PHENOTYPIC AND PROTEOMIC ANALYSIS ASSOCIATED WITH MACROCYCLIC LACTONES-IVERMECTIN EXPOSURE IN *Caenorhabditis elegans*

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by

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
GDP	Gross Domestic Product
IPG	Immobilized pH gradient
Ident.	Identity
IVM	Ivermectin
kDa	Kilodalton
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Fourth-stage larvae
L-Broth	Luria Broth
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NGM	Nematode Growth Medium
No.	Number
nr	Non-redundant
pI	Isoelectric point
rpm	Revolutions per minutes

SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
spp.	Species
TEMED	Tetramethylethylenediamine

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
±	Plus-minus
=	Equal to
<	Less than
µg	Microgram
µL	Microliter
cm	Centimeter
g	Gram
L	Liter
M	Molar
mg	Milligram
mL	Milliliter
mm	Millimeter
ng	Nanogram
nm	Nanometer
pH	Potential of hydrogen
V	Voltage
w/v	Weight per volume
X	Times

FENOTIP DAN ANALISIS PROTEOMIK BERHUBUNG KAIT DENGAN PENDEDAHAN MACROCYCLIC LACTONES-IVERMEKTIN DALAM *Caenorhabditis elegans*

ABSTRAK

Rintangan antelmintik telah dilaporkan dalam hampir semua spesies parasit ternakan dan melibatkan semua kelas utama spektrum luas dadah antelmintik termasuk ivermektin. Oleh kerana kajian rintangan ivermektin dalam nematod parasit adalah terhad, maka, *Caenorhabditis elegans* yang hidup bebas telah digunakan sebagai sistem model dalam kajian ini. Ujian kepekaan ivermektin telah dijalankan untuk menyiasat tindak balas strain *C. elegans* N2 yang jenis liar kepada pendedahan ivermektin. Enam ujian *in vitro* telah dijalankan; ujian penetasan telur, ujian perkembangan larva, ujian membiak, ujian gerakan, ujian motiliti, dan ujian pengepaman faring dengan strain *C. elegans* DA1316 yang rintang IVM sebagai kawalan. Keputusan menunjukkan bahawa ivermektin adalah berkesan pada larva tetapi tidak pada telur strain *C. elegans* N2 yang jenis liar. Ivermektin menghalang perkembangan larva, perilaku pengeluaran telur, gerak alih, dan perilaku pemakanan oleh strain *C. elegans* N2 yang jenis liar. Berikutan ujian kepekaan ivermektin, pendekatan proteomik telah dijalankan bagi mengenal pasti kemungkinan ekspresi protein yang diatur naik dan diatur turun oleh strain *C. elegans* N2 yang jenis liar kepada pendedahan ivermektin. Gel elektroforesis dua dimensi telah dijalankan untuk membandingkan peta protein antara strain *C. elegans* N2 yang rintang IVM (kumpulan rawatan) dan strain *C. elegans* N2 yang jenis liar (kumpulan kawalan). Antara 25 tompok protein yang dipilih, 18 tompok protein telah diatur naik dan tujuh tompok protein telah diatur turun dalam kumpulan rawatan berbanding kumpulan

kawalan. Analisis selanjutnya oleh spektrometri jisim MALDI-TOF telah mengenal pasti 12 protein daripada 18 tompok protein yang diatur naik dan satu protein daripada tujuh tompok protein yang diatur turun. Protein yang diatur naik terdiri daripada protein kejutan haba 70 kDa A, calreticulin, aktin-2, aktin-3, aktin-4, ATP sintase subunit alpha, ATP sintase subunit beta, kemungkinan jenis proton V ATPase subunit B, kemungkinan kinase arginin, faktor permulaan transkripsi IIB, adenosylhomocysteinase, dan vitelogenin. Protein yang diatur turun adalah kemungkinan S- adenosylmethionine sintase. Protein ini; yang terlibat dalam caperon molekular, motiliti sel, metabolisma dan penghasilan tenaga, ungkapan regulasi gen, penyimpanan lipid, dan sebagai protein prekursor; mungkin berkaitan dengan rintangan ivermektin yang diperolehi oleh *C. elegans*. Sebagai penemuan asas untuk mekanisme penyesuaian dan perlindungan oleh *C. elegans* untuk mempertahankan terhadap ancaman alam sekitar dan kerosakan selular, kajian ini akan membantu menyampaikan peranan sistem saraf dan laluan tambahan untuk meneroka rawatan jangkitan helmin dalam ternakan.

PHENOTYPIC AND PROTEOMIC ANALYSIS ASSOCIATED WITH MACROCYCLIC LACTONES-IVERMECTIN EXPOSURE IN *Caenorhabditis elegans*

ABSTRACT

Anthelmintic resistance has been reported in almost all species of parasites of livestock and involved all the major classes of broad-spectrum anthelmintic drug including ivermectin. Due to the study of ivermectin resistance in parasitic nematode is limited, thus, a free-living *Caenorhabditis elegans* was used as a model system in this study. The ivermectin sensitivity assay was carried out to investigate the response of wild-type *C. elegans* N2 strain to ivermectin exposure. Six in vitro assays were conducted; egg hatch assay, larval development assay, reproduce assay, thrashing assay, motility assay and pharyngeal pumping assay with IVM-resistant *C. elegans* DA1316 strain as a control. The results showed that ivermectin is effective on larvae but not on the eggs of wild-type *C. elegans* N2 strain. Ivermectin inhibits the larval development, egg-laying behavior, locomotion and feeding behavior of wild-type *C. elegans* N2 strain. Following the ivermectin sensitivity assay, the proteomic approach was carried out to identify the possible up-regulated and down-regulated protein expression of wild-type *C. elegans* N2 strain to ivermectin exposure. Two-dimensional gel electrophoresis was performed to compare the protein maps of the IVM-resistant *C. elegans* N2 strain (treated group) and wild-type *C. elegans* N2 strain (control group). Among the 25 selected protein spots, 18 protein spots were up-regulated and seven protein spots were down-regulated in the treated group compared to the control group. A subsequent analysis by MALDI-TOF mass spectrometry has identified 12 proteins of the 18 up-regulated

protein spots and a protein of the seven down-regulated protein spots. The up-regulated proteins consist of heat shock 70 kDa protein A, calreticulin, actin-2, actin-3, actin-4, ATP synthase subunit alpha, ATP synthase subunit beta, probable V-type proton ATPase subunit B, probable arginine kinase, transcription initiation factor IIB, adenosylhomocysteinase, and vitellogenin. The down-regulated protein is probable S-adenosylmethionine synthase. These proteins; which are involved in molecular chaperone, cell motility, metabolism and energy production, gene regulation expression, lipid storage, and as a precursor protein; might be related in the acquired ivermectin resistance in the *C. elegans*. As a fundamental discovery for adaptive and protective mechanisms in *C. elegans* to defend against the environmental threat and cellular damage, this study will help to cast light on the role of the nervous system and the additional pathways to explore for the treatment of helminth infection in livestock.

CHAPTER 1

INTRODUCTION

The livestock industry is a significant factor in the agricultural sector in Malaysia. It provides employment opportunities and producing food sources of animal protein for the population. This industry can be classified into two sectors which are ruminant and non-ruminant. The non-ruminant sector such as poultry and swine has been able to commercial and efficient in production (Ariff et al., 2015). However, the ruminant sector such as buffalo, cattle, goat, and sheep is not well production explored in which the production is less than demand and need to be imported from abroad (Jamaludin et al., 2014; Ariff et al., 2015).

There are many factors affect the livestock production and productivity, which are climate changes, health aspects, and nutrition supply (Thornton, 2010; Lamy et al., 2012). However, the factor that gives most impact on the livestock production is the health aspects involving diseases and parasites such as helminthiasis and pneumonia (Chandrawathani et al., 2009). The livestock usually gets infected with parasitic nematode through the free contact with their eggs or larvae (Bethony et al., 2006). These parasites live and reproduce by sucking the nutrient in the livestock and causes the animal getting sick, lose weight, and decrease in production and productivity (Florian et al., 2013). It caused direct loss to farmers due to decline in production and death of livestock (Jabbar et al., 2006).

The used of chemotherapy agent such as anthelmintic drug was managed to minimise these infections for a while. However, a new problem has emerged because

the parasitic nematodes have become resistant to the anthelmintic drug. The resistance is said to be developed when the previously effective drug is no longer kill the parasitic population at the therapeutically recommended dosages (Jackson, 1993). The resistance has been reported in nematode parasites of almost all species of livestock and included all the major classes of broad-spectrum anthelmintic drug (Jabbar et al., 2006). In Malaysia, the frequently used of the drugs with no control has triggered the emergence of drug resistance in the small ruminant population (Chandrawathani and Nurulaini, 2012).

In order to maintain anthelmintic drug efficacy, it is essential to disclose the mechanism of action of the drug and the molecular mechanism employed by the parasitic nematode that results in resistance. The development of molecular-based test gives the opportunity to understand the mechanisms of anthelmintic adaptation in parasitic nematode that turn into resistance. This study is focused on ivermectin drug as it remains a major global anthelmintic (Crump and Omura, 2011). Moreover, the study of ivermectin resistance in parasitic nematode is very limited due to the complex life cycle of parasitic nematode and also the lack of appropriate functional genomic assay (Jones et al., 2005; Britton and Murray, 2006; Holden-Dye and Walker, 2007). Thus, the *Caenorhabditis elegans* was used as a model organism for the parasitic nematode as they share some physiological and pharmacological characteristics (Geary and Thompson, 2001; Jones et al., 2005; Britton and Murray, 2006; Holden-Dye and Walker, 2007).

Ivermectin kills the *C. elegans* at the therapeutic concentration that making the *C. elegans* as a useful model system to examine the mechanism of ivermectin toxicity and resistance (Dent et al., 2000). The data from this study will expand our understanding of how cellular remodeling might occur in *C. elegans* to ivermectin

stress response and the survival of *C. elegans* at the protein level.

1.1 Research objectives

The objectives of this study are as follows:

i. To investigate the response of *C. elegans* to ivermectin exposure.

ii. To identify the possible up-regulated and down-regulated protein expression of *C. elegans* to ivermectin exposure.

CHAPTER 2

LITERATURE REVIEW

2.1 Livestock industry in Malaysia

Agriculture is one of the main contributors to the Malaysian economy. This sector contributed 8.9% (RM94.1 billion) to the Gross Domestic Product (GDP) in 2015 (Department of Statistics Malaysia, 2016) and showed an increased about 1.2% from previous year (Department of Statistics and Ministry of Finance Malaysia, 2016). Of the total, 10.7% was from the livestock industry which consists of poultry meat, chicken/duck egg, cattle milk, beef, mutton and swine (Department of Statistics Malaysia, 2016). According to Department of Veterinary Services (2016), the production of poultry meat, chicken/duck egg, and cattle milk showed an increased in 2015 about 2.6%, 6.5%, and 0.9% compared to the previous year, however, the production of beef, mutton, and swine showed a decreased about 4.5%, 2.2%, and 0.8%. The poultry meat and chicken/duck egg recorded self-sufficiency ratio more than 100% which is 104.5% and 116.7%, while the beef, mutton, swine and cattle milk recorded self-sufficiency ratio less than 100% which is 23.5%, 11.5%, 94.6% and 7.0% (Department of Veterinary Services, 2016). The slower growth of production and higher growth of consumption is probably the cause of the decline in self-sufficiency ratio of beef, mutton, and swine.

2.2 The parasitic nematodes of livestock

Nematodes are typically worm-like which have a body that is long, narrow and threadlike, except they are un-segmented (Kiontke and Fitch, 2013). They are simple in body plan, lacking appendages and colorless (Kiontke and Fitch, 2013). Basically, the body form is similar to a flexible cylinder with a rounded head and a pointed tail. They have been characterized as a tube within a tube because of the alimentary canal which extends from the mouth on the anterior end to the anus located near the tail (Kiontke and Fitch, 2013). They possess the digestive, nervous, excretory and reproductive systems but lack a discrete circulatory or respiratory system (Kiontke and Fitch, 2013). They move by contraction of the dorsal and ventral longitudinal muscles which is thrashing back and forth, and at least a film of water is required for dynamic movement (Kiontke and Fitch, 2013).

Many of them are free-living and play critical ecological roles as decomposer and predator on microorganisms (Blaxter et al., 1998; Dorris et al., 1999). However, there are also including parasitic species which are cause diseases on animals, plants and humans such as roundworms, hookworms, trichina, pinworms and filarial worms (Blaxter et al., 1998; Dorris et al., 1999). These species can be distinguished by the body size and feeding structure (Kiontke and Fitch, 2013). The bacterial-feeding nematodes have no teeth or stylet like the plant-feeding nematodes that have a hypodermic needle-like stylet to puncture plant cell walls and ingest food (Kiontke and Fitch, 2013). While the predatory nematodes have large teeth to slice open their prey (Kiontke and Fitch, 2013) and the parasitic nematodes have large hook-like teeth (Blaxter and Koutsovoulos, 2015). Most of them are microscopic in size, however, some species that parasite to animals are much larger and can grow to several centimeters in length (Dorris et al., 1999). Their sizes are the range in 0.2 mm

to over 6 meters depends on species (Blaxter and Koutsovoulos, 2015).

Nematodes are the most abundant type of animal on earth and can be found in most habitats; within or on host animals and plants, from the Arctic ice to the hot springs (Coghlan, 2005; Kiontke and Fitch, 2013; Blaxter and Koutsovoulos, 2015). There are nearly 30, 000 described species have been classified in the phylum Nematoda (Kiontke and Fitch, 2013). Scientific classification of Nematoda is shown in Figure 2.1.

Kingdom	:	Animalia
Super phylum	:	Ecdysozoa
Clade	:	Cycloneuralia
Phylum	:	Nematoda

Figure 2.1: Scientific classification of Nematoda.

Nematoda is divided into two classes, namely Secernentea and Adenophorea (Blaxter et al., 1998; Dorris et al., 1999). Secernentea includes a parasitic and free-living group in terrestrial habitats, while Adenophorea includes a wide range of marine, freshwater and soil nematodes (Dorris et al., 1999). Five major clades are identified in phylum Nematoda (Blaxter et al., 1998; Dorris et al., 1999). Two major clades (I and II) are found within Adenophorea and three major clades (III, IV, and V) are found within Secernentea (Blaxter et al., 1998; Dorris et al., 1999). Figure 2.2 shows the phylogenetic structure of the phylum Nematoda. Clade I groups the vertebrate-parasitic order Trichocephalida, the insect-parasitic order Mermithida, the plant-parasitic order Dorylaimida and the free-living order Mononchida (Blaxter et al., 1998; Dorris et al., 1999). Clade II groups the plant-parasitic order

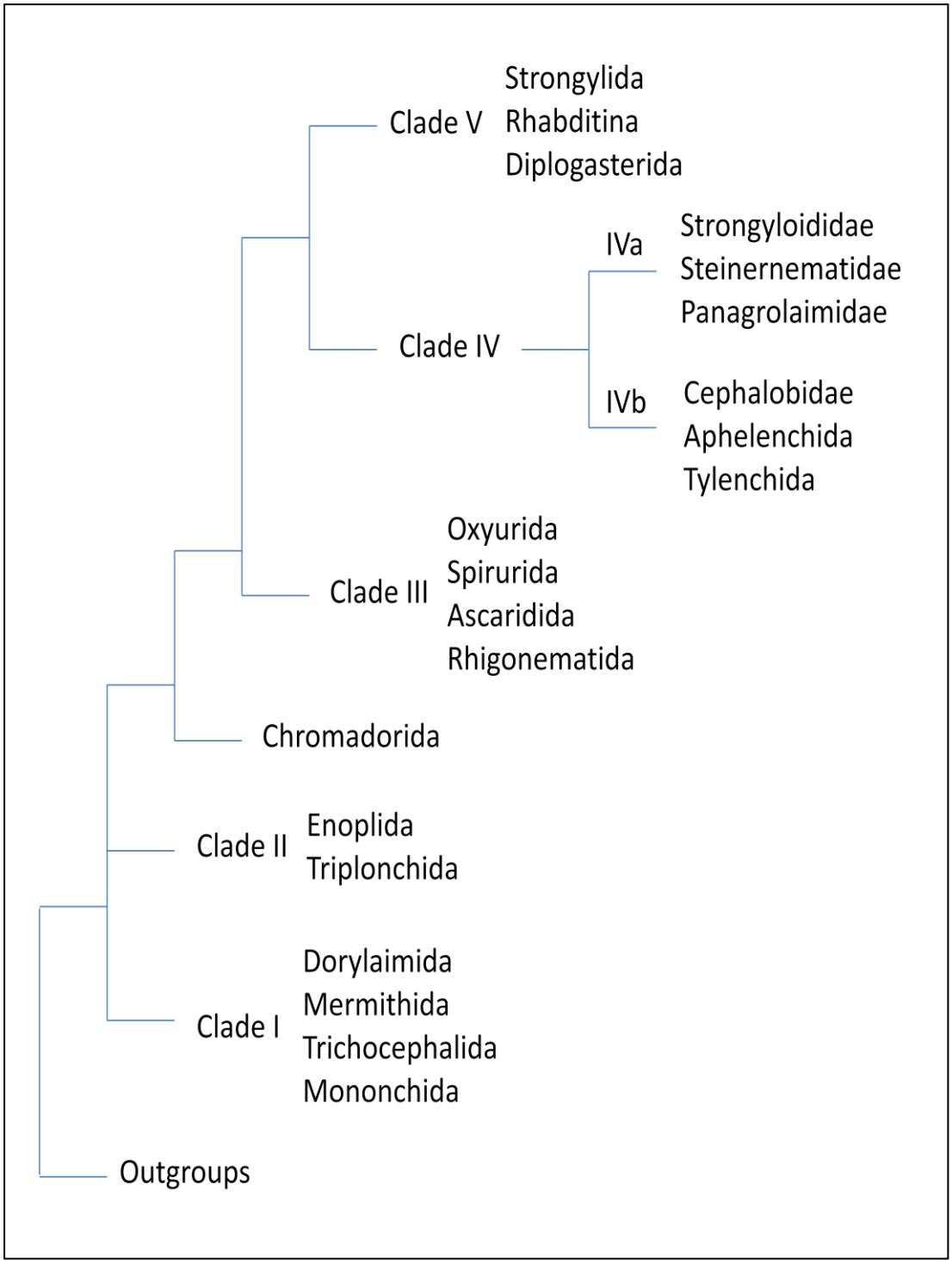

Figure 2.2: The phylogenetic structure of the phylum Nematoda. Five major clades are identified in the Nematoda and labeled I to V. (Source: Dorris et al., 1999).

Triplonchida and the free-living order Enoplida (Blaxter et al., 1998). Clade III groups the vertebrate-parasitic from orders Ascaridida, Spirurida, and Oxyurida with the arthropod-parasitic order Rhigonematida (Blaxter et al., 1998; Dorris et al., 1999). Clade IV groups the plant-parasitic from orders Tylenchida and Aphelenchida, the vertebrate-parasitic order Strongyloididae, the insect-parasitic order Steinernematidae and the free-living from orders Cephalobidae and Panagrolaimidae (Blaxter et al., 1998; Dorris et al., 1999). The last clade, clade V, groups the free-living order Rhabditina, the vertebrate-parasitic order Strongylida and the insect-parasitic order Diplogasterida (Blaxter et al., 1998; Dorris et al., 1999).

In phylum Nematoda, five orders are free-living nematodes, four orders are parasites on plants and ten orders are parasites on animals (Blaxter et al., 1998; Dorris et al., 1999). The animal-parasitic nematodes are parasites for humans, domestic animals, and wildlife (Blaxter et al., 1998; Dorris et al., 1999). The animal-parasitic nematodes are often characterized in a larger group as helminths which consist of nematodes, cestodes, and trematodes (James et al., 2009). They are a major limiting factor in livestock sector worldwide. Their infections on livestock cause significant economic losses. These parasitic nematodes have the ability to survive the immunological attack and can live in an infected individual for years (Coghlan, 2005).They reduce weight gain and productivity in meat, milk, and wool, incur the cost for anthelmintic treatments, and cause animal mortality (Sissay et al., 2007). However, the impact of the infection depends on the type of host species, the pathogenicity of the parasite species, the host and parasite interaction, and the infective dose (Over et al., 1992).

Livestock usually infected with gastrointestinal nematodes. In small ruminants like sheep and goats, the major parasitic nematode which frequently

reported is from family Trichostrongylidae such as *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Waller, 2006; Roeber et al., 2013). In large ruminants like cattle and horses, the parasitic nematode species mostly detected is such as *Ostertagia* spp., *Cooperia* spp., *Bunostomum* spp., *Strongyloides papillosus*, *Oesophagostomum* spp., and *Trichostrongylus axei* (De Graef et al., 2013; Roeber et al., 2013). *Ostertagia* spp. and *Cooperia* spp. represent important pathogenic genera, and when they are present in the large numbers, they can cause serious losses. These gastrointestinal nematodes infections are associated with poor farm management, ingestion of contaminated food and water, nutritional deficiencies, age of the ruminants and the climate (Gwaze et al., 2009; Mat Yusof and Md Isa, 2016).

Infestation with parasitic nematodes is generally termed as helminthiasis. All species of animals can be infected with parasitic nematodes; however, the infection is worst in the very young and the very old animals (Bamaiyi, 2012). Helminthiasis can cause mortality and morbidity (Norakmar et al., 2010; Nor-Azlina et al., 2011). However, the effects are dependent on the number of parasitic nematodes in the infected animal and the species of nematodes. Common clinical signs include weight loss, anemia, diarrhea, severe dehydration, reduced feed intake, lethargy, rough hair coat, reduced milk production, uncoordinated movement and reduced growth. Untreated severe cases can lead to death.

Haemonchus contortus, a bloodsucker nematode, is the most important parasite infecting small ruminant in Malaysia and the second most important cause of mortalities in goat and sheep of all ages mostly in poorly managed farms (Sani et al., 2004; Chandrawathani et al., 2009; Norakmar et al., 2010). Chandrawathani et al. (1999) reported that *H. contortus* had predominated the goats and sheep in Peninsular

Malaysia. Studies on the prevalence of parasitic infestation of small ruminant reported that *H. contortus* as the most prevalent nematodes species infected the goats and sheep in Penang Island (Wahab and Adanan, 1992), Perak (Zainalabidin et al., 2015) and Terengganu (Mat Yusof and Md Isa., 2016). They cause several subclinical effects on small ruminant such as growth depression, reduction in milk yield, hypoproteinemia, digestive inefficiency and loss of appetite (Mat Yusof and Md Isa, 2016).

2.3 The use of anthelmintic drugs against parasitic nematodes

The most common method used to control parasitic nematode infection in livestock is under treatment with anthelmintic drugs. Anthelmintic drugs are chemical substances that serve as an antiparasitic agent to eradicate parasitic worm infections from the body either in the intestine or tissues of other organs. Infections in the intestines are treated by the drug kill or paralyze the worms, which then eliminate them through the feces. Worm infections of other organs are directly killed by the drug to prevent them absorbing the essential nutrient from the body. Usually, these drugs do not give the side effects but sometimes it causes diarrhea, headaches, and dizziness in several individuals.

Phenothiazine is the first anthelmintic drug that was introduced in 1940, followed by piperazine in 1954 and bephenium in 1959. These drugs are effective to treat specific parasite species with few side effects to the host. Between 1960 and 1980, the safer broad-spectrum drugs were introduced namely benzimidazoles, imidazothiazoles and macrocyclic lactones. These drugs are more effective than the earlier drugs and had significantly lower dose rates (Kohler, 2001; McKellar and Jackson, 2004). Figure 2.3 shows the chemical structure of these drugs.

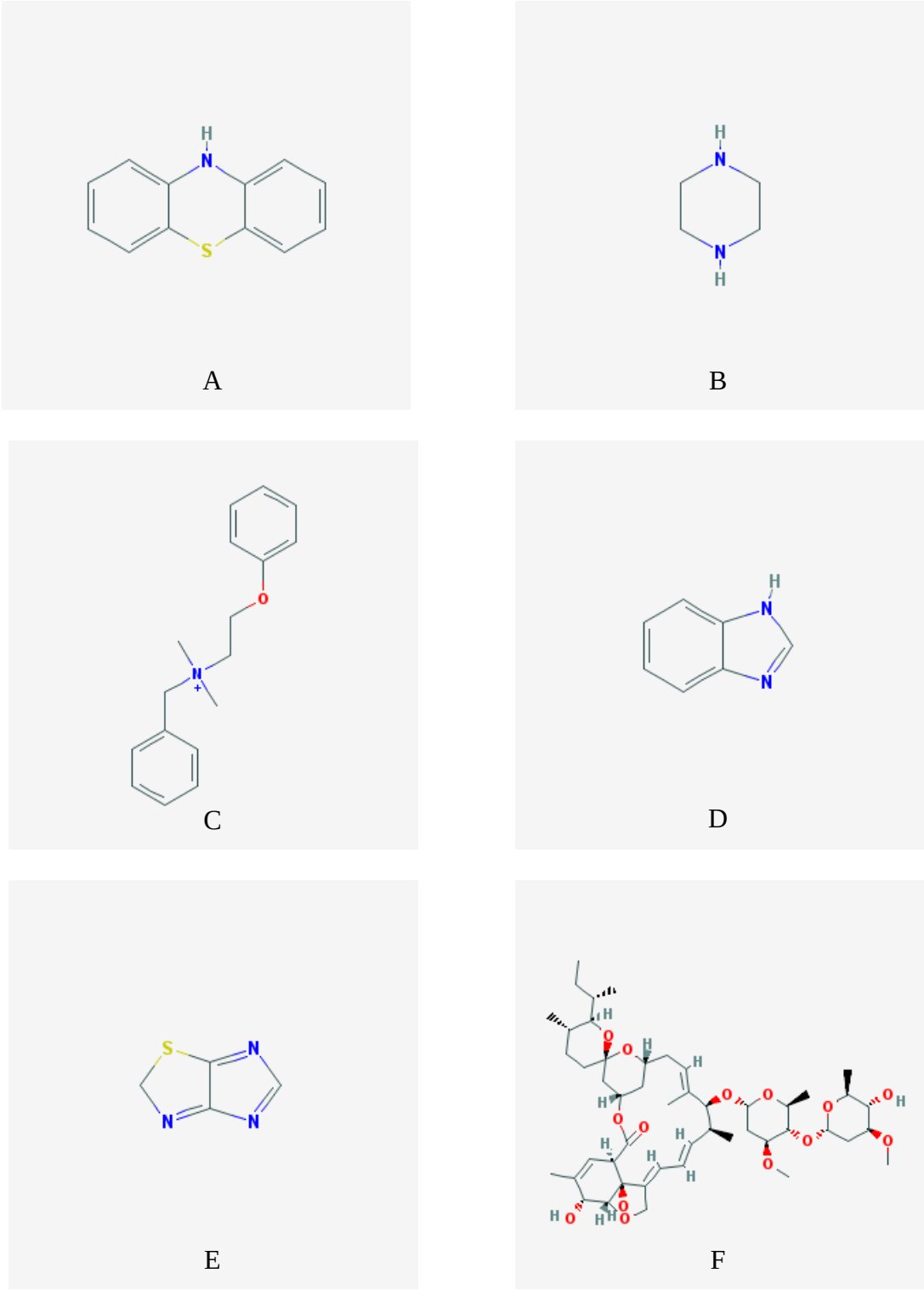

Figure 2.3: Chemical structure of anthelmintic drugs. (A) Phenothiazine ($C_{12}H_9NS$), (B) Piperazine ($C_4H_{10}N_2$), (C) Bephenium ($C_{17}H_{22}NO^+$), (D) Benzimidazole ($C_7H_6N_2$), (E) Imidazothiazole (C_4H_3S) and (F) Macrocyclic lactone, ivermectin ($C_{48}H_{74}O_{14}$).
Source: NCBI. PubChem Compound Database (https://pubchem.ncbi.nlm.nih.gov/compound/CID).

Benzimidazoles were introduced in 1961 started with thiabendazole, the first broad-spectrum anthelmintic produced, then followed by albendazole and fenbendazole (Kohler, 2001; Holden-Dye and Walker, 2007; De Graef et al., 2013). The biochemical basis of benzimidazole's action is they bind selectively to the microtubule subunit protein, β-tubulin, and inhibit the microtubule formation that affects on locomotion and reproduction (Martin, 1997; Kohler, 2001; Jabbar et al., 2006; Gilleard, 2006; Holden-Dye and Walker, 2007).

After benzimidazoles, a new anthelmintic class of imidazothiazoles was introduced in 1970 which consists of levamisole, pyrantel, and morantel (Kohler, 2001; Holden-Dye and Walker, 2007; De Graef et al., 2013). These drugs are nicotinic receptor agonists that bind to the recognition site of the excitatory nicotinic acetylcholine receptors on body wall muscle and cause depolarization and spastic muscle paralysis (Martin, 1997; Kohler, 2001; Gilleard, 2006; Jabbar et al., 2006; Holden-Dye and Walker, 2007).

In 1981, the remarkable potent anthelmintic drugs, macrocyclic lactones, which consist of avermectins and milbemycins, were introduced into market (Kohler, 2001; Holden-Dye and Walker, 2007). They act as an agonist of invertebrate-specific inhibitory chloride channels family that is activated by glutamic acid (Kohler, 2001; Gilleard, 2006; Jabbar et al., 2006). The binding of this drug results in irreversible chloride ion currents that cause depolarization of the cell membrane and muscle paralysis (Kohler, 2001; James et al., 2009). This group elicits a potent and persistent paralysis of nematode pharyngeal and body wall musculature (Martin, 1997; Holden-Dye and Walker, 2007).

2.4 Macrocyclic lactones-ivermectin as novel chemotherapeutic

Macrocyclic lactones are a class of anthelmintic drugs that are widely used to treat parasitic nematode infections in animal and human (Ardelli et al., 2009). It consists of two subclasses; the avermectins such as ivermectin, abamectin, doramectin, and eprinomectin, and the milbemycins such as MOX (Ardelli et al., 2009). They are produced naturally by fermentation of soil-dwelling actinomycetes from the genus *Streptomyces* which was originally isolated from soil in Japan (Crump and Otogura, 2005). The strains of *Streptomyces* spp which produces milbemycin-type compounds are commonly found in soil samples, instead of the strains of *Streptomyces* spp which produces avermectin-type compounds are rare and are only produced by the strain of *Streptomyces avermitilis* (Vercruysse and Rew, 2002).

Ivermectin was the first macrocyclic lactone developed and approved for use in animals (Vercruysse and Rew, 2002; Ardelli et al., 2009). It is a semi-synthetic derivate of a naturally occurring fermentation product, avermectin (Holden-Dye and Walker, 2007; Canga et al., 2009; James et al., 2009). Its chemical structure is almost similar to the avermectin, selectively reducing one double bond in avermectin using Wilkinson's catalyst produced ivermectin (Figure 2.4) (Canga et al., 2009). This small change in molecular shape makes the ivermectin is more safety to host and more effective to fight the parasitic nematode infections (Geary, 2005; Holden-Dye and Walker, 2007; Liebig et al., 2010).

After being introduced as an antiparasitic agent in 1981 by Merk and Dohme (Holden-Dye and Walker, 2007; Omura, 2008; Canga et al., 2009), ivermectin has become the mainstay of livestock parasite control (Laing et al., 2012; Demeler et al., 2013) and has been increasingly used in community-wide treatment programs over

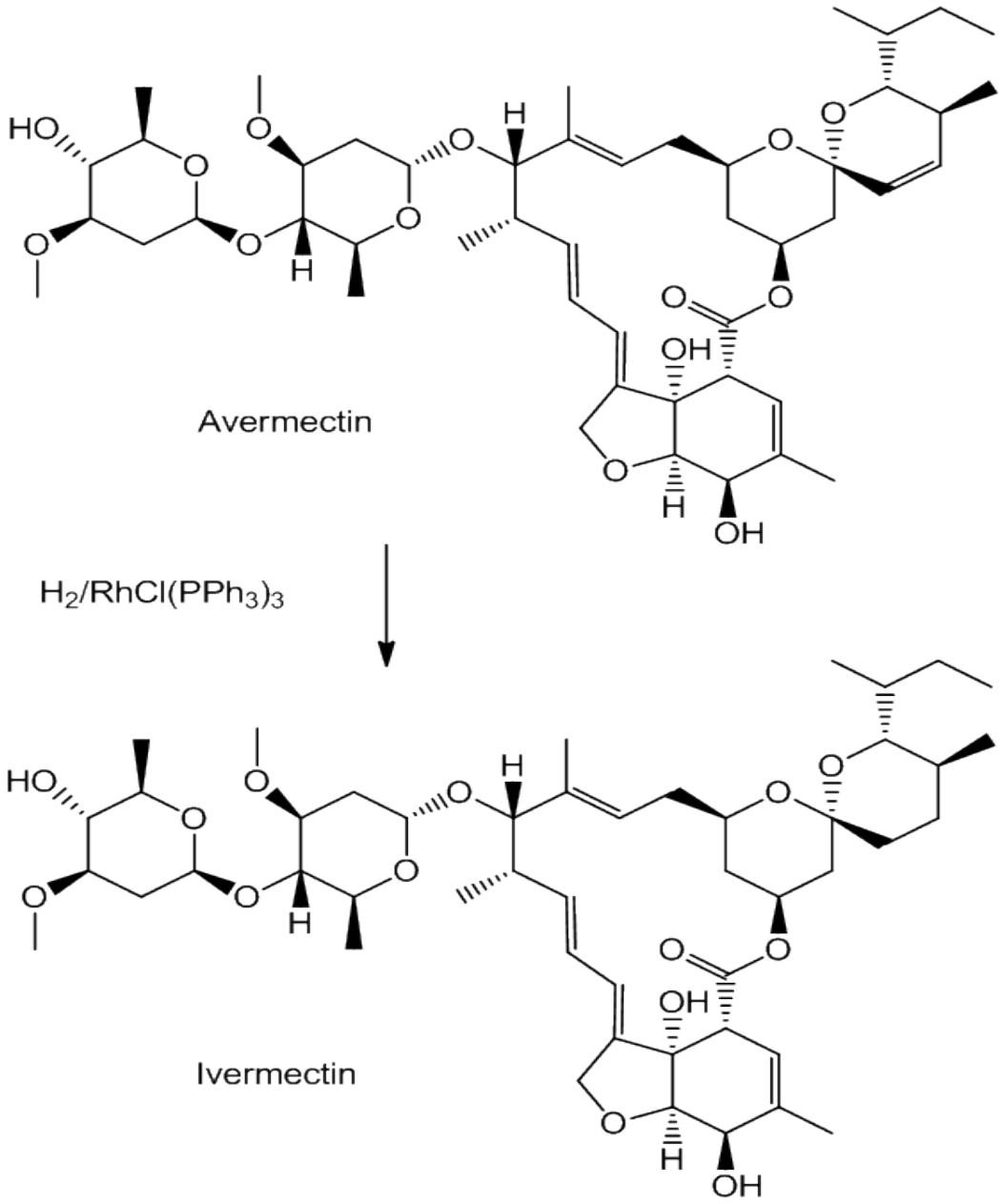

Figure 2.4: Chemical structure of ivermectin. Ivermectin is a derivate of avermectin. Selectively reducing one double bond in avermectin using Wilkinson's catalyst produced ivermectin. (Source: Canga et al., 2009).

the last decade (Laing et al., 2012). It becomes one of the most important and successful anti-parasitic drug (Geary, 2005; James and Davey, 2009; Laing et al., 2012) and widely used for the treatment of endo- and ectoparasites in human and animal including inserts, arthropods, and nematodes such as *Onchocerca volvulus, Haemonchus contortus, Trichosrongylus colubriformis, Cooperia oncophora*, and *Dirofilaria immitis* (Geary, 2005; Omura, 2008). In humans, ivermectin has been used intensively since 1987 to control endemic onchocerciasis (river blindness) caused by infection with the parasitic worm *O. volvulus* in Africa and Latin America (Dourmishev et al., 2005).

Ivermectin is characterized by slow absorption process, a broad distribution of the organism, low metabolism and slow excretion (Canga et al., 2009). It shows good bioavailability and enterohepatic recycling which makes it very effective for the long-term (Dourmishev et al., 2005). The ivermectin accumulates in the liver, edible tissues and fat of the host in unaltered ivermectin form as a drug reservoir (Canga et al., 2009). The drug was mainly excreted in the feces and urine. Due to ivermectin undergoes little metabolism, most of the dose is excreted unchanged (Canga et al., 2009).

2.5 The emergence of anthelmintic resistance

Anthelmintic resistance has emerged as a significant problem in the livestock industry. The resistance occurs when the susceptible parasite populations that normally affected by a given dose of the anthelminitic drug are managed to survive the treatment (James et al., 2009; De Graef et al., 2013). The decline in the response of the parasites to the anthelmintic drug can be indicated through the reduction in the efficacy of the drug against the population of parasites or the increasing duration of

treatment with requiring the additional treatments (James et al., 2009). Resistance is heritable (Sangster, 1999; Gilleard and Beech, 2007; De Graef et al., 2013). The parasites can be resistant to one or more drugs at a time. Resistance to drugs in the same class is known as side-resistance, whereas the resistance to two or various classes is known as cross-resistance and multi-drug-resistance (Sangster, 1999; De Graef et al., 2013).

The anthelmintic resistance was reported is more severe in small ruminant than in large ruminant (Waller, 1997; De Graef et al., 2013; Shalaby, 2013). The parasitic nematodes of sheep and goats have reported resistance to all of the major classes of broad-spectrum anthelmintic drugs (Waller, 1997; James et al., 2009; Shalaby, 2013). In contrast, there are reports of cattle nematodes resistant to the range of broad-spectrum anthelmintic drugs, however, the evolution of resistance in these parasites is slower than in sheep and goat nematodes (Waller, 1997; Sutherland and Leathwick, 2011; Shalaby, 2013). The management systems that used an extensive grazing area and few anthelmintic treatments help in controlling and delaying the development of resistance in cattle nematodes (Coles, 2002; Shalaby, 2013).

The resistance much going on parasitic nematodes that live in sheep and goats (Waller, 1997) then was developed among the small and large ruminants (Kaplan, 2004). To date, the resistance was reported to spread rapidly among the parasites in most classes of livestock to all the commercially available anthelmintic drugs (Sangster, 1999; Gilleard and Beech, 2007). The development of resistant to anthelmintic drugs have been reported shortly after their introduction into the market (Table 2.1) (James et al., 2009; De Graef et al., 2013).

Table 2.1: The development of resistance to anthelmintic drugs

Class	Drug	Introduction Date	Resistance Reported
-	Phenothiazine	1940	1957
-	Piperazine	1954	1966
Benzimidazole	Thiabendazole	1961	1964
	Cambendazole	1970	1975
	Oxibendazole	1970	1985
	Mebendazole	1972	1975
	Albendazole	1972	1983
	Fenbendazole	1975	1982
	Oxfendazole	1976	1981
	Triclabendazole	1983	1998
Imidazothiazole	Levamisole	1970	1979
	Pyrantel	1974	1996
	Oxantel	1976	-
	Morantel	1970	1979
Macrocyclic lactone	Abamectin	Late 1970's	2001
	Ivermectin	1981	1988
	Moxidectin	1991	1995
	Doramectin	1993	2007
	Eprinomectin	1996	2003

The resistance was reported to all the commercially available anthelmintic drugs. The development of resistant to anthelmintic drugs was reported shortly after their introduction into the market.
Source: Anthelmintic resistance of gastrointestinal cattle nematodes (De Graef et al., 2013).

The anthelmintic resistance was begun in the middle of 1950s (Jabar et al., 2006). The first reports of anthelmintic resistance were to phenothiazine in *Haemonchus contortus* of sheep (Kaplan, 2004; Jabbar et al., 2006) and then in cyathostomins (small strongyles) of horses (Kaplan, 2004). Phenothiazine was introduced to the market in 1940 and the resistant populations were reported in 1957 (James et al., 2009; De Graef et al., 2013).

Similar trends occurred with the classes of broad-spectrum anthelmintic drugs. Resistance to thiabendazole (benzimidazoles), levamisole (imidazothiazoles) and ivermectin (macrocyclic lactones) was reported in 1964, 1979 and 1988 after the drugs being introduced to the market in 1961, 1970 and 1981. The first resistance of thiabendazole and ivermectin were reported in *H. contortus* of sheep (Shoop, 1993; Kaplan, 2004) while the first resistance of levamisole was reported in *H. contortus* and *T. colubriformis* of sheep (Sangster, 1999). The resistance to all these three major drug classes has become a common thing nowadays (Gilleard, 2006; Pomroy, 2006; Sargison et al., 2007).

2.6 Ivermectin resistance

Ivermectin resistance was first reported in South Africa in 1988, after 33 months the drug was officially introduced to the market there and the first species detected resistance to this drug is *Haemonchus contortus* in the sheep (Shoop, 1993). Intensive use of ivermectin over several years has led to the widespread development of ivermectin resistance (Shoop, 1993; Gilleard, 2006; Laing et al., 2012). The resistance was developed after only a few generations of parasites were exposed to the drug (Coles et al., 2005). It has been spread out rapidly and was reported in a number of parasites including the trichostrongylid nematodes (Waller, 1999).

The mechanism of ivermectin resistance is complex with alleles at several loci contributing to the resistance phenotype (Gilleard, 2006). Mutagenesis experiments have uncovered over 30 different loci that give rise to resistance but at relatively low levels (Starich et al., 1995). However, studies using the mutagenic drug treatment in the model nematode *C. elegans* have shown that mutations in subunits of glutamate-gated chloride channel receptor render worms highly resistant to ivermectin (Dent et al., 1997, 2000; Gilleard, 2006; Gilleard and Beech, 2007). These studies identified several potential genes that may be associated with ivermectin resistance, demonstrating that the genetics of ivermectin resistance are complex (Gilleard, 2006).

Ivermectin resistance required null mutations in three different subunits of the target glutamate-gated channels which are *avr-14* (GluClα3), *avr-15* (GluClα2) and *glc-1* (GluClα1) (Dent et al., 2000; Gilleard, 2006). The mutation confers extremely high levels of resistance to ivermectin whereas mutating any two of these genes does not cause the resistance (Dent et al., 2000; Gilleard, 2006). However, mutating of only one of the channel subunits can inhibit the pharyngeal pumping of nematode (Dent et al., 2000; Gilleard, 2006). This is because each of these receptor subunits is involved in parallel pathways which is *avr-15* acts in pharyngeal muscle whereas *avr-14* and *glc-1* act in neurons regulating pharyngeal function (Dent et al., 2000; Gilleard, 2006).

2.7 Limitation study of parasitic nematodes

Haemoncus contortus had been used as a model parasitic nematode for the study of anthelmintic resistance. It is one of the parasitic nematode species which is resistant to anthelmintic drugs and relatively closed to other important parasitic

nematode species in livestock (Kaplan, 2004; Gilleard, 2006). However, being a parasite makes it impossible to undertake the experiment in the laboratory without using the artificial laboratory animal hosts (Jones et al., 2005). Parasitic nematodes are difficult to culture and analyze independently of their hosts (Blaxter et al., 1998). This is a major problem faced by researchers working on parasitic nematodes which is the lack of in vitro culture systems and the requirement to propagate life-cycles in vivo. Moreover, the studies of this parasite in their natural hosts were limited by ethical, time-consuming, labor-intensive and cost consideration (Gilleard, 2006; Wimmersberger et al., 2013).

Most parasitic nematodes have a direct life cycle. The life cycle has two distinct phases; within the host and the free-living stage where the parasite is developing in the environment. The fertilized adult female worms in the digestive tract lay the eggs which are then passed in the feces onto pasture (Roeber et al., 2013). The eggs hatch to first-stage larvae and feed on bacteria contained in the fecal pellet and then undergo two molts to develop to the ensheathed third-stage larvae (Roeber et al., 2013). The sheath protects the L3 stage from environmental conditions but prevents it from feeding (Roeber et al., 2013). The L3, the most resistant of the free-living stages, then migrates under moist conditions onto the pasture and need to survive until ingested by the ruminant host (Roeber et al., 2013). Following ingestion, the larvae undergo two more molts as L4 and L5 in the gastric glands before maturing into adult worms and emerge from the glands onto the surface of the abomasal mucosa. The adult's mate and the female worm will then produce a large number of fertilized eggs which are passed out of the animal in the feces.

Apart from the complex life cycle of parasitic nematodes, another constraint is the study of gene function in parasitic nematodes is still limited (Gilleard, 2006). These parasites lack homogeneity in their parental genetic backgrounds due to the parental populations are likely to be highly polymorphic (Gilleard, 2006). This situation may lead to misleading results in the detailed analysis of cross progeny (Gilleard, 2006). *Brugia malayi* is the only parasitic nematode which essentially complete genome sequence is available, however full assembly and annotation have not been completed (Ghedin et al., 2004; Britton and Murray, 2006; Blaxter and Koutsovoulos, 2015).

Until now, the technology and genomic resources for parasitic nematode are still lagging behind those available for *C. elegans*. Even there is some recent progress in developing RNAi for *H. contortus* but there are problems for reverse genetic analysis in these parasites species (Gilleard, 2006) and optimization on this technique will be required before it can be used as a reliable functional genomics tool in parasitic nematodes (Britton and Murray, 2006). Given the difficulty in passaging parasitic nematodes, the limited success of transgenics and RNAi, and the absence or poorly annotated genomes of most helminths, *C. elegans* stay as a good alternative (James et al., 2009).

2.8 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans (Plate 2.1) is a small free-living nematode usually found in the temperate soil environments and feed on bacteria such as *Escherichia coli*. The name of *Caenorhabditis elegans* is made up of a combination of Greek and Latin words. *Caenorhabditis* is a Greek word which means recent rod-like and *elegans* is a Latin word which means elegant or nice. At the beginning, the

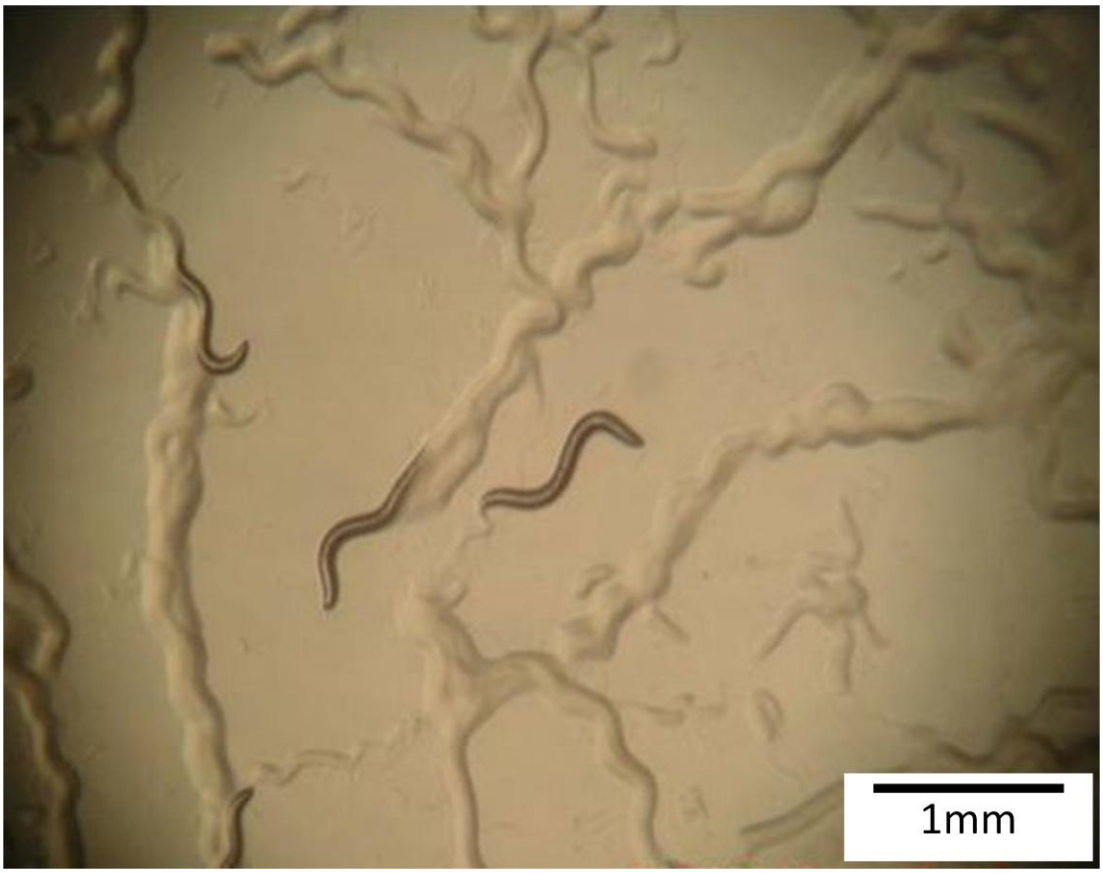

Plate 2.1: Adult hermaphrodite of *C. elegans*.

worm was named *Rhabditis elegans* by Maupas in 1900 that collected it from rich humus soil in Algeria, North Africa. However, it was then placed in the subgenus *Caenorhabditis* by Osche in 1952 and raised to generic status by Dougherty in 1955 (Riddle et al., 1997). Scientific classification of *C. elegans* taxonomy is shown in Figure 2.5.

Kingdom	:	Animalia
Phylum	:	Nematoda
Class	:	Chromadorea
Order	:	Rhabditida
Family	:	Rhabditidae
Genus	:	*Caenorhabditis*
Species	:	*C. elegans*

Figure 2.5: Scientific classification of *C. elegans* taxonomy tree.

Caenorhabditis elegans has emerged as one of the most popular and well-characterized model organisms after being introduced by Brenner in 1963 (Ankeny, 2001; Jones et al., 2005). Table 2.2 shows the major events in *C. elegans* research. Sydney Brenner is the person who is doing research about genetics and developmental of *C. elegans* in 1974 (Ankeny, 2001) and John Sulston is the person that responsible for completing the cell lineage of *C. elegans* in 1980. The genetic sequence of *C. elegans* was essentially completed in 1998 (Ankeny, 2001; Britton and Murray, 2006). The information greatly helped in the study of development and morphology in the living organisms.

Table 2.2: Major events in *C. elegans* research

Year	Event
1900	Organism that becomes known as *C. elegans* identified by Maupas.
1948	*Caenorhabditis* has been proposed as the target for genetic research (Dougherty and Calhoun).
1963	June: Brenner writes the letter to Perutz proposing a change in research direction. October: Brenner obtains the sample of *C. elegans* from Dougherty.
1964	Brenner continuous to investigate other organisms for the project.
1966	Genetic work on *C. elegans* begins at the Laboratory of Molecular Biology (LMB). Mutagenic agents used on worms for genetic studies.
Late 1960s	Neurobiological studies begun at the LMB on *C. elegans*. Sequence length determined by Brenner and Sulston.
1974	Brenner publishes results of genetic studies on *C. elegans*. Post-embryonic developmental lineages published.
1983	Complete embryonic cell lineages published in "The Mind of a Worm" (nervous system wiring diagram).
Mid 1980s	Mapping of the *C. elegans* genome. The Human Genome Project formally begins: *C. elegans* included among the 'model organisms'.
1998	Genetic sequence completed.

Source: Timeline: The natural history of *C. elegans* research (Ankeny, 2001).