The role of neutrophils against *Pythium insidiosum*

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พิเธียม อินซิดิโอซุ่ม เป็นจุลชีพที่มีลักษณะคล้ายคลึงกับเชื้อราแต่ถูกจัดให้อยู่ในไฟลัมโอโอไมโคตา (oomycota) ก่อให้เกิดโรคพิธิโอซิสในสัตว์เลี้ยงลูกด้วยนม ได้แก่ สัตว์ในปศุสัตว์ สุนัข แมว และมนุษย์ ประเทศไทยพบอุบัติการณ์การติดเชื้อและมีอัตราการเสียชีวิตมากที่สุด โดยพบว่ามีความสัมพันธ์กับผู้ป่วยที่มีโรคประจำตัวเกี่ยวกับความผิดปกติของเม็ดเลือดแดง สาเหตุของการติดเชื้อ คือผู้ป่วยได้รับซูโอสปอร์ ซึ่งเป็นระยะติดต่อที่เชื้อสร้างขึ้นอยู่บริเวณพื้นที่ชื้นแฉะหรือแหล่งน้ำสะอาดตามธรรมชาติ เนื่องจากองค์ความรู้เกี่ยวกับพยาธิสภาพของโรคพิธิโอซิสยังมีการศึกษาไม่มากเท่าที่ควร ประกอบกับเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลซึ่งมีบทบาทสำคัญในการตอบสนองต่อการติดเชื้อรา การศึกษานี้จึงมุ่งเน้นศึกษาเกี่ยวกับความสามารถของนิวโทรฟิลในการทำลายเชื้อพิเธียม อินซิดิโอซุ่ม ได้แก่ ความสามารถในการฆ่าเชื้อ กระบวนการจับกินเชื้อเพื่อนำเข้าไปทำลายภายในเซลล์ และปล่อยเส้นใยดีเอ็นเอเพื่อจับและทำลายตัวเชื้อ นำเชื้อพิเธียม อินซิดิโอซุ่มจำนวน 6 สายพันธุ์ มากระตุ้นให้เกิดการสร้างซูโอสปอร์ เพื่อนำมาทดสอบกับนิวโทรฟิลที่แยกได้จากอาสาสมัครสุขภาพดีจำนวน 6 คน ผลการศึกษาพบว่านิวโทรฟิลสามารถลดจำนวนเชื้อลงได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับสภาวะที่ไม่มีนิวโทรฟิล ($p < 0.001$) โดยวัดจากปริมาณเชื้อที่เจริญอยู่บนอาหารเลี้ยงเชื้อชนิด blood agar เปรียบเทียบกับซูโอสปอร์ที่ถูกย้อมสีเพื่อดูเซลล์เป็นหรือตายด้วยกล้องจุลทรรศน์ ต่อมาศึกษากระบวนการจับกินเชื้อโดยใช้หลักการโฟลว์ไซโตเมทรี พบว่านิวโทรฟิลสามารถจับกินซูโอสปอร์ได้อย่างมีนัยสำคัญทางสถิติเพียงแค่ 2 สายพันธุ์เท่านั้น ($p < 0.01$) แต่การศึกษาสุดท้ายกลับพบว่าซูโอสปอร์จำนวนทั้งหมด 6 สายพันธุ์สามารถกระตุ้นให้นิวโทรฟิลปล่อยเส้นใยดีเอ็นเอได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับสภาวะที่นิวโทรฟิลไม่ถูกกระตุ้น ($p < 0.001$) โดยประเมินจากการย้อมด้วยสารเรืองแสงเพื่อดูใต้กล้องฟลูออเรสเซนต์และวัดปริมาณดีเอ็นเอที่ถูกปล่อยออกมา จากผลการศึกษาครั้งนี้ถือเป็นข้อมูลใหม่ที่สามารถบ่งชี้ให้เห็นว่านิวโทรฟิลที่ได้จากอาสาสมัครสุขภาพดีนั้นปล่อยเส้นใยดีเอ็นเอออกมาเพื่อตอบสนองต่อการติดเชื้อพิเธียม อินซิดิโอซุ่ม

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Pythium insidiosum (*P. insidiosum*) is an oomycetes fungus-like microorganism causing pythiosis infection in humans. The zoospores (infective stage) of this pathogen contaminate aquatic environments such as moist soil and swampy area, which is a natural habitiat. Humans are infected with *P. insidiosum* via cutaneous route which is the main entry of the zoospores. Recent studies have shown that patients with pythiosis shows high morbidity and mortality rates particularly those with hematologic diseases. As neutrophils are the first line of immune defense during fungal infections, this study therefore investigated the activity of neutrophils in response to *P. insidiosum*. The neutrophil killing activity, phagocytosis, and neutrophil extracellular trap (NET) formation were determined. Zoospores from six different strains of *P. insidiosum* were randomly selected and incubated with isolated healthy neutrophils (n=6). The results showed that human neutrophils significantly decreased number of zoospores as observed in both colony counts on blood agar and live/dead cell staining ($p < 0.001$). Phagocytosis measured by flow cytometry showed only two strains of zoospores (pHrodo-labeled) were significantly phagocytosed by neutrophils ($p < 0.01$). In contrast, all six strains of heat-killed zoospores significantly induced NETs ($p < 0.001$) detected by immunofluorescence staining and picogreen assay. Our findings suggests that human neutrophils produce NET formation as a main mechanism rather than phagocytosis in response to *P. insidiosum*.

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Apichaya Sriwarom

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LIST OF ABBREVIATIONS

A. fumigatus	*Aspergillus fumigatus*
APC	Allophycocyanin channel
APCs	Antigen-presenting cells
ATCC	American type culture collection
BA	Blood agar
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
C. albicans	*Candida albicans*
CD	Cluster of differentiation
CHS	Chitin synthase gene
CLRs	C-type lectin receptors
CMA	Corn meal agar
COX2	Cytochrome C oxidase II gene
CRs	Complement receptors
CTL	Cytotoxic T lymphocytes
DAPI	4',6-Diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
etc.	et cetera
EVs	ELISA values
*exo*1	*exo-1,3-β-glucanase* gene
FACs	Flow cytometry staining buffer
FBS	Fetal bovine serum
FMLP-R	Formyl-methionyl-leucyl-phenylalanine receptor
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GMPs	Granulocyte-monocyte progenitors
H_2O_2	Hydrogen peroxide
HSC	Hematopoietic stem cells
IFN-γ	Interferon-gamma

IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGS	Intergenic spacer region
IL-10	Interleukin 10
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
ITS	Internal transcribed spacer region
KOH	Potassium hydroxide
LAD	Leukocyte adhesion deficiency
LCB	Lactophenol cotton blue
LMPPs	Lymphoid-primed multipotent progenitors
LPS	Lipopolysaccharide
MMPs	Matrix metalloproteinase
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MPP	Multipotent progenitor cells
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase system
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NO	Nitric oxide
NOD-like receptor	Nucleotide-binding oligomerization domain-like receptors
NOX2	NADPH oxidase 2
P. insidiosum	*Pythium insidiosum*
P. verrucosa	*Phialophora verrucosa*
PAD4	Peptidyl arginase deaminase 4
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral mononuclear cells
PBS	Phosphate-buffered saline

PDA	Potato dextrose agar
PE	phycoerythrin channel
PI3K	Phosphatidylinositol 3-kinases pathway
PIA	*Pythium insidiosum* antigen
Pi-Ab	anti-*P. insidiosum* antibodies
PMA	phorbol-12-myristate 13-acetate
PMN	Polymorphonuclear cells
PNH	Paroxysmal nocturnal hemoglobinuria
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SSC	Side scatter
SYK	Spleen tyrosine kinase pathway
TBS	Tris-buffered saline
Th0	naïve T helper cells
Th1	T helper 1 cells
Th2	T helper 2 cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TPK	Therapeutic penetrating keratoplasty

CHAPTER I INTRODUCTION

Pythium insidiosum (*P. insidiosum*) is a member of oomycetes. *P. insidiosum* is a fungus-like pathogen found in moist soil and swampy areas [1, 2] in tropical and subtropical climate zones [3, 4]. The typical characteristic is similar to true fungi, including cell wall structure, life cycle, hypha development, etc. As the molecular analysis has classified in the kingdom stramenopila, *P. insidiosum* is therefore related to diatoms and brown algae [5, 6]. This organism is a significant pathogen in mammals, especially cattle, dogs, cats, and humans [7-9]. The infectious agent is an asexual reproductive stage, zoospores, that can invade injured skin and soft tissue, causing pythiosis [10]. After the adhesion in host tissue, the germinated hyphae from encysted zoospore will trigger the host immune response within a few hours [11-13]. Four forms of clinical manifestation in human pythiosis include vascular, cutaneous/subcutaneous, ocular, and disseminated types [8, 14, 15]. Without early and efficient treatments, patients always suffer from the infection and become a chronic/sub-chronic disease with a high mortality rate or loss their sight [16]. Most of the underlying disorders in vascular pythiosis involve hematological diseases, including thalassemia and paroxysmal nocturnal hemoglobinuria (PNH) [17, 18].

The equine model revealed the study of the immunological response of pythiosis. It demonstrated that after the adherence of the infectious encysted zoospore, geminated hyphae invade the host. These hyphae activate the T helper 2 (Th2) response, affecting interleukin-4 (IL-4) and IL-5 cytokines release, resulting in hypersensitivity and tissue damage, indicating a poor prognosis. When the infected horses receive *Pythium insidiosum* antigen (PIA) as immunotherapy, the immunogens drive the Th1 response, interferon-gamma (IFN-γ), and IL-2 stimulate the cell-mediated immune response. Then pathogens are eliminated finally in non-chronic infected equines [13].

Our previous investigation demonstrated the increasing levels of IFN-γ, IL-10, and IL-17 in 50 Thai pythiosis patients after receiving the PIA immunotherapy (PIAI). Interestingly, IL-17 is the critical cytokine that can activate neutrophil accumulation at the infection site, as found in this experiment [19]. Moreover, the rabbit model showed moderate neutrophilia when

injection of zoospores via subcutaneous and intraperitoneal routes was administered [20]. We hypothesize that neutrophils are associated with the pathogenesis of pythiosis.

At present, the immune response against *P. insidiosum* infection remains poorly understood. Neutrophils are the most abundant polymorphonuclear leukocytes in human circulation [21], which are one of the innate immune cells that play essential roles during the first step of the infection [19]. Evidence has shown that neutrophils are the first cells that migrate through the site of infection and eliminate the various type of pathogens [22, 23]. The functions of neutrophils include phagocytosis, degranulation, reactive oxygen species (ROS) production, and neutrophil extracellular trap (NET) formation [24, 25], which can play vital roles in the clearance of various fungi, including *Candida albicans* (*C. albicans*) [26, 27], *Cryptococcus neoformans* [28], *Paracoccidioides brasiliensis* [29], *Phialophora verrucosa* (*P. verrucosa*) [30], *Aspergillus fumigatus* (*A. fumigatus*) [31]. Since no data are currently available demonstrating the neutrophil functions during pythiosis infection, this study investigates the *in vitro* killing ability and mechanisms of neutrophils in response to *P. insidiosum*.

CHAPTER II OBJECTIVES

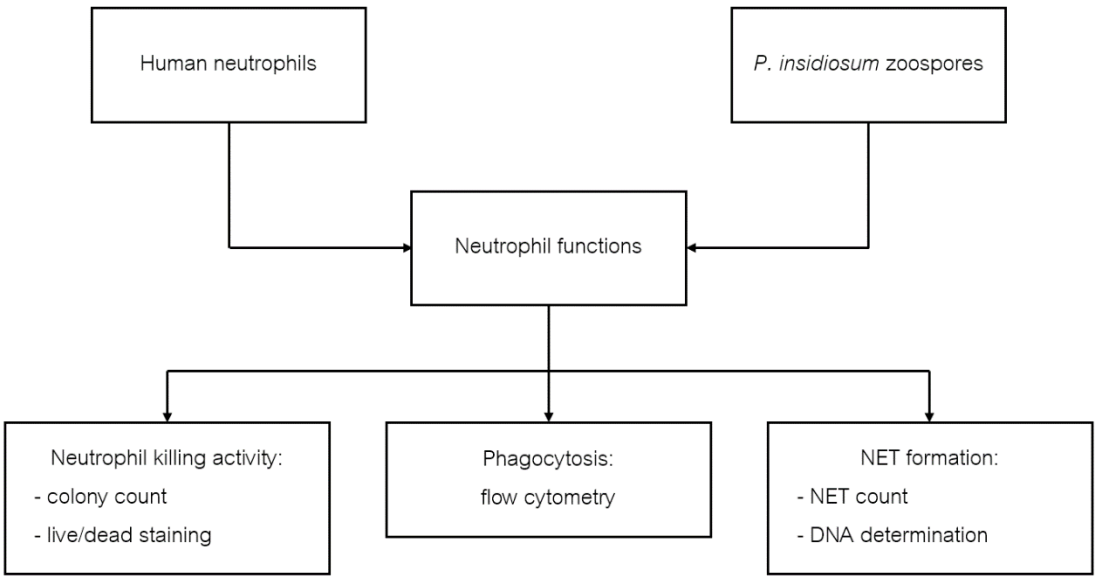
Hypothesis

Neutrophils can eliminate *Pythium insidiosum* zoospores by phagocytosis and NET formation.

Objective

To investigate the neutrophil functions against zoospores of *P. insidiosum.*

Research plan



CHAPTER III LITERATURE REVIEWS

Background of *Pythium insidiosum*

The oomycetes are a group of eukaryotes classified in the phylum Oomycota, which includes ecologically significant plant and animal pathogens [32]. These organisms are found in wet environments, including moist soil, aquatic reservoirs, or swampy agricultural areas [1]. They have similar characteristics to true fungi, including cell wall synthesis, nutrient acquisition, life cycle, and filamentous production [2, 33]. Thus, “water mold” has been commonly known. The molecular phylogeny showed an evolution of oomycetes related to diatoms and brown algae in the kingdom stramenopila [5, 6]. The comparison of typical features between oomycetes and true fungi is shown in Table 1. The taxonomic arrangement is classified into two large groups because an oomycete is highly diverse in morphological characteristics. The first group is mainly saprotrophic, composed of Eurychasmales, Leptomitales, and Saprolegniales. Another group is the plant and animal pathogens, composed of Rhipidiales, Pythiales, and Peronosporales [34]. The order Pythiales includes *Pythium* and *Pilasporangium* genus are the important pathogens in several hosts, animals, plants, fungi, and algae [35, 36].

Pythium genus is a common pathogen that can cause disease in plants. They are therefore known as plant pathogens. Current studies have shown that *Pythium insidiosum* (*P. insidiosum*) can cause infection in mammals, especially horses, dogs, cats, and humans, called “pythiosis”. This infection occurs when the host contacts zoospores in environments, the infected form of *P. insidiosum*, via a wound in the appendages (leg and hand), eye, and soft tissues [7]. Pythiosis is a non-transmissible and emerging disease that has been reported in the wetland of tropical, subtropical, and temperate areas, including Australia, Japan, India, the USA, Brazil, South East Asia, etc. [3, 4].

In 1884, the infection was first reported in the cutaneous granulomatous lesion in horses by a British veterinarian who worked in India. However, the pathogen could not be isolated [37]. The infection was subsequently named “Hyphomycosis destruens” in 1901 and modified to “Hyphomycosis destruens equi” in 1902 by De Haan and Hoogkamer [38]. Until 1961, the causative agent was isolated from horses and named *Hyphomycosis destruens* (*H.*

destruens), derived from Hyphomycosis destruens infection [39]. In 1974, the zoospore production in an aqueous medium was observed by Austwick and Copland. They proposed that *H. destruens* was related to an oomycete genus *Pythium* [1]. The infection has been known under various other names: “Bursattee” or “Bursate” which means rainy season derived from the Indian word, “Espundia” in Latin America, “Equine phycomycosis” in Australia and USA, “granular dermatitis” in Japan, “Hyphomycosis destruens equi” in Indonesia, “Leeches” in the USA, “Summer sore” in Australia, Latin America and USA, and “Swamp cancer” in Australia and USA [40-42]. Until 1980, The term “pythiosis” was proposed by Chandler *et al*. [43].

In 1985, human pythiosis was first described in two thalassemic patients with cutaneous infection at Siriraj Hospital, Thailand [44]. The second case of five systemic infections was reported from Ramathibodi Hospital, Thailand, from 1987 to 1988 [45]. After that, sporadic human infection has been described in many countries, including Australia, Brazil, New Zealand, and the USA [46, 47].

Table 1 The typical features between oomycetes and true fungi.

(Modified from Jamie McGowan and David A. Fitzpatrick, 2020 [48].)

Features	Oomycetes	True fungi
Kingdom	Straminipila	Fungi (Eumycota)
Neighboring taxonomic groups	Diatoms and brown algae	Animals
Size of genome	50 – 250 Mb	10 – 40 Mb
Cell wall	Cellulose and β-glucans (with little chitin)	Chitin and β-glucans
Cell membrane	Incomplete ergosterol synthesis pathway	Complete ergosterol synthesis pathway
Hyphal structure	Multinucleated in non-septate hyphae	Uni-nucleated/multinucleated compartment in non-septate/septate hyphae
Sexual reproductive structure	Oospore	Zygospore, Ascospore and Basidiospore
Asexual reproductive structure	Zoospore	Conidia/spore
Pigmentation	Unpigmented	Commonly pigmented

Biology of *P. insidiosum*

1. Cell structure

The cell wall is an external barrier surrounding the fungal and oomycete cells with many vital functions, including protecting and maintaining the cell. Moreover, the structure of cell walls in pathogenic fungi and oomycete contains many biological molecules in host recognition, adhesion, and colonization [49]. In true-fungi, cell wall components are composed of β-glucan (β-1,3-D-glucan and β-1,6-D-glucan) synthesized by a glucan synthase complex, the target of various immune cells and antifungal drugs [50]. Minor components are chitin (β-1,4-N-acetylglucosamine polymer) and mannan (also called phosphopeptidomannan) [51]. Oomycetes' cell walls are composed of β-glucan and cellulose with little chitin (Table 1) [52]. Cellulose is a polymer of β-1,4-linked glucosyl residues and is considered to have a similar function as chitin in fungi [53]. In filamentous fungi, chitin is a significant fraction of the cell wall responsible for the rigidity of this structure, synthesized by the chitin synthase (CHS) gene [54]. Previous studies have shown that no chitin was present in *P. insidiosum* cell walls. In 2020, Kammarnjassadakul P *et al*. reported the expression of the *CSH2* gene in *P. insidiosum,* which is probably related to chitin synthesis in the cell wall of *P. insidiosum* [55].

Sterols are the essential lipid compounds in the eukaryotes cell membrane that display many roles in biological processes, including selection and permeabilization, signal transduction, cytoskeleton reorganization, etc. [56]. Sterols are diverse in different organisms; for example, cholesterol is the principal sterol found in animals, whereas fungi contain ergosterol in their membrane [57]. Sterol synthesis is a significant target of several antifungal drugs, which inhibit enzymes in the sterol biosynthesis pathway. The cell membrane structure of *P. insidiosum* and some oomycetes lack ergosterol [58]. In general, there are 14 essential enzymes in the sterol biosynthesis pathway, which include ERG20, ERG9, ERG1 (Terbinafine targeted enzyme), ERG7, ERG11 (Itraconazole targeted enzyme), ERG24, ERG25, ERG26, ERG27, ERG6, ERG2, ERG3, ERG5, and ERG4, resulting in ergosterol end products. At the same time, *P. insidiosum* consists of only six enzymes leading to a failure in the production of ergosterol in their cell membrane (Figure 1) [59]. The incomplete ergosterol biosynthesis pathway affects the efficacy of some antifungal drug treatments in pythiosis.

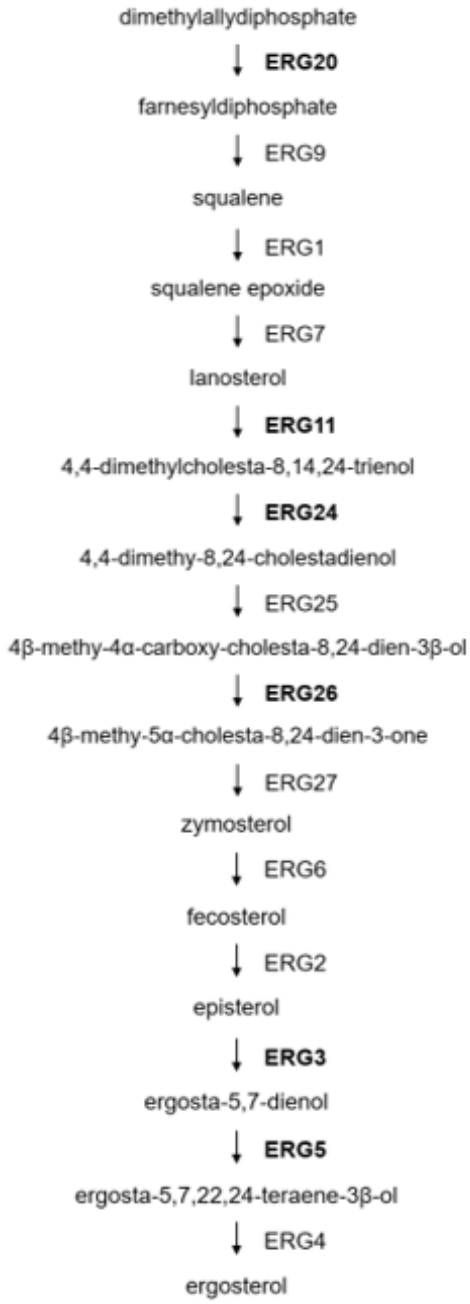


Figure 1 Diagram shows enzymes and substrates in the sterol biosynthesis pathway

Bold texts represent *P. insidiosum* contained enzymes: ERG3, ERG5, ERG11, ERG20, ERG 24, and ERG 26, resulting in incomplete ergosterol production. (Modified from T. Lerksuthirat *et al*., 2017) [59]

2. Morphology

P. insidiosum develops mycelia-like fungal growth, which can be rapidly observed within 1-2 days on a culture medium. The common mediums used for the cultures in mycology laboratories include blood agar (BA), sabouraud dextrose agar (SDA), sabouraud dextrose broth (SDB), corn meal agar (CMA), and potato dextrose agar (PDA). The macroscopic morphology on the solid medium showed submerged hyphae, colorless to white colonies (Figures 2A and 2B). The colorless short mycelium with an irregular radiate pattern is shown in the liquid medium (Figure 2C) [10]. *P. insidiosum* has an optimum temperature for growth at 34-36ºC, which relates to the susceptible host (mammals) body temperature [60].

Microscopic examination in 10% potassium hydroxide (KOH) preparation and lactophenol cotton blue (LCB) wet mount showed the sparsely septate hyphae size between 4-12 μm in diameter with 90° perpendicular lateral branches (Figure 3A and 3B) [8]. The biflagellate zoospores of size between 8-12 μ m are generated in a water environment and then release their flagella to become the encysted form (Figure 3C), which is an asexual reproductive stage structure produced from zoosporangia (Figure 3D) [11].

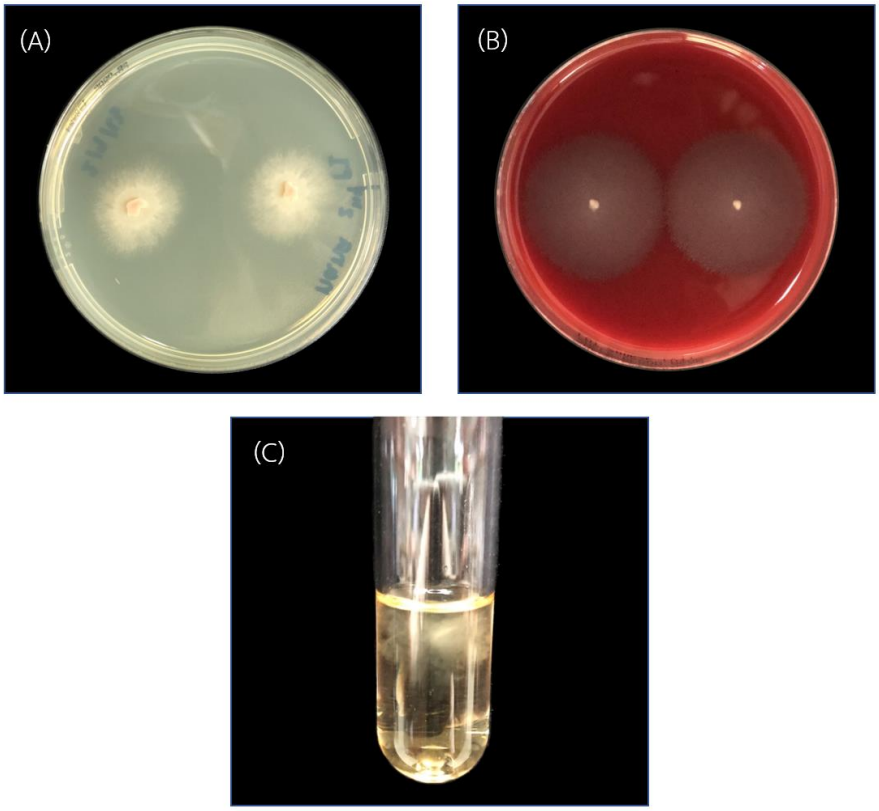


Figure 2 Macroscopic morphology of *P. insidiosum*

Representative pictures show the colorless colonies with submerged hyphae on sabouraud dextrose agar (A) and blood agar (B) and an irregular radiate pattern mycelium in sabouraud dextrose broth (C), incubated at 37ºC for 48 h.

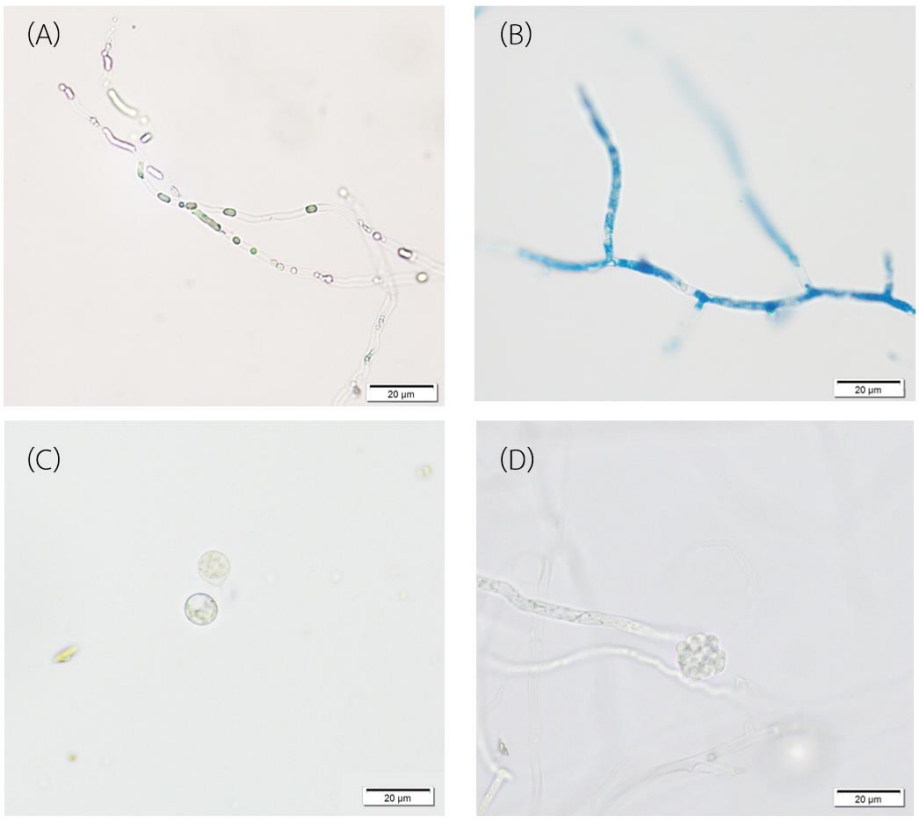


Figure 3 Microscopic characteristics of *P. insidiosum*

Representative pictures show sparsely septate hyphae in 10% KOH preparation (A) and LCB wet mount (B). After being induced with the induction medium, the encysted zoospores (C) and zoosporangium (D) were observed under the light microscope, 400x magnification.

3. Life cycle of *P. insidiosum*

The reproductive stage of *P. insidiosum* includes the sexual and asexual stages dependent on environmental conditions, like other oomycetes (Figure 4).

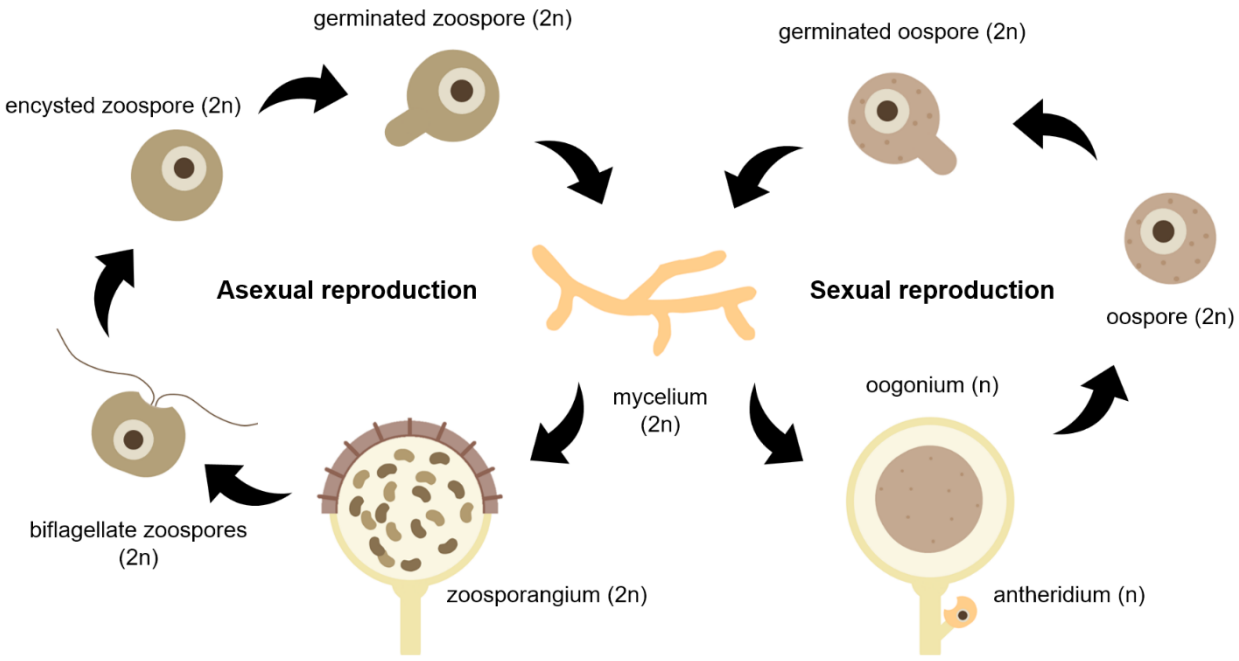


Figure 4 The Life cycle of typical oomycetes, including *P. insidiosum*.

Mycelia generate Oogonium and zoosporangium. The right part represents a sexual reproductive stage: fertilization occurs when the male antheridia transfer nuclei to the female oogonia. The oogoniums develop a thick wall, becoming mature oospores that can be resistant to various environmental conditions. The left part shows an asexual reproductive stage: the differentiation of zoosporangium into the mature stage leads to zoospores release. The biflagellate zoospores swim and lose their flagella when adhered to the host tissue, becoming the encysted form, germinated form, and hyphal form or mycelium, respectively [10]. (Modified from https://istudy.pk/oomycota/).

3.1 Sexual reproduction

Sexual reproduction of oomycetes occurs among male and female reproductive cells called gametangia. The large, round thin-walled structure contains one to several egg-like organelles known as female oogonia and smaller male reproductive cells known as antheridia (Figure 5). The general reproduction in fungi and oomycetes occurs when two gametangia come in contact, and nuclei transfer from the male antheridia to the female oogonia via a fertilization tube [61]. Oomycetes display in both heterothallic and homothallic reproduction. Heterothallic species require two different types of gametangia that develop from separate compartments, whereas homothallic species fertilize by self-originated gametangia. All species of *Pythium* are homothallic [62]. After fertilization, the oogonial cytoplasm becomes rich in lipid, protein, and β-linked glucose polymers. Then, thick-walled layers develop in mature oospores, which are essential for protection against environmental stress and microbial degradation [63].

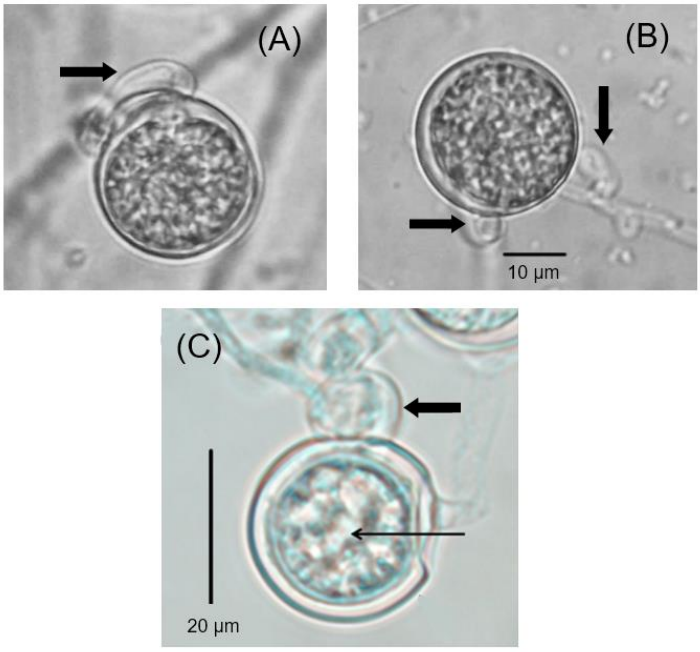


Figure 5 *Sexual reproductive cells of* Pythium *spp.*

Female oogonium containing oospore with male antheridium of *P. delawarii* (A and B) [64] and *P. aphanidermatum* (C) [65]. The fat arrows represent antheridium, and the thin arrow represents oospore.

3.2 Asexual reproduction

Zoospore is an asexual reproductive structure of oomycetes produced from zoosporangia (Figures 3C and 3D). The formation of undifferentiated zoosporangia starts with the protoplasm flowing to the hyphal tip and forming into the vesicle. Then, the differentiation of protoplasm into zoospores within the vesicle (zoosporangia) is processed. During the mature stage, the zoosporangial membranes are ruptured, and biflagellate zoospores are released into the environment (Figure 6) [10]. The zoospore is typically kidney-shaped, with two flagella that emerge from a lateral groove. A whiplash-type is a posteriorly directed flagellum responsible for the zoospore movement through water; another is the anteriorly shortened flagellum, a tinsel-type [7]. The cell surface of a zoospore is composed of various peripheral vesicles surrounded by a cell membrane. All these peripheral structures function in the encystment process.

The encystment is performed when the zoospore attaches to injured plant or animal tissue. In an initial encystment process of zoospores, the flagella are detached, the peripheral vesicles are fused with the cell membrane as mentioned above, and the adhesive glycoprotein from these vesicles is released. Then, the encysted cell wall is completely produced, a germ tube is germinated, and a hypha is elongated to the environment [66, 67].

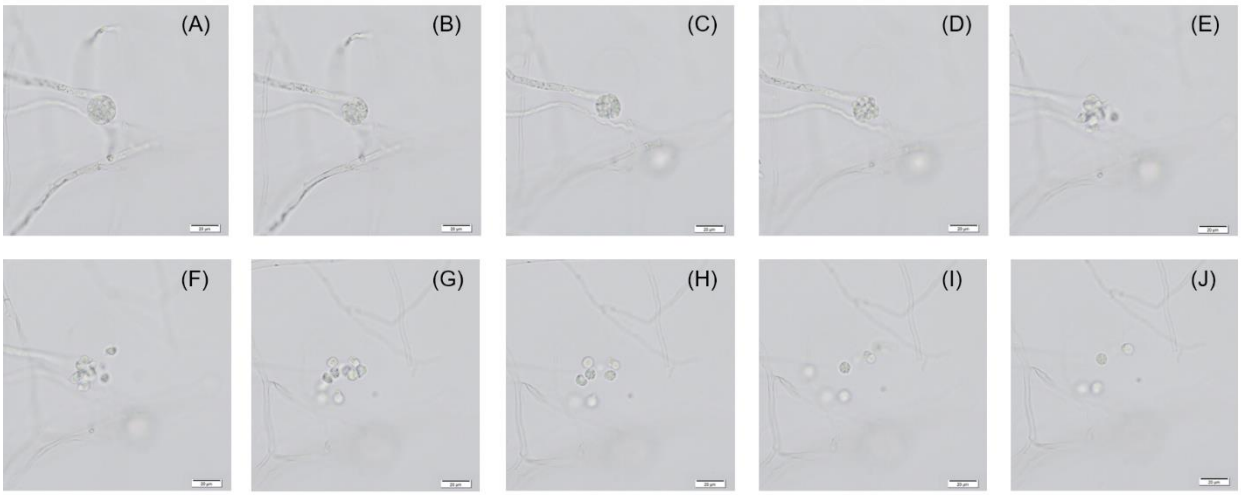


Figure 6 An asexual reproduction of *P. insidiosum* on a grass leaf

The representative pictures show the differentiation of zoosporangium (A-D) and the release of zoospore into the environment (E-J), 400x magnification.

Pathogenesis

During an asexual reproductive stage of *P. insidiosum*, biflagellate zoospores are produced in an aquatic environment. Zoospores display chemotaxis towards plant and animal tissues, such as hair, wounds, cornea, and injured skin [8]. Susceptible hosts are infected by exposure to the natural habitat of *P. insidiosum,* especially patients who live or work in agricultural areas [7]. When zoospore contacts damaged tissues, the flagella are shed, and a sticky amorphous glycoprotein is secreted, such as protease. These substances facilitate the adhesion of zoospores and relate with a general feature of other pathogenic oomycetes [10]. The encysted zoospores develop a germ tube into the infected tissue, stimulated by the host's body temperature [11]. Then, the hypha invades the host tissue and infiltrates blood vessels leading to thrombosis and aneurysm.

Clinical manifestation

1. Vascular pythiosis

The majority of human vascular cases have been reported in Thailand. Previous studies showed that patients with vascular pythiosis had a high mortality rate of 10-40% [14]. The most common organ involvement in vascular infection is the lower extremities, including the legs and thighs. Typical clinical and pathological features of vascular pythiosis are gangrenous ulcers due to the occlusion of an infected artery. Histological findings reveal the invasion of hyphae into arterial lumens. Eosinophilic migration and infiltration, focal suppurative granuloma, and giant cells surrounding the hyphae are observed. Then, the progression of pathogenesis results in the vascular aneurysm and arterial occlusion (thrombus or fibrosis). Angiograms have been reported to detect and monitor vascular lesions, which can observe an occlusion or expansion of fungal hyphae (Figure 7) [68, 69]. The current treatment for vascular infection is radical surgery in combination with antifungal agents, including itraconazole, terbinafine, and immunotherapy [15].

Many studies have reported that vascular pythiosis causes more severe infection in patients with hematological disorders, such as thalassemia and paroxysmal nocturnal hemoglobinuria (PNH) [17]. Iron overload can interfere with immune cell activities, especially phagocytic cells, affecting cytokine production. Moreover, Ud-naen *et al*. reported the decreasing level of IFN-γ that was produced by monocytes/macrophages derived from thalassemia patients after *P. insidiosum* zoospores exposure [18]. This study might indicate that iron overload is a significant factor that induces immune dysregulation during infection [70]. In addition, the transcriptome analysis found that *P. insidiosum* carries a gene encoding the ferrochelatase enzyme required for the final step of heme biosynthesis in both prokaryotes and eukaryotes, which is necessary for the survival of microorganisms inside the host [71]. These data support that ferrochelatase affects *P. insidiosum* infection in patients with hematological disorders [72].

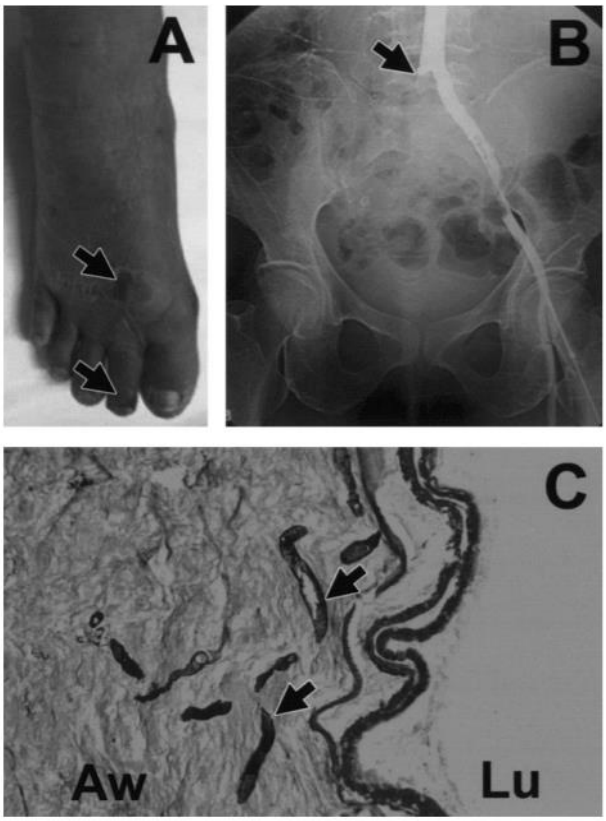


Figure 7 Clinical and pathological features of vascular pythiosis

Gangrenous ulcer (A), arterial occlusion detected by angiography (B), and hyphal branching in artery detected by Gomori methenamine silver staining (C). Aw, arterial wall; Lu, arterial lumen [14].

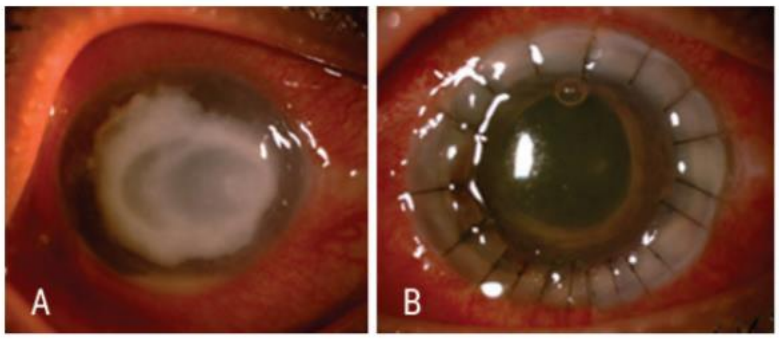


Figure 8 Clinical presentation of ocular pythiosis

The representative pictures show the full thickness near-total corneal infiltration, which is the typical lesion of ocular pythiosis (A) and post-therapeutic penetrating keratoplasty (TPK) in pythiosis patients [73].

2. Ocular pythiosis

Ocular pythiosis is the second most common clinical feature in patients with pythiosis in Thailand [8]. Most cases of ocular form are often caused by accidental contact, including eye rubbing and contact lens use, with contaminated water in the eyes [74, 75]. Moreover, hematological disorders are not a risk factor for ocular form. The symptoms of ocular lesions are a corneal ulcer, infective keratitis, corneal irritation, impaired visual acuity, swelling of the eyelids, and conjunctival injection [76]. Eye examination typically reveals “tentacle-like” or “dot-like” corneal infiltrates, full-thickness central large corneal ulcer, and endophthalmitis (Figure 8) [73]. The treatment of choice for ocular pythiosis includes surgical intervention with therapeutic penetrating keratoplasty (TPK), which is corneal transplantation in patients who have received severe infective keratitis, and a combination with antifungal drugs and immunotherapy [77].

3. Cutaneous/Subcutaneous pythiosis

Skin and subcutaneous tissue infection is the early form of vascular pythiosis commonly found in humans and animals [8]. The common clinical manifestations of subcutaneous pythiosis are pain and swelling, granulomatous, tumor-like lesions, infiltrative lumps, chronic ulcers, or necrotizing cellulitis [78]. The typical histopathology finding shows the granulomatous reaction with many inflammatory cells, predominantly neutrophils and eosinophils, surrounding the pathogens, which is called the “Splendore-Hoeppli phenomenon” (Figure 9A) [79]. This characterization presents the radiating, star-like asteroid or club-shaped granulocytic material around the infectious agent, the unique reaction in subcutaneous fungal infection, including basidiobolomycosis, conidiobolomycosis, aspergillosis, and candidiasis [80]. In addition, the unique characteristic of pythiosis in horses showed the necrotic tissue with yellow-white material, referred to as “kunkers” [7]. The kunkers or necrotic masses occur by the degranulation of eosinophils and mast cells damaging tissue, resulting in loss of exudates through the ulcerated skin lesion (Figure 9B) [9].

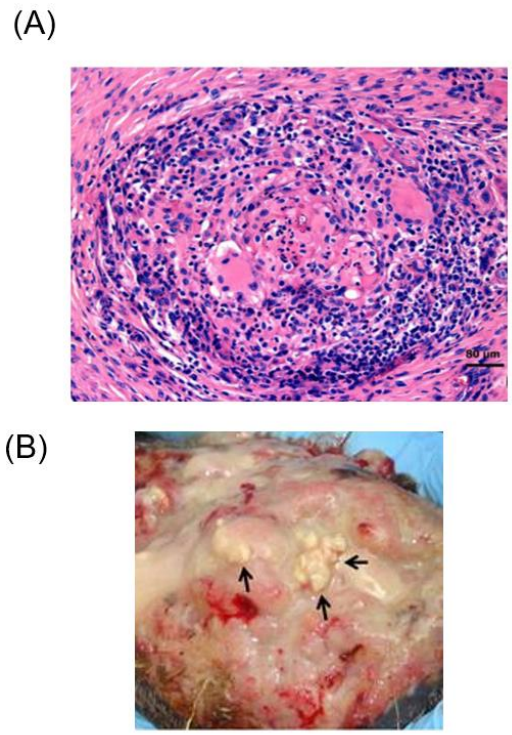


Figure 9 The histopathological and clinical presentation of cutaneous/subcutaneous pythiosis.

Representative pictures show the Splendore-Hoeppli phenomenon, observed by H&E staining, magnification ×200 (A) [3], and the kunkers from horse tissue; arrows represent the lesion with white-yellowish exudates (B) [9].

4. **Disseminated pythiosis**

Disseminated pythiosis is rare and found in only 3% of patients in Thailand [14]. This infection usually involves internal organs such as the lungs, heart, brain, intestine, and bones. Most patients with disseminated pythiosis have underlying hematological diseases [8].

Epidemiology

Pythiosis has been reported in some countries in tropical, subtropical, and temperate regions of the world (Figure 10) [3, 4, 7]. Most cases have been reported in mammals, especially horses, dogs, and humans. In 2006, Krajaejun *et al*. summarized that approximately 100 cases of human pythiosis were found in Thailand, which is associated with the hemoglobinopathy underlying disease (85%), agricultural occupation (75%), and mortality rate (40%) [14].

Phylogenetic analysis using the internal transcribed spacer (ITS) region, mitochondrial cytochrome C oxidase II (COX2) gene, intergenic spacer (IGS) region, and *exo-1,3-β-glucanase* gene (*exo1*), categorizes *P. insidiosum* into three clades, which are associated with geographic regions. Clade I or A_{TH} is an American strain, clade II or B_{TH} is an American, Asian, and Australian strain, and clade III or C_{TH} is mostly a Thai and American strain [6, 81, 82].

Figure 10 Geographic distribution of pythiosis.

The colored representative picture of tropical, subtropical, and temperate regions where pythiosis cases are found [37].

In 2006, the retrospective epidemiological study of human pythiosis in Thailand from January 1985 to June 2003 from 9 tertiary care hospitals was reported by Krajaejun *et al*. The criteria: isolation of *P. insidiosum* and zoospore induction positive, anti- *P. insidiosum*

antibodies presentation in blood samples, and demonstration of pathological features, were recruited in this study. They revealed that the cases of 120 Thai patients with pythiosis included 59% vascular form, 33% ocular form, 5% cutaneous/subcutaneous, and 3% disseminated form (Figure 11A). Human pythiosis cases in Thailand were found in the central region (46%), northeastern region (27%), northern region (16%), southern region (8%), and eastern region (3%) (Figure 12). Moreover, human pythiosis was associated with agricultural occupation, hemoglobinopathy underlying disease including Thalassemia (69%) and non-thalassemia (11%), and male sex (71%) (Figure 11B) [14]. As human pythiosis has been increasingly reported in Thailand, in 2019, Lohnoo *et al.* explored the seroprevalence of anti-*P. insidiosum* antibodies in 2,641 Thai individuals who lived in 21 provinces across Thailand from August 2008 to March 2009. They found that serum samples of 0.15% were identified with anti-*P. insidiosum* antibodies, which have no pythiosis history, suggesting subclinical infections [83].

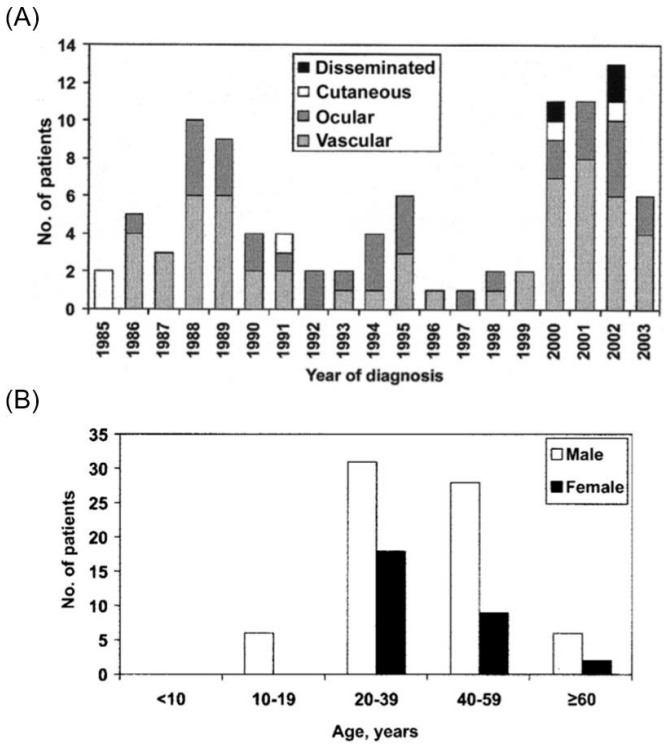


Figure 11 The retrospective data of human pythiosis in Thailand was diagnosed from January 1985 to June 2003.

Representative pictures show the various forms of pythiosis (A) and the sex of patients with pythiosis (B) [14].

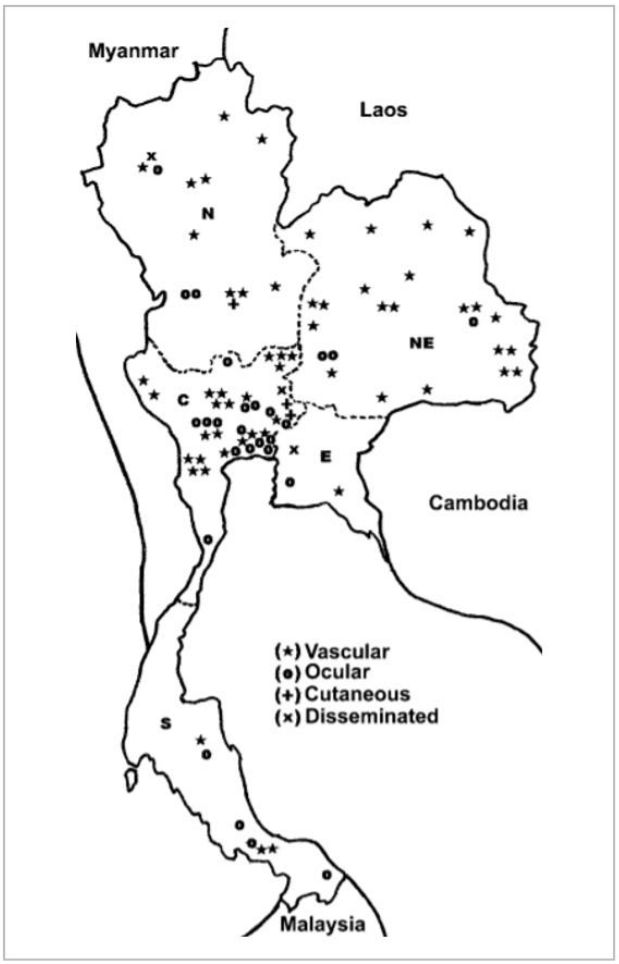


Figure 12 The distribution of human pythiosis in Thailand

C, central region; E, eastern region; N, northern region; NE, northeastern region; S, southern region [14].

Immune response against *P. insidiosum*

Understanding immune responses to *P. insidiosum* in humans and animals is still unclear. Previous studies showed that infected animals, including horses and dogs, generate anti-*P. insidiosum* antibodies that can be detected by an immunodiffusion and complement fixation test [12, 84, 85]. In humans, Imwidthaya *et al.* developed an in-house immunodiffusion test for the diagnosis of subcutaneous and systemic pythiosis [86]. In 1991, an immunodiffusion test was used to diagnose and monitor patients with pythiosis in Thailand [87]. Moreover, the detection of anti-*P. insidiosum* antibodies with enzyme-linked immunosorbent assay (ELISA) have recently been used for diagnosis, monitoring, and epidemiological studies in humans and animals [16, 83, 88, 89]. These serological studies suggested that *P. insidiosum* antigen recognizes the humoral immune response or antibodies during the infection.

As anti-*P. insidiosum* antibodies are developed by the different host species, the recognition of different dominant antigens by antibodies is presented. This hypothesis was confirmed by Chindamporn *et al*. [90]. They found that serum samples collected from various hosts, including cats, cattle, dogs, horses, and humans displayed different recognition with various molecular masses of *P. insidiosum* proteins, which were detected by western blot analysis. Worasilchai *et al.* reported that the ELISA values (EVs) of *P. insidiosum*-specific-immunoglobulin-G antibody (*Pi*-Ab) in 140 serum samples from patients with pythiosis were significantly increased when tested with the antigens prepared from clinical isolates in comparison with the equine-type strain, which in-house ELISA determined at Mycology laboratory service of King Chulalongkorn Memorial Hospital, Thailand [91]. This finding indicates that the binding affinity of specific *Pi*-Ab depends on the antigen type. Furthermore, EVs of *Pi*-Ab and serum β-1,3-D-glucan levels are significant biomarkers for monitoring vascular pythiosis [16].

Cellular immunity is processed by activating various inflammatory cells, including macrophages, eosinophils, mast cells, etc., which is observed by the histological analysis of infected tissues [7, 78, 80]. Degranulation of eosinophils and mast cells affects the severe pruritis, kunkers formation, and development of tumor-like necrotic tissue in horses [9]. The

appearance of yellow-white exudates in kunkers lesions might suggest that the polymorphonuclear cells (PMNs) or neutrophils play a role in infection and inflammation against *P. insidiosum*. In addition, Miller and Campbell reported that significantly progressive leukocytosis with moderate neutrophilia was observed in rabbits who received motile zoospores injected by subcutaneous and intraperitoneal routes [20].

Based on equine models, Mendoza and Newton proposed cytokine production in response to *P. insidiosum* infection [13]. They demonstrated that when a zoospore develops a germ tube and generates hypha, these components act as the exogenous antigen that can recognize the hosts' antigen-presenting cells (APCs). Then, APCs process and present the antigens to naïve T helper cells (Th0), affecting Th2 polarization by releasing interleukin-4 (IL-4). The differentiation of the Th2 subset results in increasing levels of IL-4 and IL-5, which can stimulate B cells to produce IgG, IgM, and IgE molecules. Migration and degranulation of eosinophils and mast cells occur at the site of infection, causing hypersensitivity and tissue damage in the infected hosts. This finding is supported by hypersensitivity testing on the skin of infected horses [92]. The infected horses were vaccinated with *P. insidiosum* antigen (PIA), a crudely extracted protein from *P. insidiosum* cultures. These immunogens are processed and presented to APCs in a different pathway of the natural infection. These PIA-APCs activate Th0 and turn into the Th1 subset, affecting the release of interferon-gamma (IFN-γ) and IL-2. The switching of host immune response stimulates cell-mediated immunity, including cytotoxic T lymphocytes (CTL), which can eliminate the pathogen and decrease hypersensitivity reactions in the infected horses (Figure 13). Similar to the immune response in humans, Wanachiwanawin *et al*. reported significantly increased levels of IL-2 in vascular pythiosis patients after receiving the PIA immunotherapy [93]. In addition, unpublished data documented by Worasilchai *et al.* demonstrated the rising levels of IFN-γ, IL-10, and IL-17 produced by peripheral mononuclear cells (PBMC) derived from 50 Thai pythiosis patients who received PIA immunotherapy. Interestingly, increased levels of IL-17 were observed after immunotherapy. This finding might indicate that neutrophils can recruit and eliminate *P. insidiosum* due to IL-17 being the key cytokine to activate neutrophil accumulation into the infection site.

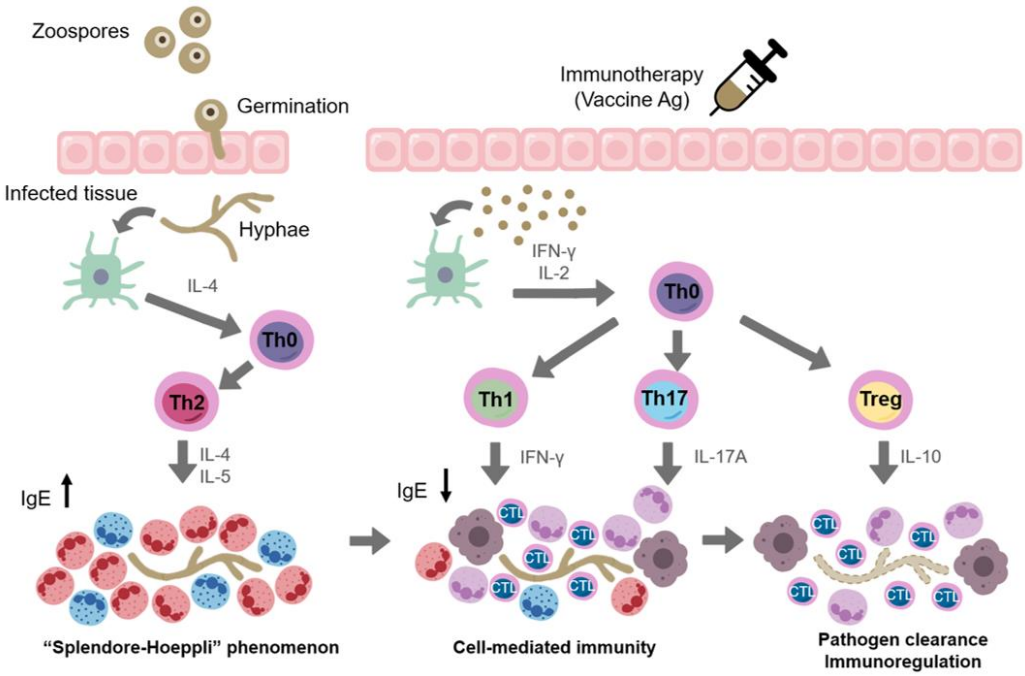


Figure 13 The cytokine production in response to the natural P. insidiosum infection (right) and treatment with P. insidiosum antigen (PIA) immunotherapy (left) in equine models.

The encysted zoospores attach to the host and then germinate hyphae. Antigen-presenting cells (APCs) recognize PIA as an immunogen and become activated. IL-4 released by activated APCs induces naïve T helper cells (Th0) to Th2 polarization. Th2 cells further produce and increase levels of IL-4 and IL-5, which thereby stimulate B lymphocytes. Productions of IgG, IgM, and IgE trigger mast cell and eosinophil migration to the site of infection, causing tissue damage and hypersensitivity reaction. In contrast, PIA injection is recognized by APCs. The activation of APCs drives Th0 cells into Th1 cells. The release of IFN-γ and IL-2 stimulates the cell-mediated immune response, particularly cytotoxic T lymphocytes (CTLs), which induce the destruction of *P. insidiosum* [13, 94]. (Modified from Yolanda H, 2021)

Neutrophils

Neutrophils are classified as PMNs and phagocytic leukocytes. These innate immune cells are the most abundant circulating leukocytes and are found in approximately 50–70% of total white blood cells in humans. During infection, the percentage of neutrophils may increase to 80% or more [21]. Their lifespan is approximately 6-8 h in blood circulation, and they can last up to 7 days in tissue [23]. Mature neutrophils have an average diameter of 7-10 μm, composed of segmented nucleus and granules or protein vesicles accumulated in the cytoplasm (Figure 14) [24]. They are the first cells of the immune system to migrate to a site of inflammation and play an essential role in cytokine production and pathogen elimination [19].

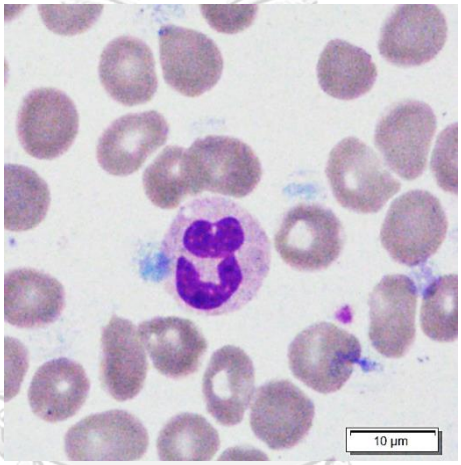


Figure 14 Neutrophil in peripheral blood smear

Representative picture observed by Wright's staining, light microscope, 1,000x magnification.

1. **Neutrophil maturation and granulation**

More than 10^{11} neutrophils are released daily in the bloodstream and are generated in the bone marrow about 14 days from hematopoietic stem cells (HSC) [95]. HSCs differentiate into multipotent progenitor (MPP) cells and then transform into lymphoid-primed multipotent progenitors (LMPPs). After that, LMPPs differentiate into granulocyte-monocyte progenitors (GMPs) regulated by the granulocyte colony-stimulating factor (G-CSF), resulting in neutrophil generation from the myeloblast. The myeloid-controlled process by G-CSF is produced in response to IL-17A synthesized by T cells that regulate neutrophils [19]. The cells then follow a maturation process that includes the stages of promyelocyte, myelocyte, metamyelocyte, band form, and mature neutrophil (Figure 15) [96]. During differentiation, neutrophils change their nucleus from a round-shaped into a banded and segmented morphology, respectively, expressing the various receptors in each formation stage.

During the maturation process, neutrophils contain specific enzymes, vesicles, granules, and other protein molecules, formed at four particular differentiation stages (Figure 16).

Primary or azurophilic granules (peroxidase-positive granules) are the largest oval-shaped in morphology, first formed during neutrophil maturation, including myeloblast and promyelocyte stage. The term “azurophils” refers to the affinity for taking up the basic dye azure A, a dark blue dye often used in Giemsa stain [21]. The characteristic of these granules is the presence of myeloperoxidase (MPO) which is essential for respiratory burst [97]. Primary granules also contain other protein molecules, including defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), neutrophil elastase (NE), proteinase 3, and cathepsin G [98].

Secondary or specific granules are presented at the myelocyte and metamyelocyte stages, characterized by the glycoprotein lactoferrin’s presence. Lactoferrin's activity interferes with the iron acquisition of several pathogenic bacteria and fungi during infection [21]. The other protein molecules in specific granules, such as matrix metalloproteinase (MMPs), are the compartments of phagocytic vacuoles and a plasma membrane stored as inactive proenzymes. During phagolysosome formation, MMPs are activated, and the integral membrane components of phagocytosed microorganisms are degraded by this enzyme [99].

Tertiary or gelatinase granules are the third class of neutrophil granules produced in the banded stage, containing a high gelatinase concentration [21]. Dewald *et al*. demonstrated a relationship between gelatinase secretion and the induction of respiratory bursts from human neutrophils. They found that superoxide production in response to various stimuli did not parallel gelatinase release. This data suggests that gelatinase secretion is not dependent on respiratory burst [100].

The secretory protein vesicles are detected in mature neutrophils. These vesicles contain plasma-derived proteins, including albumin, important for membrane-bound molecules during neutrophil migration [25]

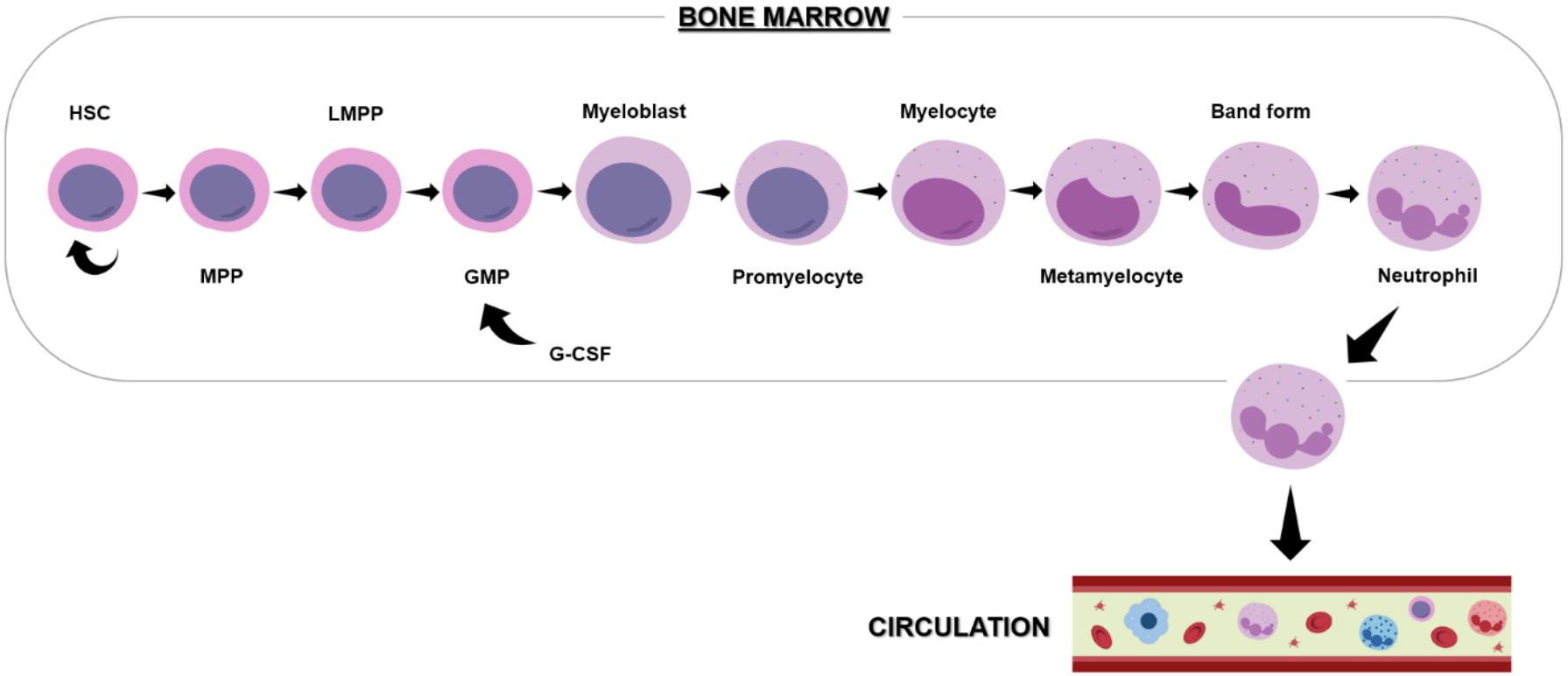


Figure 15 Granulopoiesis or neutrophil differentiation

The representative picture shows the differentiation of neutrophils in bone marrow from hematopoietic stem cells (HSC), multipotent progenitor (MPP), lymphoid-primed multipotent progenitors (LMPP), and granulocyte-monocyte progenitors (GMP), which is controlled by granulocyte colony-stimulating factor (G-CSF) for transform into neutrophil generation. Myeloblast follows a maturation process: promyelocyte, myelocyte, metamyelocyte, band form, and mature neutrophil [22]. (Modified from Rosales C, 2018)

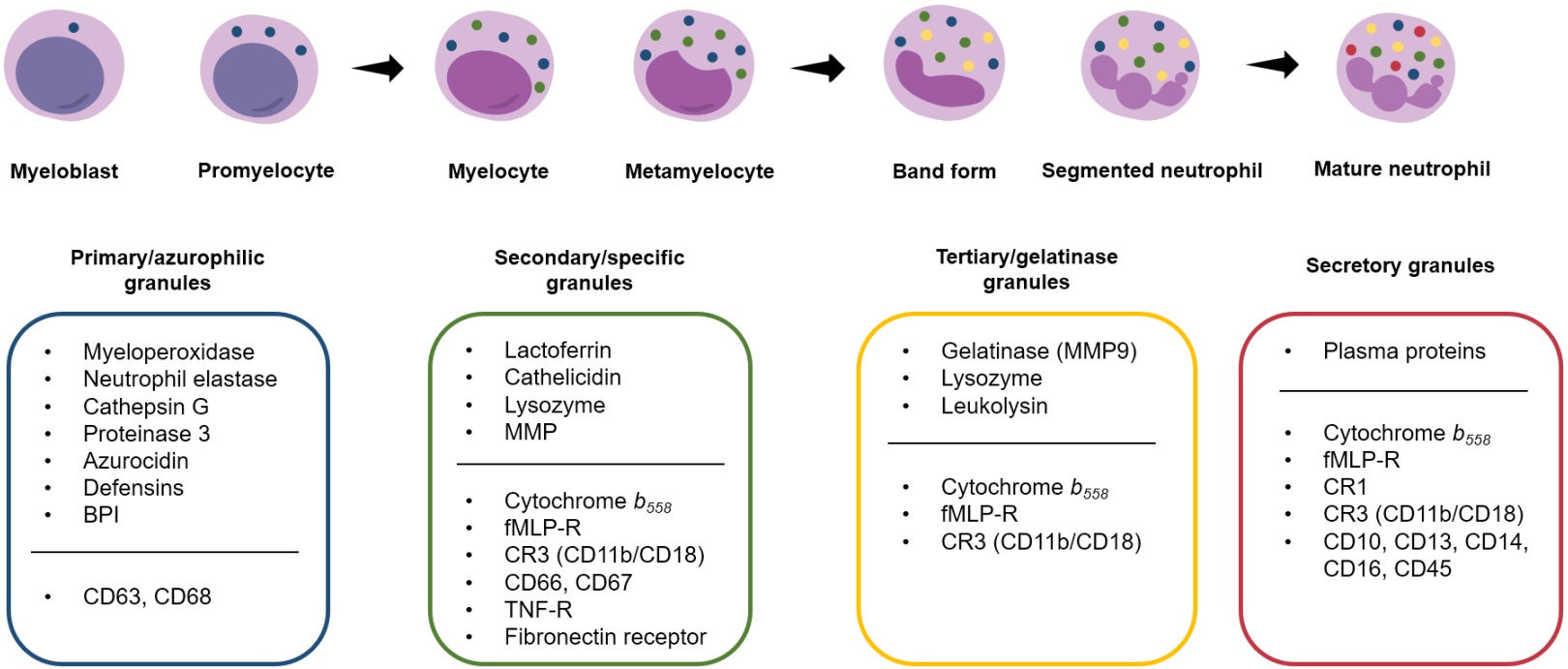


Figure 16 Neutrophil granules and protein-containing molecules

The representative picture shows granules and protein molecules of neutrophil during the maturation process that include primary or azurophilic granules are contained in the stage of myeloblast and promyelocyte, secondary or specific granules are contained in the stage of myelocyte and metamyelocyte, tertiary or gelatinase granules are contained in the stage of banded and segmented form, and secretory granules are contained in a mature stage. BPI, Bactericidal/permeability-increasing protein; FMLP-R, formyl-methionyl-leucyl-phenylalanine receptor; CR, complement receptor; CD, cluster of differentiation; MMP, matrix metalloproteinase; TNF-R, tumor necrosis factor receptor [21, 101]. (Modified from Fiedler and Brunner, 2012)

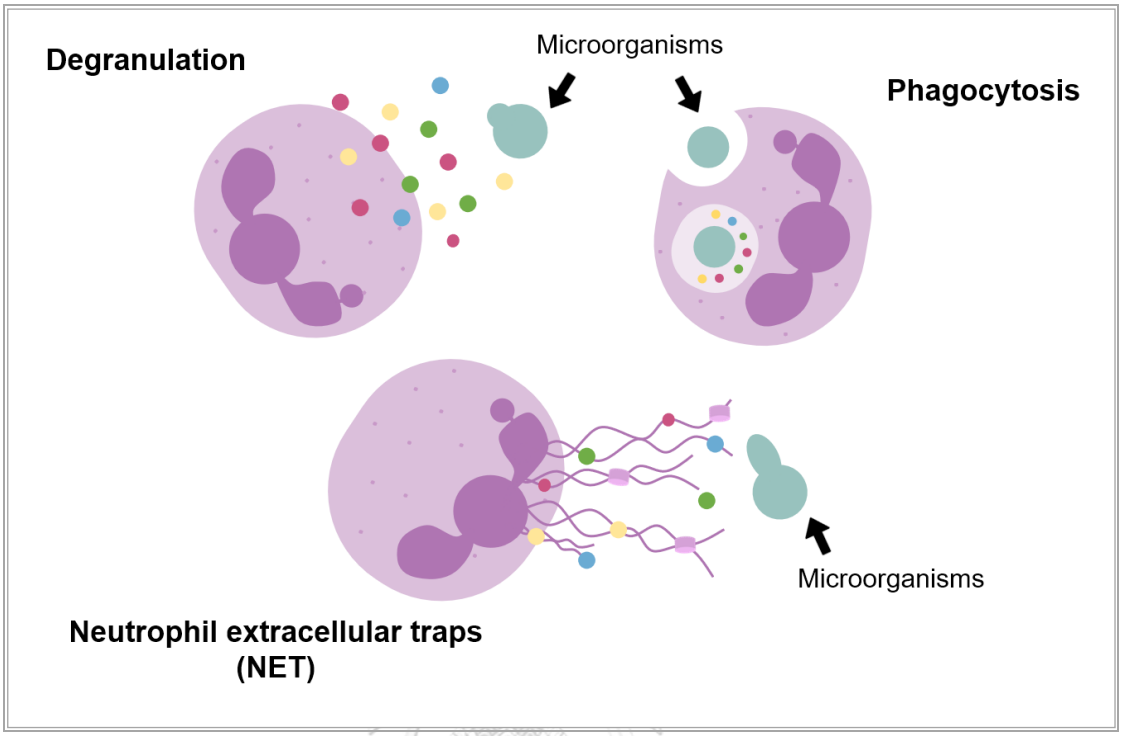


Figure 17 Mechanisms of neutrophils against invading pathogen

The representative picture demonstrates the killing activity of neutrophils, including phagocytosis, degranulation, and neutrophil extracellular traps (NET). During infection, neutrophils recognize the pathogen by their cell surface receptors. Phagocytosis occurs when the microorganisms are engulfed in the cytoplasm. Then, neutrophil forms the phagosome, generates a phagolysosome by fusing their granule vesicles, and produces a superoxide substance. Degranulation is the secretion of granules, enzymes, or protein vesicles to destroy the infected pathogen. NETs are the extracellular DNA and protein components activated by microorganisms, particularly those with large pathogens [22]. (Modified from Rosales C, 2018)

Killing mechanisms and antifungal activities

Neutrophils are the first line of defense against invading pathogens, which eliminates by their intra- and extracellular killing mechanisms. When microorganisms penetrate the host, neutrophils attach to the endothelium of blood vessels through various interactions by their receptor and adhesion molecules. After passing through the endothelium, neutrophils recognize and migrate to the signal source, called chemotaxis. Finally, neutrophils encounter and destroy the targeted pathogens [21]. Neutrophils display several immune response activities when encountering microbes, including phagocytosis, degranulation, and release of neutrophil extracellular traps (NETs) (Figure 17).

1. **Receptors and fungal recognition**

Neutrophils can directly recognize pathogen-associated molecular patterns (PAMPs), which are microbial molecules that share a lot of different general structures [102]. Components of fungal cell walls act as PAMPs such as β-glucan, chitin, mannans, and fungal nucleic acid [103]. These PAMPs are targeted with neutrophils via pattern recognition receptors (PRRs) on the cell surface. PRRs can recognize several domains of microbes and induce downstream events designed to eliminate pathogens from the host (Figure 18).

The major PRRs for fungal pathogens are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), galectin family proteins, nucleotide-binding oligomerization domain (NOD)-like receptors, and NALP3 (NOD-, LRR and pyrin domain-containing protein 3) inflammasome [104]. Several studies have reported that complement receptor 3 (CR3, CD11b/CD18) is the crucial receptor on human neutrophils for the recognition of β-glucan. Bruggen *et al*. demonstrated that CR3 of neutrophils mediates phagocytosis of *Saccharomyces cerevisiae* (*S. cerevisiae*) [105]. Gazendam *et al*. reported that recognition of *Aspergillus fumigatus* (*A. fumigatus*) triggered CR3 through the phosphatidylinositol 3-kinases (PI3K) pathway to kill conidia. Additionally, *Aspergillus* hyphae recognize Fcγ receptors (FcγR), proceed with the signaling via spleen tyrosine kinase (SYK), PI3K, and protein kinase C, then trigger the production of ROS and MPO [31]. These data indicate that patients with a leukocyte adhesion deficiency I (LAD-I) with the invasive fungal infection lack CR3 integrin expression failing to bind with a blood vessel of phagocytes. Moreover, TLRs are classified into transmembrane receptors (TLR1, 2, 4, 5, 6, and 10) and intracellular receptors (TLR3, 7, 8, and 9), that

recognize both fungal cell wall components and nuclear components, such as DNA and RNA [106].

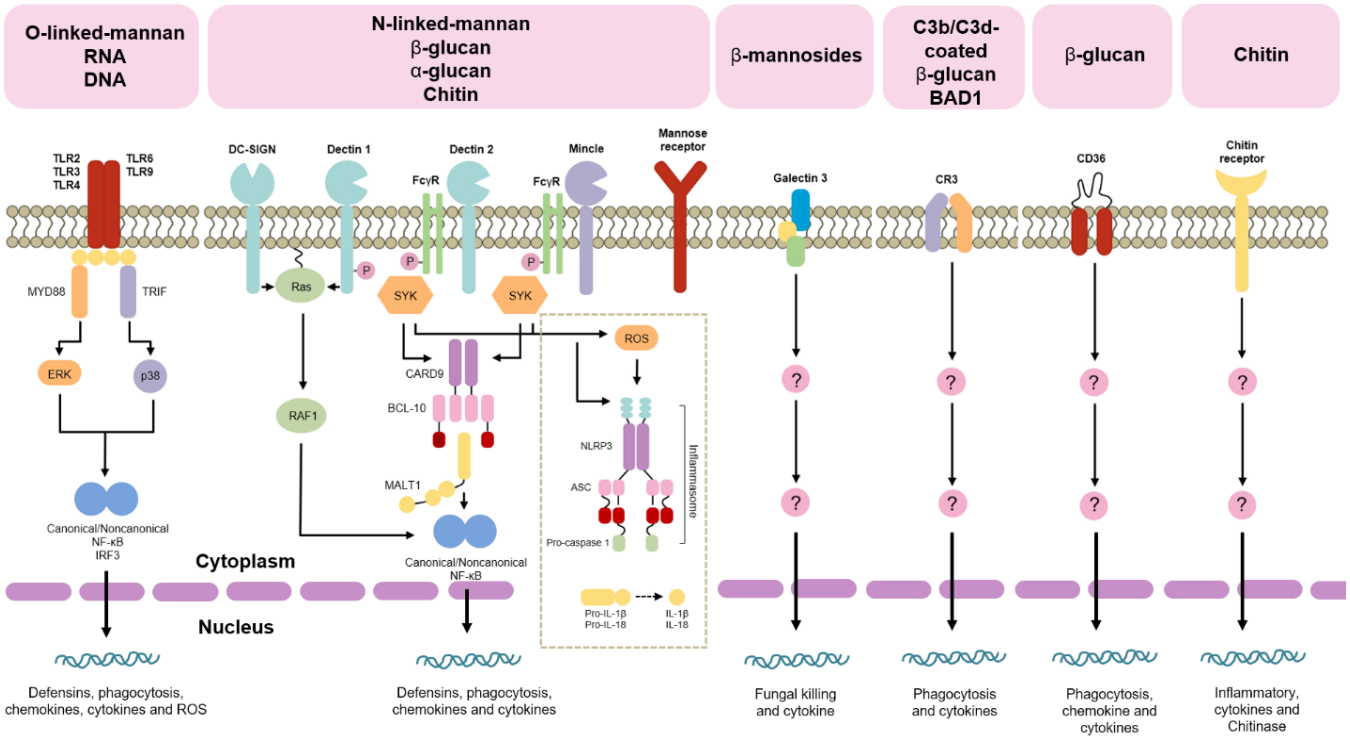


Figure 18 Signaling pathways in fungal recognition

The representative picture shows the fungal components or PAMPs that can recognize PRRs molecules exposed on the neutrophil surface, and stimulate various signaling pathways in the host immune response. ASC, apoptosis-associated speck-like protein containing a CARD; BCL 10, B cell lymphoma 10; CARD 9, caspase recruitment domain-containing protein 9; ERK, extracellular signal-regulated kinase; IRF3, IFN-regulated factor 3; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MYD88, myeloid differentiation primary response protein 88; CR3, complement receptor 3 [103]. (Modified from Cunha *et al*, 2012)

2. Phagocytosis

Phagocytosis is the primary mechanism for engulfing and eliminating pathogens by intracellular mechanisms [107]. When neutrophils encounter microorganisms, they recognize PAMPs, small molecules found on microbial cells that can induce neutrophils via PRRs and opsonic receptors [108]. Antigens (microorganisms) can be labeled for phagocytosis by opsonin, which are host-derived proteins that bind specific receptors on phagocytic cells. The opsonin-mediated receptors include FcγR, and CRs, which depend on IgG- and complement-opsonized microbes. The process of antigen-labeled opsonin binding specific receptors on phagocytic cells is called opsonization [109]. Moreover, non-opsonic receptors include Dectin-1, Dectin-2, and Mincle, which are receptors for fungal β-glucan components that recognize microbial ligands and induce phagocytosis.

During phagocytic uptake, the polymerization of the actin cytoskeleton is processed, resulting in a change in the cell membrane that affects the depression of the membrane area. Then, pseudopods are formed around the microorganism [110]. The pathogens are engulfed in the cytoplasm of neutrophils, creating a new vesicle called a phagosome. The lysosome contains several hydrolytic enzymes, protein molecules, and antimicrobial peptides released from the neutrophil granules. The phagosome-containing microorganism and lysosome are called phagolysosome; this component finally eliminates and degrades the pathogens inside the cytoplasm [25].

In addition to other antimicrobial molecules, neutrophils can kill microbes by the generation of toxic substances such as nitric oxide (NO), superoxide, and hydrogen peroxide (H_2O_2) [111]. This process is called a respiratory burst, producing reactive oxygen species (ROS) mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. When the activation of NADPH oxidase is localized to specific granule membranes, electrons are transferred from NADPH to generate superoxide, and hydrogen peroxide is rapidly produced. Moreover, MPO can react with hydrogen peroxide affecting hypochlorous acid production, which increases bactericidal activity against pathogens [21]. Aratani *et al.* demonstrated a relationship between MPO and NADPH oxidase [112]. In mice models, they found that MPO could not play a role in host defense against A. fumigatus and C. albicans without NADPH oxidase. This data confirms that ROS production mediated by the NADPH system increases the strongest fungicidal activity of neutrophils.

3. Degranulation

One of the crucial roles during the infection of neutrophils is the release of antimicrobial substances and other molecules from secretory vesicles, which is called degranulation. The different types of granules demonstrate various properties in response to infection signals. During passing through the endothelium, neutrophils are exposed to the microbial activation signals affecting the release of gelatinase granules called metalloproteinase. Delclaux *et al*. demonstrated that gelatinase B could degrade type IV collagen, which plays the major factor in neutrophil migration across the basement membrane, activated by NE [113]. When neutrophils are activated, stimulation of the oxidative burst and mobilization of the azurophilic and specific granules are initiated. These granules fuse with the phagosome or plasma membrane, resulting in NADPH oxidase stimulation and ROS production [114]. Moreover, Lefkowitz *et al*. found that MPO released from neutrophils activates macrophages and induces microbicidal activity against *C. albicans* [115]. This investigation suggests that the released components from neutrophils have synergism with other immune cells.

Neutrophils can release antimicrobial proteins that are contained in matured stage secretory vesicles. The main types of antimicrobial molecules include cationic peptides, enzymes, and proteins [25]. The neutrophil cationic antimicrobial peptides such as defensin and cathelicidin that can bind to microbial membranes inhibit bacterial cell wall synthesis. BPI is the lipopolysaccharide (LPS) binding protein that increases bacterial permeability and hydrolysis of the bacterial phospholipid [116, 117]. Neutrophils also contain proteolytic enzymes such as lysozyme, a serine protease, and NE that can destroy the bacterial cell wall and cleave some pathogenic virulence factors of bacteria [118]. The protein-containing vesicles have unique properties by interfering with the sequestrating of essential nutrients by microorganisms, including lactoferrin and calprotectin, which are iron and zinc chelators, respectively [119].

4. **Neutrophil extracellular traps (NETs)**

Neutrophil extracellular traps (NETs) were first described in 1996 and characterized as the release of nuclear contents and morphological changes of neutrophils after treatment with phorbol-12-myristate 13-acetate (PMA) [120]. Many studies showed that the structure of NETs was composed of nuclear chromatin, antimicrobial peptides, and neutrophil enzymes; the process of the released components is called NETosis (Figure 19) [121].

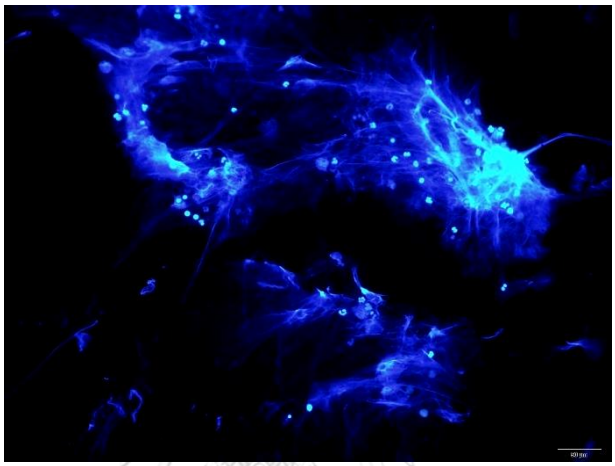


Figure 19 Neutrophil extracellular traps (NETs)

The representative picture shows dsDNA release by human neutrophils after treatment with Phorbol myristate acetate (PMA), observed by 4',6-diamidino-2-phenylindole (DAPI) staining, fluorescence microscope, 400x magnification.

The main signaling pathway of NETosis was demonstrated by several *in vitro* studies. Various stimulants include PMA, the cell-permeable activator of protein kinase C (PKC), calcium ionophores, hydrogen peroxide, and LPS, which can induce NETosis [122]. After PMA treatment, neutrophils are stimulated via PKC and Raf-MEK-ERK signaling pathways, resulting in the activation of NADPH oxidase 2 (NOX2) and the induction of NET formation by ROS production [123]. The formation of ROS promotes MPO and NE translocation into the nucleus. MPO then converts hydrogen peroxide to hypochlorous acid, resulting in NE activation, which degrades the nuclear membrane's cytoskeleton and affects the NET expansion [122]. Moreover, histone deamination and chromatin decondensation are promoted by peptidyl arginase deaminase 4 (PAD4) initiated calcium ionophores, which increase the intracellular calcium level that can activate PAD4 [124].

On the other hand, NETs are triggered by recognizing stimuli through TLRs and CRs, which occur independently of ROS production. These processes promote the release of DNA via nuclear morphological changes, resulting in the loss of the multinucleated shape of the nucleus, separation of the nuclear membrane, and the release of DNA through a small area on the cell surface [125]. After releasing DNA from the nucleus, neutrophils can still engulf the pathogen, and their lifespan is not affected by DNA loss [126].

As the complicated structures of fungi, including the variation in size and shape (yeast, hyphae, pseudohyphae, spore, etc.), special components (capsule, spherule, etc.), that affect the fungicidal activity of neutrophils [127], neutrophils, therefore, demonstrate the release of NETs in response to those of pathogens. Branzk *et al*. found that human neutrophils phagocytose the yeast form of *C. albicans*, whereas the hyphal form induces NETosis [26]. Gazendam *et al*. demonstrated that *A. fumigatus* hyphae induce NET formation, whereas the killing of conidia depends on the intracellular killing of human neutrophils [31]. Qui Liu *et al*. reported that NETs are the important neutrophil activity for controlling *Phialophora verrucosa* (*P. verrucose*) conidia [30]. These investigations support that neutrophil displays NETs in controlling and evading fungal infection.

CHAPTER IV MATERIALS AND METHODS

Blood collection and neutrophil isolation

Blood samples from volunteers were collected by phlebotomy with their written informed consent. Whole blood from healthy donors (n=6) was collected in a heparinized tube, and the sample size was calculated by G Power Software version 3.1.9.2 (G*Power, Germany). The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 572/2020, IRB No. 045/63).

For neutrophil isolation, fresh heparinized blood was layered on Polymorphprep™ (Axis-shield, Norway) with a 1:1 ratio and centrifuged at 1,800 rpm for 30 min at 25˚C. A sterile pipette removed the plasma and PBMC layers. The neutrophil layer was collected and washed with RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). The cells were centrifuged at 1,800 rpm for 5 min at 25˚C. The supernatant was removed, and contaminated red blood cells were lysed by red cell lysis buffer with a ratio of 1:9 (cell: lysis buffer). Then, the cell suspension was centrifuged at 1,800 rpm for 5 minutes at 25˚C, and the pellets (neutrophils) were resuspended in 1 ml RPMI 1640 medium with 10% FBS. Cell purity and viability were determined by Wright-Giemsa and trypan blue staining (Gibco, USA), respectively [128].

P. insidiosum culture and zoospore induction

P. insidiosum isolates in this study were collected from different sources, as shown in Table 2.

Table 2 The list of *P. insidiosum* isolates in this study.

No.	*P. insidiosum* isolates	Source	clade
1	ATCC 58643 (CBS 574.85)	Equine	I
2	CBS 101039	Indian patient	II
3	CBS 777.85	Equine	II
4	PC10	Thai patient *	II
5	ATCC 90586	American patient	III
6	PEC1	Water reservoir **	III

* Clinical isolated from a vascular pythiosis patient in King Chulalongkorn Memorial Hospital

** Environmental isolated from water in Pasak Chonlasith Dam, Saraburi, Thailand

All isolates were grown on sabouraud dextrose broth (SDB, Oxoid, UK) and incubated at 37˚C for 18-48 h [10]. The zoospore induction method was slightly modified from Mendoza *et al*. [11]. *P. insidiosum* isolates were inoculated on sterile grass leaves (*Axonopus compressus*), which were placed on the surface of corn meal agar (CMA, Becton-Dickinson, USA) and incubated at 37˚C [129]. After 2-3 days, grass leaves with hyphae were transferred into 15 ml of the induction medium to generate zoospores at 37ºC [130]. After 18 h, the induction medium containing encysted zoospores was centrifuged at 5,000 rpm for 10 min at 10˚C. The supernatant was removed, and the pellet was washed three times with 1x phosphate-buffered saline (PBS, Biolegend, USA). The encysted zoospores were counted by hemocytometer.

Killing assay

As the reference study of neutrophil killing against *P. insidiosum* has been uninvestigated, this study optimized the co-culture conditions. Opsonization and the ratio between neutrophils and zoospores were examined. The killing of zoospores was modified from Gazendam *et al*. [31]. Isolated neutrophils were incubated with zoospores in MOI (multiplicity of infection) 100:1, 10:1, 1:1, and 1:10, respectively, as shown in Table 3.

Table 3 Neutrophil and zoospore ratio

MOI (Neutrophil: zoospore)	Neutrophil (cells)	Zoospore (cells)
100:1	2×10^5	2×10^3
10:1	2×10^5	2×10^4
1: 1	2×10^5	2×10^5
1: 10	2×10^4	2×10^5

After zoospore induction, the fresh encysted zoospores were opsonized with heat-inactivated pooled healthy serum at 37˚C for 30 min and washed three times with 1xPBS. Zoospores were incubated with the neutrophil suspension in a 96-U bottom well cell culture plate (SPL Life Sciences, Korea) with the ratio mentioned in Table 3 for 2 h at 37ºC in a 5% CO_2 incubator. Every 15 min, the suspension was mixed gently. After the incubation, cells were centrifuged at 10˚C, 5,000 rpm for 10 min. Then, sterile dH_20 was added to the suspension, and neutrophils were lysed. To determine the viability of zoospores, the fungal colony numbers and the live/dead-stained zoospores were compared. All experiments were processed in triplicate independently.

To observe the fungal colonies, the suspension after the neutrophil lysis was diluted in 1xPBS. Next, 100 µl of the mixture was transferred to sheep blood agar (SBA, Oxoid, UK) and then spread using a sterile spreader. After incubation at 37˚C for 15 h, the colony count was performed.

To determine the live/dead zoospores under the microscope, cell suspension in a 96-U bottom well cell culture plate was centrifuged at 5,000 rpm for 10 min. After that, the supernatant was removed, and the cell was stained with 0.4% trypan blue dye. The unstained/live and stained/dead zoospores were differentiated under a 40x light microscope (Olympus BX50, Japan).

Phagocytosis activity

1. **Heat-killed and pHrodo labeled zoospore preparation**

To study phagocytosis activity, zoospore labeling with pHrodo was performed. The pHrodo staining protocol was modified from Shintaku T *et al*. [131]. All six strains of *P. insidiosum* zoospores were heated at 80˚C for 30 min with a shaker. Then, the heat-killed zoospores were centrifuged at 5,000 rpm for 10 min, and the pellet was resuspended with 1 µM pHrodo-succinimidyl ester (Invitrogen, USA) in 1x PBS. The mixture was incubated with a shaker for 30 min at room temperature, protected from light. After staining, pHrodo-labeled zoospores were washed three times with 1x PBS and resuspended in RPMI 1640 medium with 10% FBS.

2. **Phagocytosis assay**

To determine the ability of zoospore-induced neutrophil phagocytosis, neutrophils were seeded in a 96-U bottom well cell culture plate. The pHrodo-labeled zoospores were added and mixed gently. To perform the opsonization process, heat-inactivated healthy serum was added to the cell suspension (final serum concentration of 5% v/v) and incubated at 37ºC in a 5% CO_2 incubator for 30 min in the dark. The negative control was incubated with 10 µg/ml cytochalasin D (Sigma-Aldrich, Israel). After the incubation, the mixture was centrifuged at 1,800 rpm for 5 min at room temperature. The supernatant was removed, and the pellet was resuspended in 90 µl of flow cytometry staining (FACS) buffer supplemented with 10 µl of human AB serum for blocking. The suspension was incubated at 4˚C for 20 min. To stain neutrophil, 0.2 mg/ml APC anti-mouse/human CD11b antibody (Biolegend, USA) was added as a surface marker and incubated at room temperature for 15 min in the dark. APC rat IgG2b, k (Biolegend, USA) was chosen as an isotype control. Next, the suspension was washed with FACs buffer and centrifuged at 1,800 rpm for 5 min at room temperature. The supernatant was removed, and the pellet was resuspended in 100 µl of FACS buffer. Cells were stored on ice and measured by flow cytometer (FACsAria II, BD Biosciences, USA) with a maximum delay of 2 h [132]. Flow cytometry phagocytosis data were analyzed by FlowJo (Version X 10.0.7).

Neutrophil extracellular trap formation

An immunofluorescence staining was performed to assess the ability of zoospore-induced NETs by neutrophils. The sterile round glass coverslip was coated with poly-L-lysine (Sigma-Aldrich, USA) in a 24-well cell culture plate (Jet Bio-Filtration, China) overnight at 4ºC. After the incubation, poly-L-lysine was removed, and the glass coverslip was washed with 1xPBS. Neutrophils were seeded on a glass coverslip and incubated for 1 h at 37ºC in a 5% CO_2 incubator. The heat-killed zoospores were added and incubated for 2 h. 100 ng/ml Phorbol myristate acetate (PMA, Sigma-Aldrich, USA) was chosen as a positive control. After the incubation, the supernatant was aspirated for DNA quantification by Quant-iT™ PicoGreen® (Invitrogen, UK). The glass coverslips were fixed with 1% formaldehyde for 5 min and washed with 1xPBS. Next, the cells were permeabilized with 1X Tris-buffered saline (TBS) in 0.05% tween 20 for 1 min and washed with 1xPBS. After that, glass coverslips were blocked with 1xTBS with 2% bovine serum albumin (BSA, Himedia, India) for 30 min and washed with 1xPBS.

Immunofluorescence revealed the NET formation, and the staining protocol was modified from Sae-khow *et al*. [133]. Rabbit anti-Neutrophil Elastase and mouse anti-Myeloperoxidase (Abcam, UK) as primary antibodies were added (at 1:200 dilution) and incubated overnight at 4ºC. After washing with 1xTBS, goat anti-rabbit IgG Alexa fluor 488 and goat anti-mouse IgG Alexa fluor 647 (Abcam, UK) as secondary antibodies were added (at 1:200 dilution) and incubated at room temperature for 1 h, protected from light. The cells were washed with 1xTBS and stained with 1 µg/ml of 4',6-Diamidino-2-phenylindole (DAPI, Thermo fisher scientific, USA) for 10 min at room temperature in the dark. After that, the coverslips were washed with 1xTBS and dried. Antifade mounting media (Invitrogen, USA) was dropped, and coverslips were placed on the slide before identification by fluorescence microscope (Olympus IX81, Japan) and confocal laser scanning microscope (ZEISS LSM800, Germany).

Quantification of NET formation (Quant-iTTM PicoGreen®) [134]

As mentioned in NET formation, the supernatant was prepared to determine the levels of dsDNA induced by zoospores. After the incubation, 0.1 M $Cacl_2$ and 50 U/ml micrococcal nuclease (Sigma-Aldrich, USA) were added to the supernatant and incubated at 37ºC for 10 min. After that, the reaction was stopped by 0.5 M ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1,800 rpm for 5 min to remove cell debris. The supernatant was collected and stored at -80 ºC.

Quant-iT™ PicoGreen® kits (Invitrogen, UK) were used for cell-free DNA determination. The sample and PicoGreen® reagent were added in a 1:1 ratio to a 96-flat bottom well plate (Corning, USA). The suspension was mixed and incubated at room temperature for 5 min in the dark. The ten-fold dilution of the DNA standard (1 µg/ml) was prepared for a standard curve [135]. Finally, the DNA level in the mixture was measured at an excitation wavelength of 480 nm and emission wavelength of 530 nm by spectrofluorometer (Varioskan Flash, Thermo-fisher scientific, Finland).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Data were evaluated for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni analysis to compare the groups. The results are presented as the mean ± standard error of the mean (SEM), and differences with a *p-value* < 0.001 were considered statistically significant.

CHAPTER V RESULTS

Neutrophil killing assay

1. Killing assay optimization

As the reference protocol for neutrophil killing against *P. insidiosum* is unpublished, this study conducted the optimization of the neutrophil killing assay. The type strain of *P. insidiosum* CBS 777.85 was randomly selected for the optimization protocol.

To compare neutrophil killing activity between opsonization and un-opsonization, zoospores and neutrophil isolated from two healthy donors were incubated at 37ºC in a 5% CO_2 incubator for 2 h. The ratio between neutrophils and zoospores is described in Table 2. Heat-inactivated pooled healthy serum was added in the opsonized condition during incubation, whereas RPMI 1640 culture medium was added in the unopsonized condition. Colony count was used to determining the zoospores' survival rate after co-incubation with neutrophils. Neutrophils from healthy donors showed a significantly higher killing capacity against opsonized zoospores (73.9 ± 5.0%) than unopsonized (89.1 ± 3.6%) ($p < 0.001$, $n = 2$). No significant differences in the neutrophil killing activity against opsonized zoospores between MOI 1:1 (70.4 ± 5.9%) and 1:10 (77.5 ± 5.0%) were found ($p > 0.05$) (Figure 20).

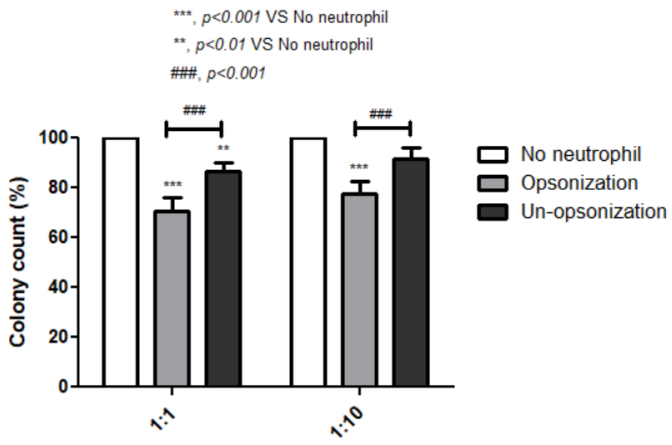


Figure 20 Comparison of neutrophil killing capacity between opsonization and un-opsonization

Serum-opsonized (white bar) and un-opsonized (black bar) *P. insidiosum* zoospores CBS 777.85 were incubated with neutrophils in MOI (neutrophil: zoospore) 1:1 and 1:10. The survival rate of zoospores was assessed with colony count. $n = 2$. Mean ± SEM. *** $p < 0.001$ and ** $p < 0.01$ compared with the no neutrophil; ### $p < 0.001$ comparison between opsonization and un-opsonization.

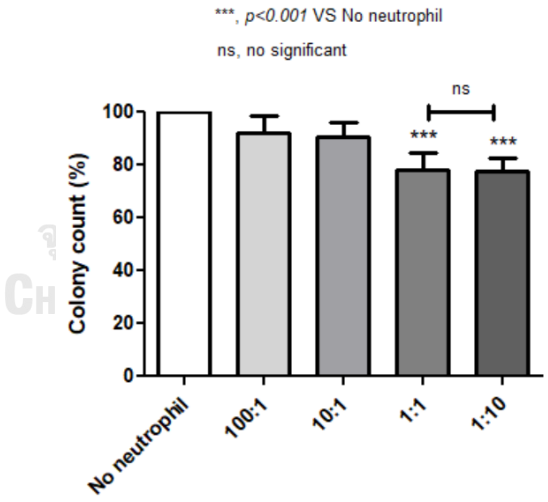


Figure 21 Neutrophils and zoospores optimal ratio

Opsonized-*P. insidiosum* zoospores CBS 777.85 were incubated with neutrophils in MOI (neutrophil: zoospore) 100:1, 10:1, 1:1, and 1:10, respectively. The survival rate of zoospores was assessed with colony count compared with the incubation without neutrophils. $n = 2$. Mean ± SEM. ***, $p < 0.001$ compared with the no neutrophils; ns = no significant between MOI 1:1 and 1:10.

Next, we investigated the optimal ratio between neutrophils and zoospores for neutrophil-killing assay. As neutrophils significantly displayed the role in killing capacity against opsonized-*P. insidiosum* zoospores, CBS 777.85 zoospores were opsonized with serum and co-incubated with neutrophils isolated from two healthy donors with the ratio as shown in Table 3. Colony count evaluated the antimicrobial activity of neutrophils after incubation for 2 h. Colony count on BA showed a significantly decreased percentage of *P. insidiosum* colonies observed in MOI 1:1 (78.0 ± 6.5%) and 1:10 (77.5 ± 5.3%), respectively, compared with the incubation of zoospores without neutrophils ($p < 0.001$, $n = 2$). Moreover, no significant differences in neutrophil killing capacity were found between MOI 1:1 and 1:10 ($p > 0.05$) (Figure 21).

2. Neutrophil killing against *P. insidiosum* zoospores

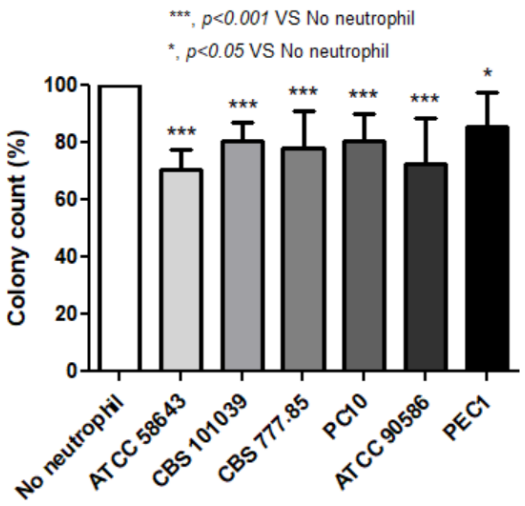
To evaluate neutrophil killing capacity against *P. insidiosum* zoospores, neutrophils from six healthy donors were incubated with fresh zoospores from different sources, as mentioned in Table 2. The preliminary results indicated that opsonization with serum enhanced the killing capacity of neutrophils when incubated with zoospores in both MOI 1:1 and 1:10. Therefore, pooled serum from healthy donors was added to the experiments. Colony count determined the neutrophil killing ability after incubation with zoospores compared with live/dead cell staining.

To observe the *P. insidiosum* colonies, suspension of zoospores after incubation with neutrophils was spread on BA (Figure 30, Appendix B). Neutrophils significantly decreased the percentage of colony count when co-incubated with all six strains of *P. insidiosum* zoospores compared to the incubation of zoospores without neutrophils. Treatment of neutrophils with zoospore ATCC 58643, CBS 101039, CBS 777.85, PC10, and ATCC 90586 displayed a higher significantly decreased the survival rate ($p < 0.001$, $n = 6$) than treatment of neutrophils with zoospore PEC1 ($p < 0.01$, $p < 0.05$, $n = 6$), which was observed in both MOI 1:1 and 1:10 (Figure 22). The data of neutrophils incubated with zoospores observed by colony count are summarized in Table 4.

To determine *P. insidiosum* viability under a microscope, zoospores were stained with trypan blue dye to indicate live/dead cells. Neutrophils were lysed with sterile dH_20, and

zoospores were stained with 0.4% trypan blue. Then, unstained zoospores were counted to indicate the viable cells. All six strains of *P. insidiosum* zoospores showed a significantly decreased viability rate when co-incubated with neutrophils, compared with total zoospore count per well ($p < 0.001$, $n = 6$) (Figure 23). The data of zoospore viability observed by live/dead cell staining are summarized in Table 5.

A



B

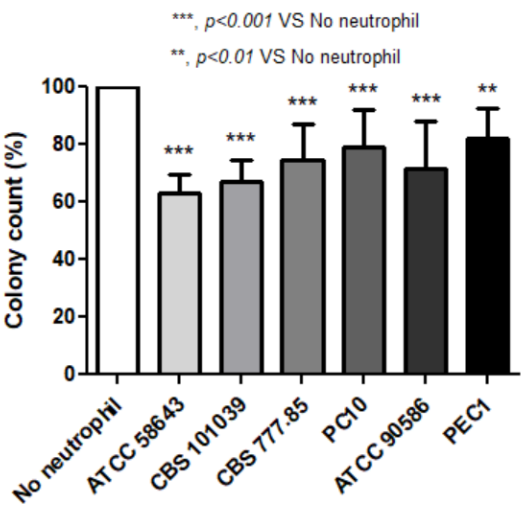
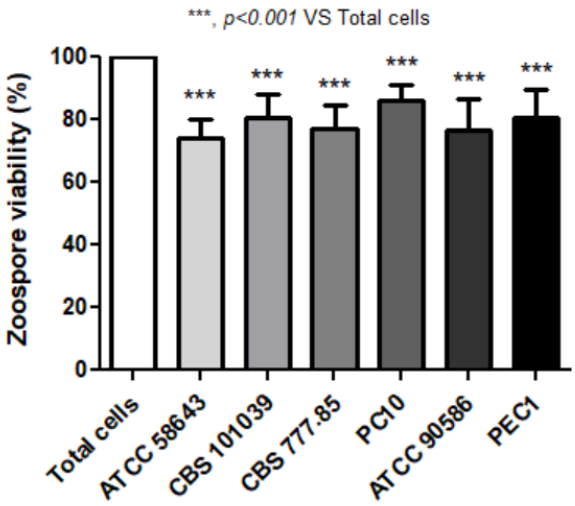


Figure 22 All six strains of *P. insidiosum* zoospores were killed by neutrophils, as observed by colony count.

Co-culture 2.0 × 10^5 serum-opsonized zoospores incubated with 2.0 × 10^5 and 2.0 × 10^4 neutrophils in MOI (neutrophil: zoospore) 1:1 (A) and 1:10 (B), respectively. Zoospore viability was assessed by colony count, compared with the incubation without neutrophils. n = 6. Mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with no neutrophils.

A



B

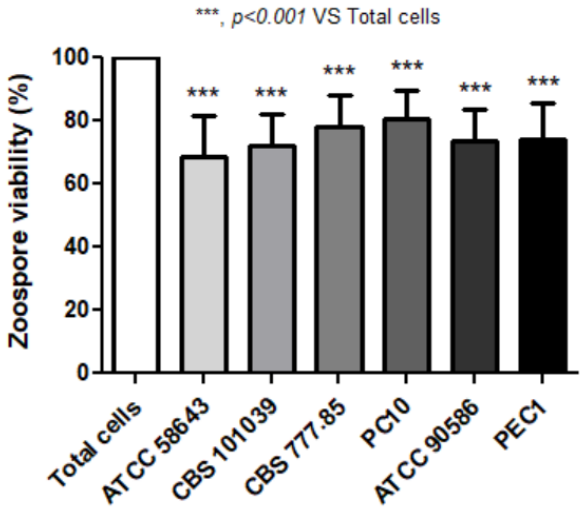


Figure 23 All six strains of *P. insidiosum* zoospores were killed by neutrophils, as observed by live/dead cell staining.

Treatment 2.0 × 10^5 serum-opsonized zoospores incubated with 2.0 × 10^5 and 2.0 × 10^4 neutrophils in MOI (neutrophil: zoospore) 1:1 (A) and 1:10 (B), respectively. After neutrophil lysis, zoospores were stained with trypan blue, and un-stained/lived cells were observed under a light microscope, 400x magnification, calculated relative to total cell count. n = 6. Mean ± SEM. *** $p < 0.001$ compared with total cells.

Table 4 The percentages of colony count, observed in both MOI 1:1 and 1:10

No.	*P. insidiosum* isolates	Mean ± SEM (%)	
		MOI 1:1	MOI 1:10
1	ATCC 58643 (CBS 574.85)	70.7 ± 6.7	63.1 ± 6.3
2	CBS 101039	80.4 ± 6.4	67.2 ± 7.1
3	CBS 777.85	78.2 ± 13.1	74.4 ± 12.6
4	PC10	80.6 ± 9.7	78.9 ± 13.0
5	ATCC 90586	72.4 ± 16.3	71.6 ± 16.5
6	PEC1	85.8 ± 11.7	81.9 ± 10.8

Table 5 Zoospore viability, observed in both MOI 1:1 and 1:10.

No.	*P. insidiosum* isolates	Mean ± SEM (%)	
		MOI 1:1	MOI 1:10
1	ATCC 58643 (CBS 574.85)	74.0 ± 6.1	68.4 ± 13.3
2	CBS 101039	80.3 ± 7.8	71.9 ± 10.4
3	CBS 777.85	77.1 ± 7.7	77.9 ± 10.1
4	PC10	86.1 ± 5.2	80.4 ± 9.2
5	ATCC 90586	76.6 ± 10.2	73.4 ± 10.2
6	PEC1	80.4 ± 9.3	74.3 ± 11.2

Neutrophil phagocytosis

The percentages of neutrophil phagocytosis of *P. insidiosum* zoospores were evaluated by flow cytometry after incubation for 30 min. As described in the methods, neutrophils were labeled with the anti-CD11b antibody observed in the allophycocyanin (APC) channel at 660 nm wavelength. Single cells were gated (Figure 24A), the neutrophil population was determined by forward scatter (FSC-A), and cell size and cell complexity were determined by the side scatter (SSC-A), respectively (Figure 24B). All six strains of heat-killed zoospores were stained with 1 µM pHrodo™ red detected in the phycoerythrin (PE) channel at 575 nm wavelength. Based on the principle, pHrodo™ dye is a non-fluorescent dye outside the cell but fluorescence brightly when pH is dropped from neutral to acidic. Zoospore-phagocytosed neutrophils excited the red dye of pHrodo™ due to an acidified environment inside a neutrophil [136].

The main parameter of interest in the phagocytosis assay was the percentage of PE-positive cells representing the neutrophil population in co-culture conditions. CD11b positive cells were first gated to indicate only neutrophils, and then PE positive cells were determined in neutrophil gating, which was demonstrated by PE fluorescence histogram (Figure 24C). Treatment of the co-culture with cytochalasin D, a negative control, showed the absence of PE fluorescence signals (Figure 24D).

Zoospores induced from type strains, ATCC 58643 and CBS 777.85 of *P. insidiosum* were significantly phagocytosed by neutrophils, compared with negative control ($p < 0.01$, $p < 0.05$, $n = 6$) (Figure 25). In addition, all six strains of zoospore treatment showed significantly decreased phagocytosis activity when compared with *C. albicans* as a positive control ($p < 0.001$, $n = 6$) (Figure 36, Appendix B). The data on neutrophil phagocytosis are summarized in Table 6.

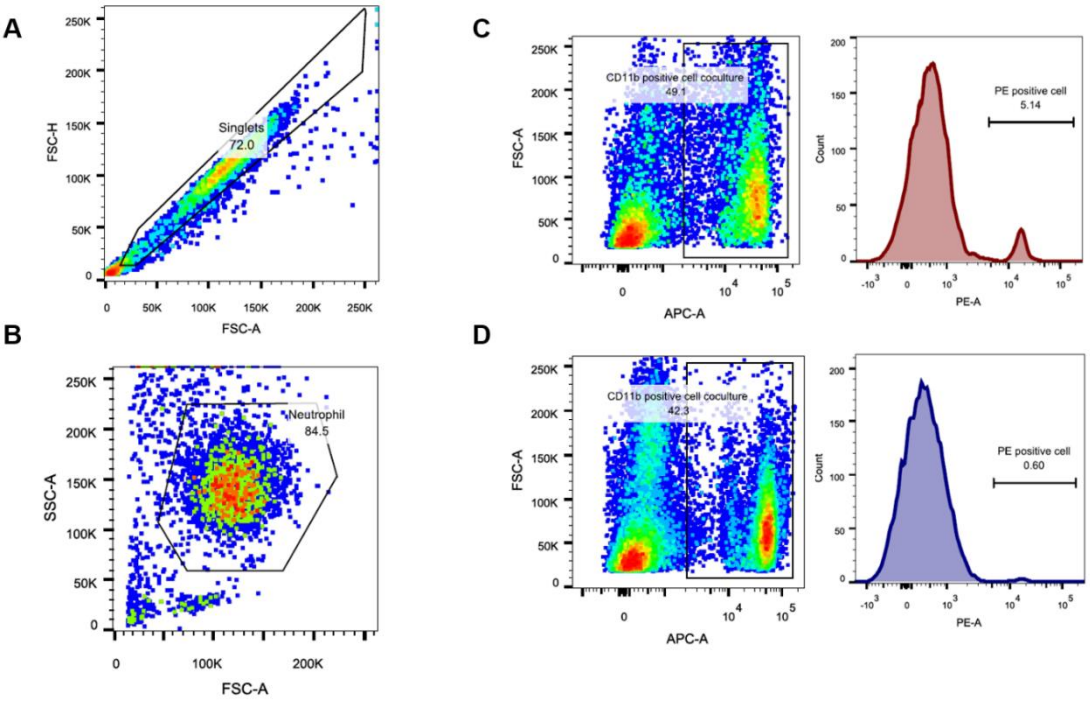


Figure 24 Representative flow cytometry results of neutrophil phagocytosis of *P. insidiosum* zoospores after 30 min incubation.

Gating neutrophils in single cells (A) and neutrophil populations (B). Neutrophil-phagocytosed zoospores were detected by gating neutrophils-labeled anti-CD11b (APC) antibody and selection of PE-positive cells, which demonstrated the peak of PE signals by the histogram (C). PE signals were absent when treated with cytochalasin D as a negative control (D).

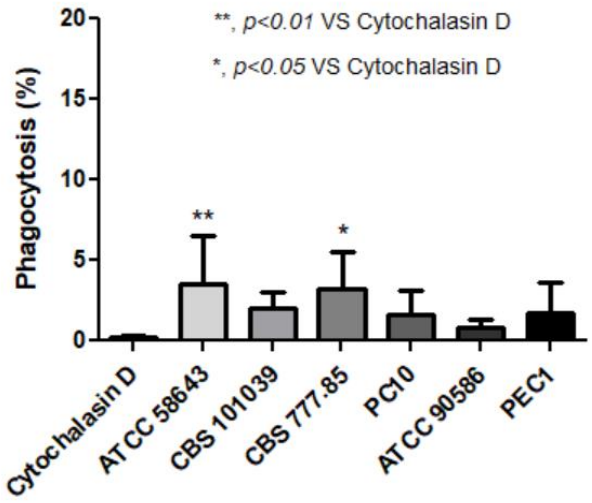


Figure 25 Phagocytosis activity of neutrophils incubated with six strains of *P. insidiosum* zoospores.

Co-culture 2.0×10^5 serum-opsonized zoospores incubated with 2.0×10^5 neutrophils. After 30 min incubation, flow cytometry assessed phagocytosis activity compared with cytochalasin D treatment. $n = 6$. Mean ± SEM. ** $p < 0.01$, * $p < 0.05$ compared with cytochalasin D.

Table 6 Neutrophil phagocytosis of *P. insidiosum* zoospores, measured by flow cytometry

No.	*P. insidiosum* isolates	Mean ± SEM (%)
1	Cytochalasin D (Negative control)	0.2 ± 0.1
2	ATCC 58643 (CBS 574.85)	3.6 ± 3.0
3	CBS 101039	2.0 ± 1.1
4	CBS 777.85	3.2 ± 2.3
5	PC10	1.7 ± 1.4
6	ATCC 90586	0.8 ± 0.5
7	PEC1	1.7 ± 1.8

NET formation

1. Qualification of NET formation

After incubating human neutrophils and *P. insidiosum* zoospores for 2 h, cells were stained and preserved on a slide. The preliminary results showed that neutrophils formed NETs after zoospores CBS 777.85 and CBS 101039 treatments, which were observed under a confocal microscope (630x original magnification). PMA treatment as a positive control resulted in more robust NET-formed neutrophils than treatment with zoospores, which was observed as the DNA web-like structure (blue) with elastase (green) and MPO (red) granules. Whereas unstimulated neutrophils produced a minimal NET structure (Figure 26).

The percentage of NET-released neutrophils was observed after the incubation of *P. insidiosum* zoospores. NETs formed by neutrophils were stained with DAPI and counted per 100 cells using a fluorescence microscope (400x magnification). All six strains of zoospore treatment demonstrated a significantly increased percentage of NET formation compared with negative control ($p < 0.001$ and $p < 0.01$, $n = 6$), which was observed in both MOI 1:1 and 1:10 (Figure 27). The data on *P. insidiosum* zoospore-induced NET formation are summarized in Table 7.

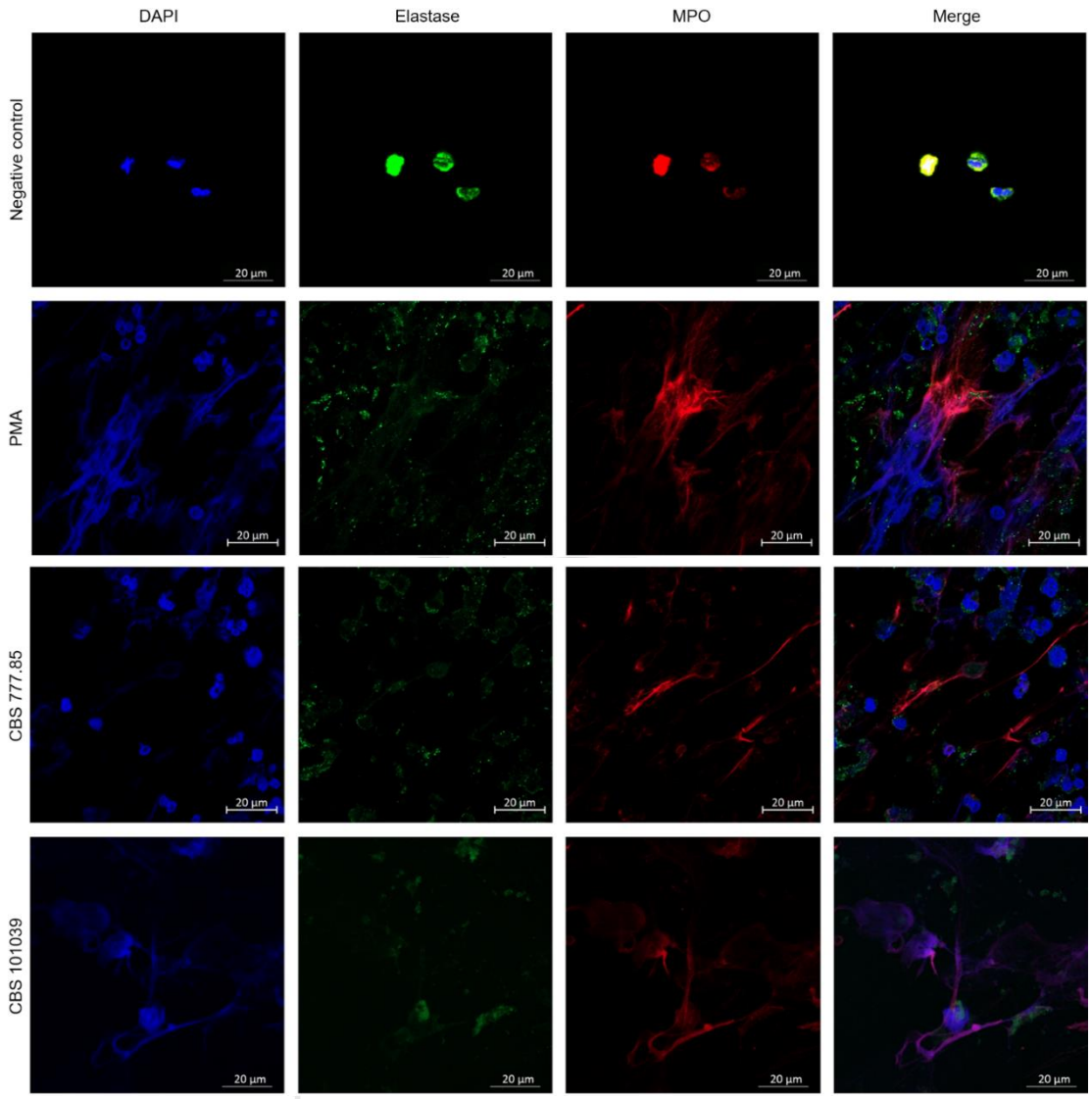
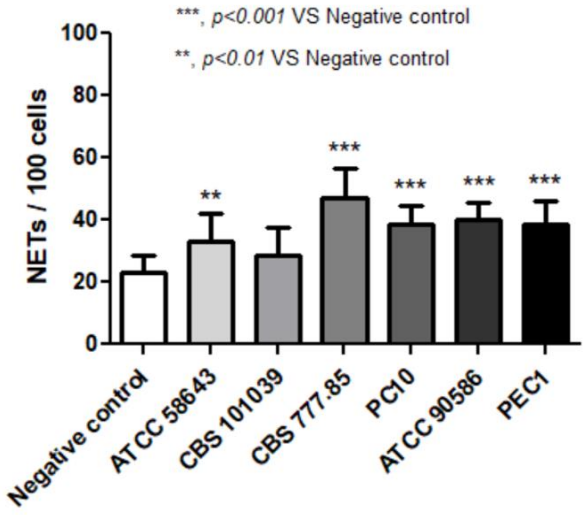


Figure 26 Representative NET formation pictures visualized by immunofluorescence confocal microscope.

Neutrophils isolated from healthy donors (2.0×10^5 cells) were stimulated with 100 ng/ml PMA as a positive control, and zoospore CBS 777.85 and CBS 101039 (2.0×10^5 cells), compared with unstimulated neutrophils as a negative control. The released nucleus from neutrophils was detected by DAPI (blue) and released enzymes of elastase (green) and myeloperoxidase (MPO; red), which was observed under a confocal laser scanning microscope, 630x original magnification.

A



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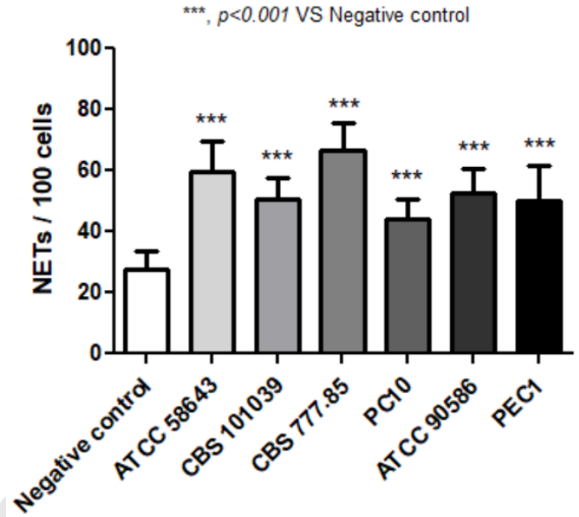


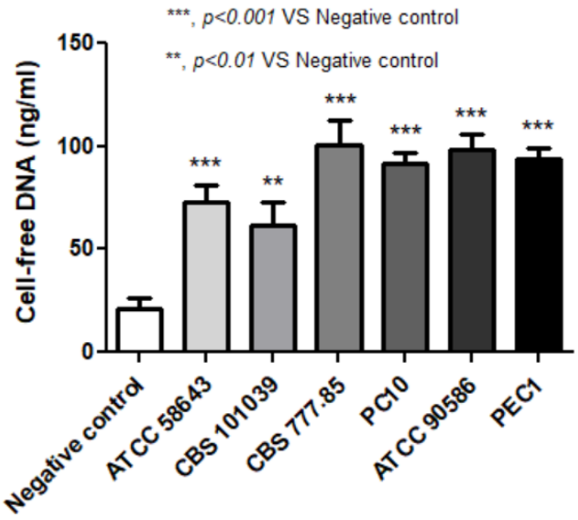
Figure 27 *P. insidiosum* zoospores induced NET formation by human neutrophils

Treatment 2.0 × 10^5 heat-killed zoospores incubated with 2.0 × 10^5 and 2.0 × 10^4 neutrophils in MOI 1:1 (A) and 1:10 (B), respectively. The number of NETs was counted per 100 cells using a fluorescence microscope. n = 6. Mean ± SEM. *** $p < 0.001$ and ** $p < 0.01$ compared with negative control.

2. Quantification of dsDNA levels

After zoospore-induced NETs incubation for 2 h, DNA components released from the nucleus of neutrophils were digested by micrococcal nuclease to cleave the dsDNA contained in the supernatant. Then, the culture medium was aspirated to determine cell-free DNA by Quant-iT^{TM} PicoGreen® assay. The results showed significantly increased levels of dsDNA for all six strains of zoospores compared with unstimulated neutrophils ($p < 0.001$ and $p < 0.01$, $n = 6$), which was observed in both MOI 1:1 and 1:10 (Figure 28). As *P. insidiosum* is a eukaryote, the determination of cell-free DNA released from zoospores was performed. The incubation of heat-killed zoospores without neutrophils showed a minimal concentration of cell-free DNA, which were a range of 0 – 10 ng/ml (data not shown). The data on cell-free DNA levels after *P. insidiosum* zoospore treatment are summarized in Table 8.

A



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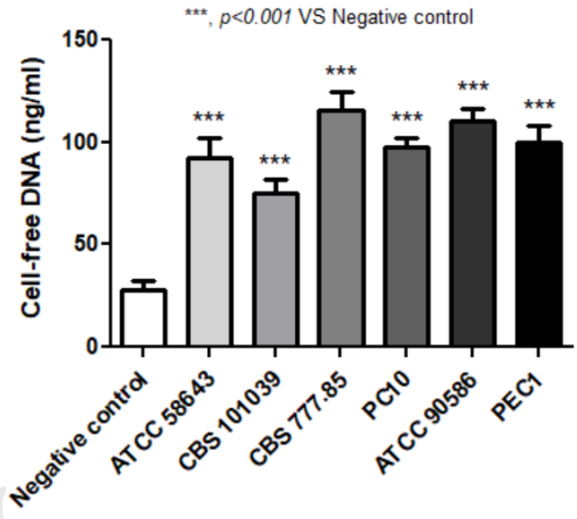


Figure 28 *P. insidiosum* zoospores induced NETs released in culture medium.

Treatment 2.0 × 10^5 heat-killed zoospores incubated with 2.0 × 10^5 and 2.0 × 10^4 neutrophils in MOI 1:1 (A) and 1:10 (B), respectively. The level of cell-free DNA was quantified by Quant-iT™ PicoGreen® assay. n = 6. Mean ± SEM. *** $p < 0.001$ and ** $p < 0.01$ compared with negative control.

Table 7 NET formation detected under a fluorescence microscope

No.	*P. insidiosum* isolates	Mean ± SEM (%)	
		MOI 1:1	MOI 1:10
1	ATCC 58643 (CBS 574.85)	33 ± 9.3	60 ± 10.1
2	CBS 101039	29 ± 8.9	51 ± 6.9
3	CBS 777.85	48 ± 9.3	67 ± 9.2
4	PC10	39 ± 6.3	45 ± 6.4
5	ATCC 90586	41 ± 5.6	53 ± 7.7
6	PEC1	39 ± 7.5	51 ± 11.0
Negative control (neutrophils)		23 ± 5.9	28 ± 5.7
Positive control (PMA)		90 ± 5.2	90 ± 5.8

Table 8 Cell-free DNA in culture medium detected by Quant-iT^{TM} PicoGreen®

No.	*P. insidiosum* isolates	Mean ± SEM (ng/ml)	
		MOI 1:1	MOI 1:10
1	ATCC 58643 (CBS 574.85)	72.70 ± 8.4	92.11 ± 10.2
2	CBS 101039	61.53 ± 11.0	74.78 ± 7.4
3	CBS 777.85	100.80 ± 12.2	115.80 ± 9.0
4	PC10	91.65 ± 4.9	97.26 ± 5.0
5	ATCC 90586	98.73 ± 7.1	110.60 ± 5.7
6	PEC1	93.68 ± 5.4	99.77 ± 8.1
Negative control (neutrophils)		21.12 ± 5.2	27.69 ± 4.8
Positive control (PMA)		201.60 ± 71.8	185.10 ± 26.9

CHAPTER VI DISCUSSION

Pythiosis is a life-threatening fungal infection caused by *P. insidiosum*, an oomycetes fungus-like pathogen. The challenging diagnosis and treatment have been associated with poor prognosis and loss of host organs with a high mortality rate [14]. Evidence has shown that neutrophils are one of the innate immune cells that display essential roles in response to several fungi [19, 22]. The reference studies of neutrophils in response to *P. insidiosum* are scarce. This study demonstrates the *in vitro* neutrophil killing activities against *P. insidiosum* with killing ability, phagocytosis capacity, and NET formation.

As the protocol for neutrophil killing against *P. insidiosum* has been uninvestigated, this study optimized the killing capacity when incubating neutrophils and zoospores (infective stage). The type strain, CBS 777.85 *P. insidiosum,* was randomly selected for the optimization protocol. First, we investigated the role of serum components (opsonin) in enhancing the killing activity of neutrophils, which is called opsonization. Heat-inactivated pooled serum from healthy donors was added to the wells during incubation. Serum-opsonization demonstrated a higher killing capacity of neutrophils than the incubation without serum (Figure 20). This finding indicates that opsonization enhances the killing of *P. insidiosum* by neutrophils. Opsonization is the process of recognizing and targeting invading pathogens, which facilitates phagocytosis [109, 137]. IgG, complement component, and other serum factors are the opsonin that can bind the pathogens and activate phagocytes, including neutrophils, monocytes, and macrophages [138]. However, complements did not appear during the incubation because serum was heated at 56ºC for 30 min for complement inactivation. Thus, natural IgG contained in serum displays as an opsonin in response to *P. insidiosum* zoospores and facilitates neutrophil killing activities.

Next, we investigated neutrophils and zoospores ratio (MOI) for the killing activity of neutrophils. Neutrophils showed an optimal killing capacity when incubated with zoospores in a ratio (neutrophils: zoospores) of 1:1 and 1:10, observed by colony count. No significant difference in the killing capacity between MOI 1:1 and 1:10 was found (Figure 21). Therefore, both neutrophils and zoospores ratios of 1:1 and 1:10 were used in the neutrophil-killing assay.

After optimization, the capacity of neutrophils for *P. insidiosum* elimination was first investigated. The survival rate of zoospores indicated neutrophil killing capacity after co-incubation, evaluated by colony count on BA and live/dead zoospore staining. Trypan blue dye was used to distinguish cell viability under a light microscope. Live cells cannot take up the dye due to the integral cell membrane resulting in unstained cells. The dye can be absorbed in dead cells, displaying blue cells [139]. This assay counted the unstained or viable cells to compare with *P. insidiosum* colonies grown on BA after challenge with neutrophils. All six strains of zoospores demonstrated colony numbers and viable cells of more than 60% in both MOI 1:1 and 1:10 (Figures 22 and 23). These results indicated that neutrophils from healthy donors significantly eliminated the zoospores by around 40%.

A previous study reported that common fungal pathogens, such as *C. albicans,* were killed by human neutrophils [104, 140]. therefore, this study investigated the viability of *C. albicans* and found that neutrophils significantly inhibited yeast by more than 40% when compared with the condition without neutrophils (Figures 31 and 32, Appendix B). These results indicated that the viability of *C. albicans* was much lower than *P. insidiosum* after incubation with neutrophils, which supported that *P. insidiosum* zoospores were hardly eliminated by neutrophils.

One of the important mechanisms of neutrophils in response to invading pathogens is phagocytosis. This study further investigated the phagocytosis of neutrophils in *P. insidiosum*, determined by the percentage of pHrodo-positive cells. Based on the principle, pHrodo fluorescent dye can express brightness in an acidified environment inside phagocytic cells due to the accumulation of phagosomal enzymes and acidic granules [25, 131, 136]. When a neutrophil engulfs a zoospore, the intensity of pHrodo labeled zoospores is increased, detected in the PE channel by a flow cytometer. Zoospore-phagocytosed neutrophils were gated in the neutrophil population that excited the PE positive signals, as demonstrated in the PE fluorescence histogram. Cytochalasin D was chosen as a negative control to inhibit neutrophil phagocytosis. Cytochalasin D is a chemical extract that can bind the plus end of microfilament, which inhibits actin polymerization and blocks cell movement [110]. Incubation

of neutrophils and zoospores treated with cytochalasin D resulted in few PE-positive signals (Figure 24).

Only two strains of *P. insidiosum* showed significantly increased neutrophil phagocytosis: the heat-killed ATCC 58643 and CBS 777.85 *P. insidiosum* zoospores. In the other strains, no significant difference was observed compared with the negative control. These results might indicate that phagocytosis is probably not the primary killing mechanism of *P. insidiosum,* and the phagocytosis capacity of neutrophils might depend on the microbial size. These hypotheses were supported by the determination of neutrophil phagocytosis in *C. albicans.* The results showed that heated-killed yeast-formed *C. albicans* were engulfed by neutrophils as the percentage of phagocytosis was significantly increased compared with the negative control (Figure 36, Appendix B). For fungal phagocytosis, the cell size and shape of engulfed fungi affect the phagocytosis activity of neutrophils [127]. Human neutrophils are 10-15 μm in diameter, while *P. insidiosum* zoospores are 8-12 μm in diameter, larger than the yeast form of *C. albicans* (approximately 2-3 μm in diameter) [141]. Branzk N *et al*. demonstrated that neutrophils could induce phagocytosis of the yeast-locked strain of *C. albicans*, whereas hyphal formed *C. albicans* resulted in NET formation [26]. Moreover, Sulvatori O *et al*. reported hyphal form of C. albicans (length of > 10 μm) reduced the phagocytosis index of human neutrophils [142]. These data support that neutrophil phagocytosis capacity depends on the microbial size. Moreover, neutrophils might release NETs to entrap larger pathogens.

As mentioned above, large-size pathogens affect the phagocytosis activity of neutrophils [127]. Thus, neutrophils may display another important killing mechanism, particularly NETs, in pathogen elimination [22, 122]. This study also evaluated the ability of *P. insidiosum* zoospores to induce NETs. First, zoospores from type strains, CBS 101039 and CBS 777.85 *P. insidiosum,* were randomly selected to evaluate the capacity of zoospores inducing NET formation by immunofluorescence staining. Two strains of selected zoospores showed the activation of neutrophils releasing dsDNA, elastase, and myeloperoxidase (Figure 26). In addition, our qualification and quantification analysis of NET demonstrated that all zoospore isolates significantly induced NET released by neutrophils (Figures 27 and 28),

whereas a negative control showed minimal NETs. These results suggest that neutrophil-activated NETs may display a key mechanism during *P. insidiosum* infection. Many studies have demonstrated that neutrophils release NETs in response to fungi. Qui Liu *et al*. demonstrated that *P. verrucosa* conidia triggered NET and elastase releasing neutrophils [30]. Gazendam *et al*. reported *A. fumigatus* hyphae induced NET but did not have a role in the killing activity, whereas both NADPH oxidase and MPO were required for *Aspergillus* elimination [31]. These data support our result that *P. insidiosum* can induce the production of NET, which is one of the important killing mechanisms of neutrophils.

Neutrophils play not only an important role in the control of infectious diseases but also an essential role in the inflammatory response [19, 21, 22, 25]. Animal pythiosis demonstrates the necrotic mass with a yellowish exudate appearance, which is the unique characteristic of the cutaneous and subcutaneous lesion [7, 9, 78, 80]. Vascular pythiosis manifests arterial occlusion that composed of fungal hyphae, thrombus, and inflammatory cells [68, 69]. These characteristics can be assumed that granulocytes, especially neutrophils, may recruit and demonstrate inflammation during *P. insidiosum* infection. The releasing of several intracellular mediators from NETosis, such as DNA components, histone, proteins and enzymes, toxic substances, etc. displays the important activator in the inflammatory response [111, 121-123]. Nitric oxide (NO) produced from nitric oxide synthase enhanced cell death during inflammation [143]. Histones released from NETs display the cytotoxic component on the lung epithelial cells [144]. Moreover, chronic infection of the lung reveals fibrotic areas, neutrophil proteins, and DNA, which are associated with NET formation [145]. These data suggest that the accumulation of NET mediators can promote inflammatory response in pythiosis.

This is the first study of *in vitro* neutrophil killing activities in response to *P. insidiosum*. Neutrophils from healthy donors showed the release of NETs to entrap zoospores, which were the infective stage induced by various strains of *P. insidiosum.* Nevertheless, these findings could not represent the exact pathogenesis of neutrophils during infection because neutrophils isolated from healthy donors were used in this study. Further study of neutrophils

derived from pythiosis patients is needed to explain the pathogenesis during *P. insidiosum* infection.

CHAPTER VII SUMMARY

This study investigated the *in vitro* killing activities of neutrophils in response to *P. insidiosum*. We demonstrated that zoospores, the infective stage, induced the formation of neutrophil extracellular traps (NETs), observed in both qualification (the percentage of NETs) and quantification (the levels of dsDNA) of the NET assay. After co-incubation with live zoospores, neutrophils from healthy donors showed a zoospore killing capacity of around 40%, observed by the survival rate of zoospores (colony count and lived-cell staining). Moreover, we found that only two strains of six *P. insidiosum* zoospores could induce phagocytosis by neutrophils. Nevertheless, this study first observes neutrophil killing against *P. insidiosum*. Neutrophils isolated from pythiosis patients are needed in future studies.

APPENDIX

APPENDIX A

BUFFERS AND REAGENTS

Reagents for neutrophil isolation

1. RPMI 1640 medium with 10% Fetal bovine serum (FBS)

- RPMI 1640 medium (Gibco, USA)	5.000	ml
- Heat-inactivated FBS (Gibco, USA)	45.000	ml

2. Red cell lysis buffer

- NH_4Cl_2	8.290	g
- $KHCO_3$	1.000	g
- EDTA	0.037	g
- Distilled water (Dw)	1,000.000	ml

Sterilization by autoclave at 121˚C for 15 min

Reagents for zoospore induction

1. Induction medium solution A

- K_2HPO_4	87.090	g
- KH_2PO_4	48.050	g
- $(NH_4)_2HPO_4$	46.040	g
- Dw	500.000	ml

Sterilization by autoclave at 121˚C for 15 min

2. Induction medium solution B

- $MgCl_2 \cdot 6H_2O$	25.420	g
- $CaCl_2$	118.380	g
- Dw	500.000	ml

Sterilization by autoclave at 121˚C for 15 min

3. Working solution of Induction medium

- Solution A	500.000	µl
- Solution B	100.000	µl
- Sterile Dw	1,000.000	ml

4. 1x Phosphate buffered saline (PBS)

- 10x PBS (Biolegend, USA)	100.000	ml
- Dw	900.000	ml

Sterilization by autoclave at 121˚C for 15 min

Reagents for flow cytometry

1. FACs buffer (2% FBS in 1x PBS)

- 1x PBS	49.000	ml
- Heat-inactivated FBS	1.000	ml

Reagents for Quantification of NET formation

1. 0.1 M $CaCl_2$

- $CaCl_2 \cdot 2H_2O$	0.735	g
- Sterile Dw	50.000	ml

2. 0.5 M Ethylenediaminetetraacetic acid (EDTA)

- EDTA	46.750	g
- Sterile Dw	250.000	ml

3. 50 U/ml micrococcal nuclease (CAS: 9013-53-0, Sigma-Aldrich, USA)

- 200 U/ml micrococcal nuclease (stock solution)	250.000	µl
- 1x PBS	750.000	µl

Reagents for immunofluorescence staining

1. 1% Formaldehyde

- 10% formaldehyde	10.000	ml
- 1x PBS	90.000	ml

2. 1X Tris-buffered saline (TBS)

- Tris base (Thermo Fisher scientific, USA)	6.100	g
- NaCl	8.800	g
- Sterile Dw	1,000.000	ml

3. 1X TBS with 0.05% tween 20

- 1x TBS	50.000	ml
- Tween 20	25.000	µl

4. 1xTBS with 2% bovine serum albumin (BSA)

- 1x TBS	50.000	ml
- BSA (Himedia, India)	1.000	g

Culture medium preparation

1. Sabouraud dextrose broth (SDB)

- SDB powder (Oxoid, UK)	30.000	g
- Dw	1,000.000	ml

Sterilization by autoclave at 121˚C for 15 min

2. Corn meal agar (CMA)

- CMA powder (Becton-Dickinson, USA)	17.000	g
- Dw	1,000.000	ml

Sterilization by autoclave at 121˚C for 15 min

APPENDIX B

NEUTROPHIL ISOLATION RESULTS

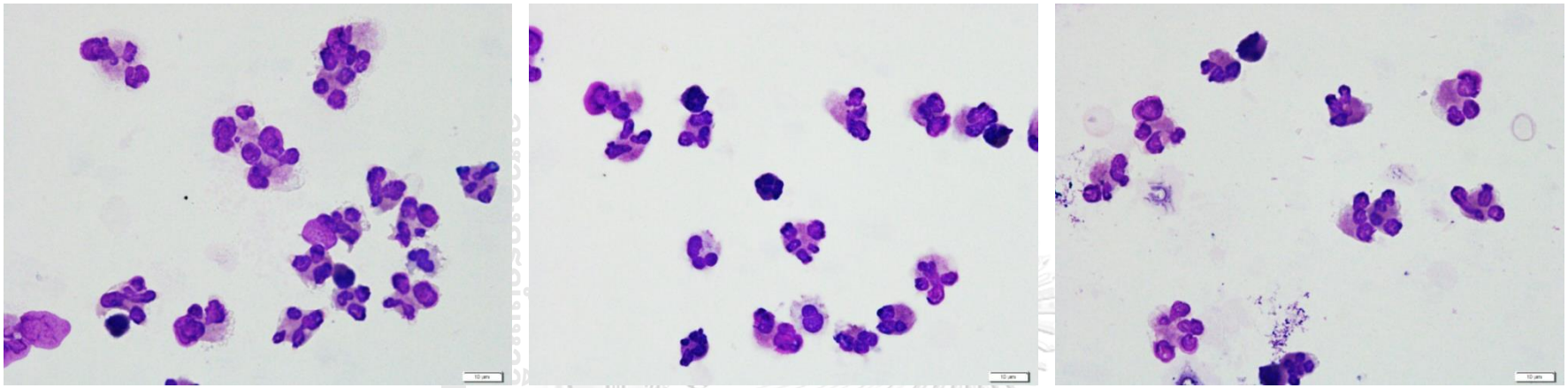


Figure 29 Neutrophil purity measured by Wright-Giemsa staining. Neutrophil-derived from healthy donors were isolated by Polymorphprep™ reagent and then stained with Wright-Giemsa. To observe purity, neutrophils were differentiated per 100 white blood cells under a light microscope, 1,000x magnification.

NEUTROPHIL KILLING RESULTS

Figure 30 Representative pictures of *P. insidiosum* colonies, counted on BA. Co-culture 2.0 × 10^5 serum-opsonized zoospores incubated with 2.0 × 10^5 neutrophils for 2 h. Then, neutrophils were lysed and zoospore suspension was spread on BA to observe the viable colonies. After 15 h of incubation, *P. insidiosum* colonies were counted and the percentage of colony count was compared with the incubation without neutrophils.

Table 9 Raw data of killing assay optimization

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
100: 1	No neutrophil	31	27	29	1,860	1,620	1,740	100.0		
100: 1	1	27	28	24	1,620	1,680	1,440	93.1	96.6	82.8
100: 1	2	25	27	29	1,500	1,620	1,740	86.2	93.1	100.0
10: 1	No neutrophil	45	52	49	13,500	15,600	14,700	100.0		
10: 1	1	40	46	43	12,000	13,800	12,900	82.5	94.5	88.4
10: 1	2	48	45	42	14,400	13,500	12,600	98.6	92.5	86.3
1: 1	No neutrophil	68	71	70	102,000	106,500	105,000	100.0		
1: 1	1	60	55	53	90,000	82,500	79,500	86.1	78.9	76.1
1: 1	2	49	59	50	73,500	88,500	75,000	70.3	84.7	71.8
1: 10	No neutrophil	68	71	70	102,000	106,500	105,000	100.0		
1: 10	1	53	59	56	79,500	88,500	84,000	76.1	84.7	80.4
1: 10	2	48	55	53	72,000	82,500	79,500	68.9	78.9	76.1

Table 10 Neutrophil killing activity comparison between opsonization VS un-opsonization

Condition (Neutrophil: zoospore)		Samples	Colony count (colonies)			Average colony calculation (CFU/ml)			Colony count (%)		
No neutrophil			70	68	66	105,000	102,000	99,000	100.0		
Opsonization	1: 1	1	49	53	51	73,500	79,500	76,500	72.1	77.9	75.0
	1: 1	2	45	42	47	67,500	63,000	70,500	66.2	61.8	69.1
	1: 10	1	58	53	49	87,000	79,500	73,500	85.3	77.9	72.1
	1: 10	2	51	55	50	76,500	82,500	75,000	75.0	80.9	73.5
Un-opsonization	1: 1	1	58	60	57	87,000	90,000	85,500	85.3	88.2	83.8
	1: 1	2	63	59	56	94,500	88,500	84,000	92.6	86.8	82.4
	1: 10	1	63	66	62	94,500	99,000	93,000	92.6	97.1	91.2
	1: 10	2	57	62	64	85,500	93,000	96,000	83.8	91.2	94.1

Table 11 Raw data of neutrophil killing against *P. insidiosum* ATCC 58643 (CBS 574.85), observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		69	72	74	103,500	108,000	111,000	100.0		
1: 1	1	53	48	51	79,500	72,000	76,500	74.0	67.0	71.2
1: 1	2	49	57	47	73,500	85,500	70,500	68.4	79.5	65.6
1: 1	3	51	50	43	76,500	75,000	64,500	71.2	69.8	60.0
1: 1	4	60	57	55	90,000	85,500	82,500	83.7	79.5	76.7
1: 1	5	49	53	41	73,500	79,500	61,500	68.4	74.0	57.2
1: 1	6	52	47	49	78,000	70,500	73,500	72.6	65.6	68.4
1: 10	1	51	40	48	76,500	60,000	72,000	71.2	55.8	67.0
1: 10	2	43	39	51	64,500	58,500	76,500	60.0	54.4	71.2
1: 10	3	43	51	44	64,500	76,500	66,000	60.0	71.2	61.4
1: 10	4	40	51	39	60,000	76,500	58,500	55.8	71.2	54.4
1: 10	5	48	45	50	72,000	67,500	75,000	67.0	62.8	69.8
1: 10	6	50	41	47	75,000	61,500	70,500	59.8	57.2	65.6

Table 12 Raw data of neutrophil killing against *P. insidiosum* CBS 101039, observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		63	71	67	94,500	106,500	100,500	100.0		
1: 1	1	49	53	51	73,500	79,500	76,500	73.1	79.1	76.1
1: 1	2	60	51	58	90,000	76,500	87,000	89.6	76.1	86.6
1: 1	3	60	57	55	90,000	85,500	82,500	89.6	85.1	82.1
1: 1	4	61	48	53	91,500	72,000	79,500	91.0	71.6	79.1
1: 1	5	55	52	48	82,500	78,000	72,000	82.1	77.6	71.6
1: 1	6	58	52	49	87,000	78,000	73,500	86.6	77.6	73.1
1: 10	1	42	45	49	63,000	67,500	73,500	62.7	67.2	73.1
1: 10	2	45	39	47	67,500	58,500	70,500	67.2	58.2	70.1
1: 10	3	53	47	42	79,500	70,500	63,000	79.1	70.1	62.7
1: 10	4	49	51	39	73,500	76,500	58,500	73.1	76.1	58.2
1: 10	5	51	37	46	76,500	55,500	69,000	76.1	55.2	68.7
1: 10	6	38	47	44	57,000	70,500	66,000	56.7	70.1	65.7

Table 13 Raw data of neutrophil killing against *P. insidiosum* CBS 777.85, observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		75	63	69	112,500	94,500	103,500	100.0		
1: 1	1	66	57	60	99,000	85,500	90,000	95.7	82.6	87.0
1: 1	2	62	50	68	93,000	75,000	102,000	89.9	72.5	98.6
1: 1	3	63	38	49	94,500	57,000	73,500	91.3	55.1	71.0
1: 1	4	57	61	47	85,500	91,500	70,500	82.6	88.4	68.1
1: 1	5	48	52	58	72,000	78,000	87,000	69.6	75.4	84.1
1: 1	6	51	35	49	76,500	52,500	73,500	73.9	50.7	71.0
1: 10	1	70	47	53	105,000	70,500	79,500	100.0	68.1	76.8
1: 10	2	53	55	60	79,500	82,500	90,000	76.3	79.7	87.0
1: 10	3	67	49	55	100,500	73,500	82,500	97.1	71.0	79.7
1: 10	4	43	52	49	64,500	78,000	73,500	62.3	75.4	71.0
1: 10	5	49	31	52	73,500	46,500	78,000	71.0	44.9	75.4
1: 10	6	43	50	47	46,500	75,000	70,500	62.3	72.5	68.1

Table 14 Raw data of neutrophil killing against *P. insidiosum* PC10, observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		81	53	66	121,500	79,500	99,000	100.0		
1: 1	1	51	60	57	76,500	90,000	85,500	76.5	90.0	85.5
1: 1	2	63	47	59	94,500	70,500	88,500	94.5	70.5	88.5
1: 1	3	57	45	48	85,500	67,500	72,000	85.5	67.5	72.0
1: 1	4	65	51	60	97,500	76,500	90,000	97.5	76.5	90.0
1: 1	5	50	57	49	75,000	85,500	73,500	75.0	85.5	73.5
1: 1	6	50	56	42	75,000	84,000	63,000	75.0	84.0	63.0
1: 10	1	49	64	60	73,500	96,000	90,000	73.5	96.0	90.0
1: 10	2	41	48	53	61,500	72,000	79,500	61.5	72.0	79.5
1: 10	3	49	72	51	73,500	108,000	76,500	73.5	100.0	76.5
1: 10	4	67	45	49	100,500	67,500	73,500	100.0	67.5	73.5
1: 10	5	43	70	55	64,500	105,000	82,500	64.5	100.0	82.5
1: 10	6	45	43	52	67,500	64,500	78,000	67.5	64.5	78.0

Table 15 Raw data of neutrophil killing against *P. insidiosum* ATCC 90586, observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		61	75	70	91,500	112,500	105,000	100.0		
1: 1	1	33	48	51	49,500	72,000	76,500	48.1	69.9	74.3
1: 1	2	32	36	49	48,000	54,000	73,500	46.6	52.4	71.4
1: 1	3	56	60	38	84,000	90,000	57,000	81.6	87.4	55.3
1: 1	4	64	70	56	96,000	105,000	84,000	93.2	100.0	81.6
1: 1	5	51	63	55	76,500	94,500	82,500	74.3	91.7	80.1
1: 1	6	34	51	49	51,000	76,500	73,500	49.5	74.3	71.4
1: 10	1	60	31	51	90,000	46,500	76,500	87.4	45.1	74.3
1: 10	2	37	52	49	55,500	78,000	73,500	53.9	75.7	71.4
1: 10	3	51	36	44	76,500	54,000	66,000	74.3	52.4	64.1
1: 10	4	63	53	47	94,500	79,500	70,500	91.7	77.2	86.4
1: 10	5	67	64	49	100,500	96,000	73,500	97.6	93.2	71.4
1: 10	6	30	37	51	45,000	55,500	76,500	43.7	53.9	74.3

Table 16 Raw data of neutrophil killing against *P. insidiosum* PEC1, observed by colony count

Condition (neutrophil: zoospore)	Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil		73	77	65	109,500	115,500	97,500	100.0		
1: 1	1	70	51	60	105,000	76,500	90,000	97.7	71.2	83.7
1: 1	2	71	76	58	106,500	114,000	87,000	99.1	100.0	80.9
1: 1	3	58	70	60	87,000	105,000	90,000	80.9	97.7	83.7
1: 1	4	70	53	65	105,000	79,500	97,500	97.7	74.0	90.7
1: 1	5	45	66	58	67,500	99,000	87,000	62.8	92.1	80.9
1: 1	6	50	73	58	75,000	109,500	87,000	69.8	100.0	80.9
1: 10	1	59	47	60	88,500	70,500	90,000	82.3	65.5	83.7
1: 10	2	68	57	63	102,000	85,500	94,500	94.9	79.5	87.9
1: 10	3	43	59	57	64,500	88,500	85,500	60.0	82.3	79.5
1: 10	4	70	63	55	105,000	94,500	82,500	97.7	87.9	76.7
1: 10	5	68	63	59	102,000	94,500	88,500	94.9	87.9	82.3
1: 10	6	67	52	47	100,500	78,000	70,500	93.5	72.6	65.6

Table 17 Raw data of neutrophil killing against *C. albicans* ATCC 14053, observed by colony count

Samples	Colony count (colonies)			Colony calculation (CFU/ml)			Colony count (%)		
No neutrophil	101	89	112	151,500	133,500	168,000	100.0		
1	60	44	48	90,000	66,000	72,000	59.6	43.7	47.7
2	62	50	55	93,000	75,000	82,500	61.6	49.7	54.6
3	54	62	78	81,000	93,000	117,000	53.6	61.6	77.5
4	63	68	51	94,500	102,000	76,500	62.6	67.5	50.7
5	66	64	73	99,000	96,000	109,500	65.6	63.6	72.5
6	61	58	55	91,500	87,000	82,500	60.6	57.6	54.6

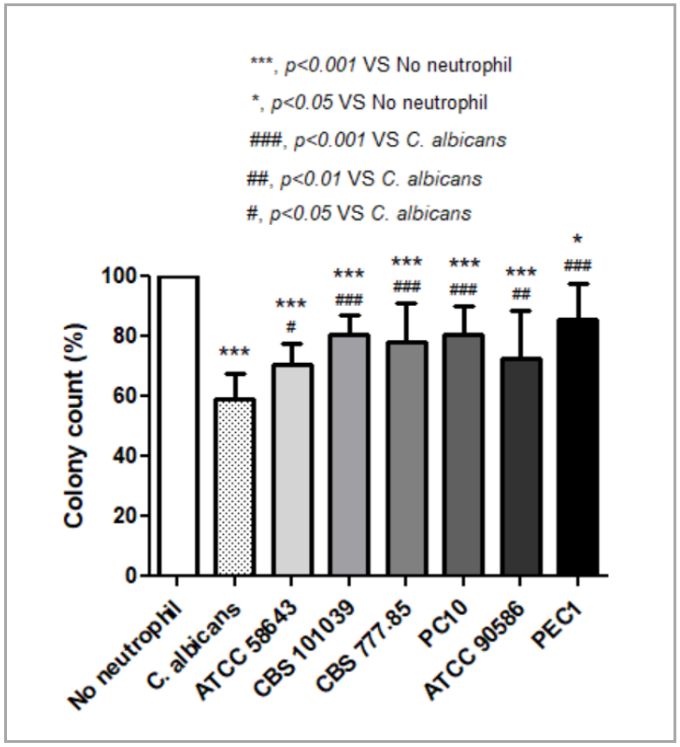


Figure 31 *C. albicans* and all 6 strains of *P. insidiosum* zoospores were killed by neutrophils, as observed by colony count. Co-culture 2.0×10^5 serum-opsonized microorganisms incubated with 2.0×10^5 neutrophils in MOI (neutrophil: microorganisms) 1:1. Fungal viability was assessed by colony count, compared with the same strain without neutrophils. $n = 6$. Mean ± SEM. *** $p < 0.001$, and * $p < 0.05$ compared with no neutrophils; ### $p < 0.001$, ## $p < 0.01$, and # $p < 0.05$ compared with *C. albicans*.

Table 18 Raw data of neutrophil killing against *P. insidiosum* ATCC 58643 (CBS 574.85), measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	7.5	6.4	6.9	5.6	4.6	5.1	74.7	71.9	73.9
1: 1	2	6.4	8.4	7.6	4.9	6.5	5.0	76.6	77.4	65.8
1: 1	3	6.6	6.8	5.3	5.0	4.9	3.5	75.8	72.1	66.0
1: 1	4	6.1	6.0	4.6	5.0	4.1	3.6	82.0	68.3	78.3
1: 1	5	5.6	7.1	6.4	4.0	5.1	5.3	71.4	71.8	82.8
1: 1	6	5.9	6.1	6.0	5.1	3.9	4.4	86.4	63.9	73.3
1: 10	1	6.1	4.4	5.9	4.5	2.6	3.8	73.8	59.1	64.4
1: 10	2	6.3	6.0	5.6	5.3	3.6	3.1	84.1	60.0	55.4
1: 10	3	7.9	6.4	6.1	6.1	3.8	3.8	77.2	59.4	62.3
1: 10	4	5.6	4.8	5.1	5.0	2.5	2.9	89.3	52.1	56.9
1: 10	5	4.6	5.3	5.6	4.1	2.9	3.4	89.1	54.7	60.7
1: 10	6	5.9	6.3	6.0	5.3	3.9	4.9	89.8	61.9	81.7

Table 19 Raw data of neutrophil killing against *P. insidiosum* CBS 101039, measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	5.8	6.0	6.4	5.6	5.0	5.3	96.6	83.3	82.8
1: 1	2	7.1	7.5	6.6	5.8	5.1	5.0	81.7	68.0	75.8
1: 1	3	6.4	6.1	5.9	5.9	4.9	4.5	92.2	80.3	76.3
1: 1	4	4.4	5.0	4.0	3.4	3.9	2.6	77.3	78.0	65.0
1: 1	5	7.2	6.5	6.0	6.4	5.6	5.0	88.9	86.2	83.3
1: 1	6	3.5	4.9	5.1	2.6	3.8	4.0	74.3	77.6	78.4
1: 10	1	6.6	5.9	6.5	5.6	4.1	4.3	84.8	69.5	66.2
1: 10	2	6.1	6.3	4.9	5.3	5.3	2.9	86.9	84.1	59.2
1: 10	3	4.0	4.9	4.6	2.4	3.9	3.6	60.0	79.6	78.3
1: 10	4	5.4	6.3	5.6	3.4	4.8	3.4	63.0	76.2	60.7
1: 10	5	6.4	6.1	6.6	5.4	3.1	4.8	84.4	50.8	72.7
1: 10	6	4.6	5.4	5.6	3.5	3.9	3.9	76.1	72.2	69.6

Table 20 Raw data of neutrophil killing against *P. insidiosum* CBS 777.85, measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	5.1	4.1	4.4	3.4	3.6	2.8	66.7	87.8	63.6
1: 1	2	3.4	4.8	4.5	2.6	3.8	3.6	76.5	79.2	80.0
1: 1	3	4.4	6.1	3.4	3.0	5.3	2.4	68.2	86.9	70.6
1: 1	4	5.4	3.4	3.9	4.6	2.4	3.5	85.2	70.6	89.7
1: 1	5	4.8	5.6	4.0	3.6	4.1	3.4	75.0	73.2	85.0
1: 1	6	4.4	2.9	5.4	3.4	2.3	3.9	77.3	79.3	72.2
1: 10	1	2.4	4.6	4.9	2.0	3.9	3.6	83.3	84.8	73.5
1: 10	2	4.4	5.1	2.6	3.5	2.6	2.3	79.5	51.0	88.5
1: 10	3	4.8	2.6	6.3	3.8	2.3	5.5	79.2	88.5	87.3
1: 10	4	3.1	4.5	5.1	2.4	3.8	3.8	77.4	84.4	74.5
1: 10	5	5.6	5.3	3.4	4.9	4.1	2.0	87.5	77.4	58.8
1: 10	6	3.1	2.6	4.1	2.5	2.0	2.8	80.6	76.9	68.3

Table 21 Raw data of neutrophil killing against *P. insidiosum* PC10, measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	4.4	5.0	4.8	3.4	3.9	3.8	77.3	78.0	79.2
1: 1	2	6.8	6.1	6.4	6.1	5.6	5.9	89.7	91.8	92.2
1: 1	3	7.4	6.5	6.1	6.9	5.9	5.3	93.2	90.8	86.9
1: 1	4	5.9	4.6	5.6	4.9	3.6	5.0	83.1	78.3	89.3
1: 1	5	6.9	7.6	6.6	5.9	6.5	5.9	85.5	85.5	89.4
1: 1	6	6.4	5.9	5.3	5.4	5.0	4.8	84.4	84.7	90.6
1: 10	1	5.3	5.6	6.0	4.0	4.1	3.9	75.5	73.2	65.0
1: 10	2	5.6	6.1	5.3	5.1	4.8	4.9	91.1	78.7	92.5
1: 10	3	6.1	6.3	5.9	4.5	3.9	5.1	73.8	61.9	86.4
1: 10	4	6.3	7.1	5.6	4.3	6.1	4.9	68.3	85.9	87.5
1: 10	5	6.4	6.6	6.1	5.4	6.0	5.4	84.4	90.9	88.5
1: 10	6	5.3	4.9	4.6	4.5	3.9	3.6	84.9	79.6	78.3

Table 22 Raw data of neutrophil killing against *P. insidiosum* ATCC 90586, measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	6.0	4.0	4.4	4.9	2.6	3.8	81.7	65.0	86.4
1: 1	2	3.6	6.4	4.1	2.3	5.5	3.5	63.9	85.9	85.4
1: 1	3	4.0	3.9	5.8	2.6	3.1	5.0	65.0	79.5	86.2
1: 1	4	4.4	5.1	4.9	3.4	4.1	3.4	77.3	80.4	69.4
1: 1	5	5.3	4.5	4.0	2.6	3.8	3.3	49.1	84.4	82.5
1: 1	6	4.8	4.4	6.6	3.8	3.4	5.3	79.2	77.3	80.3
1: 10	1	4.9	6.4	5.1	2.6	5.4	4.1	53.1	84.4	80.4
1: 10	2	4.0	3.4	2.9	2.6	2.3	2.3	65.0	67.6	79.3
1: 10	3	5.6	4.1	5.3	4.9	3.4	3.8	87.5	82.9	71.7
1: 10	4	5.4	3.9	4.4	3.4	2.8	3.5	63.0	71.8	79.5
1: 10	5	5.3	6.3	4.5	4.6	5.0	2.6	86.8	79.4	57.8
1: 10	6	3.5	2.6	4.4	2.5	1.6	3.4	71.4	61.5	77.3

Table 23 Raw data of neutrophil killing against *P. insidiosum* PEC1, measured by live/dead cell staining

Condition (neutrophil: zoospore)	Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Zoospore viability (%)		
1: 1	1	4.9	3.5	5.1	4.4	2.4	4.9	89.8	68.6	96.1
1: 1	2	5.0	4.5	3.4	3.8	4.1	2.5	76.0	91.1	73.5
1: 1	3	4.6	6.4	5.6	3.6	5.1	4.3	78.3	79.7	76.8
1: 1	4	3.9	5.3	4.4	3.4	3.9	3.8	87.2	73.6	86.4
1: 1	5	4.8	4.1	6.0	3.8	3.3	5.3	79.2	80.5	88.3
1: 1	6	3.6	4.5	2.3	3.1	3.6	1.3	86.1	80.0	56.5
1: 10	1	2.4	3.9	4.4	1.9	2.6	2.9	79.2	66.7	65.9
1: 10	2	5.0	6.9	4.1	3.6	6.1	3.5	72.0	88.4	85.4
1: 10	3	4.4	3.4	5.6	3.6	2.6	4.1	81.8	76.5	73.2
1: 10	4	5.6	4.1	2.3	4.6	3.5	1.4	82.1	85.4	60.9
1: 10	5	5.4	2.1	6.3	4.2	1.6	2.9	77.8	76.2	46.0
1: 10	6	6.6	3.5	2.6	5.9	2.4	1.6	89.4	68.6	61.5

Table 24 Raw data of neutrophil killing against *C. albicans* ATCC 14053, observed by live/dead cell staining

Samples	Total cell count (× 10^4 cells)			Live cells (× 10^4 cells)			Yeast viability (%)		
1	4.8	5.1	5.6	2.8	3.5	3.4	58.3	68.6	60.7
2	5.3	4.4	7.9	3.9	2.4	4.1	73.6	54.5	51.9
3	7.1	6.1	5.6	4.0	3.1	3.4	56.3	50.8	60.7
4	6.0	6.4	5.6	3.4	3.8	2.9	56.7	59.4	51.8
5	7.6	5.6	6.1	4.4	3.9	3.0	57.9	69.6	49.2
6	4.9	5.4	6.0	2.6	3.1	3.0	53.1	57.4	50.0

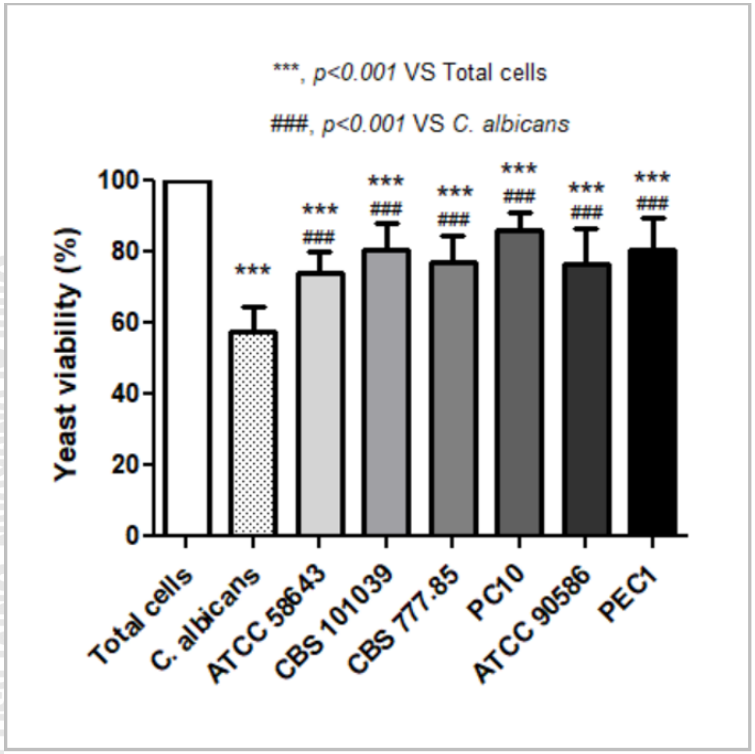


Figure 32 *C. albicans* and all 6 strains of *P. insidiosum* zoospores were killed by neutrophils, as observed by live/dead cell staining. Co-culture 2.0 × 10^5 serum-opsonized microorganisms incubated with 2.0 × 10^5 neutrophils in MOI (neutrophil: microorganisms) 1:1. After neutrophil lysis, microorganisms were stained with trypan blue and observed un-stained/live cells under a light microscope, 400x magnification, calculated relative to total cell count in each microorganism. n = 6. Mean ± SEM. *** $p < 0.001$ compared with Total cells; ### $p < 0.001$ compared with *C. albicans*.

PHAGOCYTOSIS RESULTS

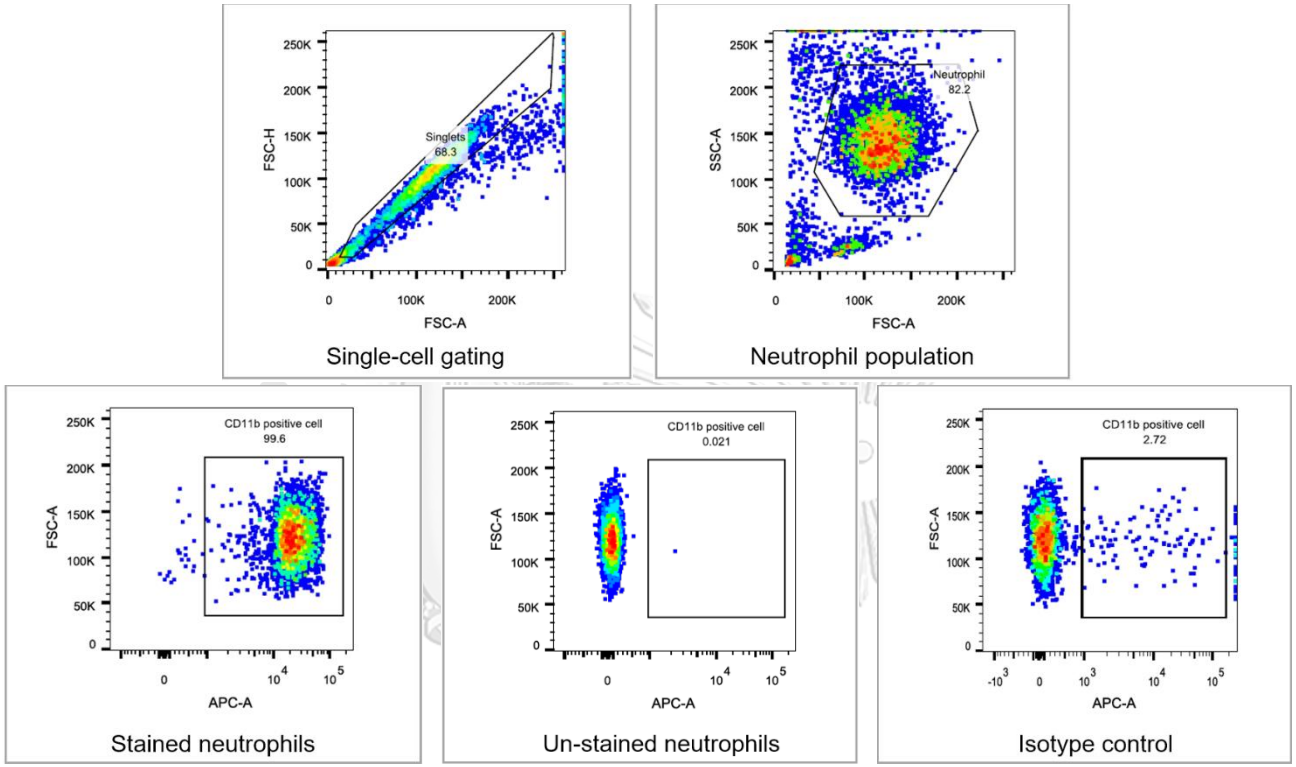


Figure 33 The representative neutrophil population gating. Isolated 2.0 × 10^5 neutrophils were stained with anti-CD11b and anti-IgG2b, **K** to indicate neutrophil surface marker and isotype control, respectively. After that, stained neutrophils were observed in the APC channel, measured by a flow cytometer, compared with un-stained neutrophils. Flow cytometry results were analyzed by FlowJo (Version X 10.0.7).

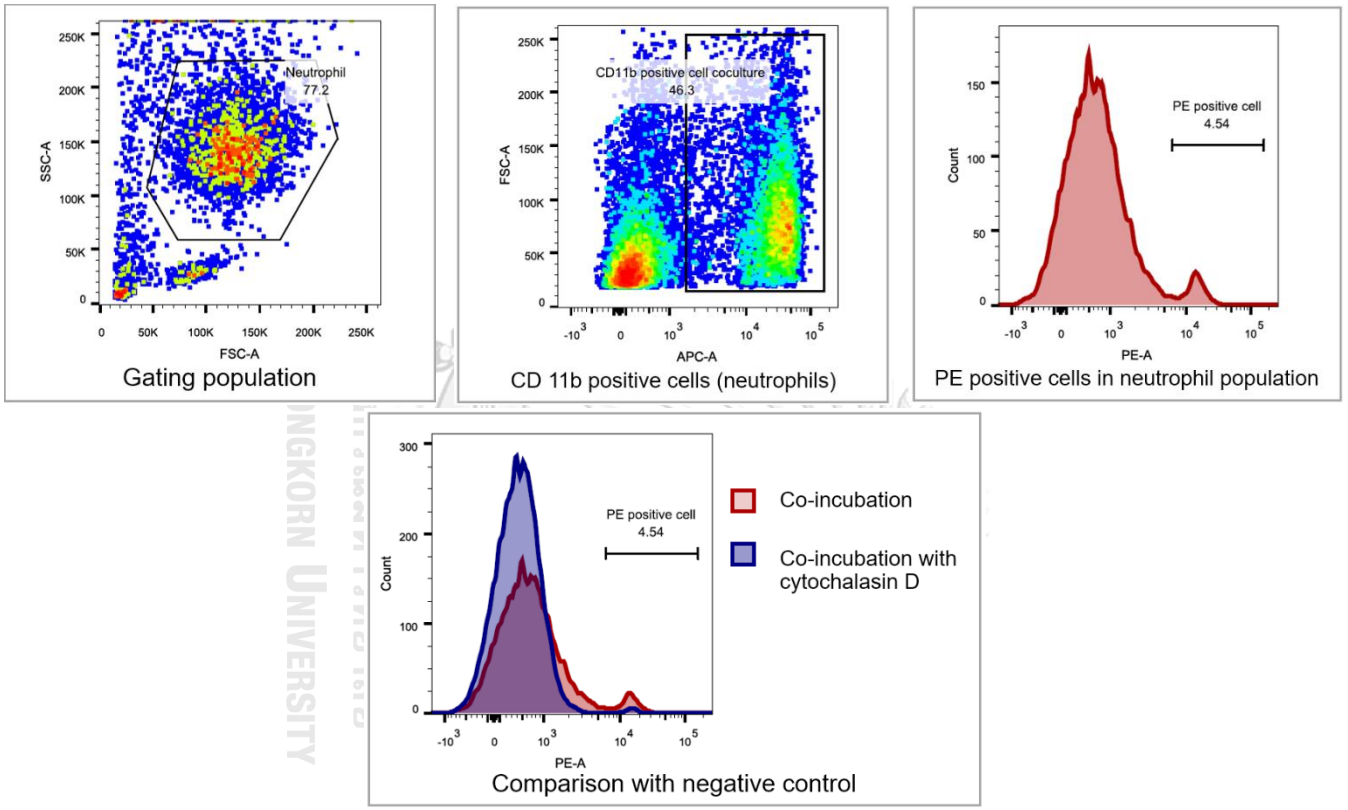


Figure 34 The representative phagocytosis results. Co-culture 2.0 × 10^5 pHrodo labeled zoospores incubated with 2.0 × 10^5 neutrophils for 30 min. After that, neutrophils were stained with anti-CD11b and zoospore-phagocytosed neutrophils were measured by a flow cytometer. The neutrophil population was first gated and PE (pHrodo) positive cells were observed in the neutrophil population to indicate the ability of neutrophil phagocytosis. PE positive signals were shown in histogram (red), compared with negative control (cytochalasin D) (blue).

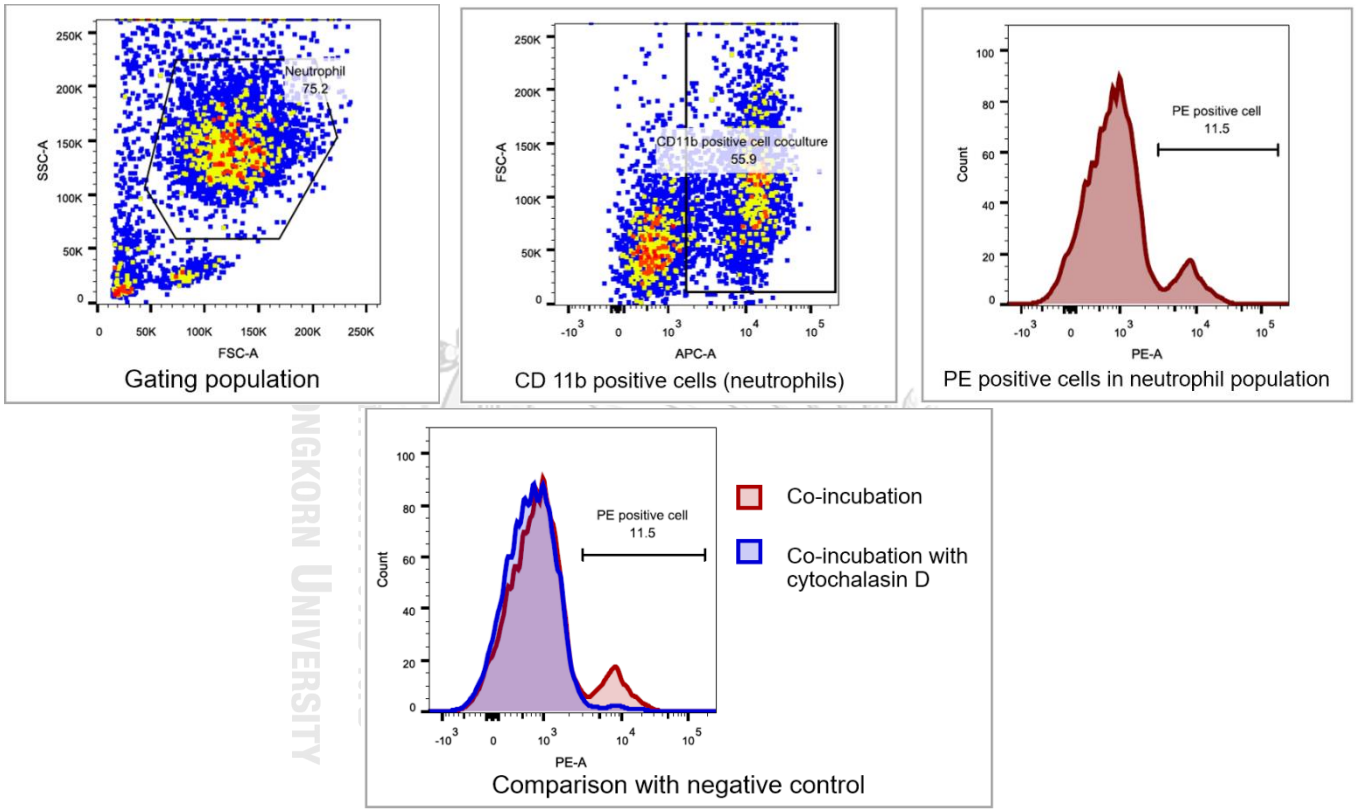


Figure 35 The representative phagocytosis positive control results. Co-culture 2.0 × 10^5 pHrodo labeled *C. albicans* incubated with 2.0 × 10^5 neutrophils for 30 min. After that, neutrophils were stained with anti-CD11b and yeast-phagocytosed neutrophils were measured by a flow cytometer. The neutrophil population was first gated and PE (pHrodo) positive cells were observed in the neutrophil population to indicate the ability of neutrophil phagocytosis. PE positive signals were shown in histogram (red), compared with *C. albicans* negative control (cytochalasin D) (blue).

Table 25 The percentages of neutrophil phagocytosis

No	Samples	Phagocytosis (%)					
		1	2	3	4	5	6
1	Negative control	0.2	0.1	0.3	0.1	0.0	0.3
2	*P. insidiosum* ATCC 58643 (CBS 574.85)	1.5	4.8	1.4	2.8	1.7	9.1
3	*P. insidiosum* CBS 101039	1.3	2.0	1.2	2.4	1.2	3.8
4	*P. insidiosum* CBS 777.85	1.2	5.7	1.8	6.5	1.4	2.8
5	*P. insidiosum* PC10	0.5	1.4	0.4	3.0	0.7	3.9
6	*P. insidiosum* ATCC 90586	0.2	0.9	0.4	1.0	0.5	1.7
7	*P. insidiosum* PEC1	0.3	2.4	0.3	1.8	0.4	5.1
8	*C. albicans*	15.2	13.2	15.7	12.1	11.9	12.8

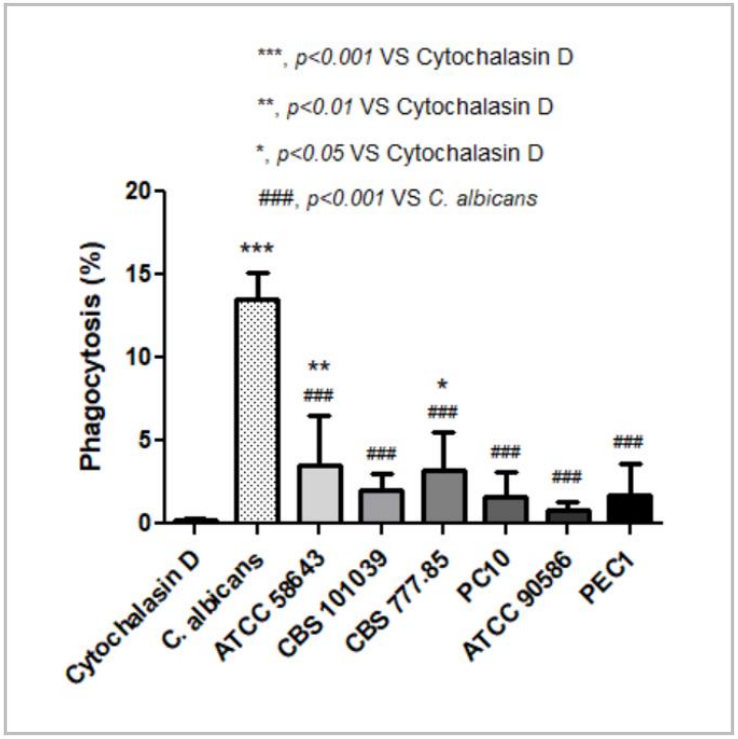
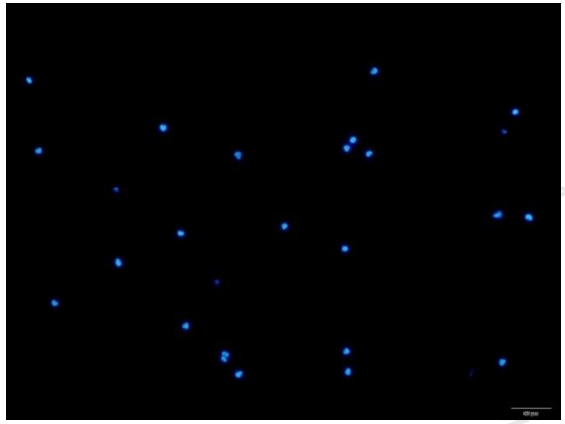


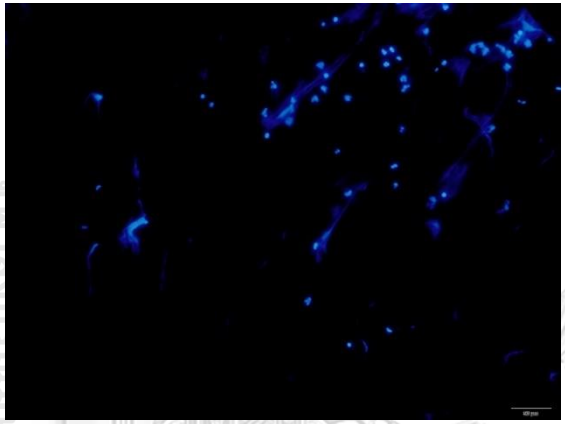
Figure 36 Phagocytosis activity of neutrophils incubated with 6 strains of *P. insidiosum* zoospores and *C. albicans*. Co-culture 2.0×10^5 serum-opsonized zoospores incubated with 2.0×10^5 neutrophils. After 30 min incubation, phagocytosis activity was assessed by flow cytometry, compared with cytochalasin D treatment and *C. albicans* incubation as a negative and positive control, respectively. $n = 6$. Mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with cytochalasin D; ### $p < 0.001$ compared with *C. albicans.*

NET FORMATION RESULTS

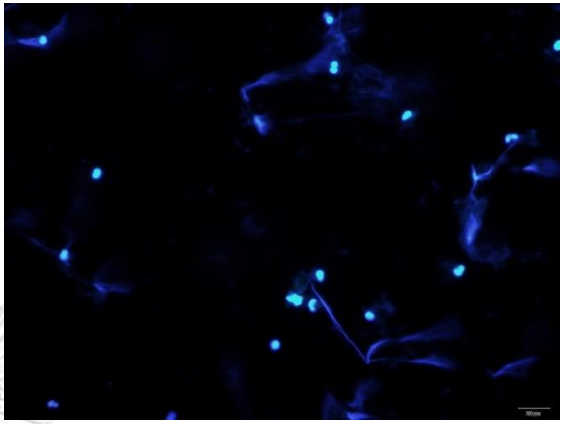
Negative control



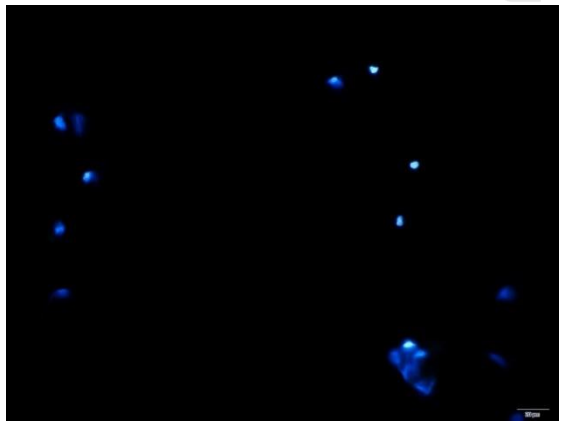
Positive control (PMA)



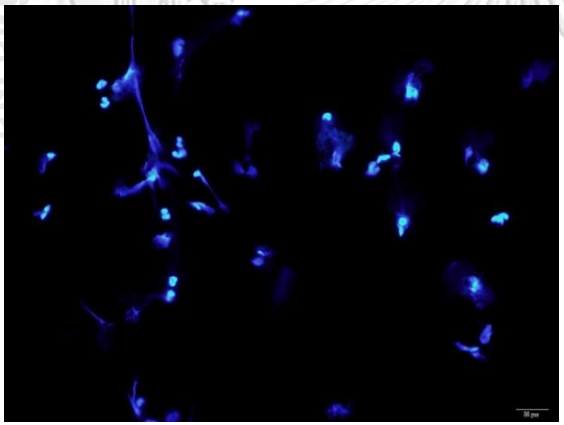
ATCC 58643 (CBS 574.85)



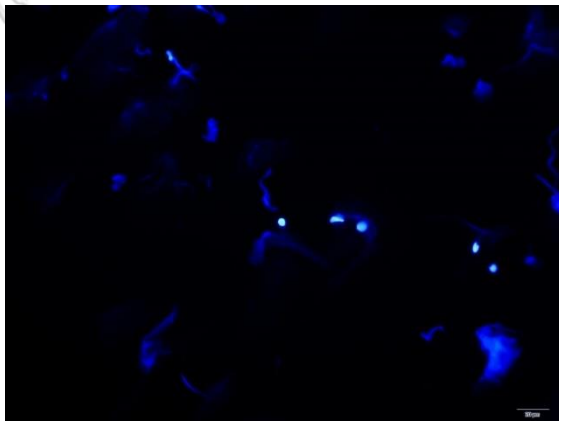
CBS 101039



CBS 777.85



PC10



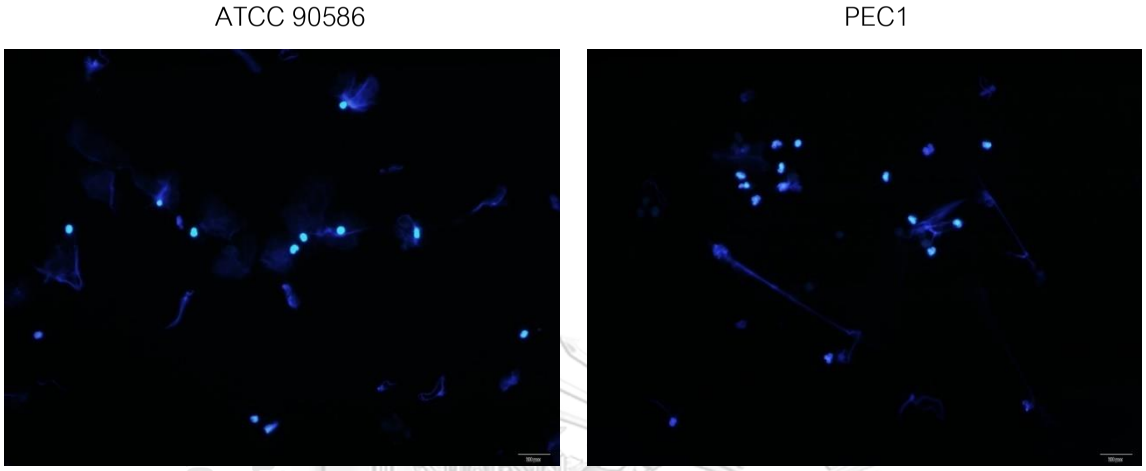
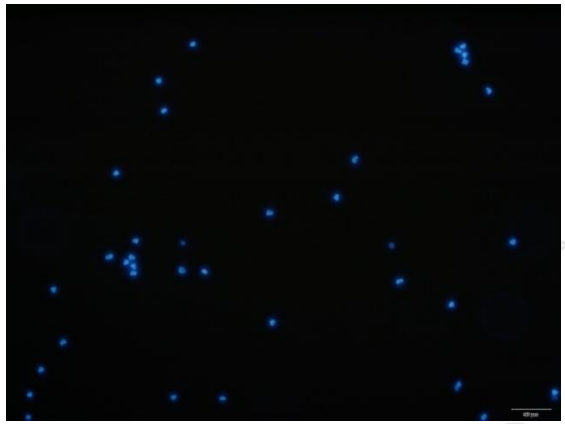


Figure 37 The representative immunofluorescence pictures of NET formation. Treatment 2.0 × 10^5 heat-killed zoospores incubated with 2.0 × 10^5 neutrophils in MOI 1:1. NETs were stained with DAPI to indicate nucleus and released DNA (blue), observed under a fluorescence microscope, 400x magnification.

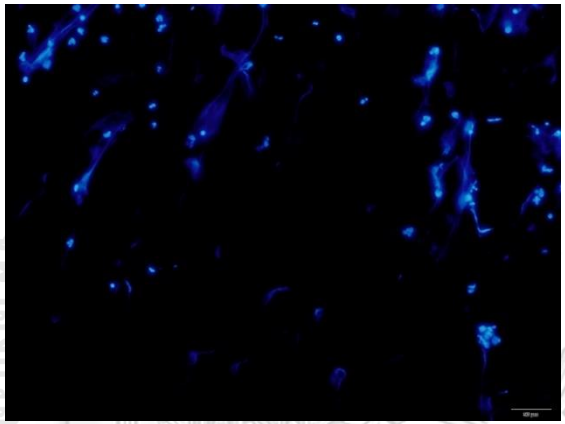
Table 26 The percentages of NET formation MOI 1:1

No	*P. insidiosum* isolate	NET formation (%) n=6																	
		1			2			3			4			5			6		
1	ATCC 58643 (CBS 574.85)	33	55	38	26	31	42	25	27	23	19	37	28	41	28	30	49	33	28
2	CBS 101039	21	29	22	29	20	21	22	19	23	27	50	41	32	28	45	34	27	26
3	CBS 777.85	41	52	38	58	48	55	35	38	49	56	57	33	39	34	61	56	50	48
4	PC10	32	37	35	35	39	41	47	38	39	32	54	41	38	29	31	41	36	47
5	ATCC 90586	48	33	39	49	32	37	42	31	44	41	37	50	38	43	40	43	35	41
6	PEC1	31	41	39	35	38	28	40	39	36	43	60	41	31	45	29	38	47	35
7	Negative control (neutrophils)	19	15	21	28	16	18	22	31	25	30	32	27	25	18	30	22	13	21
8	Positive control (PMA)	94	91	87	93	97	81	97	86	79	95	87	92	89	96	90	85	90	86

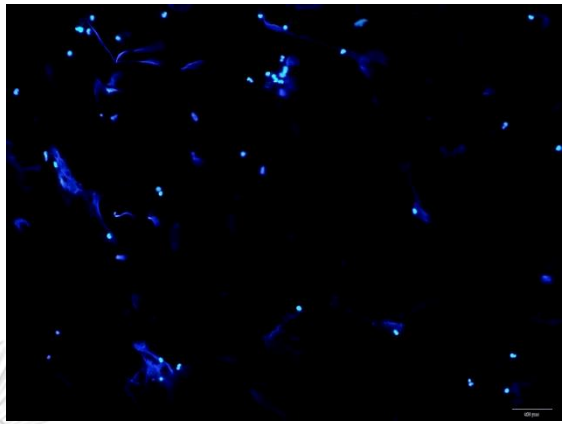
Negative control



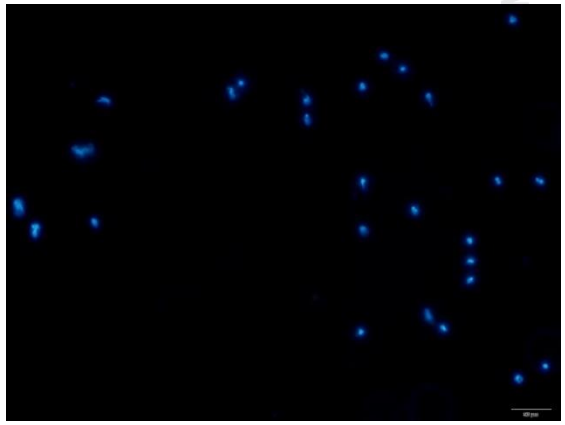
Positive control (PMA)



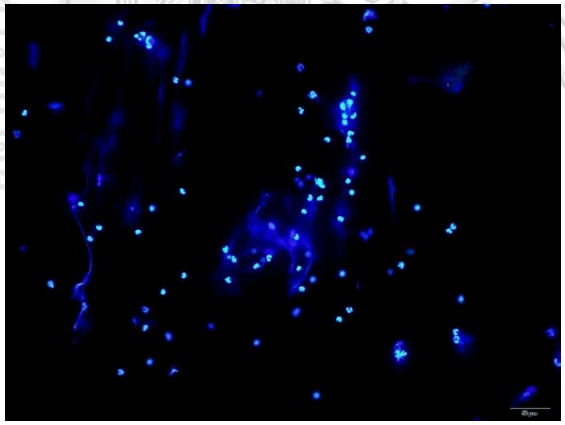
ATCC 58643 (CBS 574.85)



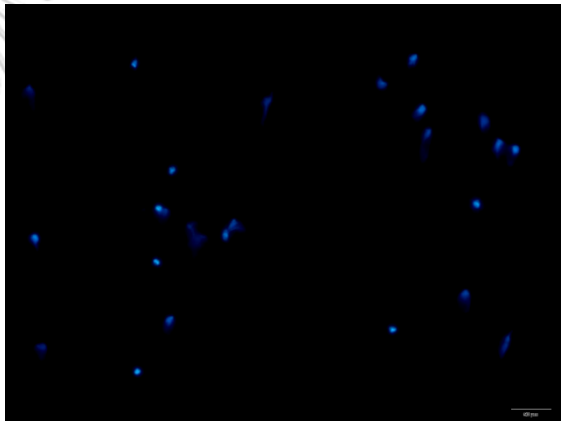
CBS 101039



CBS 777.85



PC10



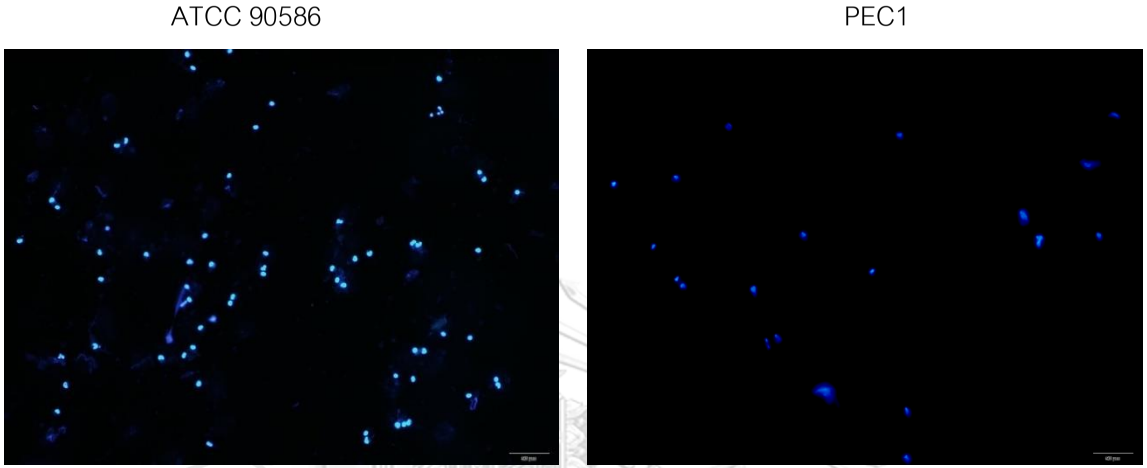


Figure 38 The representative immunofluorescence pictures of NET formation. Treatment 2.0 × 10^5 heat-killed zoospores incubated with 2.0 × 10^4 neutrophils in MOI 1:10. NETs were stained with DAPI to indicate nucleus and released DNA (blue), observed under a fluorescence microscope, 400x magnification.

Table 27 The percentages of NET formation MOI 1:10

No	*P. insidiosum* isolate	NET formation (%) n=6																	
		1			2			3			4			5			6		
1	ATCC 58643 (CBS 574.85)	37	65	49	61	55	70	65	59	45	66	68	71	63	41	69	59	65	61
2	CBS 101039	52	58	39	49	49	55	58	46	60	65	53	48	49	45	52	38	50	49
3	CBS 777.85	77	79	68	65	71	80	66	58	49	65	55	78	72	54	65	66	56	71
4	PC10	43	37	41	50	46	38	47	41	51	61	43	50	33	39	49	42	44	43
5	ATCC 90586	55	59	61	47	51	48	41	51	60	56	58	65	60	40	55	57	39	48
6	PEC1	65	53	39	36	48	51	41	37	57	67	49	70	67	43	44	38	52	49
7	Negative control (neutrophils)	30	28	31	24	32	27	26	16	21	22	28	32	25	21	34	35	39	29
8	Positive control (PMA)	94	91	89	93	97	90	97	86	78	95	87	91	89	96	79	85	90	81

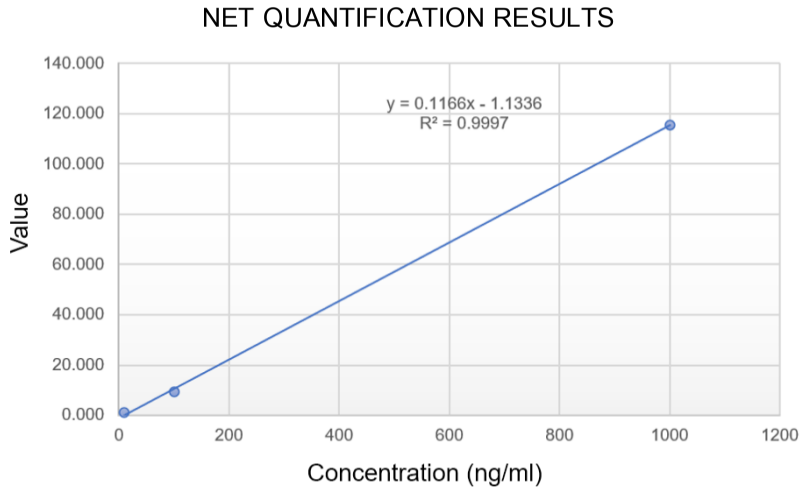


Figure 39 DNA level standard curve for NET quantification. The ten-fold dilution of the DNA standard from Quant-iT™ PicoGreen® kits (1 µg/ml, 100 ng/ml, and 10 ng/ml) was incubated with PicoGreen reagent. The levels of dsDNA were measured by spectrofluorometer. The X-axis represents fluorescent intensity values and Y-axis represents dsDNA concentration (ng/ml)

Table 28 DNA standard concentration for NET quantification

Standard concentration (ng/ml)	Value	Calculated
1,000 (1 µg/ml)	115.6	999.64
100	9.4	81.50
10	1.1	8.98

Table 29 Quantification of NET formation in MOI 1:1

No	*P. insidiosum* isolate	Cell-free DNA level (ng/ml) n=6																	
		1			2			3			4			5			6		
1	ATCC 58643 (CBS 574.85)	70.68	66.65	69.23	76.20	61.25	71.32	61.11	65.28	71.30	76.41	89.04	75.81	85.53	82.24	84.65	64.21	72.40	65.36
2	CBS 101039	43.77	43.41	39.19	70.50	79.93	69.40	64.41	51.78	57.63	67.62	69.32	72.23	64.30	61.02	60.18	61.31	60.22	71.25
3	CBS 777.85	97.05	106.63	92.44	116.13	115.99	123.39	95.42	109.35	104.65	93.38	88.23	72.18	98.74	108.48	97.61	90.11	95.98	108.72
4	PC10	92.45	95.27	86.34	92.56	96.38	86.70	94.72	93.80	101.37	82.64	87.75	89.90	96.00	95.09	93.75	85.02	93.71	86.16
5	ATCC 90586	105.57	96.47	106.86	95.15	96.36	97.96	93.34	93.76	104.89	105.64	103.91	106.19	87.23	82.62	94.83	96.31	105.59	104.39
6	PEC1	99.58	95.48	104.74	91.83	84.04	83.54	95.05	93.59	91.07	94.39	90.21	94.26	98.25	100.41	97.57	88.59	92.01	91.63
7	Negative control (neutrophils)	13.61	21.36	19.75	21.80	18.98	20.75	17.41	27.84	28.91	16.47	10.34	26.69	17.60	26.90	26.18	17.45	21.76	26.28
8	Positive control (PMA)	203.09	200.80	197.05	228.59	247.60	220.20	110.84	109.70	119.40	261.86	284.96	285.53	201.44	199.47	207.30	191.72	170.48	188.02

Table 30 Quantification of NET formation in MOI 1:10

No	*P. insidiosum* isolate	Cell-free DNA level (ng/ml) n=6																	
		1			2			3			4			5			6		
1	ATCC 58643 (CBS 574.85)	75.17	82.13	74.14	90.51	99.76	95.67	89.17	95.54	90.36	94.55	82.45	84.83	90.50	91.24	110.13	110.00	99.86	101.91
2	CBS 101039	82.98	95.35	72.16	78.33	67.49	69.75	69.28	73.91	73.52	71.91	72.19	61.88	78.30	74.32	72.31	83.04	70.04	79.35
3	CBS 777.85	124.38	128.24	118.31	115.36	115.09	126.12	115.63	113.46	112.98	98.74	97.15	104.00	128.45	124.83	114.50	116.57	113.90	116.06
4	PC10	94.52	96.57	95.95	102.71	101.51	97.98	96.65	103.56	95.68	95.21	90.66	109.52	87.85	94.39	98.05	100.00	97.74	92.07
5	ATCC 90586	123.95	115.71	118.29	105.54	106.45	107.82	115.20	107.79	106.69	114.65	106.67	107.01	110.13	115.60	115.01	104.52	105.84	103.73
6	PEC1	106.79	110.96	102.39	94.95	99.78	99.37	108.08	114.01	102.40	113.32	99.86	94.47	91.14	92.73	90.16	90.51	92.43	92.48
7	Negative control (neutrophils)	25.59	29.77	21.79	35.37	23.08	38.42	25.26	28.13	20.81	27.48	25.20	35.60	26.53	31.91	25.27	24.39	26.68	27.20
8	Positive control (PMA)	207.07	188.50	191.54	186.49	197.48	181.67	135.71	124.20	176.00	215.66	220.90	215.90	146.09	167.91	186.60	199.11	197.89	192.40

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