Thesis progression

Thesis title: The impairment of neutrophil functions of Beta-thalassemia in *Pythium insidiosum* infection

Thesis progression title: Role of neutrophils in *P. insidiosum* infection in healthy donors.

Student: Miss Pasinee Sangsiwarit

Student ID: 675070027-5

Advisor: Dr. Pratsanee Hiengrach

Co-advisor: Asst. Prof. Dr. Wisitsak Phoksawat, Assoc. Prof. Dr. Nattiya Teawtrakul

Date: 18th March 2026

---

1. Introduction

Pythium insidiosum is an oomycete, a fungal-like microorganism commonly found in tropical and subtropical swampy regions (Gurnani et al., 2022) It is the causative agent of pythiosis, a life-threatening disease characterized by high morbidity and mortality rates. Pythiosis affects various mammalian hosts, including horses, dogs, and humans. Although it is a rare, non-transmissible condition, it has a high incidence in temperate, subtropical, and tropical regions, especially Thailand.

Most cases of human pythiosis, including cutaneous, ocular, vascular, and disseminated forms, must be recognized in patients with thalassemia hemoglobinopathy syndrome. Interestingly, iron overload conditions, particularly in β-thalassemia patients, impair innate immune cell functions, including macrophages and monocytes, by reducing TNF-α and IFN-γ production (Ud-naen et al., 2019). Similarly, neutrophils in β-thalassemia patients exhibit diminished phagocytosis, chemotaxis, and NET formation.

Previously, many reports showed that immune dysfunctions might result from ferritin-associated iron; however, the precise mechanisms underlying neutrophil dysfunctions in β-thalassemia remain unclear. Hence, this study aims to investigate the role of neutrophils in *P. insidiosum* zoospore infections in β-thalassemia patients.

2. Objective

2.1 To demonstrate the roles of neutrophils against *P. insidiosum* zoospores infection in healthy donors.

3. Materials and methods

3.1Blood collection

Healthy donors aged 18-60 years old will be included in this study. Neutrophils will be isolated from whole blood within 2–3 hours of collection.

3.2 Neutrophil isolation

To separate and purify human neutrophils from whole blood, a standard density gradient separation method using Polymorphprep^{TM} (Axis-shield, Norway). has been published (Mendoza et al., 1988).

3.3 *Pythium* zoospore production

P. insidiosum, isolated from human pythiosis, will be grown in Sabouraud dextrose broth (SDB, Oxoid, UK) overnight. The microorganism will be cultured on sterile blanket grass (*Axonopus compressus*) at 37°C for 48 h. All blanket grass with *Pythium* hyphae will be transferred to the induction medium. The zoospores will be washed with 1X phosphate-buffered saline (1X PBS, pH 7.4) and counted before use.

3.4 Role of neutrophils in *P. insidiosum* infection

To investigate the role of neutrophils in the response to *P. insidiosum* zoospore infection in healthy donors. Neutrophils and *Pythium* zoospores will be co-incubated at different concentrations. The culture supernatant will be collected to assess neutrophil activity.

3.4.1 Killing activity uses a fungicidal assay, plated onto Blood agar (Oxoid), and incubated at 37ºC, overnight. The colony enumeration will be counted.

3.4.2 NET formations and degranulation will be evaluated via neutrophil extracellular trap-associated genes and immunofluorescence staining, detected with antibodies against myeloperoxidase (MPO; Abcam), neutrophil elastase (NE; Abcam), and citrullinated histone H3 (CitH3; Abcam), and nuclear morphology will be stained with DAPI (Sigma-Aldrich). Fluorescent images will be captured at high magnification from 10 randomly selected fields.

3.5 Data analysis

All experiments will be performed in triplicate, and a Two-Way analysis of variance (ANOVA) will be used to compare differences among multiple groups, and a Wilcoxon signed-rank test for two-group analysis. A *p-value* < 0.05 will be considered statistically significant. Statistical analyses will be performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

4. Results

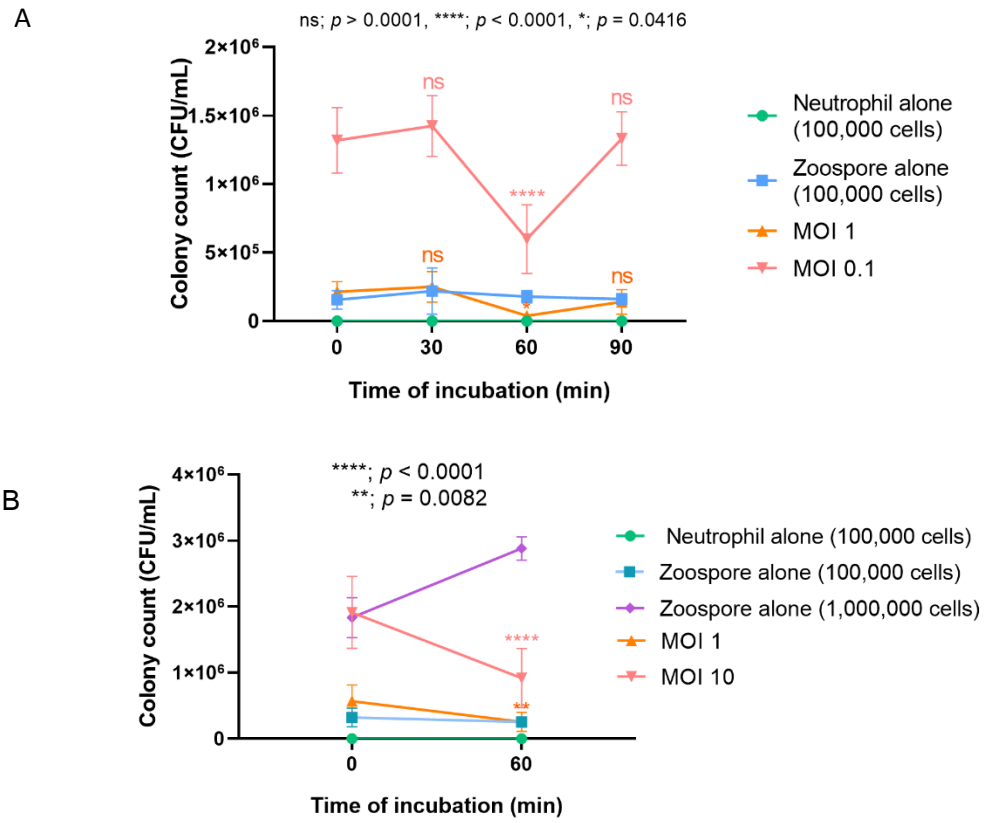

Figure 2. Killing activity of neutrophils against *P. insidiosum* zoospores from healthy donors. Time variation in killing activity (A) and killing activity after 60 minutes of incubation (B) were analyzed using two-way ANOVA. (n=5)

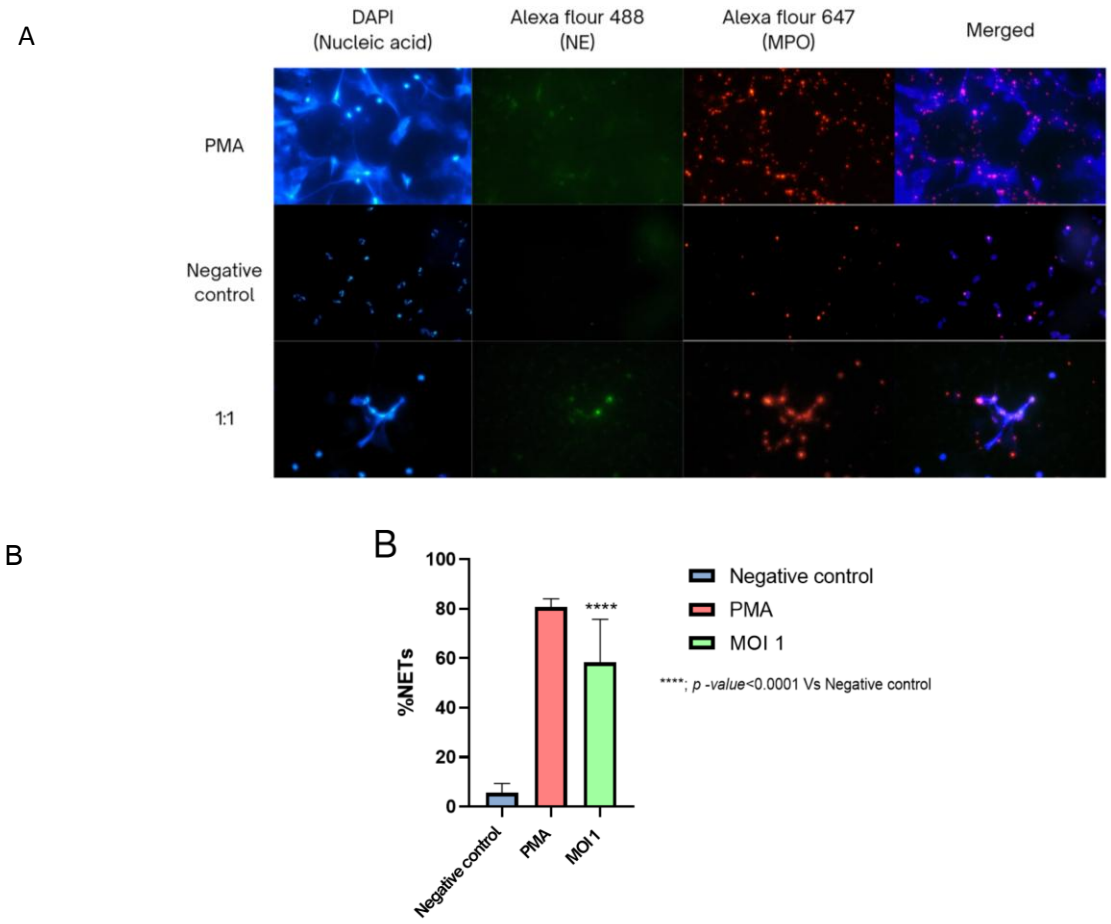

Figure 4 Representative NET formation pictures visualized by an immunofluorescence microscope.(A) Neutrophils were incubated with treatment heat-killed zoospores in MOI 1 and 100 ng/mL PMA as a positive control, compared with unstimulated neutrophils as a negative control.The number of NETs was counted per 100 cells using a fluorescence microscope.(B) **** $p < 0.0001$ compared with negative control. Magnification ×20 (n=5), Wilcoxon signed-rank test use as statistical analysis.

The results showed that when neutrophils isolated from healthy donors were co-incubated with the pathogen at MOIs of 1 and 0.1 for 60 min, the number of zoospores was significantly lower than at 0 min of incubation ($p = 0.0416$ and $p < 0.0001$, respectively). However, no reduction in zoospore numbers was observed after 30 min of incubation. In addition, regrowth of *Pythium insidiosum* was observed after 90 min of incubation. (Figure 1). Furthermore, the Immunofluorescence assay showed that neutrophils were significantly stimulated to produce neutrophil extracellular traps (NETs) ($p < 0.0001$) after exposure to *Pythium*'s zoospores (Figure 2).

5. Conclusion and discussion

The results demonstrated that co-incubation of neutrophils with the *zoospore* at both MOI of 1 and 0.1 for 60 min resulted in a significant reduction in the number of organisms compared with time 0. Additionally, MOI 1 showed the magnitude of reduction was lower than that observed at MOI 0.1. This difference may be attributed to the higher pathogen burden present at MOI 0.1, which likely increases the probability of interaction between neutrophils and the pathogen, thereby promoting stronger activation of neutrophil immune responses compared with MOI 1. Furthermore, neutrophils exhibited a marked increase in NET formation compared with the negative control group, which consisted of unstimulated neutrophils. The proportion of NET-forming cells was significantly higher following exposure to the pathogen ($p < 0.0001$), even at an MOI of 0.1. Interestingly, no reduction in pathogen numbers was observed at 30 min in the killing assay. This finding suggests that the physical characteristics of the zoospores of *P. insidiosum* may influence neutrophil activation and their subsequent antimicrobial response. In particular, the relatively large size and structural properties of the zoospores, compared with particles typically internalized by neutrophils, may limit the efficiency of phagocytosis during the early phase of interaction (within 30 minutes). Under such conditions, neutrophils may preferentially respond through an alternative defense mechanism, such as NETs release, which function to entrap and restrict the extracellular dissemination of pathogens.

6. References

Gurnani, B., Kaur, K., Venugopal, A., Srinivasan, B., Bagga, B., Iyer, G., Christy, J., Prajna, L., Vanathi, M., Garg, P., Narayana, S., Agarwal, S., & Sahu, S. (2022). *Pythium insidiosum* keratitis - A review. In *Indian Journal of Ophthalmology* (Vol. 70, Issue 4). https://doi.org/10.4103/ijo.IJO_1534_21

Mendoza, L., & Prendas, J. (1988). A method to obtain rapid zoosporogenesis of *Pythium insidiosum*. *Mycopathologia*, *104*(1). https://doi.org/10.1007/BF00437925

Ud-naen, S., Tansit, T., Kanistanon, D., Chaiprasert, A., Wanachiwanawin, W., & Srinoulprasert, Y. (2019). Defective cytokine production from monocytes/macrophages of E-beta thalassemia patients in response to *Pythium insidiosum* infection. *Immunobiology*, *224*(3). https://doi.org/10.1016/j.imbio.2019.02.002