

THE IMPAIRMENT OF NEUTROPHIL FUNCTIONS OF β -THALASSEMIA PATIENTS IN *PYTHIUM INSIDIOSUM* INFECTION

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ABSTRACT

Patients with β -thalassemia are more susceptible to *Pythium insidiosum* infection, leading to human pythiosis, potentially due to iron overload, although the underlying mechanisms remain unclear. This study aimed to investigate the functional responses of neutrophils from β -thalassemia patients upon exposure to *P. insidiosum*. Neutrophils were isolated from patients and healthy controls and co-cultured with *P. insidiosum* zoospores at varying multiplicities of infection. Neutrophil functions, including killing activity and neutrophil extracellular trap (NET) formation, were assessed to evaluate pathogen clearance capacity. The results showed that β -thalassemia patients had significantly reduced fungal clearance, indicated by higher fungal burdens at both MOIs 1 and 0.1 ($p < 0.05$). In addition, NET formation was significantly lower compared to healthy controls ($p < 0.05$). These findings highlight a defect in neutrophil-mediated innate immunity and provide new insights into the increased susceptibility to infection in β -thalassemia patients.

Keywords: Pythiosis, *Pythium insidiosum*, β -thalassemia, Neutrophil

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INTRODUCTION

Pythium insidiosum is an oomycete pathogen that causes pythiosis, a life-threatening disease affecting both animals and humans. It is commonly found in aquatic environments, where it produces motile zoospores that can actively swim, adhere to damaged tissue, and initiate infection. Pythiosis remains underdiagnosed and is associated with high morbidity and mortality. Between 1980 and 2021, 4,203 human cases were reported, with approximately 94.3% occurring in Thailand (Yolanda & Krajaejun, 2022). Notably, β -thalassemia patients are particularly susceptible to *P. insidiosum* infection, likely due to iron overload resulting from defective hemoglobin synthesis. Excess iron negatively affects immune cell function and increases vulnerability to opportunistic infections (Nairz & Weiss, 2020). Neutrophils, the most abundant circulating leukocytes in the human body, are essential components of innate immunity. They act as a first line of defense against pathogens through mechanisms such as phagocytosis, reactive oxygen species (ROS) production, cytokine secretion, degranulation, and NET formation (Louis & Parkos, 2015). However, iron overload in β -thalassemia has been shown to impair neutrophil functions, including reduced phagocytosis and NET formation (Buttari et al., 2020).

Despite these findings, the specific responses of neutrophils to *P. insidiosum* infection in β -thalassemia patients remain unclear. Therefore, this study aims to investigate neutrophil functions in β -thalassemia patients compared to healthy controls, focusing on pathogen killing activity and NET formation. This work will provide insights into immune dysfunction and its role in susceptibility to *P. insidiosum* infection.

LITERATURE REVIEWS

Pythium insidiosum is an oomycete pathogen in the kingdom of *Stramenopila*. Although it shares morphological features with fungi, such as filamentous hyphae, it is phylogenetically distinct and more closely related to algae. Its cell wall mainly contains cellulose and β -glucans, with minimal chitin, and it lacks ergosterol in the cell membrane. This explains the limited effectiveness of conventional antifungal drugs against this organism (Gaastra et al., 2010). *P. insidiosum* grows rapidly (1-2 days) at 28-37°C and forms hyaline, non-septate hyphae. It can be cultured on media such as Sabouraud Dextrose Agar and blood agar. Zoospores, the infective stage, can be induced in specific media and are essential for host invasion. The organism reproduces sexually (oospore) and asexually (zoospore). Zoospores are motile via flagella, allowing them to reach host tissue, encyst, and germinate into invasive hyphae, which contribute to disease progression (Gaastra et al., 2010; Walker & van West, 2007).

Pythiosis refers to the severe disease affecting humans and animals, first described in horses. It presents in various forms depending on the host, including cutaneous and gastrointestinal infections in animals. In humans, most cases occur in tropical regions, particularly Thailand. Human pythiosis is classified into four forms, including ocular, which causes corneal ulcers and vision loss, cutaneous/subcutaneous, which presents granulomatous lesions, vascular, the most common and severe, associated with arterial insufficiency, and disseminated, rare but highly fatal, affecting internal organs (Jindayok et al., 2009). Treatment is challenging because *P. insidiosum* lacks ergosterol. Antifungal drugs are often ineffective, making surgical removal the main treatment. Immunotherapy and some antibiotics show potential but have inconsistent outcomes (Chitasombat et al., 2020; Sussaengrat et al., 2019). The major concern is that human pythiosis mostly occurs in thalassemia patients, especially β -thalassemia (Krajaejun et al., 2006). Thalassemia is a genetic disorder causing reduced hemoglobin production and chronic anemia (Angastiniotis & Lobitz, 2019). It is prevalent in Thailand and classified into α - and β -thalassemia, with β -thalassemia often requiring regular blood transfusions and leading to iron overload. β -thalassemia is associated with immune dysfunction due to iron overload and chronic inflammation. Neutrophils show impaired function, including reduced ROS

production, altered receptor expression, and decreased antimicrobial activity. Cytokine responses are also weakened, increasing susceptibility to infections (Buttari et al., 2020). Neutrophils employ several key mechanisms to eliminate pathogens and regulate immune responses. NET formation enables the trapping and killing of large pathogens. Phagocytosis allows neutrophils to internalize and degrade microbes within phagolysosomes. In addition, neutrophils contribute to immune regulation by producing cytokines. Degranulation facilitates the release of antimicrobial enzymes, while the oxidative burst generates ROS that are essential for effective pathogen killing (Ud-naen et al., 2019).

Although neutrophil dysfunction in β -thalassemia and immune responses to *P. insidiosum* have been studied separately, their interaction remains unclear. Therefore, this study aims to investigate neutrophil function in patients with β -thalassemia in response to *P. insidiosum* infection compared with healthy controls.

RESEARCH METHODOLOGY

Zoospore induction of *P. insidiosum*

P. insidiosum from a human pythiosis patient was obtained from Srinagarind Hospital and cultured overnight on blood agar (BA; Oxoid, UK). The method of zoospore production applied from Mendoza & Prendas, 1988. In brief, the microorganism was cultured on cornmeal agar (Oxoid) with sterile blanket grass (*Axonopus compressus*) at 37°C for 48 h. All the blanket grass with *Pythium* hyphae was then transferred to the induction medium to induce the encysted zoospores. The zoospores were washed with 1X phosphate-buffered saline (1X PBS, pH 7.4) and counted before use.

Blood collection

The 10 mL of whole blood was collected from β -thalassemia and healthy donors from Srinagarind Hospital (n = 4). All participants provided informed consent under an IRB-approved protocol by the ethics committee in the Center for Ethics in Human Research, Khon Kaen University (KKUEC).

Neutrophil separation

Neutrophil was separated by the standard density gradient separation method with Polymorphprep™ (Axis-Shield, Norway). Following centrifugation at $400 \times g$ for 30 mins at 20-25°C, the neutrophil layer was collected and resuspended in RPMI1640 that contain 10% heat-inactivated fetal bovine serum (FBS) to stabilize the cells. Neutrophils are then used in co-culture steps.

Killing activity

To determine the capacity of neutrophils for clearance of *P. insidiosum*, the killing activity was performed by co-culturing human neutrophils with zoospores of *Pythium* in MOI 1 and 0.1 in 0 for 60 mins at 37°C in a humidified 5% CO₂ incubator. Then, the supernatant was collected to do a spread plate and colony enumeration to assess the fungal viability.

NET formation

The formation of NETs was visualized using immunofluorescence staining. The separated human neutrophil was co-cultured with zoospores of *Pythium* at an MOI of 1 for 60 mins at 37°C in a humidified 5% CO₂. Neutrophil degranulation and NET formation were detected by antibodies against myeloperoxidase (MPO; Abcam), and nuclear morphology was stained with DAPI (Sigma-Aldrich). The fluorescent-detected images were snapped with 20× magnification in 10 randomly selected fields.

Statistical analysis

Data was analyzed using GraphPad Prism 8. Normality of the data distribution was assessed. If the data were normally distributed, One-way ANOVA and Two-way ANOVA were applied. A p-value < 0.05 was considered statistically significant.

RESEARCH RESULTS

Killing activity

The results demonstrated that neutrophils isolated from healthy volunteers significantly reduced the number of *P. insidiosum* after co-incubation at MOI 1 and 0.1 for 60 mins compared with baseline (0 min) ($p < 0.0001$), indicating effective pathogen-killing activity. In contrast, neutrophils from β -thalassemia patients showed an increase in fungal burden after 60 mins of incubation at both MOIs. This increase was statistically significant when compared with the healthy control group ($p = 0.0001$ and $p < 0.0001$), reflecting impaired antimicrobial function (Figure 1).

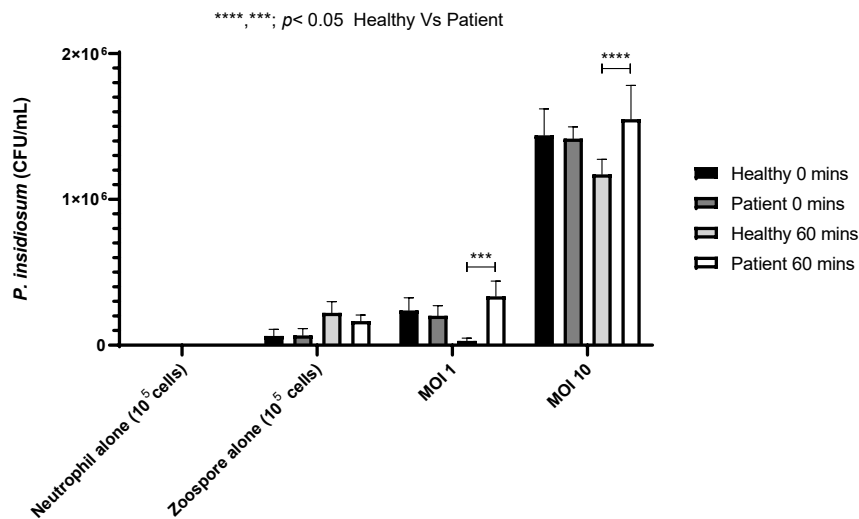


Figure 1 The quantity of *P. insidiosum* after co-incubation with neutrophils from β -thalassemia patients was compared with that of healthy controls. Statistical differences were analyzed using two-way ANOVA ($n = 4$), ***, **** p -value < 0.05 .

NET formation

Regarding the NET formation, unstimulated neutrophils exhibited low levels of NET production, with no significant difference between the two groups. However, upon stimulation with PMA (positive control) or *P. insidiosum* zoospores, NET formation was significantly increased compared to the negative control. Notably, neutrophils from β -thalassemia patients produced significantly lower levels of NETs than those from healthy individuals ($p < 0.01$) (Figure 2A-C).

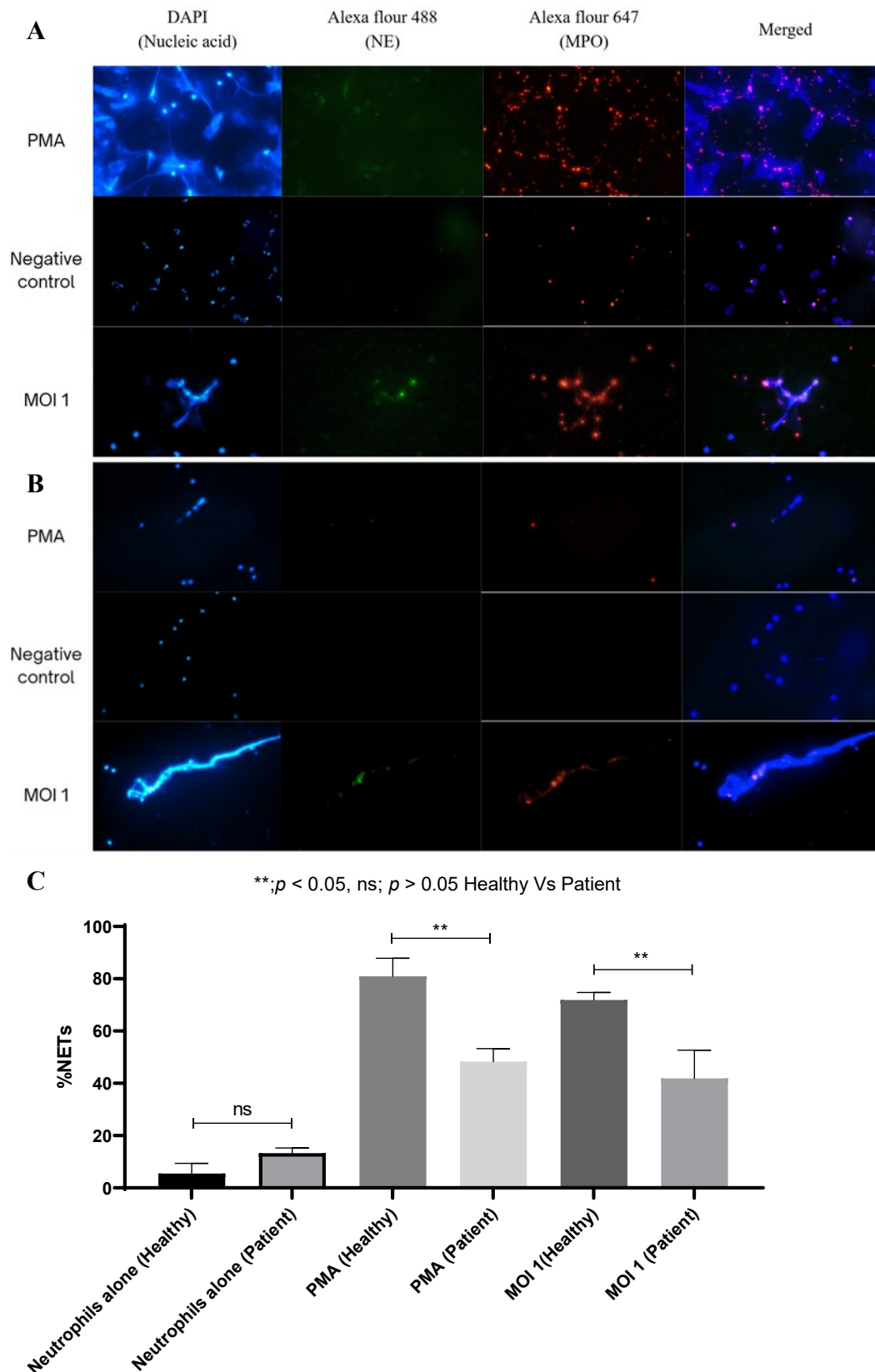


Figure 2 Representative images of neutrophil extracellular trap (NET) formation in healthy volunteers (A) compared with β -thalassemia patients (B). Images were randomly captured at $20\times$ magnification, and the percentage of NET formation was quantified (C). Statistical differences were analyzed using one-way ANOVA ($n=4$), **, ** p -value < 0.05

DISCUSSION & CONCLUSION

This study demonstrates that the killing activity of neutrophils from β -thalassemia patients is significantly impaired, with a reduced capacity to eliminate *P. insidiosum* compared to healthy controls, particularly at 60 minutes across all MOI conditions. The increased fungal burden observed in the patient group indicates ineffective pathogen clearance, whereas healthy neutrophils efficiently control fungal growth. This impaired killing activity is further supported by defects in NET formation. While both groups showed low baseline NET levels under unstimulated conditions, stimulation with PMA or *P. insidiosum* zoospores markedly enhanced NET production in healthy neutrophils but not in β -thalassemia patients. Together, these findings suggest that impaired NETosis contributes to defective neutrophil-mediated killing and overall innate immune dysfunction in β -thalassemia patients.

These findings are consistent with previous reports suggesting that iron overload in β -thalassemia patients adversely affects neutrophil function, potentially by disrupting ROS production and impairing enzymes involved in NET formation, ultimately reducing pathogen clearance efficiency (Buttari et al., 2023). Additionally, the relatively large size of *P. insidiosum* zoospores and hyphal structures suggests that NET-mediated mechanisms may play a crucial role in host defense (Sriwarom et al., 2023). This impairment may therefore contribute to increased pathogen proliferation in patients.

Overall, this study highlights that neutrophil dysfunction in β -thalassemia patients, particularly in pathogen killing and NET formation, is a key factor underlying their increased susceptibility to *P. insidiosum* infection. However, the small sample size ($n = 4$) limits the statistical power of the study, which may reduce the sensitivity to detect subtle differences in neutrophil responses and increase the possibility that the observed effects are influenced by inter-individual variability. In addition, this limited cohort may restrict the generalizability of the findings to the broader β -thalassemia population, as patient heterogeneity in clinical status, iron burden, and treatment history could not be fully represented. Further investigations into underlying mechanisms, such as ROS production, surface receptor expression, and cytokine responses, are warranted to better understand disease pathogenesis.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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