaria digitata*. While there is no gold standard method to detect microfilariae in blood, blood parasitology, such as thick blood smear and Knotts technique, is the preferred method traditionally (Melchers et al., 2020).

In this study, several factors such as sex, age group, breed category, water source availability, and cattle farm exposure were tested to see if they were associated with PCR positivity. Based on Fisher's Exact Test, none of these variables showed a significant relationship with the PCR results. One of the main reasons for this may be the small number of positive cases in the dataset (only 3 out of 31 horses). With such a low number of positives, it becomes difficult to detect any real differences between groups. Although Fisher's Exact Test is suitable for small samples, it still cannot overcome the lack of variation in the data. For example, Arabian-line horses had more positive cases than non-Arabian horses, but the difference was not statistically significant. This could be due to limited sample size rather than the absence of a true association.

Some variables also did not have enough variation to be analyzed properly. In the case of Cattlefarm, all horses came from farms where cattle were present, so no comparison could be made. Similarly, the detailed breed categories contained too many small groups, which made statistical testing unsuitable. Previous studies have suggested that factors like breed, environment, and management practices can influence infection risk, but larger and more diverse samples are usually needed to detect these trends. Therefore, the lack of significant results in this study does not necessarily mean these factors have no effect. Instead, it may simply reflect the limitations of the sample used.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study represents an early attempt to investigate the molecular prevalence of *Setaria digitata* among horses in Kelantan using two molecular markers, 12S rDNA and COI. The findings revealed a suspected prevalence of 9.7%, with three samples showing amplification consistent with *Setaria digitata* using the 12S rDNA marker. However, all samples tested negative for the COI gene. These conflicting results are most likely attributed to biological differences between markers, low parasite load, and the presence of PCR inhibitors, particularly guanidine contamination, reflected in the low A260/A230 ratios of several samples.

Furthermore, statistical evaluation showed no significant association between infection status and sex, age group, breed category, water sources, or proximity to cattle farms, although these outcomes are strongly influenced by the small number of positive samples and limited sample size. The restricted number of horses, limited geographical representation, and reliance solely on blood-based PCR may have contributed to the underestimated detection rate.

Despite these limitations, the detection of suspected positives indicates that *Setaria digitata* may be present at low levels within the equine population in Kelantan. The findings highlight the need for improved diagnostic protocols and more comprehensive surveillance to better understand the epidemiology and potential impact of equine setariasis in the region.

6.2 RECOMMENDATIONS

Based on the findings of this study, several recommendations are proposed to improve future research on the molecular detection of *Setaria digitata* in horses. Increasing the sample size and expanding sampling to all districts in Kelantan would provide a more accurate and representative estimate of prevalence. Improving DNA extraction quality through additional purification steps is strongly advised, as the presence of contaminants such as guanidine may inhibit PCR amplification, particularly for longer gene targets like COI.

Future studies should also incorporate additional diagnostic methods, such as Knott's test, thick blood smear examination, or sampling of aqueous humor in ocular cases, to enhance detection sensitivity, especially when microfilarial levels in blood are low. Samples that produce suspected positive bands should be submitted for nucleotide sequencing to allow definitive species confirmation and avoid misinterpretation due to non-specific amplification.

Furthermore, collecting more detailed environmental and management data, such as mosquito population density, vector control practices, and stable management systems, would provide greater insight into potential risk factors. Finally, increasing awareness among horse owners and veterinarians regarding early clinical signs of setariasis and emphasizing the importance of vector control and routine health monitoring will help reduce the risk of infection within equine populations in Kelantan.

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APPENDIX

Appendix A:

Approval from the Animal Ethics Committee, Faculty of Veterinary Medicine of Universiti Malaysia Kelantan under the approval code of UMK/FPV/ACUE/FYP/012/2025.

UNIVERSITI MALAYSIA KELANTAN
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UNIVERSITI MALAYSIA KELANTAN

FAKULTI PERUBATAN VETERINAR
Faculty of Veterinary Medicine

Ruj. Kami (*Our Ref.*) : UMK.A06.800-1/2/8 (12)
Tarikh (*Date*) : 1st SEPTEMBER 2025

DR. TAN LI PENG
Main Supervisor
Faculty of Veterinary Medicine

Dear Dr.,

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) TO CONDUCT RESEARCH INVOLVING ANIMALS

We are pleased to inform you that your application for approval to conduct research from Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University Malaysia Kelantan has approved. Please refer the table below for approval code:

Approval Code	***UMK/FPV/ACUE/FYP/012/2025***
Title	**Molecular Prevalence of Setaria Digitata and its Associated Risk Factors Among Horses in Kelantan**
Duration of Research	**04 September 2025 – 10 January 2026**
Number of Sample/Strain/Gender	**50/ Equus Ferus caballus (Domesticated Horse)/**
Name of Student	**ISABEL SIO HWEE LING – D21B0111** **NUR AMIRA SYAFIEQA BINTI YA'AKOB – D21A0129**

You are advised to always follow "3R" (REDUCE, REFINE, & REPLACE) and all animal ethics and animal welfare principles to reduce suffering in animal.

Thank you.

"ISLAM MEMBIMBING, RAJA MEMIMPIN, NEGERI DIBERKATI"
"MALAYSIA MADANI"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

(DR. MOHAMMED DAUDA GONI)
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine

ENTREPRENEURSHIP IS OUR THRUST

Appendix B: Table of Demographic Data of Horse Samples (n=31)

Name	Breed	Sex	Age	Cattle farm	Water	PCR
Mira	Arabian line	Female	>5	Present	Present	Positive
Cassandra	Arabian line	Female	>5	Present	Present	Negative
Rizikizul	Arabian line	Male	>5	Present	Present	Negative
Joker	Non-Arabian line	Female	>5	Present	Present	Negative
Wawa	Arabian line	Female	>5	Present	Present	Negative
Felia	Arabian line	Female	>5	Present	Present	Negative
Puteri	Non-Arabian line	Female	>5	Present	Present	Negative
Rayyan	Arabian line	Male	>5	Present	Present	Negative
Mirzan	Arabian line	Male	>5	Present	Absent	Negative
Black Putera	Arabian line	Male	>5	Present	Absent	Negative
KY Putera	Arabian line	Male	>5	Present	Absent	Negative
Black Boy	Arabian line	Male	<5	Present	Absent	Negative
Marie	Arabian line	Male	>5	Present	Absent	Negative
Angela	Arabian line	Female	>5	Present	Absent	Positive
KJ Putera	Arabian line	Female	>5	Present	Absent	Negative
Dubai	Arabian line	Male	<5	Present	Absent	Negative
Bobby	Arabian line	Male	<5	Present	Present	Positive
Q	Arabian line	Female	<5	Present	Present	Negative
Flower	Non-Arabian line	Female	>5	Present	Present	Negative

Icarus	Non-Arabian line	Male	>5	Present	Present	Negative
King	Arabian line	Male	>5	Present	Present	Negative
Pokchek	Non-Arabian line	Male	>5	Present	Present	Negative
Bespoke	Arabian line	Male	>5	Present	Present	Negative
Lady	Non-Arabian line	Female	>5	Present	Present	Negative
Kabir	Non-Arabian line	Female	<5	Present	Present	Negative
Angle	Non-Arabian line	Female	>5	Present	Present	Negative
TM	Non-Arabian line	Female	>5	Present	Present	Negative
Makmo	Non-Arabian line	Female	>5	Present	Present	Negative
Yatim	Non-Arabian line	Female	>5	Present	Present	Negative
Kukuburuk	Non-Arabian line	Female	<5	Present	Present	Negative
W1	Non-Arabian line	Female	<5	Present	Present	Negative

Appendix C: Table of Results of DNA Concentration and Purity Assessment

Sample	Conc (ng/µl)	A260/280	A260/230
1	52	1.8	2.0
2	40	1.8	1.9
3	41.5	1.8	1.9
4	14.5	1.7	0.9
5	34	1.8	1.8
6	29	1.8	1.1
7	41	1.8	1.4
8	40	1.8	1.5
9	20.5	1.8	1.7
10	46	1.9	1.6
11	51.5	1.9	1.9
12	28	1.8	1.6
13	26.5	1.8	1.8
14	33.5	1.9	1.9
15	31	1.9	1.7
16	34	1.8	1.5

Sample	Conc (ng/µl)	A260/280	A260/230
17	28	1.8	0.9
18	21	1.8	1.5
19	30.5	1.8	1.5
20	15	1.8	1.4
21	20.5	1.7	0.9
22	25	1.7	1.1
23	12.5	1.9	2.7
24	23.5	1.6	0.7
25	25.5	1.8	1.5
26	21.5	1.7	1.0
27	16.5	1.7	1.5
28	29.5	1.7	1.3
29	31	1.8	1.5
30	26.5	1.8	1.5
31	30.5	1.8	1.2