CHAPTER II

REVIEW LITERATURES

1. Background History

In the last few decades, the taxonomic status of oomycetes has also changed. Kreisel (65) was the first individual to exclude oomycota from the true fungi. Based on morphological differences and molecular evidence, the genetics of oomycetes are more closely related to higher organisms than true fungi (66). Vegetative nuclei of oomycetes exist primarily in a diploid state in contrast to the haploid state of true fungi (67). Apart from their lack of photosynthetic pigments, they related to diatoms and brown or golden algae features, including:

- Cell walls composed primarily of glucans, including cellulose-like polymers
- Cell membranes composed of plant sterols, in contrast to ergosterol, the characteristic fungal sterol
- Energy storage compounds similar to those of plants, in contrast to the polyols (sugar alcohols) and trehalose found in most fungi
- Different sensitivities to a range of antifungal agents
- Biflagellate zoospores and heterokont flagella (one whiplash and one tinsel flagellum)

Therefore, oomycetes are considered as "fungus – like" mycelia organism that belong to the kingdom Straminopila (68-69) which is a new one in that classification system (70). The Oomycetes are the largest group of heterotrophic Stramenopiles

which have evolved either pathogenic or saprophytic lifestyles. They are found all over the world in fresh and salt water habitats. Some of the terrestrial oomycetes are among the most important plant pathogenic organisms that may be facultative or obligatory parasitic. For the longest time, oomycetes were considered to belong to the fungi based on certain morphological similarities. Both fungi and oomycetes are characterized by filamentous vegetative growth, the production of mycelia, and formation of spores through asexual and sexual processes.

The phylum oomycota contains a single class oomycetes (3), seven orders i.e. Saproleginales, Rhipidiales, Leptomitales, Salilagenidiales, Pythiales, Peronosporales and Sclerosporales. In order Pythiales contains two most economically important genera *Phytophthora* and *Pythium*, which include some of the world most destructive plant pathogens, that account collectively for multibillion dollar losses in world cash crops (3, 71). Moreover, these are able to infect hosts ranging from algae, plants, protists, fungi and arthropods to vertebrate animals (72). In general, *Phytophthora* and *Pythium* are so intrinsically linked with the mycology and fungal plant pathology that they continue to be studied in mycological laboratories and to be taught in mycology lectures and practical classes.

The genus *Pythium* is a common inhabitant of terrestrial and distribution. More than 200 recognized species and new members have been continuously described from different parts of the world (73-76). In nature, *Pythium* species play a versatile role as either beneficial or harmful organisms. Many species of *Pythium* are known to parasitize and cause infections in the roots of crop plants and ultimately damage them (1). The only one species, *Pythium insidiosum* (*P. insidiosum*), has been implicated in

diseases of plants and mammals (horses, cattle, dogs, cats, or fish), particularly in tropical and subtropical parts of the world (11, 77). The disease caused by *P. insidiosum* is called pythiosis (78). Pythiosis in mammals is characterized by the development of cutaneous, subcutaneous, blood vessel, ocular and intestinal lesions and, less frequently, by the involvement of bones and lungs (79).

1.1. Mammals pathogen of *P. insidiosum*

At the end of 18th century, the primary well-documented equine cases, in which the 'fungal-like' nature of the infection was suggested (80-81). The pathogen was isolated from horses as early as 1901 by de Haan who working in Indonesia (82), it could not be induced to form spores in cultures. It was assumed to be a zygomycete or phycomycete. He called the disease in horses "Hyphomycosis desturens". In 1961, Bridges and Emmons (83) isolated a similar microorganism from 8 horses in Texas. They called the microorganism *Hyphomyces desturens*, and they could not induce sporulation. Until 1974 that Austwick and Copland were able to produce biflagellate zoospores from isolates obtained from horses in New Guinea. They were successful in obtaining sporulation by placing a portion of colonies grown on Sabouraud dextrose agar (SDA) in Petri dish which contained pieces of sterilized aqueous medium of rotten maize silage. So they identified the pathogen as an oomycete that was likely a member of the genus *Pythium* (84). The first case of human pythiosis in Thailand was documented from Siriraj Hospital (11, 85). The reported cases, two males, developed subcutaneous type. *P. insidiosum* was introduced by de Cock in 1987(6), who examined several isolates obtained from horses and dogs in several geographic regions (including the United States) and found them to be identical. In addition, de Cock was

able to produce sexual reproductive structures from these isolates *in vitro* and was therefore able to identify the pathogen as a new species of *Pythium*.

The disease of this microorganism was recognized by several different names (5, 79) such as bursattee, cutaneous habronemiasis, espundia, kunker, granular dermatitis, leeched, swamp cancer and phycomycosis whereas now we called "Pythiosis". To date, pythiosis has been reported in a board range of hosts (horses, cattle, dogs, cats, polar bears, jaguar and humans) (86-88). The most common host are horses and human. Human pythiosis was first document from Thailand in 1985 in 2 Thai thalassemic patients with subcutaneous granulomatous lesions (79). It is found mostly in all over Thailand, and two factors contribute to importance of pythiosis in this country: the prevalence of thalassemia and agricultural areas. There are some putative cases were found to have been misdiagnosed as mucomycosis, aspergillosis, and penicillosis (20) because most healthcare professionals are not familiar with the disease and causative agent. *P. insidiosum* has many fungus–like characteristics, but is not true fungi. A number of studies have revealed that it should be classified with the golden–brown algae and diatoms as straminopiles. This implies that *P. insidiosum* involved genetic and biochemical mechanisms for interaction with animals and plants that are different from true fungi (89).

This emerging disease is important to promote awareness of human pythiosis in other countries as well because the causative organism is found worldwide (4, 14, 16). Pythiosis with high morbidity and mortality has also been increasingly reported worldwide but a diagnosis is time consuming and requires skilled personnel.

2. Biology and Life cycle

Morphologically, *P. insidiosum* colonies are colorless to white-cream, and submerged with short aerial filaments, and a finely radiate pattern. It develops fungal-like sparsely septate hyphae (Figure 1). The hyphae range between 4 and 10 µm in diameter with perpendicular lateral branches. Cross-septa are only occasionally observed in young hyphae, but they are abundant in old viable ones. Because *P. insidiosum* is a member of oomycetes which are a major group of plant pathogens, there has been an effort to understand the mechanisms underlying their directed movement towards potentials hosts.

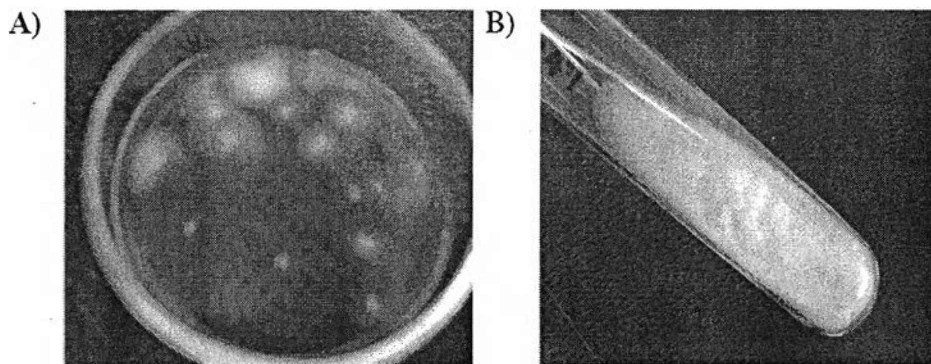

Figure 1. Colony of *P. insidiosum* (A; one day old, B; 4 days old) on SDA.

Cytoskeletal system of *P. insidiosum* consists of microtubules and actin filaments which are present near the tips of actively growing hyphae. Numerous mitochondria and Golgi bodies are behind the hyphal apex. The mitochondria with tubular cristae and the Golgi bodies consisting of multiple flattened cisternae are unique characteristics that differentiate oomycetes from fungi. These organisms

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Table 1. Important different between the Oomycetes and Fungi (93).

	Oomycetes	Fungi
Zoospores	Biflagellate; an anterior tinsel and a posterior smooth flagellum	Uniflagellate; a posterior smooth flagellum in the Chytridiomycetes
Lysine biosynthesis	Via diaminopimelic acid	Via α – aminoadipic acid
Mitochondria	Cristae tubular	Cristae plate - like
Wall polysaccharides	Cellulose present; chitin also in some species	No cellulose; chitin usually present
Wall protein	Hydroxyproline present	Proline present

3. Hyphae ultrastructure

The apex contains a Golgi–derived vesicles, responsible for modification of lipid and packaging of proteins. Large microtubules were dispersed throughout the hyphae. Endoplasmic reticula (ER) were sometimes detected near the mitochondria with multiple ribosomes around the ER. The mitochondria have tubular cisternae whereas the mitochondria of fungi have plate–cisternae. Numerous small vacuoles ranging in size and shape were also dispersed throughout the hyphae (94-95) (Figure 2).

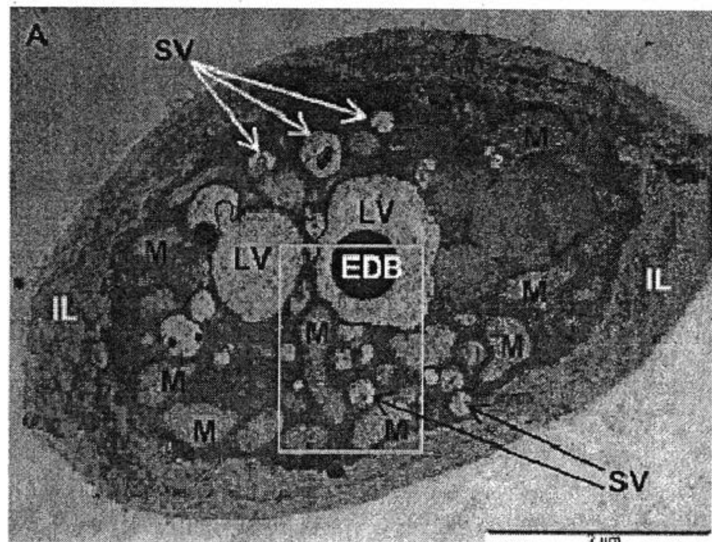

Figure 2. TEM micrograph of a transverse section of hyphae of *P. insidiosum* containing typical inner layers (IL), large vesicles (LV) with EDB, small vesicles (SV) and numerous tubular cristae mitochondria (94).

4. Life cycle

P. insidiosum can produce both asexual and sexual spores, depending on the environmental conditions.

4.1 Sexual reproduction

Most species in the genus are homothallic and can form oospores in single culture, however there are 7 species that are heterothallic which required different mating types. Gametangia involved in the sexual process may arise from the same hyphae or may be formed on different hyphae that lie close together. The oogonium (Figure 3A) is fertilized by an antheridium, resulting in the creation of thick-walled, diploid oospores. These usually exhibit constitutive dormancy, requiring a postmaturation phase during which the thick oospore wall becomes thinner by

digestion of its inner layers. This process can be hastened by keeping the spores in nutrient-poor conditions, at normal temperature and moisture levels. When oogonia develop in vitro, they are intercalary, smooth, and subglobose. They have a rigid fertilization tube measuring 23-30 μm in diameter and may have one to three diclinous antheridia per oogonium. The antheridia are attached over their entire length to the oogonim (96).

4.2 Asexual reproduction

P. insidiosum can produce asexual biflagellate zoospores (Figure 3B) which are able to swim in water for hours, using endogenous energy supplies. Motility of oomycete zoospores is achieved by the action of the two flagella that emerge from the centre of a groove along the ventral surface of the cell are unequal in length. The anterior shorter flagellum is of the whiplash type, lacks mastigonemas and directs backward. The posterior longer one is of the tinsel type, and flexing to bring about a change in swimming direction. In 1993, Mendoza and *et al.* (97) revealed the life cycle of *P. insidiosum* in nature and its relationship to infection in mammal (Figure 4). Briefly, it includes the following steps: dispersal and arrival of an infectious particle (usually a spore of some kind) in the vicinity of the host, adhesion to the host, recognition of the host (which may occur prior to adhesion), penetration into the host, invasive growth within the host, lesion development in the host, and finally production of additional infectious particles (98-99).

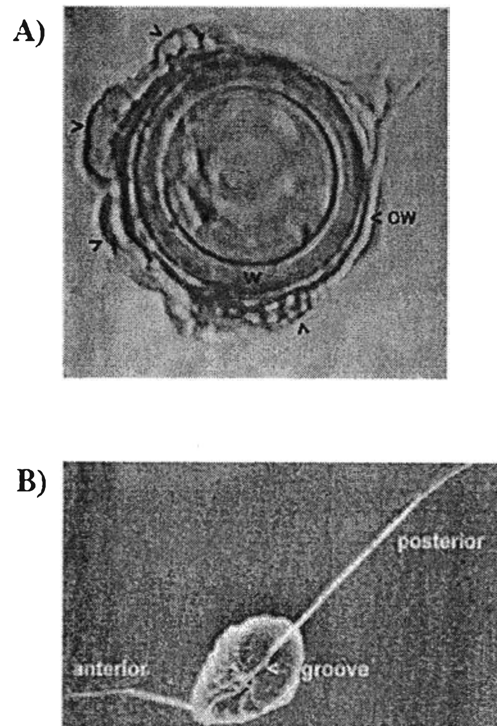

Figure 3. A developing sexual and asexual reproduction of Oomycota. A: oospore has a thick wall (w) and is contained in the outerwall (ow) of the oogonium (female reproductive cell). The arrows mark the positions of antheridia (male sex organs that fertilize the oogonium), B: zoospore with posterior whiplash flagellum and a shorter, anterior tinsel – type flagellum (92).

In general, the tactic responses are non – specific in that they guide the zoospores to both hosts and non – hosts; however, specific recognition of a chemoattractant produced by a host is known to occur (100-101). When zoospores were placed in contact with plants or animal tissues, they started to throw off the flagella, became rounded in shape, and became motionless. Adhesion of zoospores to

the surface of hosts plays as important role in a number of aspects of pathogenesis (2, 102-103). After encystment the zoospores developed germtube with the underlying host facilitates the reception of signals that guide pathogen growth on the host surface and that initiate differentiation of infection structures during pathogenesis.

In environment habitat, *P. insidiosum* zoospore is also able to attach to the Australian water lily (*Nymphaea gegantea*). Moreover, Chaiprasert and collaborators (104) showed that Thai *P. insidiosum* zoospores have chemotactic behavior to leaves of some field crops such as rice, bean and Malaysia grass (*Axonopus compressus*). To maintain its life cycle in nature, *P. insidiosum* requires a low concentration of ions, a nearly neutral pH and a plant host. Like other zoosporic fungus-like organisms, *P. insidiosum* probably uses plants to produce sporangia and release zoospores to colonize other plants and expand its ecological niche, but it has never been demonstrated to be pathogenic to plants.

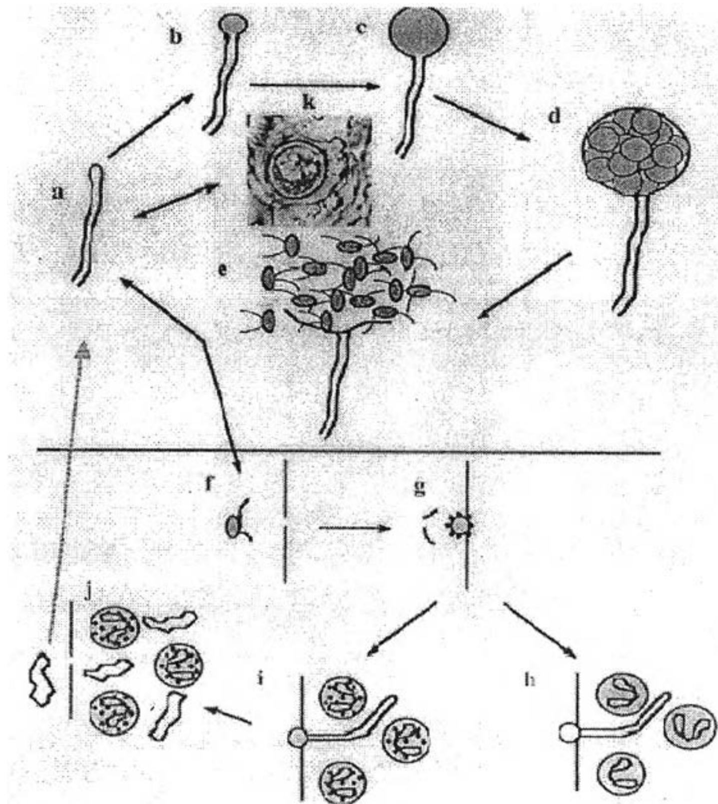

Figure 4. The life cycle of *P. insidiosum* . The upper part of this figure shows the life cycle in nature. It starts when a plant is colonized (a), and then the differentiation of sporangium into mature stages (b to d) leads to zoospore release (e). The motile zoospores swim and, directed by attractants, locate another plant to start a new cycle. If an animal or human enters this ecosystem (lower part of the figure), zoospores will also be attracted by injured tissue, as showed in this study (f). There they lose their flagella attached to tissue by using a sticky substance (g [black dots]), encyst (g), germinate (h [humans] and i [animals]), invade the host, and cause pythiosis insidiosii. In horses the formation of masses, called "kunkers," occurs (j). These masses may be expelled and may produce sporangia in wet environments, but it has no impact on the epidemiology of pythiosis insidiosii. The production of oospores may occur in nature and serve as resistant spores (k [upper panel]) (97).

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5 Epidemiology

Pythiosis is an emerging, life – threatening infections disease caused by the *P. insidiosum*. The disease has been reported in tropical, subtropical and temperate area of the world (Figure 5). Only *P. insidiosum* has been associated with animal and human diseases. Pythiosis in animals and humans, caused by *P. insidiosum* has been reported in many countries, including Australia (7, 105), New Zealand (106), USA (20, 107-108), Brazil (4), Haiti (109), Malaysia (77) and Thailand (8, 10-11, 15, 18-19, 79, 110-111). A case of intestinal pythiosis was recently reported in a dog from Venezuela (13). Verified cases of this disease have not been reported in Europe. In 2003, there are 3 cases of horse subcutaneous pythiosis with dissemination to the internal organs in USA (112). Although the geographical location and tropical climate of Africa seemingly would make it an ideal region for pythiosis, only one canine case from that continent have yet to be reported (14).

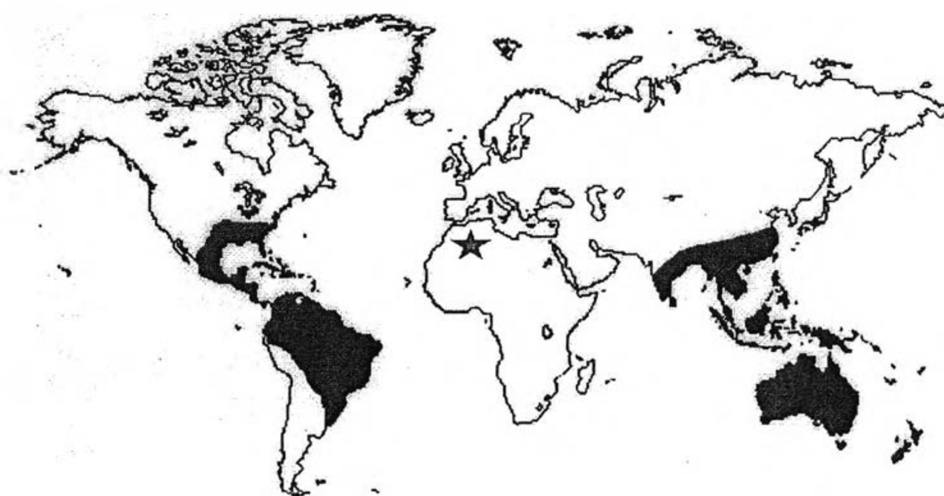

Figure 5. Distribution of pythiosis in the tropical, subtropical and temperate regions of the world (113).

In Thailand, cases of human pythiosis have been frequently found in the central and southeastern regions (Figure 6). Disseminated form pythiosis occur mostly in patient who have underlying hematologic disorder such as thalassemia (79, 110), leukemia (107) and aplastic anemia/paroxysmal nocternal hemoglobinuria (PNH) syndrome (18) whereas most ocular type pythiosis are found in healthy people (15). The relationship between pythiosis and underlying disease is unknown and needs to be explored. In the recent study (15), patients with pythiosis had the age range of 20-60 years (86% of all reported cases), were male (71%), and had agriculture–related occupations (farmer, fisherman, and domestic husbandry, 75%). In recent, human pythiosis cases have been reported from 9 tertiary care hospitals located across Thailand (15) (Table 2).

Table 2. Summary of clinical information for human patients with pythiosis diagnosed at 9 tertiary care hospitals across Thailand (15).

Study variable[a]	Form of pythiosis: Vascular	Disseminated	Ocular	Cutaneous/subcutaneous	All forms
No. (%) of patients	60 (59)	3 (3)	34 (33)	5 (5)	102 (100)
Ratio of male to female patients	2.4:1	2:1	2:1	...[b]	2.4:1
Age, mean years (range)	38 (14–71)	28 (12–44)	44 (20–78)	27 (15–40)	39 (12–78)
Occupation, n/N (%)					
Agriculture	41/50 (82)	0/3 (0)	14/20 (70)	2/3 (67)	57/76 (75)
Nonagriculture	9/50 (18)	3/3 (100)	6/20 (30)	1/3 (33)	19/76 (25)[c]
Principal diagnostic method, no. of patients					
Culture identification	26	2	34	5	67
Serodiagnosis	13	1	...	...	14
Clinicopathological features	21	...	...	...	21
Total	60	3	34	5	102
Duration of symptoms, mean days (range)	91 (7–365)	11 (1–21)	17 (1–60)	41 (4–90)	66 (1–365)
Underlying disease, n/N (%)					
Hematological disease					
Thalassemia syndrome	53/59 (90)	1/3 (33)	2/19 (11)	3/5 (60)	59/86 (69)
Nonthalassemia	6/59 (10)	2/3 (67)	0/19 (0)	2/5 (40)[d]	10/86 (11)
Nonhematological disease	0/59 (0)	0/3 (0)	1/19 (5)[e]	0/5 (0)	1/86 (1)
No underlying disease	0/59 (0)	0/3 (0)	16/19 (84)	0/5 (0)	16/86 (19)
Final treatment outcome, n/N (%)					
Cured					
All cures	26/43 (60)	0/3 (0)	19/19 (100)	3/3 (100)	48/68 (71)
Limb amputated	26/43 (60)	...	...	...	...
Eye lost	...	...	15/19 (79)	...	...
Eye saved	...	...	4/19 (21)	...	...
Died	17/43 (40)	3/3 (100)	0/19 (0)	0/3 (0)	20/68 (29)

NOTE. The participating tertiary care departments and hospitals were Faculty of Medicine, Ramathibodi Hospital, and Faculty of Medicine, Siriraj Hospital, Mahidol University (Bangkok); Faculty of Medicine, Khon Kaen University (Khon Kaen); Faculty of Medicine, Prince of Songkla University (Songkla); Faculty of Medicine, Chiang Mai University (Chiang Mai); Faculty of Medicine, Chulalongkorn University (Bangkok); Sapasittiprasong Hospital (Ubonrachathani); Lerdsin Hospital (Bangkok); and Metapracharak Hospital (Nakornpatom).

a Data were available for each study variable as follows: sex, 102 patients (100%); age, 100 patients (98%); occupation, 76 patients (75%); principal diagnostic method, 102 patients (100%); mean duration of symptoms, 79 patients (77%); underlying disease, 86 patients (84%); and final treatment outcome, 68 patients (67%).

b All patients were male.

c Nonagriculture occupations included domestic laborer, 8 patients; student, 4 patients; car driver, 2 patients; grocery seller, 1 patient; government service agent, 1 patient; and monk, 1 patient. Two patients were unemployed.

d One patient had idiopathic thrombocytopenic

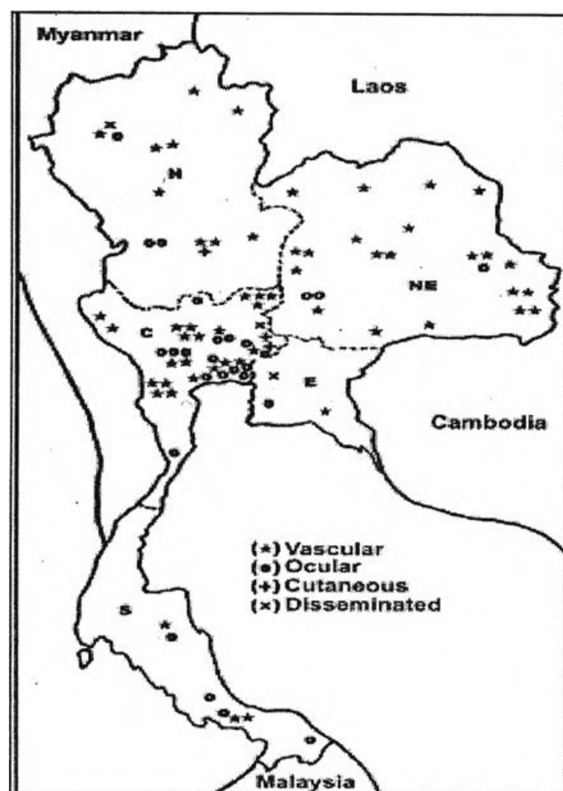

Figure 6. Geographic distribution of patients with pythiosis in Thailand. C, central region; E, eastern region; N, northern region; NE, northeastern region; S, southern region. Data were available for 88 patients (86%) (15).

6. Clinical pathogenesis

The Oomycetes is a unique group of pathogens that differs from fungi, bacteria, parasites, and viruses. Unlike most of *Pythium* species, *P. insidiosum* can grow at host body temperature (37°C), which is an essential factor for its virulence. When host enter swampy areas inhabited by *P. insidiosum,* open wounds on their skin attract zoospores that then encyst on the exposed tissue. On the basis of electronmicroscopic observations (97), the zoospores are known to secrete a sticky substance that allows them to make and maintain tightly contact with the host during the initial stages of invasion. Stimulated by the temperature of the host's skin, the encysted zoospores

develop a germ tube, often directed towards injured tissue(97), and mechanically penetrate the tissue. Ravishankar and Davis (38) experimentally established that *P. insidiosum* cannot penetrate normal skin, but has the potential to successfully penetrate injured skin. Thus, penetrating undamaged skin probably needs the combination of both mechanics and enzyme action for reducing tissue strength.

6.1 Clinical manifestation

6.1.1 Cutaneous/subcutaneous pythiosis

The patients were characterized by chronic swelling (duration, 1–3 months) and a painful, subcutaneous, granulomatous, infiltrative lump and ulcer on the arm or leg. Acute infection is also documented with a case of acute necrotizing cellulitis of both legs (10). He was a 15-year-old boy with thalassemia, and he presented with 4-day history of necrotizing cellulitis of both legs that he experienced shortly after swimming in a river. The findings of an examination of muscles and vascular tissue were unremarkable, which showed necrosis of skin and subcutaneous tissue but intact fasciae and muscle. This organism causes granulomatous disease in many kinds of animals such as horse, sheep, dog, cat and mare (Figure 7A) (105, 112, 114-122).

Histopathological examination revealed chronic infection with eosinophilia in the dermis and subcutaneous tissue. A mixed inflammatory cell infiltrate comprising eosinophils, lymphocytes, neutrophils, macrophages and mast cells were also reported. The hyphae of *P. insidiosum* were restricted to the areas of the eosinophilic granular masses. The histopathology of this clinical manifestation and that of zygomycosis (caused by *Basidiobolous* and *Conidiobolus*) is alike

(116). In addition, *P. insidiosum* initiates a cell-mediated immune response in its host, mostly in the form of eosinophils and a few neutrophils (12, 123), but the immune response cannot prevent the propagation of *P. insidiosum.* The eosinophils then, in an effort to phagocytose the microorganism, degranulate over the hyphae. The eosinophilic material (Splendore-Hoeppli-like phenomenon, Figure 7B) around *P. insidiosum* hyphae is a main feature of the infection in equines but not in the other species. In addition, a small coral-like mass known as a 'kunker' (yellow-white, coral-like necrotic masses of tissue that contain hyphae) is commonly found on the surface of the infected tissue of infected horses.

Kunkers are composed of degranulated eosinophils interlaced with the viable hyphae of *P. insidiosum*. The tissue damage observed in acute and chronic cases has been attributed to the release of chemicals from the degranulated eosinophils and mast cells (32, 123). However, similar structures are encountered in habronemiasis and subcutaneous zygomycosis caused by the nematode *Habronema* and the *Zygomycetes* (*Entomophthorales*) *Basidiobolus* and *Conidiobolus*, respectively (116, 124), which can lead to a misdiagnosis of the disease. In dogs, the presence of tumor-like masses is often confused with neoplasia (125-126).

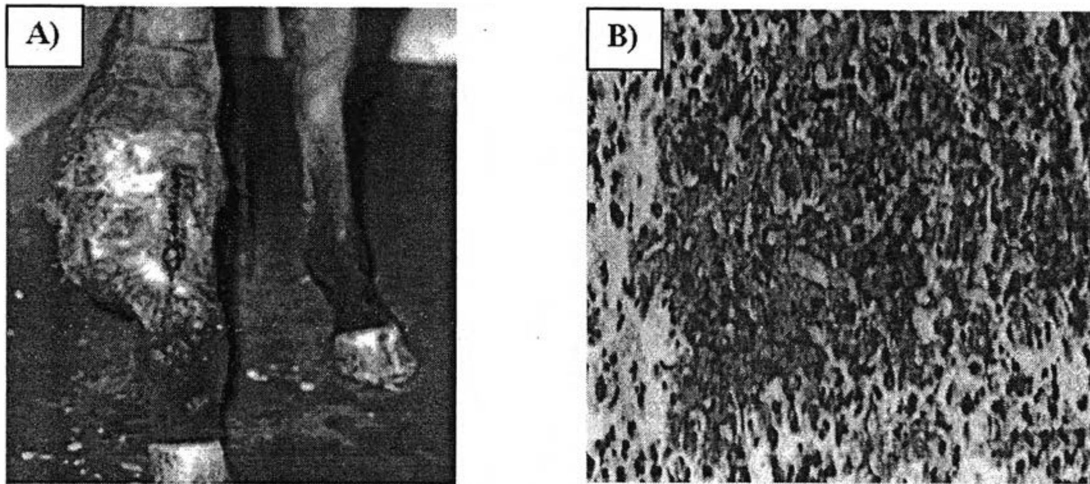

Figure 7. The pathogenesis and histopathology of cutaneous pythiosis . A) A typical lesion encountered in equine pythiosis (kunker) and B) hyphal fragment surrounded by eosinophilic material, which suggests a Splendore – Hoeppli phenomenon (H&E, magnification x 200) (32).

6.1.2 Vascular pythiosis

This pathogenesis form is the most common form in thalassemia and anemia-paroxysmal nocturnal hemoglobiruria (PNH) patients (28, 127) which affect arteries of lower extremities resulting in arterial occlusion or aneurysm. Clinical presentations varied from intermittent claudicating to gangrenous ulceration (Figure 8A). Other symptoms and signs were observed, such as fever, paresthesia, itching, vesicle/bulla, skin ulcer, cellulitis, necrotizing fasciitis, leg swelling, absence of arterial pulse, groin mass (iliac or femoral aneurysm), and abdominal mass (aortic aneurysm). The average duration time of symptoms before seeking medical care was 3 months. So the patients come to medical care late in the course. Angiographic findings showed an occlusion or dilatation of infected

medium-to-large–sized arteries of the lower extremities. In some patients with advanced pythiosis, aortic aneurysm was observed. External and internal carotid arteritis was reported in one case (128). Pathological findings revealed hyphae invading the arterial wall, eosinophil infiltration, focal suppurative granuloma, and giant cells surrounding the hyphae, as well as a blood clot containing hyphae in the arterial lumen. No infection in venous tissue was noted. The infection progressed proximally along the arterial wall and caused aneurysm or arterial occlusion from a thrombus or fibrosis. Embolism to the other leg occurred when the bifurcation of aorta was affected, as also reported in detail elsewhere (18). A patient history of exposure to swampy area shortly prior to the illness is usually obtained. The infection occurs at one or both legs. A major cause of death is from the ruptured aortic aneurysm (Figure 8B).

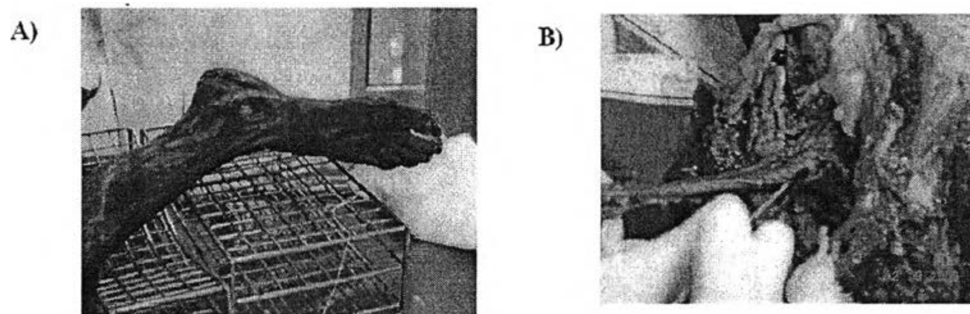

Figure 8. The pathogenesis and histopathology of vascular pythiosis. A) Gangrene on right foot, vascular pythiosis, and B) demonstrated a white clot from aortic bifurcation down to the right common iliac artery and the superficial and deep femoral arteries, surrounded by necrotic tissue.

6.1.3 Ocular pythiosis

The patients usually presented with corneal ulcer or keratitis. Pain, irritation, decreased visual acuity, eyelid swelling, conjunctival injection, corneal infiltrates, and hypo yon is observed in some cases. Most patients had no underlying disease, suggesting ocular infection may not be associated with hematolytic disease as found in cutaneous/subcutaneous and vascular pythiosis (11). The duration time for seeking in medical care is shorter than other pythiosis forms. Some patients had obvious histories of eye trauma, such as a foreign body or corneal abrasion by plant materials is usually obtained (Figure 9).

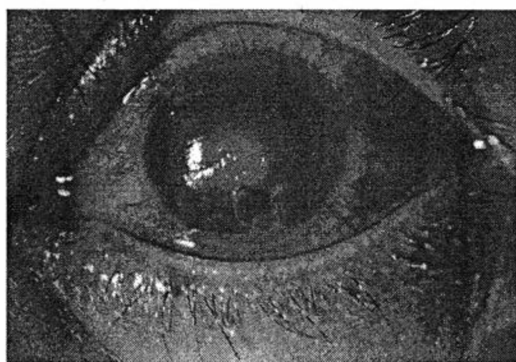

Figure 9. Clinical presentation of ocular pythiosis.

7 Laboratory diagnosis

7.1 Specimen collection

Skin biopsy and serum samples (cutaneous/subcutaneous pythiosis); and corneal scraping (ocular pythiosis) could be transported in sterile normal–saline solution. The tissue samples and serum samples from which to isolate *P. insidiosum* from human is the arterial tissue with thrombus in artery. The clinical specimens from

pythiosis patients should be sent directly to the laboratory for identification. If the samples cannot be delivered immediately, it is suggested that they should be kept in a sterile container at room temperature (should not kept at 4°C).

7.2 Culture

Clinical samples are cut into small pieces and placed on Sabouraud's dextrose agar (SDA) or broth (SDB) and incubated at 37°C for 24-48 h. After 24 – 48 h of incubation, white-cream flat colonies with no aerial hyphae normally appear from the specimens (Figure 10A and 10B). Microscopically, hyphae are branched at approximate 90 degree angles and are usually coenocytic, although occasional septa are observed in tissue sections and cultures.

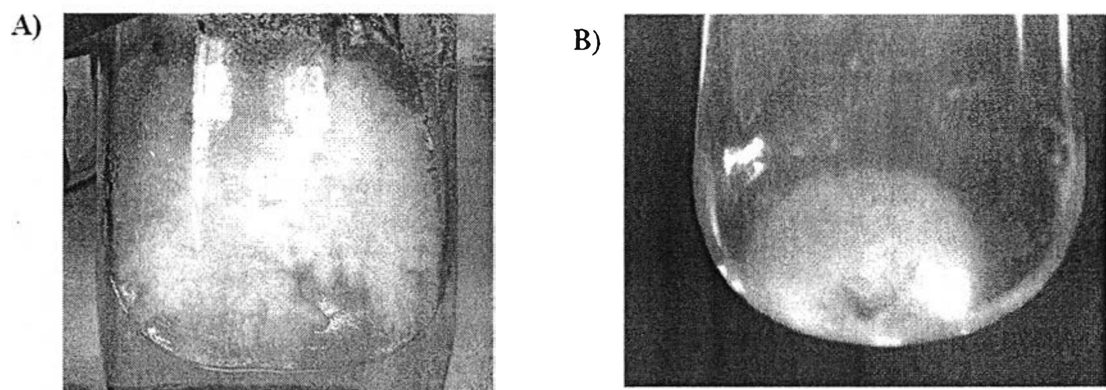

Figure 10. Colony morphology of *P. insidiosum*. A) white colonies with submerged vegetative and short aerial hyphae on SDA, and B) culture isolation of *P. insidiosum* from infected tissues in SDB.

7.3 Zoospore production

To induce zoospores, baits as boiled grass leaves or animal hair are needed. Grass leaves are placed on *P. insidiosum* cultures for 24 hr before being immersed in

dilute salt solution. The solution, called induction medium, contains calcium and magnesium ions which are essential for inducing zoospore induction. After 2-3 hr incubation, zoosporangia containing motile zoospores will be readily observed at the edges of the leaves (97). Alternatively, sterile animal hair and many pieces of 5-day old culture are submersed in the induction medium for 16-18 h at 37°C. After incubation, the zoosporangia can be observed on the hair and the pieces of culture (111). Sporangium of *P. insidiosum* is filamentous type. Morphology of zoosporangium is globose and hyaline, and measures 20-60 µm in diameter. Through progressive cleavage, biflagellate zoospores are formed inside the vesicle. In addition, the morphology of sexual spores is commonly used for *Pythium* spp. identification. However, oogonium production is very rare in *P. insidiosum*. Therefore, the specific identification is normally based on zoospore formation, serological tests and molecular methods.

7.4 Histopathology

The collected tissue from the infected areas is sent to the laboratory in sterile distilled water at room temperature. Pieces of the tissue are placed with 10% KOH (Figure 11). The finding of sparsely septate hyphae may be indicative of *P. insidiosum*, or other fungal pathogen (zygomycetes). The histopathology of *P. insidiosum* is difficult to identify morphologically. Gomori's methenamine – silver nitrate stain (GMS) and Periodic Acid-Schiff (PAS) showed 4–10 µm in diameter sparsely short septate hyphae with perpendicular branching are rarely visible, but those found in case of zygomycosis (*Basidiobolus ranarum* and *Conidiobolus coronatus* are wider, about 5 to 15 µm in diameter) (129). The most important cultural

identification feature is the biflagellate zoospores production, but this is relatively nonspecific and may take weeks to observe, and for some strains it is difficult to induce zoospore production in culture (130).

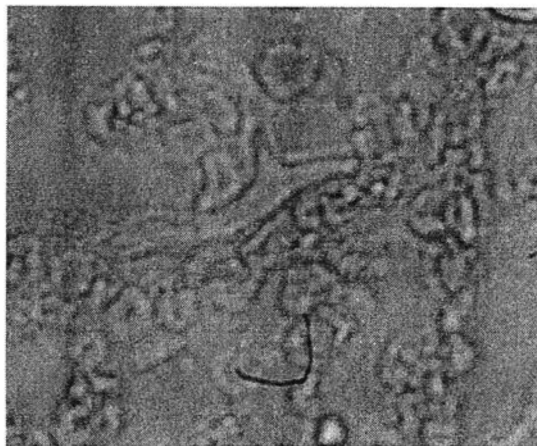

Figure 11. Direct examination from tissue biopsy from human vascular pythiosis. The biopsy of lesion showed non septate hyphae.

7.5 DNA – based diagnostic assays

Recent advances in molecular analyses of fungi provide extensive DNA sequence information that is of great benefit in molecular detection and diagnostics. Molecular analyses are now commonly used for fungal identification, especially for large genera whose species have overlapping morphological characteristics. Several specific identification methods of *P. insidiosum* using DNA – based assays have been developed, based on Polymerase chain reaction (PCR) method, nested PCR, DNA sequencing and hybridization techniques. Badenoch and his colleges (77) identified *P. insidiosum* from a keratitis patient by using DNA sequencing analysis. In 2002, Grooters and Gee (27) developed nested primers that specifically amplified 105 base

pairs form *P. insidiosum*'s internal transcribed spacer (ITS) region of genomic DNA. Moreover, Reis and *et al.* (112) obtained several amplicons of *P. insidiosum*'s 18S small–subunit rDNA sequences from three cases of horses with systemic pythiosis. The PCR product can be sequenced and blasted against the NCBI genome database for determining the species of *Pythium* (77). In 2004, dot blot hybridization using 530 – bp *Hinf*I fragment from the ribosomal DNA intergenic spacer (IGS) was developed for *P. insidiosum* identification by Schurko and *et al.* (26). These studies indicated that molecular techniques are ideal to identify *P. insidiosum* from tissues biopsies and cultures, and could be successfully used more frequently in the future.

7.6 Serological method

The serodiagnostic tests including the immunodiffusion (ID) test (23, 131-132), enzyme-linked immunosorbant assay (ELISA) (24, 34, 133), and Western blot method (134), have been developed to facilitate the diagnosis of pythiosis. Several methods have been documented and widely used as either an early diagnostic test or monitoring test. The tests showed various advantages and disadvantages. The early developed ID test could detect pythiosis in early stages (3 days) but failed in chronic infection equine cases. The improved version of this test became more sensitive, stable and useful, not only in diagnosing the disease in many varieties of hosts, but also in monitoring response to treatment. Western blot is useful for both diagnosis and determining prominent antigens. Many immunodominant antigens have been reported and it was suggested that they may play an important role in the immunotherapy of pythiosis. Western blot test is highly specific and also useful for analyzing immunoglobulin classes that appear during infection. ELISA developed to detect

pythiosis in humans and animals showed 100 % of both sensitivity and specificity. This ELISA could discriminate between sera from apparently healthy individuals or heterologous infections and patients with active pythiosis. Moreover, the assays showed very high sensitivity and specificity to diagnose pythiosis and are also suitable to monitor the response to treatment.

8 Treatment

The conventional antifungal drugs are not effective in treating pythiosis, three major therapeutic methods that are currently in use include surgery, drugs and immunotherapy. Cutaneous/subcutaneous pythiosis usually has a good response after the administration of saturated solution of potassium iodide (SSKI) and amphotericin B, along with the surgical debridement. Iodides have been used in human pythiosis since 1990. Patients with arteritis caused by *P. insidiosum* did not respond to potassium iodide, but patients with subcutaneous pythiosis seemed to respond well to this drug (85). The main drawback of iodide and amphotericin B is their toxicity (135).

Concerning vascular pythiosis in Thailand, the treatment for common fungal infection, including administration of SSKI and conventional antifungal agents (such as amphotericin B, ketoconazole, itraconazole, and terbinafme), had no favorable effect. Amputation, in order to remove the infected tissue, is the effective way for curing. With consideration of the level and severity of affected arteries, infected tissue could be removed by one or a combination of the following operations: below-knee amputation, above-knee amputation, resection of infected artery, aneurysmectomy, or hip disarticulation depending on the level and extent of the affected arteries.

Thromboembolectomy is not recommended since the infection can be easily spread while an infective clot is removed out from the distal to the proximal part of the artery. Most cases of vascular pythiosis undergo the limb amputation. Forty percent of patients with vascular pythiosis die from the infection, while 60 percent survive with some handicaps (136). In ocular pythiosis, topical and systemic conventional antifungal agents and SSKI were infective. Therefore, the main treatment is also the radical surgery to remove all infected tissue. An eye removal by enucleation or evisceration is reserved following the failure to control the infection by keratectomy. After treatment, all patients survived. A majority of the patients eventually undergo an enucleation or evisceration. There is no fatal outcome in ocular pythiosis.

P. insidiosum vaccine has been recently developed which is prepared from cytoplasmic and secretory antigens of *P. insidiosum* cultured in laboratory (28, 32, 128). Immunization of equines with products derived from *P. insidiosum* cultures was reported to have curative properties in Australia (137) and Costa Rica (12). Two antigens referred to in the literature as Miller's and Mendoza's vaccines (138) have been used for immunization. The Australian vaccine was prepared from sonicated hyphal antigens, whereas the Costa Rica vaccine used precipitated proteins from culture filtrate antigens (CFAs). Immunotherapy of horses with pythiosis using Miller's vaccine alone gave a success rate of 53%. An increase in the percentage of cured cases was obtained when immunization was followed by surgical removal of cutaneous lesions. Similar results were obtained using Mendoza's vaccine. The significant reduction in the swelling, caused by the vaccine at the site of injection, an undesirable side effect of Miller's vaccine, was the main feature of Mendoza's

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vaccine. Moreover, the vaccine described by Miller was unstable, losing its curative properties after storage at 4°C. By contrast, Mendoza's vaccine was found to be effective even 18 months after its preparation.

In 2003, Mendoza and collaborators introduced new immunotherapeutic immunogens to treat pythiosis in both humans and animals. This new formulation contains exo- and endoproteins extracted from cultures of *P. insidiosum*. It has been shown that 60% of the horses, 97% of the cattle, and 33% of the dogs with pythiosis responded favorably after the administration of the vaccine (129). In human pythiosis, the vaccine has also been used in the immunotherapy for patients with inoperable vascular pythiosis. At least 2 injections of 100–200 ml each of antigen, 2 mg/mL, at a 14-day interval were given. Twelve patients with vascular pythiosis were treated with immunotherapy. Of these, 5 patients survived, 2 died, 2 still had persistent infection, and 3 had no available outcome (15). However, this method has proved to be only partially successful because most patients finally died of disseminated abdominal arteritis (18, 139). Early detection of affected vessels using angiography is important in determining the most appropriate amputation site.

Treatment with antifungal agents, such as amphotericin B, 5-fluorocytosine (5-FC), and ketoconazole, were also ineffective for Thai cutaneous/subcutaneous pythiosis. However, patients had good responses to saturated solution of potassium iodide (SSKI; 1 ml orally 3 times per day for up to 3 months). Recently, two studies evaluated in vitro susceptibility of *P. insidiosum* to caspofungin (36) and combination drug (voriconazole, terbinafine and itraconazole) (22). All of them presented that

antifungal drugs have limited fungistatic activity against *P. insidiosum*, especially for invasive pythiosis patients that are difficult to treat.

Pythiosis is marked by high rates of morbidity and mortality. Because it is an emerging disease, many health care professionals are unfamiliar with this devastating and diagnostic tools are limited, leading to under recognition and under diagnosis of the disease, delayed treatment, and a poor prognosis for pythiosis patients. In order to overcome the potential errors in identification and diagnosis, mycologists have searched for more rapid, reliable and efficient way of identification and treatment of this organism over the past few decades. Understanding the information of *P. insidiosum* (i.e. pathogenesis, broad spectrum of clinical manifestation and virulence factors) could help to prevent and control disease. The use of molecular methods in oomycetes has become increasingly important in species separation, identification and determination of their natural relationships.

9 Genomic data of *P. insidiosum*

The field of genomics has rapid growth since 1953, Crick and Watson were found the double helix structure of DNA in the cell nucleus. Then 1958, Francis Crick suggested an RNA intermediate and proposed that there is a one-way sequential flow of information from DNA to RNA to protein. This relationship of information transfer between DNA and protein became the **Central Dogma** (meaning a set of beliefs) in molecular biology. The Central Dogma (Figure 12) was modified when it was found that in special cases RNA can direct the synthesis of DNA; under artificial conditions single-stranded DNA can specify protein. This new understanding of the molecular basis for the code of life has concepts of genetics and evolution. Large and more

ambitious efforts emerged to complete the sequencing of microorganisms and human genome.

Fig. 15.1A The Central Dogma.

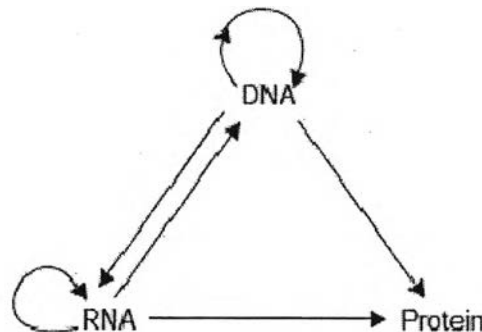

Figure 12. Diagram representing the main features of the Central Dogma

The medical interests and requires evaluation of the genetic potential of a given organism. Sequence data has to be screened for genes coding for desired enzymes involved in the pathway of interest. To meet the demand for sequence information, more and more fungal genomes have been sequenced since the first genome of the yeast model organism *Saccharomyces cerevisiae* was publish (140). Generally, genome sequencing projects continue to many organisms, resulting in more than 800 fully sequenced genomes currently being available, 25 of which fungal genomes. The study of oomycetes has reached new heights with the completion of the genome sequence drafts of five species that are among the most notorious plant pathogens (29) (Table 3).

Genome size of oomycetes varies widely, ranging from 18 – 250 Mb, based on estimates made using image analysis of nuclear Feulgen stainings, reassociation

kinetics, and contour–clamped homogeneous electric field (CHEF) gel electrophoresis (141-142). *Pythium* species may have the smallest genomes of oomycetes, with estimates ranging from 18.8-41.5 Mb based on electrophoretic karyotyping (141). The haploid chromosome counts of many oomycetes species are not exactly known due to the presence of several small chromosomes that are difficult to resolve under light microscopy. The chromosomes of eighteen *Pythium* species revealed 7–20 bands (141). A majority of oomycetes genes examined to date do not contain introns. Recent advances in genomics have improved the understanding of plant pathogenic oomycetes greatly with at least five genome-sequencing projects at or near completion (data as above).

Table 3. Oomycete genome projects and their associated web resources (29).

Species	Genome size (Mbp.)	Estimated number of genes	Resources
Hyaloperonospora parasitica	75	NA	Washington Uni., http://genome.wustl.edu/pub/organism/Fungi/Hyaloperonospora_parasitica/
Phytophthora capsici	65	12011	JGI, in progress with expected release during 2007
Phytophothora infestans	240	22658	Broad,http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html.
Phytophthora ramorum	65	15743	JGI,http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html.
Phytophthora sojae	95	19027	JGI,http://genome.jgi-sf.org/Physo1_1/Physo1_1.home.html.

P. insidiosum is a fungus–like organism that is evolutionarily related to algae which is the only one species that caused of the disease in mammals. Although less well known *P. insidiosum* is also responsible for serious human pythiosis outbreak in Thailand where it has been reported the most cases. Recently, natural occurrence of *P. insidiosum* in endemic aquatic environment from the northern part of Thailand has been evidently reported (143). In natural environments, *P. insidiosum* can be exposed to a wide range of temperatures, with daily and seasonal variations, so active growth may not always be possible. In some seasons water may be unavailable either as a result of drought, and at other times suitable substrates may be lacking. Hence a great root–rotting *Pythium* species is exacerbated when soil temperature rise to 35-40°C (144). Temperature is one of the physical parameters under constant vigilance in all living cells. Numerous processes are temperature-controlled in bacteria including the expression of heat and cold shock genes and of virulence genes to mention the most prominent examples (145). How do cells detect changes in temperature? Three different macromolecules have been identified acting as heat sensors: DNA, mRNA and proteins.

Pathogenic microorganisms are well adapted to their host ecological niche, but they must also cope with life outside the host. In the case of pathogens of human (and warm blooded animals) this means that movement occurs between a constant, thermally controlled environment of 37°C in the host and the external biosphere where the temperature fluctuates and is usually less than 37°C. Temperature influences primary and secondary metabolism in a variety of ways. At high temperatures an increase in nutritional requirements is sometimes observed. Thus *Saccharomyces*

cerevisiae requires a supply of pantothenic acid at 38°C but not at 30°C. Moreover, Cutler and Hahn (146) have identified the capacity for growth at 37°C as a minimum requirement for fungal pathogenicity against mammals. Previous studies have observed fast growth of *P. insidiosum* cultures incubated at 37°C is consistent with this organism's requirement for invasive growth mammalian body temperatures (Figure 13). That the phytopathogen *P. graminicola* has a temperature optimum above 35 °C is less intuitive (38-39).

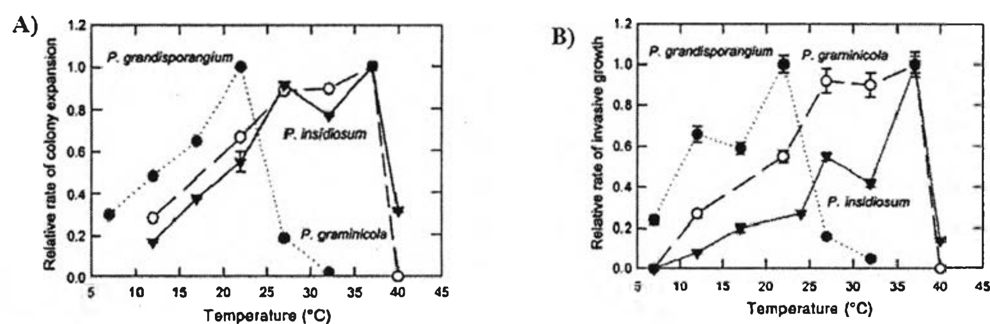

Figure 13. Relationship between incubation temperature and growth rate based on A) rate of colony expansion, and B) estimates of the volumetric expansion of hyphae. Mean rates $\pm$ S.E. are plotted relative to the maximum value for each species (39).

The important features are the ability to grow at physiological temperatures and pH values. Other features are: production of specific adhesions for attachment to host tissues, production of aggressive enzymes, mechanisms of evading host defense mechanisms and the transition from non–pathogenic saprobic form to pathogenic form. There have some problem about the different oomycete species have varying

levels of tractability and ease of use depending on the criteria examined (Table 4), and some species are particularly appropriate for certain types of analyses.

Table 4. The different oomycete species have varying levels of tractability and ease of use depending on the criteria examined (29).

Features	*H. parasitica*	*P. capsici*	*P. infestans*	*P. sojae*	*P. ramorum*
Culturing	Obligate parasite, can only be cultured on plants	Culturable, fast grower	Culturable, slow grower	Culturable, slow grower	Culturable, slow grower
Spore production	Easy but requires infected plants	Easy	Easy	Requires serial washes in buffer solutions	Not reported
Plant interaction assays, degree of difficulty	Easy, infects cotyledons and leaves	Easy, infects all plant tissues	Easy, infects leaves and potato tuber	Moderately difficult, requires root or hypocotyls	Moderately
Tractability of host plant	High, Arabidopsis	High, tomato and pepper	High, tomato and potato	Moderate, soybean	Low, woody plants
Genome complexity	Moderate	Moderate	High	Moderate to high	Moderate
DNA transformation	Currently not possible	Established through zoospore electroporation	Established through several methods	Established through several methods	Not reported but potentially feasible
Gene silencing, RNAi gene inactivation	Currently not possible	Not reported but potentially feasible	Established	Not reported but potentially feasible	Not reported

Table 4. The different oomycete species have varying levels of tractability and ease of use depending on the criteria examined (29) (cont.).

Features	*H. parasitica*	*P. capsici*	*P. infestans*	*P. sojae*	*P. ramorum*
Potential for genetic manipulation	Genetic crosses and gene mapping possible	High, highly fertile in a variety of crosses	Genetic crosses and gene mapping possible but difficult	Genetic crosses and gene mapping possible but difficult	Not possible
Genetic analysis by TILLING	Not possible	Established	Possible	Established	Not possible
Community size	Moderate	Small	Moderate	Small	Small

Advances in genetic transcription area have been made by studying fungal molecular biology, plant pathogenic oomycetes and the development of tools, coupled with the accumulation of sequence information, will undoubtedly also benefit research into the animal pathogenic *P. insidiosum* at the molecular level. The first look at the gene expression level of animal pathogenic *P. insidiosum* is performed by differential gene expression. A greater understanding of these central processes will also lead to novel control strategies against the problematic pathogenic *P. insidiosum* and might help us to exploit the pathogenesis properties of this microorganism.

10 Differentially Expressed Genes

The genome is made up of the entire DNA content of a cell. Eukaryotes and prokaryotes have quite different types of genome, but we must assume that the prokaryotic grade of organization is the primitive form from which the eukaryote

organization evolved. They have a great deal in common; the DNA of a gene is transcribed into RNA, which is called a messenger RNA (mRNA) if it is a transcript of a protein-coding gene. The mRNA is translated into protein by the ribosomes and other translation machinery, that part of a protein-coding gene sequence that is translated into protein is called the *open reading frame*, usually abbreviated to ORF. The ORF is characteristically read in the 5' to 3' direction along the mRNA, and it starts with an initiation codon and ends with a termination codon. Nucleotide sequences that occur in the mRNA before the ORF make up the leader sequence, and sequences following the ORF make up the trailer segment. Many eukaryotic genes are split into *exons* (meaningful segments) and *introns* (sequence segments that do not contribute to the protein-coding sequence). The introns are removed from the primary RNA transcript by the splicing machinery to form the functional mRNA.

Differential gene expression technologies have been applied to many biological problems. The primary considerations for selecting a technology for gene expression analysis are (i) the amount of starting material required, (ii) the sensitivity of the technique, (iii) coverage of the method, and (iv) additional follow–up costs which are often underestimated. The amount of biological starting material is primary concern because it can limit the applicability of the method. In addition, it is critical to assess the sensitivity of the method. It is estimated that 90 to 95 percent of all eukaryotic mRNA species are present at five or less copies per cell (147-148). However, these rare transcripts make up only 35 to 50 percent of the total cellular mRNA mass which is estimated to be only one to three percent of the amount of total RNA. The percentage of all possible mRNAs assayed by the selected technology (the coverage of

the method) is also important for success. In addition, the evaluation of an expression experiment requires many time-intensive investigations using additional technologies such as statistical analysis, management of huge amounts of data, and specific confirmatory follow-up investigations.

Identification and characterization of differentially expressed genes may be an important first step toward the understanding of organisms and to clarify hypothetical genes by computational annotation. Furthermore, they help to identify differences between closely related species as regards pathogenicity, secondary metabolism or other properties (149). A multitude of techniques belonging to two main categories have been developed to identify the differences in global techniques (Table 5). Whereas the selection techniques strive to identify specific differentially expressed genes, the global techniques analyze the total transcriptome or a major part of the RNA population in a defined biological material. By exploiting the known sequences of the adaptors used in suppressive subtraction hybridization techniques, a strategy named novel rescue – suppression – subtractive hybridization was developed.

Table 5. Comparison of advantage and disadvantage of gene expression profile.

Techniques	Advantage	Disadvantage
SSH	1. Relatively smaller quantities of samples 2. High efficacy, especially efficient for obtaining low bundance genes 3. High specificity	1. Generally, only two samples can be compared in one SSH 2. The results too depend on the efficacy of ligating adaptors
Microarray	1. Smallest quantities of samples 2. Highest efficacy 3. High specificity 4. More than two samples can be analyzed in one experiment	1. In general, it require some sequence information in advance 2. Low abundance genes are difficult to detect 3. High cost
RDA	1. High specificity and low percentage of false positive 2. cDNA RDA can be used in mRNA without poly A	1. Low percentage of obtaining the low abundance genes 2. Repeated procedures of enzyme but and ligation
DD PCR	1. Relatively smaller quantities of samples 2. High efficacy, and normally low abundance genes can also be obtained 3. More than two samples can be analyzed in one experiment	1. High percentage of false positive 2. The simplified products are usually in the 3'-terminal region
SH	1. Low cost 2. Relatively longer genes can be obtained in resultant library	1. Low sensitivity 2. Largest quantities of samples 3. Labor intensive

10.1 Suppression Subtractive Hybridization (SSH)

The normalization and subtraction are combined in a single step to equalize the abundance of cDNA within the target population and to exclude the sequences common to both populations (53). The dscDNAs representing transcripts from the driver and the tester populations are digested with a four – base pair restriction

enzyme. The tester cDNAs is then divided into two portions, each ligated to separate sets of adaptors. Subtractive hybridization is then performed in two steps. The first, an excess of driver is hybridized to each sample of tester molecules as well as dscDNA between single–stranded (ss) cDNA present only in the tester or in excess in the driver population. In this hybridization, the sscDNA tester fraction is normalized; meaning the concentration of high– and low– abundance sscDNAs become roughly equalized. This equalization is because the reannealing process is much faster for the more abundant molecules. Furthermore, ssDNAs representing differentially expressed mRNAs in the tester fraction are significantly enriched because common cDNAs form hybrids with the driver.

In the second hybridization step, the two separate hybridizations, containing tester with different adaptors, are mixed without denaturating, and freshly denaturated driver is added. This process increases the fraction of common tester–generated cDNAs hybridized to driver – generated cDNA molecules, and enriches the fraction of tester–unique dscDNA molecules flanked by different adaptors. The ends of the dscDNA molecules are then filled by DNA polymerase, producing different annealing sites on the differently expressed tester dscDNA molecules for primers in the following PCR step. Because of the suppression PCR effect, only dscDNA molecules with two different adaptors can be exponentially expressed dscDNA flanked by the same primer anneal to themselves, forming pan–like structures that prevents exponential amplification (150) (Figure 14).

In this way, cDNAs for differentially expressed genes of both high and low abundance are highly enriched. The SSH procedure also can be modified to identify

transcripts moderately (i.e. two–to–fourfold) regulated between the tester and driver populations (151). The main advantage with SSH is the ability to enrich both for differentially expressed transcripts with high and low expression levels. A drawback is the relatively high amounts of starting material needed; 2–4 μg of polyA+ RNA is normally suggested. Alternatively an amplification step can be incorporated, or total RNA may be used as starting material (152).

The application of SSH in microbiological research plays important roles in pathogenicity, fermentation for food or industry, and biogeochemical cycles. Having no prior requirement on sequence information, method SSH has become an important alternative of high throughput for studying the differences of gene expression in nonmodel microbiological research. Its application ranges from characteristic biologic behaviors such as sexual development (153) conidial formation and germination (154), the differences of morphology with varying physical function (155-156), to the molecular processes underlying disease development in important pathogens (157-159). To isolate genes with differential expression patterns between two samples, SSH is carried out firstly and the candidates are expected from the SSH – generated cDNA libraries. Then the analysis of Northern blot or qPRC has to be performed to exclude the false positive clones. Finally, the validation of the roles played by the differentially expressed genes depends on the functional experiments.

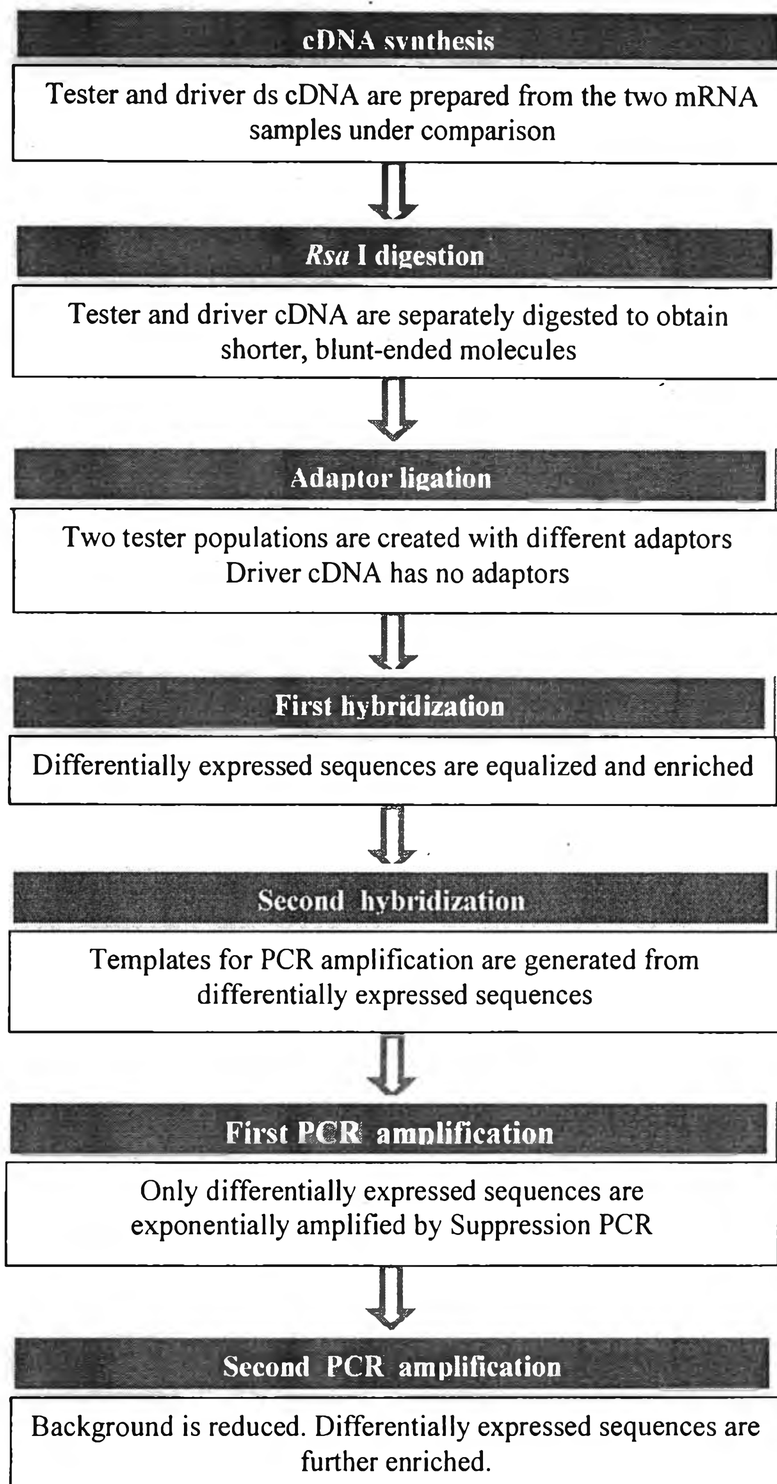

Figure 14. Overview of the PCR-Select procedure. The cDNA in which specific transcripts are to be found is referred to as **tester** and the reference cDNA is referred to as driver. If you have used the Super SMART PCR cDNA Synthesis Kit for cDNA synthesis you should begin with the Adaptor Ligation Step shown above.

To explore the genes involve in morphology determination, method SSH was employed before the genome of *A. niger* has been fully sequenced (160). This investigation aimed to identity the differentially expressed genes in *A. niger* associated with the switch from pelleted to filamentous growth, which accompanied a rapid decline in citric acid production. The screening of SSH-generated cDNA library and subsequent Northern blotting confirmed the different expression of 22 mRNAs from the estimated 14,000 total genes in *A. niger*. Fifteen genes were found highly expressed in the filamentous form, among which two were related to signal transduction pathways, six belonged to the group of amino acid synthesis and protein utilization, and there was no definite illustration to the functions of the remaining seven genes. Among the highly expressed genes in pallet cells, five of seven had no significant homology to the known sequences except that one was identical to the translated ubiquitin, and the other is homologous to an aspartic protease.

To validate the potential roles of a filament-associated gene Brsa-25 in controlling morphology and citric acid production (Brsa-25's identifier number from *A. niger* genomic sequence is XP001389968), a transformant with antisense expression was successfully constructed. The antisense strain showed an inhibition of filament formation and an increase in citrate production compared to its parent strain. These data from SSH suggested the possible mechanisms of morphogenesis and metabolite regulation, which might also be applied to construct the more powerful strains with genetic engineering.

Some modifications in the protocols are necessary to yield higher efficiency according to the characters in genomic content. In 1998, Akopyants *et al.* (161)

emphasized that three changes, while undesirable in eukaryote due to the existence of huge differences in mRNAs abundance, could improve performance and reliability in bacterial genome subtraction. First, DNA concentration can be reduced or polyethylene glycol (PEG), a hybridization enhancer used in cDNA subtraction for increasing the effective concentration of DNA in solution, can be omitted due to the low complexity of bacterial genomes. The absence of PEG also decreases the risk of fortuitous misannealing between different DNA molecules. Secondly, shortening the first hybridization time from 10 h to 1.5 h allows the increased amount of single stranded DNA that remains after the first subtractive hybridization and thereby makes the second hybridization more efficient. Lastly, a higher ratio of tester to driver DNA is needed in genomic SSH than that for eukaryotic mRNA. Furthermore, data from parallel experiments using different restriction enzymes in separate genomic SSH suggested a need to increase the coverage of positive clones significantly compared to using a single enzyme (162-163), while no available experimental evidences supported this viewpoint for the miscellaneousness in mRNA hybridization. When candidate DNA fragments are obtained, PCR amplification or Southern blot is required to confirm the results. Now it has been demonstrated that this modified SSH is effective for isolating the specific DNA region between different strains, biovars, serotypes, and species of bacteria based on the academic model and many experimental evidences.

One distinct purpose of using genomic SSH is to identify the different nucleotide sequences to serve as diagnostic markers for DNA-based detections (162-165). Some pathogenic bacteria can cause infectious disease outbreak, and thus rapid

and effective identification of putative pathogens is necessary to eliminate nonpathogenic but closely related organisms. Such identifications would be more efficient if unique fragments in pathogen are available for designing specific PCR primers. For example, *Salmonella enteric* var *enteritides* is a major cause of food-borne illness worldwide and a highly specific detection of this important pathogen requires the candidate DNA markers. Using *S. enterica* var *dublin* as driver, some different regions were subtracted in *S. enteric* var *enteritides* with genomic SSH, and these unique regions served as the pool for PCR primers. Among them, a pair of primers referred to as *Sdf* I (one region unique in *S. enterica* serovar *enteritides*) was found effective in distinguishing serovar *enteritides* from 73 non-enteritidis isolates comprising 34 different serovars with real-time PCR (162).

However, some DNA regions obtained by SSH have failed to be used as criterion for detection, although it is proved specific to the tester. DFR4, one region only present in *Yersinia pestis* biovar *Antiqua*, could be detected in only 57 of 60 *Antiqua* strains in natural plague foci, probably due to the sequence diversity or mutation (165). The characterization of DNA content specific to individual strains that confers special phenotypes is illuminating for correlation between phenotypic differences and gene differences in prokaryotic microorganisms, such as in pathogenic and nonpathogenic strains. Prokaryotic genome sequencing requires great effort and cost despite their relatively small genome. SSH, with no requirements on prior knowledge of genomic sequences, has become an increasingly important tool for comparative prokaryotic genomics. Pathogenicity islands (PAIs) are composed of the gene clusters closely related to pathogenesis and have drawn great interests from

researchers endeavoring on the mechanisms and controls of infectious diseases. In recent years, successful cases have been carried out in some important pathogens with SSH method, including *Helicobacter pylori* (161), *Salmonella typhimurium* (166), *Escherichia coli* strain 536 (167) and *Burkholderia mallei* (168), in which novel PAIs with putative function of fimbrial biosynthesis, capsule export or translocation, DNA restrictive-modification enzymes and metabolic enzymes were isolated. These investigations will undoubtedly give some new and valuable implications on the pathogenesis or even the strategy of treatments.

Method SSH can also be applied to archea, the most important inhabitants in the extreme environment, to identify a biotic stress-regulated gene transcripts of plants (169-171), in plants for the identification of disease-related (157), developmentally associated (172), tissue-specific (173), and other differentially expressed genes (171, 174-175). More recently, SSH-based methods have been employed with algae (176-177).

10.2 Real – Time Polymerase chain reaction (RT – PCR)

The procedure for performing the polymerase chain reaction (PCR) was first introduced by Kerry Mullis (178) for which he won the Nobel Prize in 1993. It is hard to think of another laboratory technique that has had a greater impact on so many different facets of biological research than PCR. In combining the reverse transcriptase (RT) reaction with the PCR, identification of a specific RNA transcript was now possible from very low copy numbers of starting material. Quantification of transcripts from sample unknowns became possible with the advent of competitive RT-PCR (179).

In this method, a truncated version of the target region of interest lies between the same primer-binding sites as the target transcript sequence within a plasmid clone. The easiest method for making a smaller competitive target was to digest the cloned region between the primer binding sites with a restriction enzyme and then ligate the resulting sticky ends, dropping out a short section of sequence. The requirements for the quantification construct were that it be a similar, but different, size than the target PCR product and quantified. The plasmid contains a T7, SP6 or T3 RNA promoter sequence up stream of the cloned target sequence. Utilizing the RNA promoter, truncated *in vitro* transcribed RNA could be made and quantified. Known amounts of the RNA product were spiked into the RT reaction and converted into cDNA along with the target sequence within the unknown sample. Subsequently, both the truncated standard and unknown target sequences were amplified using the PCR. The amplified DNAs were separated using denaturing polyacrylamide gel electrophoresis. In some methods a radioactive deoxynucleotide base was added for labeling the amplified DNA and quantified using either film or a phosphoimager. In other methods, the products on the gel were imaged following staining with ethidium bromide or SYBR® Green I. Quantification of the unknown target band was determined by comparison to signal from the spiked and quantified DNA standard. Although this method was the most accurate to date it still suffered from the detection problems mentioned earlier. However, most of the criticism centered around the spiked DNA standard. The concern was that the DNA standard was competing for reagent resources and primers with the unknown target during the PCR and might, therefore, alter the final result.

Since 1996, Applied Biosystems (ABI) made real – time PCR commercially available (180) with the introduction of the 7700 instrument. Real – time PCR (RT – PCR) has become the most accurate and sensitive method for the detection and quantification of nucleic acid yet devised. Quantitative RT-PCR is currently the "gold standard" for mRNA analysis, offering the best sensitivity, dynamic range, and reproducibility of any standard technique (181). In qRT-PCR, mRNA transcripts are first reverse transcribed into cDNA using oligo(dT), random oligomer, or gene-specific primers; the cDNAs of interest are then exponentially amplified by PCR using gene-specific primers. The concentration of amplicon in the reaction is monitored with fluorophore-conjugated hybridization probes or DNA-intercalating dyes. Template quantification is based on the number of PCR cycles required for fluorescence to reach an arbitrary threshold. Low-order multiplexing (2–5 targets) is feasible using multiple primer pairs and different-colored probes, but tends to be problematic due to formation of "primer dimer" side products and competition between assay targets (182-183). Moreover, since very small amounts of template are required, evaluation of multiple targets can usually be achieved more easily by aliquoting sample to parallel simplex qPCR reactions.

In principle, qRT-PCR can detect a single molecule of messenger RNA. With efficient reverse transcription, detection of one mRNA copy using standard qPCR dye chemistries requires about forty cycles of PCR; a typical housekeeping transcript in a single cell might be detected after around thirty cycles of amplification. However, the RNA input for gene profiling experiments is routinely scaled to support detection after around 15–25 PCR cycles, as this is considered the most trustworthy

regime for quantification. qRT-PCR-based expression profiling is therefore usually performed on homogenized samples derived from hundreds or thousands of cells. While conventional protocols have occasionally been applied to quantitate mRNA transcripts in single-cell lysates, such assays have been limited to the evaluation of a few, relatively highly expressed genes. While PCR has long set the standard for sensitivity in nucleic acid detection, the technique has only recently become highly quantitative, due to the development and commercialization of qPCR (or "real-time PCR"). In the PCR-based single-cell studies published in the nineties, amplified cDNA was evaluated on a gel at the PCR end point using ethidium bromide staining or autoradiography. PCR end-point analysis usually gives only a "positive/negative" call or, at best, a semi - quantitative readout of template abundance.

All the real-time instruments on the market today are based on the detection of a fluorescent signal. The increase in fluorescence is directly proportional to the increase in the amplified product during the PCR. Fluorescent molecules absorb light as photons within a narrow wavelength range of light. The wavelength at which the dye absorbs light maximally is called the excitation wavelength for that molecule. Following excitation, the molecule is pushed to a higher energy state. This higher energy state is transient and short lived. The excited molecule rapidly decays, falling back to the ground energy state. When this occurs a photon of light is emitted at a longer wavelength. The light that is released is at the emission wavelength. This shift between the excitation and emission wavelengths is called a Stoke's shift (184). For every fluorescent dye, there is an optimal excitation and emission wavelength. A fluorescent molecule can be excited or detected in a narrow range of wavelengths

around these optima. Fluorescent molecules with the greatest Stoke's shift are the most desirable as they allow the cleanest separation of the excitation from the emitted wavelengths of light.

All fluorescent assays used for real-time PCR achieve this delta by utilizing FRET (fluorescence resonance energy transfer) (185). FRET requires two molecules that can interact with one another, at least one of which must be capable of fluorescence. The fluorescent component is called the donor and the second molecule is called the acceptor. Some of the most common donor and acceptor (reporter and quencher) dyes currently used in real-time PCR are listed in Table 6.

10.2.1 SYBR® Green chemistry

SYBR® Green is a fluorescent dye that binds only to double-stranded DNA. Fluorescence is emitted proportionally to the amount of double-stranded DNA. In a PCR reaction, the input DNA or cDNA is minimal and, therefore, the only double stranded DNA present in sufficient amounts to be detected is the PCR product itself. As for other real-time PCR chemistries, read-outs are given as the number of PCR cycles ('cycle threshold' C_T) necessary to achieve a given level of fluorescence. During the initial PCR cycles, the fluorescence signal emitted by SYBR® Green I bound to the PCR products is usually too weak to register above background. During the exponential phase of the PCR, the fluorescence doubles at each cycle. A precise fluorescence doubling at each cycle is an important indicator of a well optimized assay. After 30 to 35 cycles, the intensity of the fluorescent signal usually begins to plateau, indicating that the PCR has reached saturation. As C_T correlates to the initial amount of target in a sample, the relative concentration

of one target with respect to another is reflected in the difference in cycle number (δC_T = C_T sample – C_T reference) necessary to achieve the same level of fluorescence.

10.2.2 Melting curve analysis

Heat dissociation (PCR product melting curve) analysis at the end of the PCR confirms whether or not a single product is amplified and that no dimers interfere with the reaction. A melting curve analysis begins with heating the PCR product at the end of the PCR reaction. As the PCR product melts and the SYBR® Green is released into the solution, its fluorescence intensity decreases. A negative first derivation curve of the fluorescence intensity curve over temperature produced by the instrument's software clearly indicates the Tm of the PCR product (peak of the –dF/dT curve) and should be quite close to the predicted Tm of the PCR product. In the example illustrated in Figure 15, the PCR product Tm is 87.5°C (curves indicated by +). Complete absence of primer-dimer is rarely achieved in the PCR negative control (curve indicated by –).

Table 6. Dyes available for use in Real - time PCR

Free dyes	Max. ab (nM)	Max. em (nM)
SYBR® Green I	497	525
EvaGreen™	497	525
BOXTO™	515	552
Reporter dyes	**Max. ab (nM)**	**Max. em (nM)**
Pulsar® 650	460	650
Fluorescein™	492	520
6-FAM	494	518
Alexa 488™	495	519
JOE™	520	548
TET™	521	536
Cal Fluor Gold 540™	522	544
Yakima Yellow™	530	549
HEX™	535	546
Cal Fluor Orange 560™	538	559
VIC™	538	554
QUASAR® 570	548	566
Cy3™	552	570
TAMRA™	565	580
Cal Fluor Red 590™	569	591
Redmond Red™	579	595
ROX™	580	605
Cal Fluor Red 635™	618	637
LightCycler® 640	625	640
Cy5™	643	667
QUASAR® 670	647	667
LightCycler® 705	685	705

Table 6. Dyes available for use in Real - time PCR (cont.)

Dark dyes	Max. ab (nM)	Max. em (nM)
DABCYL	453	None
BHQ0TH	495	None
Eclipse^{TM}	522	None
Iowa Black^{TM} FQ	531	None
BHQ1^{TM}	534	None
BHQ2^{TM}	579	None
Iowa Black^{TM} RQ	656	None
BHQ3^{TM}	680	None

As seen in this example, 1 out of 3 negative triplicates shows dimers (with a Tm of 78.5°C). The two sets of curves are usually clearly separated with a 10°C shift between Tm of primer-dimers and the specific PCR product. In an experimental negative including a template but no target, dimmers are not usually observed. The degree of an eventual primer-dimer contribution to the overall fluorescent signal of the PCR negative control can also be detected in such a dissociation analysis.

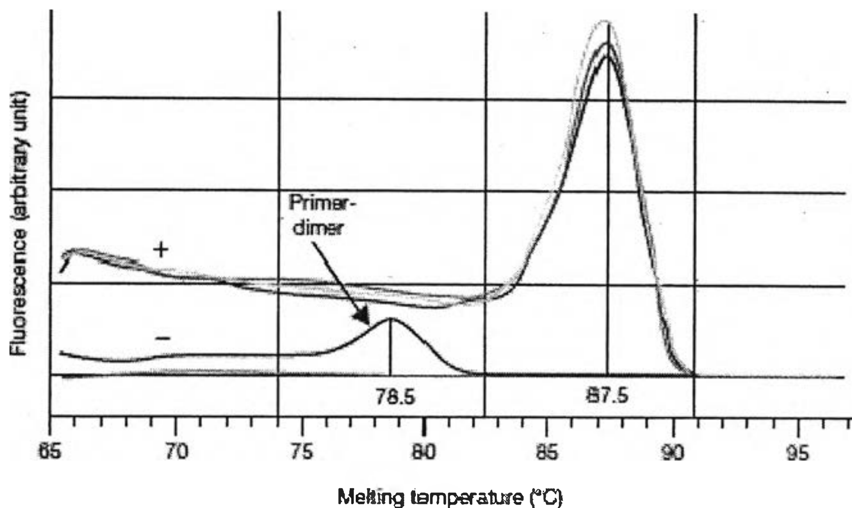

Figure 15. An illustration of the use of melting curve analysis to distinguish between specific and non – specific PCR products (186).

To understand how *P. insidiosum* has got where it has in the world and how it is able to influence mammals, it is necessary to know something about how they grow and show high ability of infection. In the pathogenic bacteria, there are special genes which promote the initial entry and permit survival of bacteria within the host, as well as genes which result in production of disease and are defined as virulent determinants (187). Such one as, the ability of a fungus to grow at host's temperature is a virulence factor for fungi that invade deep tissues and the transition to a parasitic form is essential for the pathogenicity of the dimorphic fungi (37). Up to recent time, there are five plant pathogenic oomycetes genome projects have been reported whereas there is little information available for *P. insidiosum.* In order to obtain the temperature sensitive genes, a SSH cDNA library of *P. insidiosum* at 37°C has been constructed to be used as a gene source.