CHARTER II

REVIEW OF RELATED LITERATURES

Filarial nematodes

Filarial nematodes or filariae are thread-like parasites of humans and various animal species that are transmitted by blood-sucking vectors. They refer to nematodes of the order Spirurida that have been collected into the superfamily Filarioidea (Anderson and Bain, 1976). This superfamily is consisted of two families: Filariidae and Onchocercidae. It is important to note that all the filarial nematodes that cause known diseases in humans are members of the subfamilies, Onchocercinae and Dirofilariinae, of the family Onchocercidae. The family also encompasses additional 6 subfamilies (Waltonellinae, Setariinae, Oswaldofilariinae, Icosiellinae, Splendidofilariinae, and Lemdaninae) (Bain and Chabaud, 1986). Taxonomy of the family Onchocercidae is shown in **Figure 1**.

Superkingdom:	Eukaryota
Kingdom:	Animalia
Subkingdom:	Metazoa
Phylum:	Nematoda
Class:	Chromadorea
Order:	Spirurida
Suborder:	Spirurina
Superfamily:	Filarioidea
Family:	Onchocercidae

Figure 1 Taxonomy of the family Onchocercidae.

Filarial diseases are a major health problem in many tropical and subtropical areas. The major parasites of humans are *Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus* (**Table 1**). The adult worms inhabit specific tissues where they mate and produce microfilariae, the characteristic tiny, thread-like larvae. The microfilariae infect vector arthropods, in which they mature to the 3rd-stage infective larvae.

Table 1 Major pathogenic filarial parasites of humans (Garcia *et al.*, 2001).

Species	Location of Adults	Major pathology	Location of microfilariae	Major vectors
Brugia malayi	Lymphatics	Lymphagitis, elephantiasis	Blood	Species of *Mansonia*, *Coquillettidia*, and *Anopheles* mosquitoes
Wuchereria bancrofti	Lymphatics	Lymphagitis, elephantiasis	Blood	Species of *Culex*, *Anopheles*, *Aedes*, and *Ochlerotatus* mosquitoes
Onchocerca volvulus	Subcutaneous tissues	Loss of vision, dermatitis	Fluid in the subcutaneous nodules, dermal layers of the skin, blood, and eye	*Simulium* spp. (blackflies)

Lymphatic filariasis (Elephantiasis)

Lymphatic filariasis, known as elephantiasis, is caused by the filarial parasites: *W. bancrofti*, *B. malayi*, and *B. timori* (Ottesen *et al.*, 1997; Fischer *et al.*, 2004). The majority of the infected cases are affected by *W. bancrofti* accounting for 90% of the cases, and the minority by *B. malayi* accounting for 10%, and 0.67% by *B. timori*. According to worldwide estimation, over 120 million people are infected in 83 countries, including Thailand, and 40 millions of them are seriously debilitated and disfigured by the disease (WHO, 1993; Triteeraprapab and Songtrus, 1999; Triteeraprapab *et al.*, 2001). Lymphatic filariasis is ranked by the World Health Organization (WHO) as the world's second leading cause of permanent and long-term disability (Behbehani, 1998). On the other hand, it is the world's third of the most tropical diseases leading cause of long-term disability, with disease burden estimated at 5.6 million disability adjusted life-years (DALYs: the number of healthy years of life lost due to premature death and disability) (Morel, 2000). Among the pathogens causing lymphatic filariasis, *W. bancrofti* is prevalent in tropical areas worldwide, while *B. malayi* is limited to Asia, and *B. timori* is restricted to some islands of Indonesia.

1. Life cycle

The infection is transmitted by biting of infected mosquitoes (**Figure 2**). During a blood meal, the infective larvae or third-stage larvae (L3) of lymphatic filarial parasites, penetrate into the bite wound, and pass to the lymphatic vessels and lymph nodes where they develop into an adult stage, mate, and ultimately produce microfilariae. Adult lymphatic filarial parasites have a life span of 5-10 years, while microfilariae can live long for 6-12 months (Vanamail *et al.*, 1990; TDR, 2005). Millions of the offsprings of the female adults are released into the host's blood circulation, and can infect a biting mosquito. After infection, these microfilariae shed their sheath, penetrate the stomach wall, and migrate to the thoracic muscles. Then they undergo metamorphosis into first-stage larvae (L1), and subsequently the mature infective third stage larvae. The infective larvae migrate to the mosquito's proboscis, from which they pass to another human, and the life cycle is re-initiated via the mosquito bites.

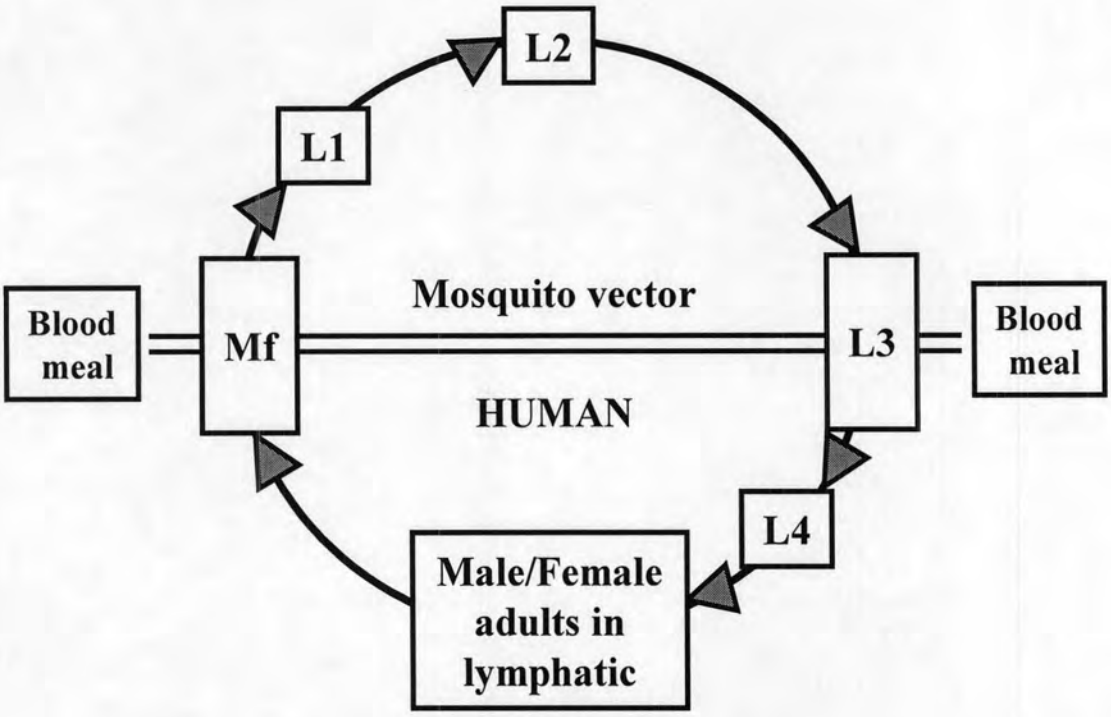

Figure 2 Life cycle of lymphatic filarial parasites; Mf, microfilaria; L1, the first-stage larva; L2, the second-stage larva; L3, the third-stage larva (infective stage); L4, the fourth-stage larva.

2. Clinical manifestations of lymphatic filariasis

There is a wide range of clinical manifestations of longstanding infection with lymphatic filarial parasites (Figueredo-Silva *et al.*, 2002). Generally, lymphatic filariasis consists of asymptomatic microfilaremia. Other patients who carry the adult worms may be amicrofilaremic and asymptomatic, or have acute lymphangitis and the chronic manifestations of the diseases (haematuria, hydrocele, chylocele, chyluria,

lymphedema and elephantiasis). Additional manifestations of the filarial infection are tropical pulmonary eosinophilia (TPE) syndrome, and drug-induced adverse reactions (Dreyer *et al.*, 1998).

2.1 Asymptomatic microfilaraemic state

In areas where lymphatic filariasis is endemic, the vast majority of infected individuals have a few overt clinical manifestations of filariasis, despite the presence of large numbers of circulating microfilariae in the peripheral blood. It has now been clearly indicated that, although they may be clinically asymptomatic, almost all patients with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease. Subclinical forms of the disease recognized in microfilaremic individuals are haematuria and/or proteinuria that reflect low-grade renal damage (Dreyer *et al.*, 1992). The renal abnormalities are found ~40% of the microfilaremic patients. Another form is from the observations on microfilaremic patients using lymphoscintigraphy to visualize the functional anatomy of lymphatic vessels (Freedman *et al.*, 1994; Dissanayake *et al.*, 1995; Suresh *et al.*, 1997). Although clinically asymptomatic, they have markedly abnormal, dilated and tortuous lymphatic vessels, and obviously atypical patterns of lymphatic flow (Freedman *et al.*, 1994; Dissanayake *et al.*, 1995; Suresh *et al.*, 1997).

2.2 Acute manifestations of lymphatic filariasis

The acute clinical manifestations of filariasis are characterized by recurrent attacks of fever associated with inflammation of the lymph nodes (adenitis) and/or lymph vessels (lymphangitis) termed adenolymphangitis (ADL). In *W. bancrofti* infection (bancroftian filariasis), in addition to the lymph nodes in the inguinal, axillaries and epitrochlear regions, the lymphatic system of the male genitalia is

frequently affected, leading to funiculitis, epididymitis or orchitis, or to a combination of these (Pani *et al.*, 1995). In brugian filariasis (*Brugia* infection), the affected lymph nodes are mostly positioned in the inguinal and axillaries, with inflammation along the course of the distal lymphatic vessels (Pani *et al.*, 1990).

The acute clinical course of lymphatic filariasis may last for several days or up to 4-6 weeks with a fulminating episode, and may result in prolonged inability to work (Gyapong *et al.*, 1996). The acute episodes are characterized by local pain, tenderness, warmth and lymphadenitis and/or lymphangitis. Other commonly associated findings include fever, oedema, constitutional complaints, and localized or ulcerated abscesses especially in areas where *Brugia* is endemic.

In endemic areas, two distinct types of acute ADL episodes are recognized: (a) ADL caused directly by the parasite infection itself; and (b) ADL secondary to bacterial or fungal infection. The former ADL is termed acute filarial lymphangitis (AFL). The most common presentation is that of a cord-like structure associated with retrograde lymphangitis in the lower or upper limps. Lymphangitis is frequently accompanied by mild fever, headache, and malaise. In the scrotal area or the breast it may present as a painful palpable nodule. Recurrence of these attacks at the same sites is common (Shenoy *et al.*, 1995; Pani *et al.*, 1995). The latter form of ADL is the most common form. It is usually recognized as a syndrome with a clinical picture that can include high fever, chills, myalgia and headache. Recent evidences have suggested that bacterial or fungal super infections of limbs with compromised lymphatic function play the primary role in triggering episodes of ADL (Montestruc *et al.*, 1960; Olszewski *et al.*, 1993), which themselves actually cause or exacerbate the chronic obstruction changes in the lymphatic vessels of affected patients. The acute process usually starts in the skin and then spreads along the lymphatic vessels to the lymph

nodes (Olszewki *et al.*, 1993). Based on the observations, this form of acute ADL is termed acute dermatolymphangioadenitis (ADLA) (Olszewki *et al.*, 1993).

2.3 Chronic manifestations of lymphatic filariasis

The chronic signs of lymphatic filariasis rarely develop before the age of 15 years, and only a small proportion of the filarial-infected population is affected. However, immigrants from areas where filariasis is not endemic tend to develop elephantiasis more often, and much sooner (sometimes within 2-3 years) than do the local population of endemic areas (Partono, 1987). Out of 120 million cases of lymphatic filariasis, 16.02 million (13.3%) cases are of lymphoedema, and 26.79 million (22.3%) cases are of hydrocele (Michael *et al.*, 1996). In bancroftian filariasis, the incidence of the major signs of chronic disease: hydrocele, chyluria, lymphoedema, and elephantiasis may differ from one area to another. The most common are hydrocele, and swelling of the testis, followed by elephantiasis of the entire lower limb, the scrotum, the entire arm, the vulva, and the breast, in descending of frequency (Pani *et al.*, 1995; 1990). In brugian filariasis, the leg below the knee is characteristically affected, and sometimes the arm below the elbow. Genital involvement has not been reported, except in areas where brugian filariasis occurs together with *W. bancrofti*.

Lymphoscintigraphic studies have shown that lymphoedema is not always the results of occlusion of lymphatic channels, but can also happen when there is extensive collateralization. Skin changes such as skin fold thickening, hyperkeratosis, hypo- or hypertrichosis, pachydermia, pigmentary changes, chronic ulceration, epidermal and sub-epidermal nodules, and clinical intertrigo may also be seen in chronic infection (Burri *et al.*, 1996).

2.4 Tropical pulmonary eosinophilia (TPE)

The usual presenting features of TPE are cough, dyspnea, wheezing similar to bronchial asthma (Spry and Kumaraswami, 1982). Microfilaraemia are almost never present in the blood, but remnants of microfilariae surrounded by aggregates of eosinophils are sometimes found in the liver, spleen, lymph nodes or lungs (Spry and Kumaraswami, 1982). Eosinophilia, and increased levels of IgE and of anti-filarial antibodies are commonly found (Ottesen and Nutman, 1992). TPE is characterized by immunological hyperresponsiveness of the human hosts to the filarial parasites, especially to the microfilariae (Ottesen *et al.*, 1979). The eosinophils found in bronchoalveolar lavage fluid of patients with TPE are degranulated and activated, and release abnormally high levels of toxic oxygen radicals (Pinkston *et al.*, 1987; Rom *et al.*, 1990). The eosinophilic granular protein, eosinophil-derived neurotoxin (EDN), associated with eosinophil trafficking is suggested to play the most important role in the pathogenesis of TPE (O'Bryan *et al.*, 2003)

2.5 Clinical manifestations of adverse reactions to treatment

When patients with lymphatic filariasis are treated with antifilarial drugs, such as DEC or ivermectin, they develop characteristic adverse reactions. Adverse reactions following treatment of lymphatic filariasis are common, and frequently severe. They are generally not caused by direct drug toxicity, but by host inflammatory responses to dying microfilariae (Ottesen, 1987). The adverse reactions can be systematic and local reactions (Babu *et al.*, 2006). Systematic reactions are headache, body ache, dizziness, decreased appetite, malaise, nausea, urticaria, vomiting, and sometimes bronchial asthma (McLaughlin *et al.*, 2003). Local reactions are lymphadenitis, funiculitis, epididymitis, orchitis, lymphangitis, abscess formation, ulceration, and transient lymphoedema. Systematic reactions and fever are positively

associated with microfilaremia, and the density of microfilariae (Turner *et al.*, 1994; Haarbrink *et al.*, 1999). They occur early during the treatment, and generally do not last for more than 3 days. Local reactions occur mainly in patients who have history of adenolymphangitis, and tend to occur later and may last longer. Mild adverse reactions will disappear spontaneously, and usually are not necessary to interrupt with treatment. However, patients with severe adverse reactions require hospitalization.

3. Pathogenesis of lymphatic filariasis

In general, the microfilaremic patients can remain asymptomatic for undetermined period of time, or progress into the chronic disease. The pathogenesis of lymphatic filariasis results from a complex interplay of factors related to adult worms (pathogenic potential, and worm burden), host immune responses (resistance, or tolerance), and secondary bacterial and fungal infections (Freedman, 1998; Dreyer *et al.*, 2000).

3.1 Subclinical lymphangiectasia caused by living adult lymphatic filarial parasites

The pathology in investigated lymphatic vessels consists of distinct histological features related to the presence of both live and dead parasites (Jungmann *et al.*, 1991; 1992). In endemic areas, the most common change in patients who carry living adult worms is subclinical lymphangiectasia (Dreyer *et al.*, 1999; Dreyer *et al.*, 2002). In such pathology, lymphatic vessels that contain living adult worms are dilated, without any inflammatory responses in the wall. Lymphatic dilatation with none of inflammatory reactions can be observed in nude mice (a mutant mouse strain that lacks a thymus gland and T lymphocytes) or severe combined immunodeficient mice (SCID

mice: mice genetically engineered to lack T and B lymphocytes) infected with *Brugia* species (Vincent *et al.*, 1984; Nelson *et al.*, 1991), and can be reversed in nude mice by removing or killing the adult worms (Vickery *et al.*, 1991). The data suggested that factors related to the parasites themselves, rather than being those immunologically mediated, contribute to lymphangiectasia. Since it can cause lymphatic dysfunction, lymphangiectasia is a major risk factor for development of chronic lymphatic disease.

3.2 Acute filarial lymphangitis (AFL) is triggered by the death of adult lymphatic filarial parasites

Acute filarial lymphangitis (AFL) is designated an acute condition that presents as a restricted inflammatory nodule or cord in a lymphatic or a lymph node of an extremity, a breast (in woman), or scrotum (in man). AFL is caused by death of adult lymphatic filarial parasites, either spontaneously or as a result of treatment with a macrofilaricidal drug (Figueredo-Silva *et al.*, 1996). It should be noted that not all natural or drug-triggered AFL episodes cause clinical illness. In many patients, granulomatous reaction develops around dead parasites in the lymphatics without any clinical outcome, and is only detected, incidentally, during physical examinations (Olszewski *et al.*, 1993). However, in other patients, AFL accompanied by local pain, swelling, and tenderness develops corresponding to the granulomatous inflammatory reaction around dying or degenerating parasites.

The studies of human biopsy specimens suggest that AFL is an acute inflammatory process that is triggered by products released from dying or disintegrating parasites, and that neither living nor completely calcified dead parasites cause the acute inflammatory changes (Lichtenberg, 1957; Cooray, 1960; Galindo *et al.*, 1962; Jungmann *et al.*, 1991; 1992).

Ultrasonographic and histopathological studies have documented an episode of AFL triggered by treatment with diethylcarbamazine (DEC) that the acute attack in this case occurs in a body site, where previously living parasites were killed by the drug (Dreyer *et al.*, 1995; Figueredo-Silva *et al.*, 1996; Noroes *et al.*, 1997). Because of the high prevalence of living adult *W. bancrofti* in the lymphatics of the spermatic cord (Noroes *et al.*, 1996a; 1996b; Dreyer *et al.*, 1996), treatment-triggered acute filariasis is particularly common in the scrotal area. The local reactions observed under histopathological investigations of biopsy samples from patients infected with *W. bancrofti* reveal mild infiltration of inflammatory cells in an early phase following parasite death by DEC treatment (Figueredo-Silva *et al.*, 2002). In the later phase, the lymphatic vessel is occluded by granulomatous inflammatory reactions around dead parasites, with variable numbers of eosinophils, lymphocytes, plasma cells and large macrophages. Rarely, neutrophils may filtrate in the center of the granuloma. The systematic responses are characterized by significantly elevated levels of TNF-α levels, and a positive correlation between its levels and the severity of the AFL (Das *et al.*, 1996).

The simultaneous death of many adult worms resulting AFL is a risk factor for development of some types of the chronic manifestations, such as hydrocele, chylocele and chyluria (Dreyer *et al.*, 2000).

3.3 Acute dermatolymphangioadenitis (ADLA) by secondary bacterial and fungal infections

Dilatation of the lymphatic vessels induced by the presence of the adult parasite finally leads to lymphatic dysfunction, and accumulation of protein-rich fluid in the tissues. The lower limbs, in particular, become predisposed to recurrent bacterial infections. Trauma, interdigital fungal infections, and onchomycosis provide

entry sites for bacteria, which multiply rapidly, and cause a reticular lymphangitis of the small collecting vessels, known as acute dermatolymphangioadenitis (ADLA) (Jungmann *et al.*, 1992). Bacteria that are generally regarded as commensal or saprophytic organisms have been isolated numerous times from the blood or tissue fluid during the acute attacks (Montestruc *et al.*, 1960; Olszewski *et al.*, 1997; Dreyer *et al.*, 1999). Early investigations emphasized the etiological role of streptococcal infections. Recurrent bacterial infections are an important co-factor in the progression to lymphedema and elephantiasis (Esterre *et al.*, 2000; Dreyer *et al.*, 2000).

Wolbachia of arthropods and filarial nematodes

Wolbachia is a genus of the class Alphaproteobacteria belonging to the order Rickettsiales (**Figure 3**). These gram-negative intracellular bacteria are found widespread in arthropods as well as in filarial nematodes (Werren, 1997; Bandi *et al.*, 1998). On the basis of 16S rDNA gene and groESL operon sequence analysis, it is organized into the family Anaplasmataceae, which also includes all the species of the genera *Ehrlichia*, *Anaplasma*, *Cowdria*, and *Neorickettsia* (Dumler *et al.*, 2001). In contrast to members of the family Rickettsiaceae, which grow in the cytoplasm or nucleus of their eukaryotic host cells, members of the Anaplasmataceae replicate while enclosed in a eukaryotic host cell membrane-derived vacuole.

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Rickettsiales
Family:	Anaplasmataceae
Genus:	*Wolbachia*

Figure 3 Taxonomy of the bacteria *Wolbachia*.

Wolbachia cannot grow in a cell-free medium. Traditional methods for bacterial species and strain determination, which largely depend on pure culture of bacterial isolates, have not been used in the genus *Wolbachia*. In the absence of a formal nomenclatural system, the *Wolbachia* community currently refers to the different lineages as supergroups (Bandi *et al.*, 2003). In addition, the species name, *W. pipientis*, remains single until new data are generated in different research areas (*e.g.* comparative genomics, molecular phylogenetics, and screening for *Wolbachia* in new hosts). The DNA-sequence-based methods, including phylogenetic analysis based on 16S rDNA, dnaA, ftsZ, gltA, groEL and wsp genes have been employed for taxonomic classification (Bandi *et al.*, 1998; Zhou *et al.*, 1998; Bordenstein and Rosengaus, 2005). At present, eight (denoted A through H) taxonomic supergroups are described for the genus *Wolbachia* by their places in molecular phylogenies. These eight supergroups are labeled alphabetically, and include A and B found in various arthropods, C (*Onchocerca* sp., and *Dirofilaria* sp.) and D (*W. bancrofti*, *Brugia* sp., and *Litomosoides* sp.) restricted to filarial nematodes, E containing *Wolbachia* from

springtails (*Folsomia candida*), and F containing *Wolbachia* from termites (*Kalotermes flavicollis* and *Microcerotermes* spp.), weevils (*Rhinocyllus conicus*), and the filarial nematode *Mansonella ozzardi* (Werren *et al.*, 1995; Bandi *et al.*, 1998; Vandekerchove *et al.*, 1999; Lo *et al.*, 2002) (**Figure 4**). The more recently proposed supergroups G and H are comprised of *Wolbachia* from Australian spiders (G), and the Pacific dampwood termites (*Zootermopsis angusticollis* and *Z. nevadensis*) (H) (Rowley *et al.*, 2004; Bordenstein and Rosengaus, 2005).

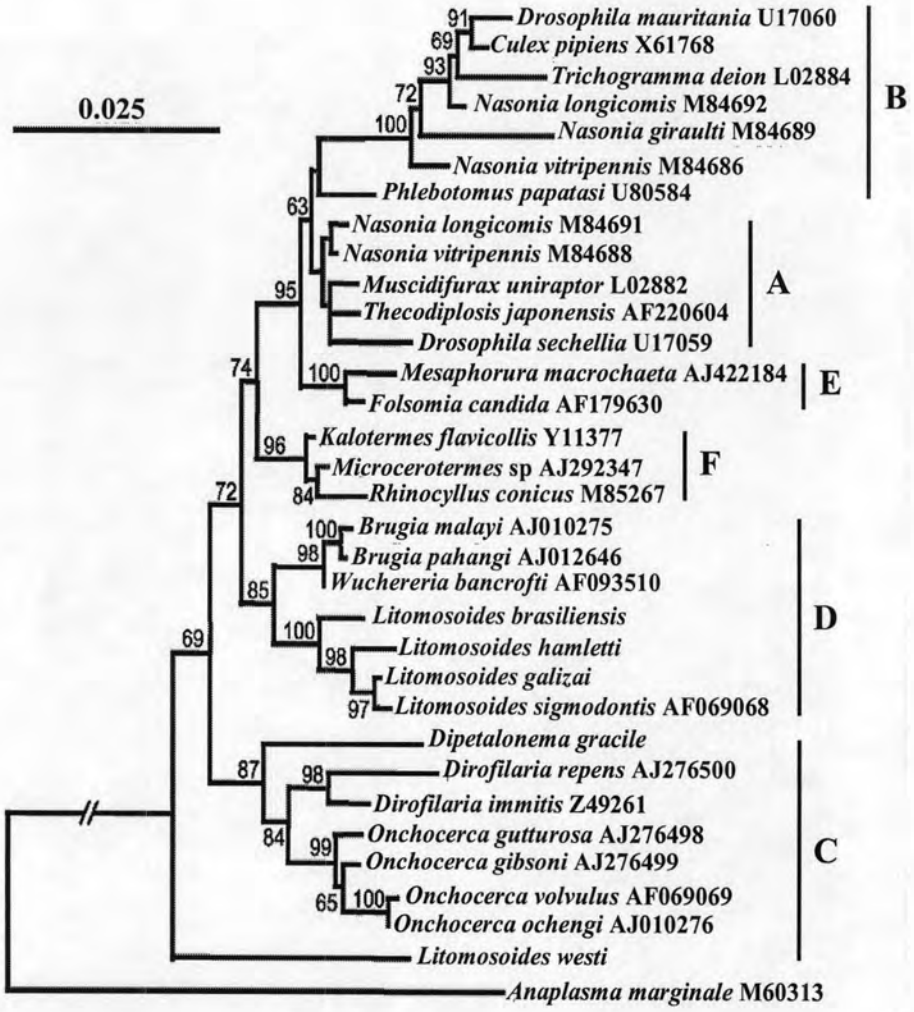

Figure 4 Phylogeny of *Wolbachia* based on 16S rDNA gene sequences (Casiraghi *et al.*, 2004). Representatives of *Wolbachia* supergroups A–F are shown. The supergroup F also includes the filarial nematode *Mansonella ozzardi* (Lo *et al.*, 2002).

1. *Wolbachia* of arthropods

In 1924, intracellular bacteria were firstly reported, as rickettsia-like microorganisms, within the ovaries and testes of the mosquito *Culex pipiens* by Hertig and Wolbach (Werren *et al.*, 1997; Stouthamer *et al.*, 1999). They were subsequently named *Wolbachia pipientis* (Werren *et al.*, 1997; Stouthamer *et al.*, 1999). Phylogenies based on 16S rDNA sequences have confirmed that morphological similarities to the Rickettsiae are based on phylogenetic relatedness (O'Neill *et al.*, 1992; Rousset *et al.*, 1992; Stouthamer *et al.*, 1993). It has been estimated that these bacteria infect at least 20% of all insect species (Haine and Cook, 2005). *Wolbachia* have also been found commonly in isopods (Rousset *et al.*, 1992) and mites (Jeyaprakash and Hoy, 2000). *Wolbachia* infecting the reproductive tissues of arthropods are transmitted maternally from infected females to their progeny via the egg cytoplasm, and have evolved to manipulate host reproduction.

Research interest in *Wolbachia* is initially sparked when it is discovered that they cause several kinds of reproductive changes in arthropod reproduction (Werren and O'Neill, 1997). These reproductive manipulations include (1) inducing embryonic lethality in insect embryos that result when uninfected females are mated to infected males (cytoplasmic incompatibility) (Sinkins *et al.*, 2004; McGraw and O'Neill, 2004; Mercot and Charlat, 2004), (2) inducing parthenogenesis in infected insects (the ability of infected unfertilized insect eggs to successfully develop into functional female adults) (Stouthamer *et al.*, 1993; Huigens *et al.*, 2004), and (3) overriding chromosomal sex determination in crustaceans to convert infected genetic males into functional phenotypic females (feminization of genetic males) (Rigaud, 1997; Moreau and Rigaud, 2003; Cordaux *et al.*, 2004). Each of these reproductive effects enhances transmission of *Wolbachia* to the arthropod population which is not infected with *Wolbachia* (Werren and O'Neill, 1997).

It has been proposed that the reproductive abnormalities induced by *Wolbachia* are of interest to applied biologists, who are looking for novel means to genetically manipulated populations of insect pests that are important for economic and health reasons (Beard *et al.*, 1993). For instance, in control of transmission of vector-borne diseases, this approach aims to express foreign anti-parasitic or anti-viral gene products in *Wolbachia* harbored by insects. Parasitoids used in biological control of insects may be more effective when infected with parthenogenesis *Wolbachia* (Stouthamer, 1993). *Wolbachia* and its hosts also are ideal candidates for the study of mechanisms of host-parasite relationship, the evolution of infectious diseases, specifically host resistance, parasite virulence, and transmission dynamics (McGraw and O'Neill, 1999).

2. *Wolbachia* of filarial nematodes

2.1 Characteristics and distribution

At the beginning in 1970s, electron microscopy studies of various filarial nematodes, including *Dirofilaria immitis*, *B. pahangi*, *B. malayi* and *O. volvulus*, have revealed the presence of the intracellular bacteria *Wolbachia* (Lee *et al.*, 1975; McLaren *et al.*, 1975; Kozek, 1977; Kozek and Marroquin, 1977). Although they occur in varying proportions between individual worms, and different developmental stages, they are found throughout all the life cycle stages of the filarial nematode hosts (Kozek, 1977; Kozek and Marroquin, 1977; Fenn and Blaxter, 2004a; McGarry *et al.*, 2004). Within the body of filarial nematodes, the bacteria are restricted in the lateral chords of adults and the reproductive tissues of the females (i.e. in the oogonia, oocytes, embryos and microfilariae) (**Figure 5A, B** and **C**). However, *Wolbachia* have not been demonstrated in the male reproductive system (Sacchi *et al.*, 2002; Kozek, 2005). These suggest that the bacteria are vertically transmitted through the cytoplasm

of the egg, and not through the sperm (Kozek, 1977; Kozek and Marroquin, 1977). Moreover, the following phylogenetic analysis reveals concomitant phylogeny of *Wolbachia* with that of the host filariae (Casiraghi *et al.*, 2001). This provides indirect evidence that transmission has been at least mainly vertical.

Their morphology are pleomorphic coccobacilli, appearing either as cocci (0.3-0.8 μm in diameter) or as short rods (up to 0.8 μm in diameter × 1.5 μm in length) (Kozek, 1977). Each *Wolbachia* cell lies in an individual vacuole enveloped by three layers of membranes. The outer layer is a host-derived membrane, followed by the outer cell wall of the bacteria; the innermost layer consists of the plasma membrane of the bacteria (Kozek and Marroquin, 1977; Taylor and Hoerauf, 1999) (**Figure 5D**). However, a few *Wolbachia* cells within a host-derived vacuole can be observed. *Wolbachia* multiply by binary fission, the most common mode of replication in bacteria. Evidence of *Wolbachia* undergoing division is always reported in adult female of filarial nematodes, especially in the reproductive tissues (Kozek, 1977; Kozek, 2005).

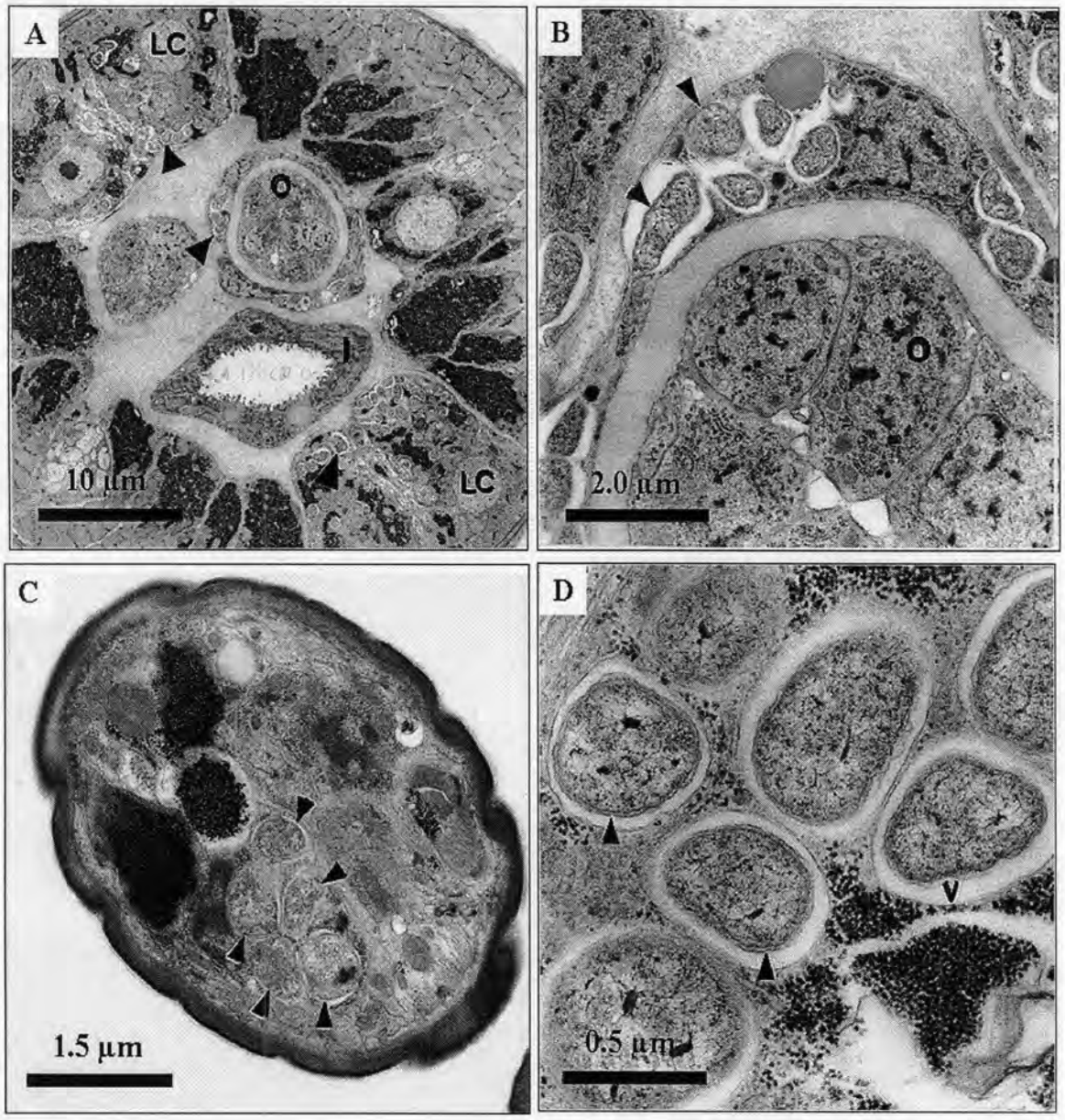

Figure 5 Electron micrographs of *Wolbachia* of *Brugia malayi* and *Wuchereria bancrofti* (Taylor and Hoerauf, 1999); A, *B. malayi* adult female, showing *Wolbachia* (arrows) in the lateral cords (LC), and the cell layer surrounding the oviduct (O) (I, intestine); B, *Wolbachia* (arrows) in the cell layer surrounding the oviduct (O) of adult female *B. malayi*; C, *Wuchereria bancrofti* microfilaria, showing a cluster of five *Wolbachia* (arrows); D, *Wolbachia* within the lateral cord of adult female *B. malayi*, showing the characteristic double membrane (arrows) and host vacuole (V).

In 1990s, two decades after the discover, based on DNA sequence data, the intracellular bacteria have been identified to be closely related to *Wolbachia*, a bacterial genus that encompasses microorganisms found in various arthropods (Sironi *et al.*, 1995; Bandi *et al.*, 1998). Besides an electron microscopy, the molecular techniques employed for surveys of *Wolbachia* are based on PCR followed by sequencing technique, and immunohistochemistry (Bandi *et al.*, 2001). It is now known that *Wolbachia* are widespread in filarial nematodes. Out of the 14 genera so far examined, *Wolbachia* have been revealed in the 8 genera of the total of 19 filarial species (**Table 2** and **3**) (Sironi *et al.*, 1995; Bandi *et al.*, 1998; Henkle-Dushrsen *et al.*, 1998; Fischer *et al.*, 2002; Casiraghi *et al.*, 2004; Egyed *et al*, 2002; Grobusch *et al.*, 2003). These include *B. malayi*, *W. bancrofti*, and *O. volvulus*, species of importance to human health, and *D. immitis* which causes dog heartworm disease (Sironi *et al.*, 1995; Bandi *et al.*, 1998; Henkle-Duhrsen *et al.*, 1998).

Table 2 Detection of *Wolbachia* in the genera of filarial nematodes.

Family	Subfamily	No. genera examined	Results for *Wolbachia*	
			Positive	Negative
Filariidae	Filarinae	1	-	1
Onchocercidae	Onchocercinae	8	5	3
	Dirofilariinae	3	1	2
	Waltonellinae	1	-	1
	Setarinae	1	-	1
	Oswaldofilariinae	0		
	Icosiellinae	0		
	Splendidofilariinae	0		
	Lemdaninae	0		

Table 3 Distribution of *Wolbachia* in filarial nematodes.

Family	Subfamily	Genus	Presence	Absence
Filariidae	Filarinae	*Filaria*	-	*F. martis*
Onchocercidae	Onchocercinae	*Brugia*	*B. malayi*	-
			B. pahangi	
			B. timori	
		Wuchereria	*W. bancrofti*	-
		Litomosoides	*L. sigmodontis*	*L. yutajensis*
			L. brasileiensis	
			L. galizai	
			L. hamletti	
		Dipetalonema	*D. gracile*	-
		Litomosa	*L. westi*	-
		Onchocerca	*O. volvulus*	*O. flexuosa*
			O. ochengi	
			O. gutturosa	
			O. gibsoni	
			O. lupi	
			O. cervicalis	
		Mansonella	*M. ozzardi*	*M. perstans*
		Acanthocheilonema	-	*A. viteae*
				A. reconditum
	Dirofilariinae	*Dirofilaria*	*D. immitis*	-
			D. repens	
		Foleyella	-	*F. furcata*
		Loa	-	*L. loa*
	Waltonellinae	*Ochoterenella*	-	*Ochoterenella sp.*
	Setarinae	*Setaria*	-	*S. equine*
				S. labiatopapillosa
				S. tundra

The presence of *Wolbachia* in filarial nematodes appears limited to the family Onchocercidae (**Table 2**). Within this family, the positive species are belonged to the subfamilies Onchocercinae and Dirofilariinae, while *Wolbachia* are found negative for the subfamilies Waltonellinae and Setarinae. However, there are both positive and negative species in the Onchocercinae and Dirofilariinae (**Table 3**). In these subfamilies, two filarial species pathogenic to humans, *Loa loa* and *Mansonella perstans*, the rodent filaria *Acanthocheilonema viteae*, the carnivore filaria *A. reconditum*, the bat filaria *Litomosoides yutajensis*, the deer filaria *O. flexuosa*, and the reptile filaria *Foleyella furcata* appear to be *Wolbachia* free (Plenge-Bonig *et al.*, 1995; Bandi *et al.*, 1998; Henkle-Duhrsen *et al.*, 1998; Casiraghi *et al.*, 2001; 2004; Buttner *et al.*, 2003; Grobusch *et al.*, 2003). For the results of screening for *Wolbachia* in nematodes outside the order Spirurida, there is no evidence for the presence of *Wolbachia* (Bordenstein *et al.*, 2003). It is consistent with the hypothesis that *Wolbachia* has entered the nematode phylum once, in an ancestral lineage of filarial nematodes.

Based on the distribution of *Wolbachia* that is placed on the taxonomy of filarial nematodes, hypotheses on their evolution could be: (1) *Wolbachia* could have been ancestrally absent from the lineages leading to *Filaria martis*, *Ochoterenella* spp., and *Setaria* spp.; (2) *Wolbachia* could have been acquired on the lineage once leading to the Onchocercinae/Dirofilariinae, and current negative species in these subfamilies are the results of secondary losses; (3) *Wolbachia* could have been acquired several times along various lineages of the Onchocercinae/Dirofilariinae; in this case, negative species in these subfamilies could represent either a primitive absence of the symbiosis or the effect of a secondary loss (Casiraghi *et al.*, 2004; Taylor *et al.*, 2005).

2.2 *Wolbachia*-nematode mutualistic relationships

In arthropods, *Wolbachia* act as a reproductive parasite in most of the known cases (Werren, 1997; Stouthamer, 1999). However, filarial nematode *Wolbachia* behave differently from arthropod *Wolbachia*. There are evolutionary aspects as well as experimental studies suggest that the association between *Wolbachia* and filarial nematodes is obligatory mutualistic (Bandi *et al.*, 2001; Fenn and Blaxter, 2004b; Fenn and Blaxter, 2006). The term "obligatory mutualism" describes the association between species living together that neither species can survive under the natural condition without the other. The phylogeny of filarial nematode *Wolbachia* is in the main congruent with that of the filarial nematode hosts (Bandi *et al.*, 1998). In another word, the bacterial phylogeny splits at the same time as the filarial nematode phylogeny. It is an evidence of a close relationship between filarial nematode *Wolbachia*, and their hosts with a stable and long association. There is also no evidence for multiple infections. In addition, in filarial species positive for *Wolbachia*, the prevalence of the infection appears 100% (Bandi *et al.*, 2001). The phylogenetic pattern and the distribution of filarial nematode *Wolbachia* appear more comparable to those generally observed in obligatory bacteria (Taylor *et al.*, 2005). Nevertheless, as reviewed above, some of the species within the *Wolbachia*-positive genus or the *Wolbachia*-positive subfamilies lack the bacteria. For example, *O. flexuosa* has no *Wolbachia*, whereas all other *Onchocerca* species do. Similarly, *Litomosoides yutajensis* is found negative for the bacteria, whereas other members of genus *Litomosoides* harbor them. These observations indicate that the filarial nematodes might not be absolutely dependent on their intracellular bacterial partners in a long-term phylogenetic sense (Fenn and Blaxter, 2004b).

The information available on the evolutionary aspects and distribution of *Wolbachia* are in general agreement that the relationship between filarial nematodes

and filarial nematode *Wolbachia* are likely dependent. *Wolbachia* have not been cultured outside their host cells. In addition, there is an experimental study implying that their habitation is species specific. Filarial nematode *Wolbachia* can be transferred from a naturally infected species, *L. sigmodontis* to a naturally uninfected one, *A. viteae*. However, the level of *Wolbachia* in *A. viteae* reduces along the time that the filarial nematodes are cultured in the Mongolian gerbils (*Meriones unguiculatus*), and *Wolbachia* cannot transmit to the filarial progeny (Hartmann *et al.*, 2003). The dependence indicates that *Wolbachia* should need some benefits from their filarial nematode hosts. On the other hands, *Wolbachia* could benefit their hosts some essentials. Investigation using antibiotics, such as tetracycline, which is known to be effective against *Rickettsiae*, have provided direct evidence for the existence of this dependence. However, the underlying molecular mechanism is largely unknown. The antibiotic showed detrimental effects on filarial nematodes which harbor *Wolbachia*, and no effects on filarial nematodes which do not have *Wolbachia* (*e.g. A. viteae*) (Hoerauf *et al.*, 1999; Bandi *et al.*, 1999).

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) and peptide mass fingerprint searching

1. Fundamental of MALDI-TOF-MS

Lasers were first utilized in ion sources during the late 1960's and early 1970's. MALDI-MS is a development of the direct laser desorption mass spectrometry of small organic molecules. It was found that laser light, incident on a pure solid or liquid sample deposited on a sample slide, could be used to create intact inorganic and organic gas-phase ions (DiGiuseppe *et al.*, 1982). The upper mass for laser desorption of biological molecules is limited to be 1000 Da. Higher-mass ions, however, require higher laser fluencies and are destroyed by the laser light.

In the late 80s, MALDI was introduced to mass spectrometry with reporting UV-laser desorption of bioorganic compounds above 10 kDa for the first time (Karas and Hillenkamp, 1988; Tanaka *et al.*, 1988). The high-mass ions could then be analyzed by time-of-flight mass spectrometry. However, ionization could be achieved much better, thus improved the sensitivity by using an organic compound as a matrix to facilitate desorption and ionization of large-biological compounds without fragmentation of the analyst ions induced by direct laser irradiation (Karas and Hillenkamp, 1988; Karas, 1996). MALDI-TOF-MS has become established as a technique for the analysis and accurate molecular weight determination of large macromolecules such as proteins, polysaccharides, nucleic acids and synthetic polymers (Harvey, 1996; Hurst *et al.*, 1996; Fenselau, 1997; Griffin *et al.*, 1997) with mass accuracy and extreme sensitivity.

The schematic set-up of a MALDI-TOF instrument (a linear mode) is shown in **Figure 6**. The key aspect of MALDI-TOF-MS is to dilute and isolate macromolecules in a suitable matrix of highly laser light absorbing small organic molecules, such as sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), and 2, 5-dihydroxybenzoic acid (DHB), and then allowing it to dry on a MALDI-target into a crystalline deposit throughout which the molecules of the analyst are dispersed. The matrix absorbs energy at the wavelength of the laser produces, most commonly with a Nitrogen UV laser (337 nm), however, Nd-YAG (266 nm) and an Erbium:YAG IR laser (2940 nm) have also been successfully applied. When the laser is fired at the target the matrix absorbs the laser light energy which vaporizes it (it desorbs from the surface) and this carries some of the sample with it. At the time that the laser is pulsed a high voltage is applied to the target plate to accelerate the ionised sample towards a time-of-flight mass analyser. The TOF can be operated in either a reflectron with isotopic resolution in the low mass range (<10 kDa), and a linear mode for high mass determination (>100 kDa).

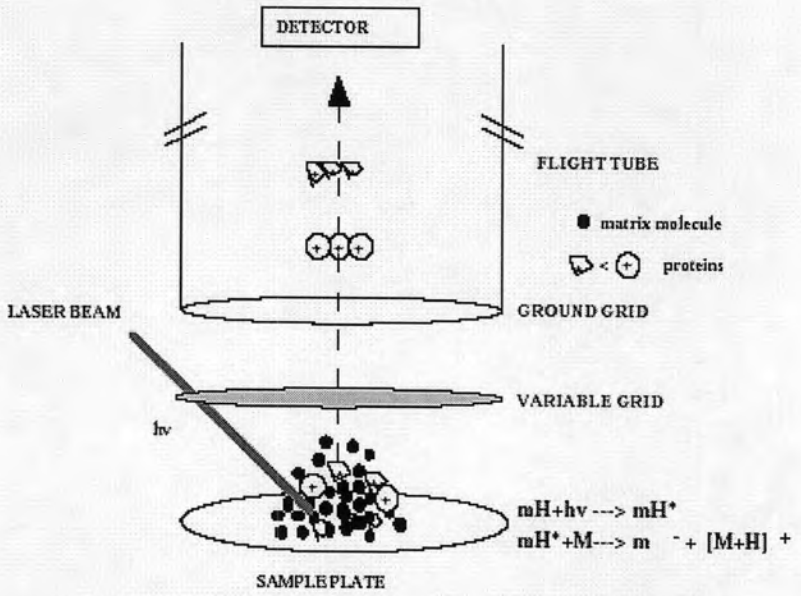

Figure 6 Principle of MALDI-TOF-MS, where mH=matrix, M=analyte, mH*= excited matrix molecule. The ions are separated in a flight tube where small ions travel faster than large ions, and thereby reach the detector first.

2. Peptide mass fingerprints

In MALDI-TOF MS, proteins and peptides are normally identified by the use of proteolytic enzymes, which cleave specifically at certain amino acids in the sequence. Mass spectrometric analysis of the enzymatic digest generates a 'mass-map' or profile which is unique to the analyzed protein. The peptide map can be used as fingerprints compared with expected peptide masses from a database by computer programs allowing unambiguous identification. This approach is known as peptide

mass fingerprinting (PMF) (Pappin *et al.*, 1993; Mann *et al.*, 1993; James *et al.*, 1993; Hentzel *et al.*, 1993).

Interpretation for protein identification includes concordance between observed MW/pI of an analyzed protein, and theoretical MW/pI of a matched protein within 20% with a significant score, and concordance between analyzed species, and matched species in the protein databases (Guillot *et al.*, 2003). However, positive protein identification can be considered based on criteria, including $\geq$ 4 matched peptides, and 20% sequence coverage.