THE ROLE OF *AMT2* GENE IN ASSOCIATION WITH VIRULENCE FACTORS AND TRANSMIGRATION PROCESS OF *CRYPTOCOCCUS NEOFORMANS* ACROSS THE BLOOD-BRAIN BARRIER

SAINAMTHIP RANGDIST

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บทบาทของยีน *AMT2* ต่อการสร้างปัจจัยความรุนแรงและกระบวนการผ่านแนวกั้นระหว่างหลอดเลือดและสมองของ *Cryptococcus neoformans*

นางสาวสายน้ำทิพย์ รังดิษฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE ROLE OF *AMT2* GENE IN ASSOCIATION WITH VIRULENCE FACTORS AND TRANSMIGRATION PROCESS OF *CRYPTOCOCCUS NEOFORMANS* ACROSS THE BLOOD-BRAIN BARRIER

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สายน้ำทิพย์ รังดิษฐ : บทบาทของยีน *AMT2* ต่อการสร้างปัจจัยความรุนแรงและกระบวนการผ่านแนวกั้นระหว่างหลอดเลือดและสมองของ *Cryptococcus neoformans* (THE ROLE OF *AMT2* GENE IN ASSOCIATION WITH VIRULENCE FACTORS AND TRANSMIGRATION PROCESS OF *CRYPTOCOCCUS NEOFORMANS* ACROSS THE BLOOD-BRAIN BARRIER) อาจารย์ที่ปรึกษา : อาจารย์ ดร.มัณฑนา แจ่มกลาง, 59 หน้า.

คำสำคัญ: เชื้อ *Cryptococcus neoformans*/ ยีน *AMT2*/ แนวกั้นระหว่างหลอดเลือดกับสมอง

เชื้อ *Cryptococcus neoformans* เป็นเชื้อก่อโรคชนิดมีแคปซูลหุ้มที่ก่อให้เกิดภัยคุกคามชีวิตด้วยการบุกรุกเข้าไปยังระบบประสาทส่วนกลางซึ่งพบได้บ่อยในกลุ่มผู้ที่มีภูมิคุ้มกันบกพร่องประกอบไปด้วย ผู้ป่วยโรคเอดส์ กลไกในการผ่านแนวกั้นระหว่างหลอดเลือดกับสมองของเชื้อ *Cryptococcus neoformans* เป็นกระบวนการที่มีความซับซ้อน จากการศึกษาก่อนหน้าในแบบจำลองแนวกั้นระหว่างหลอดเลือดและสมอง (ในหลอดทดลอง) พบว่า เชื้อ *C. neoformans* มีการแสดงออกของยีนบางกลุ่มในระดับจีโนมเพิ่มมากขึ้นขณะที่เชื้อมีการสัมผัสกับเซลล์บุผนังหลอดเลือด หนึ่งในยีนที่มีการแสดงออกมากขึ้นคือยีน ammonium transporter (*AMT2*) จากการศึกษาอื่น ๆ ก่อนหน้านี้พบว่ายีน ammonium transporter มีความเกี่ยวข้องกับการเหนี่ยวนำให้เกิดการแสดงออกทางคุณสมบัติของเซลล์ยีสต์ในการรุกรานเซลล์โฮสต์ได้ ดังนั้น วัตถุประสงค์ของงานวิจัยนี้คือ เพื่อตรวจสอบบทบาทของยีน *AMT2* ในกระบวนการผ่านเข้าสู่สมองของเชื้อ *C. neoformans* ศึกษาความเกี่ยวข้องในการสร้างปัจจัยความรุนแรงของเชื้อ และเพื่อพิจารณาความเกี่ยวข้องของยีน *AMT2* ในการเปลี่ยนแปลงทางสัณฐานวิทยาของเชื้อ *C. neoformans* ผลการศึกษาในครั้งนี้ พบว่า เชื้อ *C. neoformans* ที่ขาดยีน *AMT2* (*amt2Δ*) มีความสามารถในการผ่านเข้าสู่สมองลดลงอย่างมีนัยสำคัญเมื่อเทียบกับสายพันธุ์ดั้งเดิม (H99) ในแบบจำลองแนวกั้นระหว่างหลอดเลือดและสมอง นอกจากนี้ยังคาดว่า เชื้อ *C. neoformans* ผ่านเข้าสู่สมองด้วยกลไก transcellular ซึ่งทดสอบด้วยวิธี FITC-dextran permeability assay ในด้านการเปลี่ยนแปลงทางสัณฐานวิทยาโดยการตรวจสอบภายใต้กล้องจุลทรรศน์พบว่า เชื้อทั้ง 2 สายพันธุ์ไม่สามารถสร้างสายราเทียมภายใต้สภาวะที่ถูกจำกัดไนโตรเจนได้ อย่างไรก็ตาม ความสามารถในการสร้างปัจจัยความรุนแรงของเชื้อสายพันธุ์กลายพันธุ์ *amt2Δ* ยังคงอยู่ ได้แก่ การเจริญที่อุณหภูมิ 37°C การสร้างเมลานิน การหลั่งเอนไซม์ยูรีเอส และการหลั่งของเอนไซม์ฟอสโฟลิเพส เมื่อเทียบกับสายพันธุ์ดั้งเดิม นอกจากนี้ การเปรียบเทียบอัตราการเจริญบนอาหารที่มีปริมาณไนโตรเจนแตกต่างกันของเชื้อสายพันธุ์กลายพันธุ์และสายพันธุ์ดั้งเดิมยังคงไม่แตกต่างกัน จากผลการศึกษาในครั้งนี้สรุปได้ว่า ยีน *AMT2* มีบทบาทสำคัญในกระบวนการผ่านเข้าสู่สมองและส่งผลต่อความสามารถในการสร้างแคปซูล อย่างไรก็ตาม ยีน *AMT2* ไม่มีความสัมพันธ์กับ

ปัจจัยความรุนแรงอื่น เช่น การหลั่งเอนไซม์ยูรีเอส การหลั่งของเอนไซม์ฟอสโฟลิเพส และ การสร้างเมลานิน ของเชื้อ *C. neoformans* นอกจากนี้ เชื้อสายพันธุ์เชื้อสายพันธุ์กลายพันธุ์ *amt2Δ* ยังไม่พบความบกพร่องของการเจริญเติบโตที่อุณหภูมิ 37°C ภายใต้สภาวะที่ถูกจำกัดไนโตรเจน สำหรับทิศทางการวิจัยต่อเนื่องในอนาคตคือ การศึกษากลไกของยีน *AMT2* ในกระบวนการผ่านเข้าสู่สมองของเชื้อ *C. neoformans*

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ลายมือชื่อนักศึกษา สายน้ำทิพย์ รังสิชัย
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SAINAMTHIP RANGDIST : THE ROLE OF *AMT2* GENE IN ASSOCIATION WITH VIRULENCE FACTORS AND TRANSMIGRATION PROCESS OF *CRYPTOCOCCUS NEOFORMANS* ACROSS THE BLOOD-BRAIN BARRIER. THESIS ADVISOR : MANTANA JAMKLANG, Ph.D. 59 PP.

Keyword: *CRYPTOCOCCUS NEOFORMANS/ AMT2* GENE/ BLOOD-BRAIN BARRIER

Cryptococcus neoformans (Cn) is a pathogenic encapsulated yeast that cause life-threatening fungal invasion of the central nervous system (CNS) in immunocompromised individuals, including HIV patients. The mechanism of *Cn* in transmigration across the blood-brain barrier (BBB) is a complex process. Our previous data on cryptococcal transcriptome demonstrated that exposure of *C. neoformans* with the endothelial cells of the *in vitro* model of BBB upregulated some genes of *C. neoformans* including the amt family ammonium transporter (*AMT2*) whereas *C. neoformans* alone with no exposure to the BBB did not show the *AMT2* gene expression change. Previous studies elsewhere revealed that the *AMT2* induces an invasive phenotype expressing with pseudohyphal growth. Therefore, the aim of this study was to investigate the role of the *AMT2* gene in the transmigration process of *C. neoformans* across the BBB and whether or not the *AMT2* gene are associated with other common virulence factors as well as determine the relevant of the gene in morphological changes in *C. neoformans.*

In this study, we found that the mutant strain of *C. neoformans* lacking *AMT2* gene (*amt2Δ*) showed a significantly decreased transmigration ability compared to the wild-type strain (H99) determined by the *in vitro* model of BBB. The mechanism of *C. neoformans* was suspected to be transcellular invasion determined by FITC-dextran permeability assays. The morphological changes under nitrogen-limiting conditions were observed by light microscope did not reveal pseudohyphal growth in both tested strains. The ability to generate virulence factors including melanin production, growth rate at 37°C (thermotolerance), urease and phospholipase activity of both tested strains were comparable and the results showed the *AMT2* deletion was not associated with some virulence factors whereas the polysaccharide capsule size of the mutant

strain was significantly decreased when compared to that of the wild-type strain. In addition, the comparison of the growth rate of the mutant and wild-type strain on the ammonium depletion agar was not significantly different. In conclusion, *AMT2* gene plays a significant role in transmigration process across the BBB and capsule production of *C. neoformans*. However, *AMT2* gene was not associated with other important virulence factors such as urease production, phospholipase production and melanin pigment found in *C. neoformans*. Moreover, the mutant strain has no defect on growth at 37°C and low concentration of ammonium. Future direction is to explore how *AMT2* gene play roles in transmigration process of *C. neoformans*.

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LIST OF ABBREVIATIONS

AMT	Ammonium transporter gene
ART	Antiretroviral therapy
BBB	Blood-brain barrier
CFS	Cerebral spinal fluid
CFU	Colony forming unit
Cg	*Cryptococcus gattii*
cm	Centimeter
CM	cryptococcal meningitis
Cn	*Cryptococcus neoformans*
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
℃	Degree Celsius
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
EBM-2	Endothelial cell growth basal medium-2
ECs	Endothelial cells
FITC-Dextran	Fluorescein isothiocyanate–dextran
GalXM	Galactoxylomannan
GXM	Glucuronoxylomannan
g	Gram
g/L	Gram per liter
h	Hour
HBMEC	Human brain microvascular endothelial cells
hCMEC	Human cerebral microvascular endothelial cells
HCO_3^-	Bicarbonate
HIV	Human immunodeficiency virus

LIST OF ABBREVIATIONS (Continued)

min	Minute
mL	Milliliter
nm	nanometer
MAPK	Mitogen-activated protein kinase
OD	Optical density
PBS	Phosphate buffered saline
PRRs	Pattern recognit ion receptors
ROS	Reactive oxygen species
rpm	Revolutions per minute
TEER	Trans-endothelial electrical resistance
TLR	Toll-like receptor
TJs	Tight junctions
WT	Wild type
ZO-1	Zonula occludens-1
µg/mL	Microgram per milliliter
µL	Microliter

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Opportunistic fungal diseases have become a major concern for the national health systems worldwide. The genera *Candida*, *Aspergillus*, and *Cryptococcus* are the main causes of most of the major human fungal infections (Jacobsen, 2019). Especially in *Cryptococcus* spp., they are associated with life-threatening diseases in millions of patients worldwide in which more than 70% of these cases occur in sub-Saharan Africa, commonly in immunocompromised patients, especially patients with HIV infection (Park *et al*., 2009). There are only 2 species commonly cause human cryptococcosis including *Cryptococcus neoformans* (*Cn*) and *Cryptococcus gattii* (Cg). *Cn* and *Cg* are commonly found in the environment such as soil, pigeon dropping and in the trunks of eucalyptus trees (Ellis and Pfeiffer, 1990). The infections caused by these fungi are acquired by inhaling desiccated yeasts or basidiospores that cause pulmonary cryptococcosis, and also cause cryptococcal meningitis (CM) and skin lesion by dissemination (Maziarz and Perfect, 2016). Among the most cryptococcal infectious diseases, the CM is the most serious and has high associated mortality and economical cost, with more than 60% mortality rate within the first 3 months of infection (Beardsley *et. al*., 2016). This may be because *Cn* have the major factors to prevent and escape from host immunity, consist with polysaccharide capsule, cell-wall associated melanin, and thermotolerance (growth at body temperature). Moreover, *Cn* also produce the urease and phospholipase B which promotes brain invasion and damages endothelial cells in the brain vasculature (Kronstad *et al.*, 2011).

Brain infection caused by *Cn* occur when the fungi cross the blood-brain barrier (BBB) which plays an important role to transport nutrients and prevent the uptake of neurotoxins or pathogens into the brain (Abbott *et al*., 2010). There are three mechanisms that *Cn* can transmigrate across the BBB including paracellular, transcellular

and Trojan horse mechanism (Sabiiti and May, 2012; Liu *et al*., 2014; Sorrell *et al*., 2016). For paracellular, *Cn* damages the intracellular tight junction that introduces a weaken of endothelial vessel wall and promotes the fungal traversing. Transcellular mechanism occurs when *Cn* is able to bind receptor located on the endothelial cells of BBB in order to enhance the transmigration. For Trojan horse mechanism, *Cn* can proliferate and survive inside phagocytes to escape host immunity and subsequently across BBB. These mechanisms are complicated and associated with several molecules as well as the mechanisms in molecular level is not well understood. Several studies have attempted to elucidate the mechanism of transmigration process of *Cn*. The knowledge on complicated mechanisms of cryptococcal across the BBB may enable us to predict and develop strategies or drugs to decrease the incidence of central nervous system (CNS) infection.

Our preliminary data from RNA sequence (unpublished data) looking at the overall transcriptome of host response to *Cn* infection revealed that the transmigration process of *Cn* increased the expression of the amt family ammonium transporter that induces the invasive phenotype expressing with pseudohyphal growth and also induces dikaryon mating under the limited ammonium condition (Berg *et al*., 2019). In addition, the real-time imaging of transmigration across the BBB revealed that *Cn* are arrested in brain capillary and changed to ovoid shape for brain infection which resemble the infected by morphologic change (Shi *et al*., 2010). Therefore, this study aims to investigate the role of the amt family ammonium transporter (*AMT2*) in the transmigration process of *Cn* across the BBB using an *in vitro* model of the blood-brain barrier utilizing the human cerebral microvascular endothelial cells (hCMEC/D3) and whether or not the *AMT2* gene is associated with other common virulence factors produced from *Cn.*

1.2 Research objectives

1.2.1 To determine the association of the *AMT2* gene in the morphological changes of *C. neoformans.*

1.2.2 To determine the transmigration of the *amt2Δ* mutant strain of *C. neofromans* through the *in vitro* model of the human BBB by transcytosis assay.

1.2.3 To determine the virulence factors of the mutant strain (*amt2Δ*) of *C. neoformans*.

1.3 Research hypothesis

The ammonium transporter gene plays an important role in the morphological changes of cryptococcal cells, therefore, it facilitates the transmigration process of *C. neoformans* across the BBB.

1.4 Scope and limitations of the study

In this study, the morphological changes of cryptococcal cells were determined by an *in vitro* phenotypic analysis. The transmigration of the *amt2Δ* mutant strain was investigated and compared with the wild-type strain (H99) by the transcytosis assay using the *in vitro* BBB model. The virulence factors (urease activity, melanin production, phospholipase activity, capsule formation and growth at 37°C) of the wild-type strain of *C. neoformans* (H99) and the *amt2Δ* mutant strain were compared. The limitation of this study was the phenotypic results of *Cn* did not exhibit under the imitating condition medium. Further study of morphological change of *Cn* should be observe with the hCMEC/D3 cell.

CHAPTER II
LITERATURE REVIEW

2.1 *Cryptococcus* spp.

Cryptococcus spp. are the pathogenic encapsulated yeast in the genus of basidiomycetous fungi that cause cryptococcal disease in millions of patients worldwide more than 70% of these cases occurring in sub-Saharan Africa most commonly in immunocompromised patients including patients with HIV infection (Park *et al*., 2009). Moreover, it does not only infect in the HIV-positive population but also HIV-negative population or immunosuppressed patients is another risk group including the advancing age, liver disease, renal failure, diabetes, and hematological malignancies with chemotherapy (O'Halloran *et al*., 2017). There are only 2 species commonly cause human cryptococcosis including with *C. neoformans* (Serotype A and D) and *C. gattii* (Serotype B and C). *Cn* (serotype A) causes approximately 95% of cryptococcal infections while *Cn* (serotype D) or *Cg* (serotypes B and C) are responsible for the remaining 4% to 5% *Cn* is contained in soil and pigeon dropping and has a global distribution whereas *Cg* is usually found in decaying hollows in trunks and branches of eucalyptus tree in tropical and subtropical climates (Ellis and Pfeiffer, 1990; May *et al*., 2016). In addition, *Cg* is able to infect healthy people and animals as well (Byrnes *et al*., 2010). In tropical and subtropical regions, cryptococcal disease was so prevalent by *Cg* particularly in southern California, Hawaii, Brazil, Australia, Southeast Asia, and central Africa. Recently, *Cg* has often found in temperate climates such as Vancouver Island and the Pacific Northwest region of the United States and Europe which have a greater range of temperature as ecological shift. *Cn* and *Cg* have ability to disseminate and infect at anybody sites of immunocompromised and immunosuppressed patients such as pulmonary, central nervous system (CNS), skin and other body sites then cause pneumonia, meningitis and skin lesion, respectively (Maziar and Perfect, 2016).

Patients with 30% of CNS infections typically have lung infections (Olave *et al*., 2017). The CNS infections are the major cause of morbidity and mortality due to strong tropism of *Cryptococcus* for the CNS, which facilitates crossing of the BBB via various migration mechanisms such as paracellular, transcellular and Trojan horse (Tseng *et al*., 2015). In addition, *Cryptococcus* is able to cause disease in the meninges and brain parenchyma where fungal cells can replicate and thrive as well as cause the brain lesion and lead to cryptococcal meningoencephalitis (CM) (Vu *et al*., 2019).

Previous studies reported that the earlier detection of HIV infection and the antiretroviral therapy (ART) with high dose showed a decreased incidence of CM in HIV-positive population while the rate of cryptococcosis in HIV-negative population remained high (O'Halloran *et al*., 2017). Although, there are treatment guidelines by the combination ART with amphotericin B and fluconazole in developed countries to reduce CM, but the mortality rate is still over 30% at 10 weeks and survivors are severely disability (Beardsley *et al*., 2016). Moreover, it is still a major problem in developing countries where access to healthcare is limited. Therefore, the development of novel anticryptococcal medicines with the new mechanisms of action are needed to solve cryptococcal infection.

2.2 Virulence factor of *C. neoformans*

Many pathogenic fungi generate the virulence factors which facilitate the pathogen survive, invade and disseminate throughout the host and avoid being recognized by host immunity. Unlike to other fungal pathogens, *Candida* spp. rarely spreads through inhalation. Instead, *Candida* infections spread through filamentous growth and the development of virulence factors in host cells (Zaragoza, 2019). The virulence factors leading to *Cn* infections include the production of polysaccharide capsule, melanin pigment, and growth at 37°C (thermotolerance). Other virulence factors such as urease promote brain invasion in which urease hydrolyze urea to ammonia which destroy the tight junction of endothelial cells in the brain vasculature and disseminate to brain (Kronstad *et al*., 2011). In most cases, *Cn* can enter a chronic latent condition and remain there for several years within mammalian phagocytic cells as well as *Cn* infections typically do not exhibit early symptoms. (Hayes *et al*., 2016).

Hence, the synergistic interaction between virulence factors and host sensitivity protects yeast cells from host phagocytic cells and hiding host immune systems (Casadevall *et al*., 2019).

2.2.1 Polysaccharide capsule

The capsule is a complex polysaccharide (PS) composing with 90-95% glucuronoxylomannan (GXM), 5% galactoxylomannan (GalXM) and mannoproteins (less than 1%) that are able to be induced by severe iron deprivation and mammalian physiologic concentration of CO_2/HCO_3^- (Alspaugh, 2015). These components have immunomodulatory properties that promote *Cn* to survive and proliferate during the early stage of infection within a mammalian host (Coelho *et al*., 2014). Moreover, PS capsule has a strong negatively charged surface leading to electrostatic repulsion between cryptococcal cells and host cells, therefore, inhibiting the host-microbe interaction is required for the clearance (Nosanchunk and Casadevall, 1997).

2.2.2 Melanin and Laccase

Melanin is common in nature which is produced by all biological kingdoms and it is associated with virulence of several pathogens such as *Cryptococcus neoformans* (*Cn*), *Wangiella dermatitidis* and *Mycobacterium leprae* (Wang *et al*., 1995). Melanin is responsible for protection of *Cn* from oxidative stress caused by free radicals, ionizing radiation, and heat as well as it also binds and reduces the susceptibility of antifungal drugs such as amphotericin B (Zaragoza, 2019). Moreover, melanin offers defense against phagocytosis, which appears to be crucial for the spread from the lung to the brain (Kronstad *et al*., 2011). Melanin pigments of *Cn* are black color in the media containing phenolic substrates and also insoluble in aqueous/organic solvents and have a strong negative charge with high molecular weight (Camacho *et al*., 2019). The synthesis of melanin in *Cn* is produced through the oxidation and polymerization of phenolic/indolic precursors into quinones (extracellular enzyme) and it depends on oxidase activity of laccase enzyme that encoded by two genes, *LAC1* (expressed in cell wall) and *LAC2* (expressed in cytoplasm) (Coelho *et al*., 2014). Thus, the dual laccase activity and melanin production in cell wall will be increased the resistance of *Cn* to host defenses and

treatment by antifungal therapy. Figure 2.1 shows the structure of the fungal cell wall, capsule, melanin and cell body of melanin resides (Camacho *et al.*, 2019).

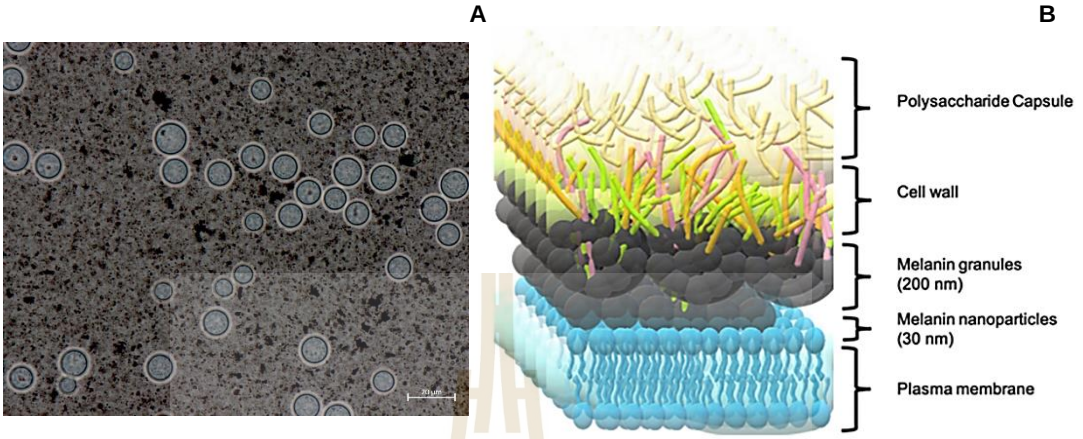

Figure 2.1 The cell wall structure of *Cn*. Capsule formation with India ink under microscope at 100X objective from our studied (A). The architecture of *Cn* cell wall (B), lipid bilayer interacts with melanin nanoparticles that aggregate to form larger melanin granules. Melanin granules are anchored to chitin, chitosan, and ß-glucan, cell-wall components (Camacho *et al*., 2019).

2.2.3 Thermotolerance

Cn is able to grow and replicate at the host temperature (37°C), therefore, *Cn* is induced for adaptation to high temperature, various nutrients sources, pH, and oxidative stress while most other nonpathogenic species of *Cryptococcus* are unable to tolerate the human body temperature leading to the inability to infect mammals (Mitchell and Perfect, 1995). Although, thermotolerance is not a virulence like capsule and laccase but it is capable of growth at body temperature that is the primary reason for *Cn*'s success as a pathogen (Coelho *et al*., 2014).

2.2.4 Urease

Urease catalyzes the hydrolysis of urea to CO_2 and ammonia and it is also important for microbial pathogenesis in humans as a virulence factor for some pathogenic fungi including *Cryptococcus neoformans* (*Cn*), *Candida albicans* and *Saccharomyces cerevisiae* (Navarathna *et al*., 2011). Urease is able to neutralize the acidic pH of the

phagolysosome which reduces the antimicrobial activity of phagolysosome with full acidification (Coelho *et al.*, 2014). In the case of *Cn*, urease production is extremely high, and it has been used to diagnose cryptococcosis (Zaragoza, 2019). Urease produces ammonia which is toxic to mammalian cells and also introduces a weaken of endothelial vessel wall that promotes the fungal traversing of epithelial barriers and enhances the ability of fungal to invade CNS, but not trapping in the capillary or growth in the CNS (Liu *et al*., 2014; May *et al*., 2016). The mechanism of urease for transmigration into brain is still unknown but could involve the ammonia production or other nitrogenous products present in host plasma that increase the expression of adhesins on the endothelial cells and affect astrocytes leading to opening the tight junctions and weakening the integrity of the BBB (Olszewski *et al.,* 2004). Cryptococci establish microemboli, proliferate and invade the brain parenchyma by disrupting the vessel wall within brain capillaries (Olszewski *et al.,* 2004). There is the evidence shown that mice infected with urease-knockout (*URE1*) strain of *Cn* not only survived significantly longer than the mice infected with the wild-type strain but the fungal burden in the brain also significantly lower. Therefore, urease activity is importantly virulence factor which involve the transmigration of *Cn* to the brain.

2.2.5 Phospholipase

Phospholipase are enzymes that are either secreted or synthesized intracellularly and act by physically damage host membrane, interfere with fungal cell signaling, and produce immunomodulatory (Djordjevic, 2010). Phospholipase has several isoforms and is able to cleave phospholipids. Two major phospholipases are Phospholipase B (*PLB*) and phosphatidylinositol (PI)-preferring phospholipase C (PI-PLC/Plc) which are secreted during *Cn* infection and enhanced fungal survival within phagocytes by non-lytic exocytosis while another isoform was found to have function in virulence through a role in thermotolerance, capsule, and cell wall synthesis (Coelho *et al.*, 2014). The activation of PLB generates glycerophospho compounds including lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) which can contribute to the detoxification of potentially cellulolytic lysophospholipids (Siafakas *et al.,* 2006). In previous studies, *PLB1*-deficient strains lose the capacity to survive in macrophages cells

that had a 50% decrease in intracellular proliferation compared to the wild-type (Alspaugh, 2015). Moreover, Phospholipase B also facilitates in the invasion of host lung tissue and dissemination into the circulation (Liu *et al.*, 2014).

2.3 Pathogenesis of *Cryptococcus neoformans* infection

Humans are frequently in contact with *Cn* in the environment. The events leading to cryptococcal diseases include the inhalation of fungal spores (basidiospores, 1-2 μm) or desiccated cells (~3 μm) to enter the alveolar spaces of the lungs and possess the physical characteristics that allow inhalation and lung deposition cause pulmonary infection in immunocompromised host, called primary infection (Botts *et al*., 2009). *Cryptococcus* can colonize on the host respiratory tract without causing symptoms; consequently, primary infection may exist in a dormant or latent state. If host immunity is compromised, the dormant form may reactivate or begins to proliferate and it must traverse through the pneumocystis cell for hematogenous or lymphatic spread then freely moving fungal cells arrested in the microcapillaries of the brain for moving out across the vessel wall (Shi *et al*., 2010 and Schaible *et al.*, 2010), that might cause hematogenous or meningitis or resulting in meningoencephalitis (Chang *et al.*, 2004). Moreover, in the cerebral spinal fluid (CSF) does not have anti-cryptococcal factors and the fungal polysaccharide capsule can also have deleterious effects on the immune response with the larger viscous capsule in solution which leads to increased intracranial pressure, possibly as a result of blocking of CSF outflow through arachnoid villi (Robertson *et al*., 2014). The patient exhibits symptoms that are not specific but are typical of neurological symptoms including headache, altered mental status, and other signs include lethargy with fever, stiff neck, nausea and vomiting (Pescador Ruschel and Thapa, 2020). Therefore, the symptoms can range from headache to severe neurological symptoms, developing coma and death.

There are 4 levels of damage scale of cryptococcosis such as molecular, cellular, tissue, and organism level (Figure 2.2). First, enzymes such as proteases, urease, phospholipase, and nuclease produced from *Cn* are responsible for molecular damage. They can modify some molecules such as cleaving proteins and directly damaging the effector cells by proteases

as well as the hydrolysis of urea to ammonia to damage the endothelial cells in the brain microvascular (Tseng *et al*., 2015; Casadevall *et al.*, 2018). Cellular damages occur from the modifications of the architecture and structure of the host cells via at least 2 major mechanisms; to damage or interfere host immune system in tissue and also the endothelial cells in the brain vasculature (Casadevall *et al.,* 2018). Next, tissue level damage causes anatomical and functional disorganization more than cellular injury. In 2003, Chen and colleague reported that the monolayer of human brain microvascular endothelial cells (HBMEC) was induced the membrane ruffling, changing in the morphology of nuclei, and swelling of the mitochondria and endoplasmic reticulum that were associated with dephosphorylation of cofilin and actin changes. Finally, the combination of these levels produces the disease of cryptococcosis at the organism level as shown in Figure 2.3. There can be little or organ damage (no necrosis) due to fungal formed granuloma that are well organized from inflammation and immune response but in patients with severe infections in the brain, the accumulation of fungi results in masses and displaces or destroys brain tissue to create the space which then causes host cell death (Casadevall *et al.*, 2018). However, the infection of *Cn* is dependent on its capacity to overcome the innate immune system of the host and cross the blood-brain barrier (BBB) (Ngamskulrungroj *et al.*, 2012).

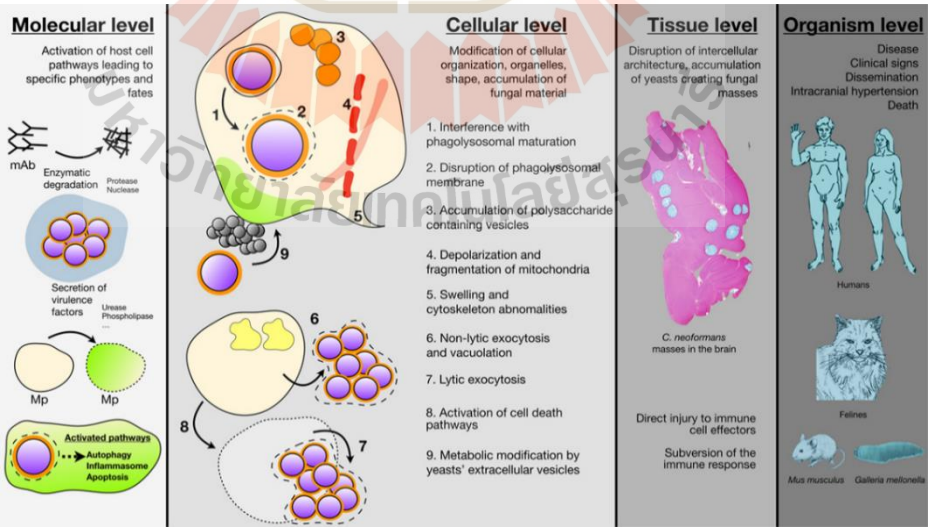

Figure 2.2 Overview of host cell damages by *C. neoformans.* There are 4 levels of cryptococcal infection including molecular, cellular, and tissue, and organism damage that can lead to cryptococcosis (Casadevall *et al.,* 2018).

2.4 The mechanisms of *C. neoformans* across the BBB

The blood-brain barrier (BBB) is selectively permeable membrane formed by endothelial cells (ECs), astrocyte end-feet, and pericytes anchored in the capillary basement membrane. ECs are joined with each other by tight junctions (TJs) to maintain the barrier function, while astrocytes and pericytes are involved in the metabolic exchange and supported or stabilized of BBB (homeostasis), respectively (Daneman *et al.*, 2010). The BBB is crucial in regulating the transport of important nutrients, ions, and hormones from the blood to the brain as well as blocking the entry of neurotoxins or pathogens as a protective shield the CNS from neurotoxic substances circulating in the blood (Abbott *et al.*, 2010) as seen from the diagram in Figure 2.3. The BBB prevents some molecules to enter to the brain, but it allows nutrients to provide energy for brain metabolism, if *Cryptococcus* attach on the transporters or signals, resulting in crossing the BBB and CNS infection (Tseng *et al.*, 2015).

There are three main mechanisms which have been proposed to describe how *Cn* passes through the BBB that called transmigration, including paracellular, transcellular and Trojan horse as shown in the diagram in Figure 2.4. Paracellular associates the weakness integrity of BBB, which pathogens enter barrier cells through loosening tight junction, and may or may not result in breakdown of the tight junction. (Sabiiti and May, 2012). One of these mechanisms involves cryptococcal *URE1* that converts urea to ammonia and effects on the extracellular matrix for promoting paracellular penetration of BBB (Tseng *et al.*, 2015). In the transcellular transmigration, cryptococcal cell are trapped on the luminal surface (blood side) and subsequently transmigrate through the endothelial cytoplasm and exit on the abluminal side (brain side) of the BBB (Sorrell *et al.*, 2016). For example, the secretion of *PLB1* interacts through lipid mediator such as PIP3 and DAG with *RAC1* that associated with STAT3 (Tseng *et al.*, 2015). Other studies demonstrated that hyaluronic acid receptor, CD44, on HBMEC is a crucial receptor which presents the adhesion and entry of *Cn* during the infection and the deficiency of this receptor decreased brain infection (Jong *et al.*, 2008). In addition, a novel metalloprotease (*MPR1*), one class of metalloprotease is required for attachment and internalization of *Cn* into the BBB both *in vitro* and *in vivo*

(Vu *et al*., 2014). Crossing the BBB via the "Trojan horse" is another route which involves *Cryptococcus* surviving in phagocytes as the horse carrying or a niche for replication and for avoiding host immunity through nonlytic exocytosis and transmigration across the BBB (Liu *et al*., 2014). These mechanisms are used by *Cn* to cross the BBB and cause CNS diseases.

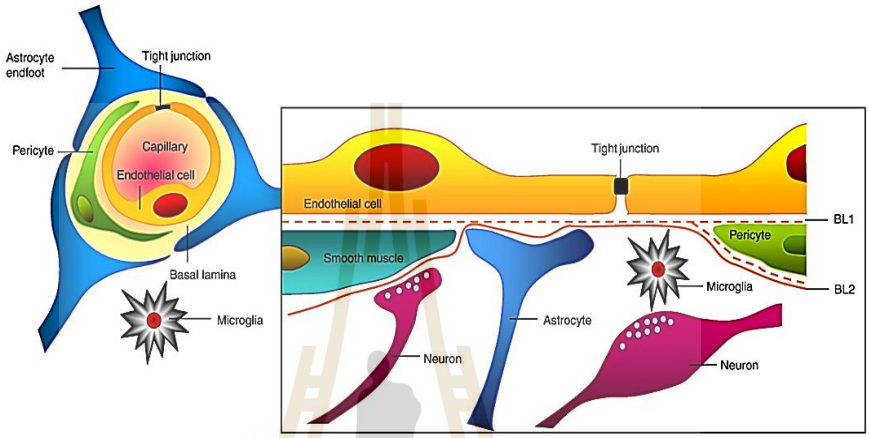

Figure 2.3 The blood brain barrier (BBB) structure. The BBB is formed by brain microvascular endothelial cells, astrocytes, pericytes, microglia and neurons (Abbott *et al.,* 2010).

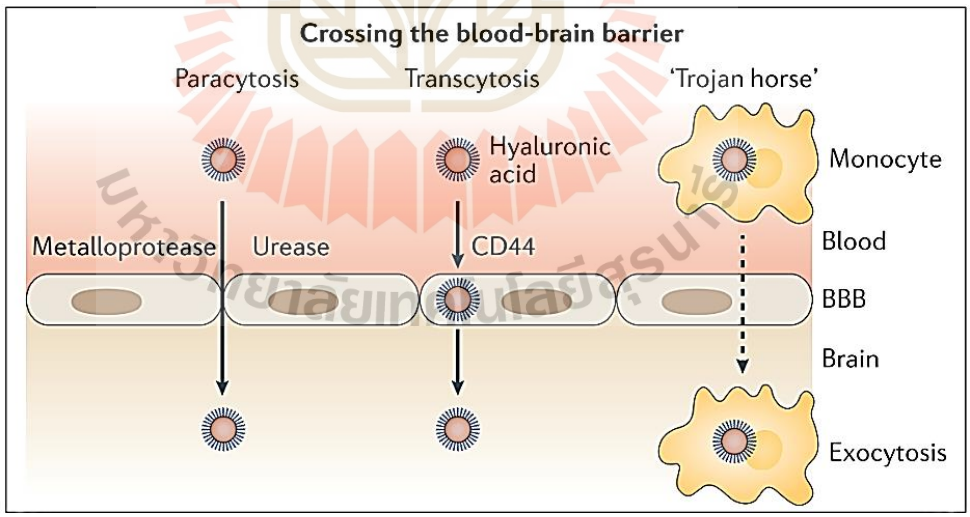

Figure 2.4 *Cn* traversal of the BBB. 1) Paracellular, which damage the intercellular tight junction. 2) Transcellular, *Cn* binds to the receptor on the endothelial cell that enhances the transmigration process. 3) Trojan horse, *Cn* can live inside phagocytes as the carrier for transmigration into the BBB (May *et al.*, 2016).

2.5 Host response

The innate immune system is the primary defense against cryptococcal infection. In healthy hosts, the infection is usually eliminated by host defense mechanisms whereas the infection is not limited to the primary site of infection in immunocompromised patients, subsequently to disseminate to the CNS (Kawakami, 2004). To defend against *Cn* infection, the host manipulates several types of innate immune cells, such as macrophages, dendritic cells (DCs) and neutrophils, which phagocytose invading fungus and secrete the reactive oxygen species (ROS) to support host protection. It is the first line of defense against pathogens, and widely protects against invading microorganisms (Lawrence and Natoli, 2011). Adaptive immunity, involves antigen-specific responses that play an important role in promoting pulmonary fungal clearance including Th1 and Th17 (Zhang *et al*., 2009). During *Cn* infection, *Cn* activates the polysaccharide capsules to prevent phagocytosis, inhibit the migration and differentiate of lymphocytes, decrease antibodies production and trigger apoptosis (Ellerbroek *et al*., 2002; Mitchell and Friedman, 1972; Monari *et al*., 2008; Murphy and Cozad, 1972). These properties avoid clearance from host immunity.

In 2012, Kawakami *et al*. reported about the pattern recognition receptors (PRRs) in sensing of *Cn*, Toll-like receptor (TLR) 9 is implicated in host defense against *Cn* specifically by the cryptococcal DNA. In addition, TLR2 and TLR4 represent PRRs for glucuronoxylomannan (GXM) that affect to inhibit the MAPK pathways which is the important pathway for proinflammatory cytokine release (Shoham *et al*., 2001). For adaptive immunity, $CD4^+$ and $CD8^+$ T cells are a crucial component of cell-mediated immunity to *Cn*, whereby $CD4^+$ plays a dominant role in recruiting macrophages and granulocytes to the lungs in pulmonary cryptococcal infection, while $CD8^+$ requires directly cell contact for releasing granulysin and its also reduces growth and survival in a lethal cryptococcal infection mode (Rohatgi and Pirofski, 2015). Therefore, the studies on the defense mechanisms against the host immune system of *Cn* are crucial because the findings will generate sufficient evidence to identify *Cryptococcus* biomarkers for better disease risk assessment, treatment and prevention using therapies and vaccines, which may increase the immunity of the host to *Cn* (Yang *et al*., 2017).

2.6 Degradation of Ammonium permease gene (*AMT2* gene)

Ammonium is a desirable nitrogen source for many fungal, bacterial, plant and human, especially in pathogenic fungi that require the nitrogen to induce both survival under adverse conditions and development processes that able to cause disease as virulence factor on a suitable host (Smith *et al*., 2003). The genes involved in ammonium transport of several fungi are the Amt/Mep/Rh family of permeases. Two permease genes, *AMT* (ammonium permeases) and *MEP* (methylammonium permeases) are the first gene cloned from *Arabidopsis thaliana* (Ninnemann *et al.*, 1994) and *Saccharomyces cerevisiae* (Marini *et al*., 1994), respectively. For *S. cerevisiae*, *MEP* play a direct role in both transport and sensing of available nitrogen and one of them, *MEP2* acts as an ammonium sensing for low ammonium availability and associates with the signal transduction cascades leading to pseudohyphae or filamentous growth (Smith *et al*., 2003). *Cn* also generates pseudohyphae during host infection by *AMT* gene including *AMT1* and *AMT2* that required under ammonium-limiting conditions. Furthermore, these gene can induce mating by producing a conjugation tube and subsequent dikaryon formation results in meiosis and sporulation (Berg *et al*., 2019). Although *AMT 1* or *AMT2* serve a similar function but the *AMT2*-deficient strains did not form invasive growth and reduced mating under the low ammonium conditions, whereas the *amt1Δ* strains exhibited the same levels of invasive growth as the wild-type (Rutherford *et al*., 2008). In 2012, Lee *et al*. reported that *AMT1 AMT2* double mutants fail to develop pseudohyphae and reveal a smooth colony whereas *AMT1* or *AMT2* single mutants are capable of forming pseudohyphae and wrinkled colonies.

However, ammonium transceptors have function to regulate fungal morphology by downstream signal transduction pathway which involves the morphology and pathogenesis of filamentous fungal (Figure 2.5).

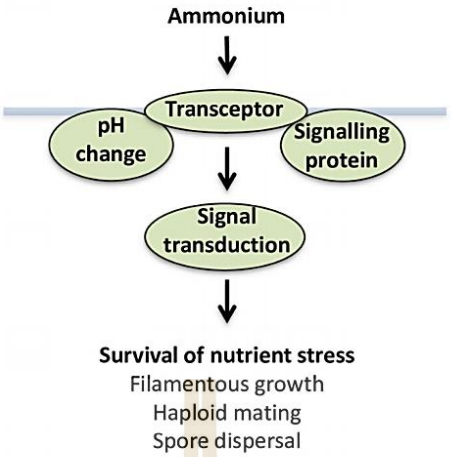

Figure 2.5 The signaling function of ammonium transceptor. The ammonium uptake changes pH in cytosolic that acts as the signal to initiate morphological change. For another mechanism involves the transceptor interacting with a signaling partner to regulate development (Berg *et al.*, 2019).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Yeast strains and cell lines

The *C. neoformans* strains were used in this study including strains H99 (serotype A, wild-type) and the *amt2Δ* mutant (04758; Ammonium permase gene). *Cryptococcus* strains have been constructed in the Hiten Madhani lab (UCSF, USA). All strains were kept at -80°C, cultured on YPD (Yeast Extract-Peptone-Dextrose) agar and incubated at 30°C for 72 h.

In the *in vitro* model of the BBB, the immobilized human cerebral microvascular endothelial cell line (hCMEC/D3) line (Figure 3.1) were used for BBB model. The cells were purchased from the American Type Culture Collection (ATCC) and provided from Asst. Prof. Dr. Oratai Weeranantanapan. The hCMEC/D3 cells were seeded on a collagen-coated culture flask until the cell were grown to confluence in EBM-2 medium with growth factors and antibiotic (gentamicin and amphotericin B) at 37°C in an atmosphere of 5% CO_2.

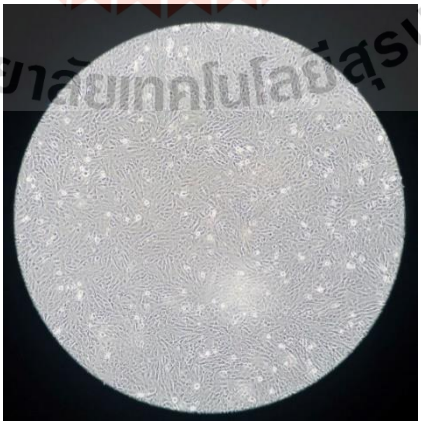

Figure 3.1 The characteristic of hCMEC/D3 cells. The hCMEC/D3 cells were observed under microscope at 400X magnification before testing with *Cn* for transmigration process in the *in vitro* model

3.1.2 Media and reagents

3.1.2.1 Bacterial culture media

Yeast Extract-Peptone-Dextrose broth (YEPD broth) medium (Himedia, India) contained 20% peptone, 10% yeast extract, and 20% dextrose (pH 6.5 ± 0.2).

Potato Dextrose agar (PDA) medium (Himedia, India) contained 200 g/L of potatoes infusion, 20% dextrose (Glucose), and 15% agar (pH 5.6 ± 0.2).

Both of YPD both and PDA medium were sterilized using an autoclave, at 121°C for 15 minutes (at a pressure of 15 psi).

Niger seed agar (NSA) (Himedia, India) contained 70% *Guizotia abyssinica* seeds, 0.780% creatinine, 10% dextrose (Glucose), 0.050% chloramphenicol, and 20% agar. The final pH was 6.7 ± 0.2 (at 25°C). Media was sterile by boiling at 100°C for 30 minutes.

Egg yolk medium (TMMEDIA, India) contained 40% proteose peptone, 25% agar, 5% disodium hydrogen phosphate, 2% dextrose (glucose), 2% sodium chloride, 1% potassium dihydrogen phosphate, 0.1% magnesium sulfate, and 0.005% hemin (pH 7.6 ± 0.2). The medium was sterile using an autoclave, at 121°C for 15 minutes (at a pressure of 15 psi).

Urea medium (Christensen medium) contained 24.01% Urea agar base, and 4% urea solution (PanReac AppliChem). For the preparation, urea agar base was sterile at 121°C for 15 minutes then filtrated urea solution was added into urea agar at 45°C.

Rapid urea broth (RUH) medium contained 4 g of urea, 0.02 g of yeast extract, 2 mg of phenol red, 0.273 g of KH_2PO_4, and 0.285 g of Na_2HPO_4 (pH 6.8), dissolved in distilled water 100 mL. Then the medium was filtrated before used (Fader *et al.,* 2015).

3.1.2.2 Cell culture media

Endothelial cell basal medium-2 (EBM-2), supplements, and growth factors were purchased from Lonza (Walkersville, MD, USA). The complete growth medium was prepared by adding growth factors and supplements (25 mL of Fetal bovine serum (FBS) , 0.2 mL of hydrocortisone, 2 mL of Human fibroblastic growth

factor-basic (hFGF-B), 0.5 mL of Vascular endothelial growth factor (VEGF), 0.5 mL of Recombinant long R insulin-like growth factor-1 (R^3IGF-1), 0.5 mL of ascorbic acid, 0.5 mL of Human epidermal growth factor (hEGF), and 0.5 mL of Gentamicin Sulfate-Amphotericin-B (GA-1,000)) into 500 mL of basal medium as 1X EBM-2. The complete medium was stored at 4°C before experiments.

3.1.2.3 Collagen Type I, Rat tail

Collagen, Type I isolation from rat tail tendons was used for coating culture plates that was purchased from EMD Millipore (Billerica, MA, USA) and stored at 2°C - 8°C.

3.1.2.4 FITC-dextrans

Fluorescein isothiocyanate–dextran (FITC-Dextran 70) powder was purchased from Sigma (Eugene, OR, USA) and stored at between -25 to 30°C until used.

3.2 Methods

3.2.1 Transcytosis assay in the *in vitro* model of the BBB

The transcytosis assay was used to analyze the transmigration of *Cryptococcus* through the *in vitro* model shown in Figure 3.2. The hCMEC/D3 was used for the transcytosis assay with the passage of less than 35. Briefly, the *in vitro* static monolayer model consisted of a transwell apparatus with the lower chamber (abluminal side) and the upper chamber (luminal side) separated by a porous membrane with a collagen-coated (8 μm; Corning). 1 x 10^4 cells of hCMEC/D3 were added on the upper chamber and were cultured for approximately 2 weeks at 37°C and 5% CO_2. During the 2-week incubation, the medium was changed from 1 to 0.5 and 0.25 strength every 3-4 days before the assay. The lower-strength medium containing growth factors was reduced for the cells to differentiate and the tight junction proteins were completely formed. Before the transcytosis assay, the integrity of the monolayer was measured as the trans-endothelial electrical resistance measurement (TEER) value by an endothelial meter. The cryptococcal cells (1 x 10^6 cells)

were added to the upper chamber and incubated overnight before collecting the migrated cryptococcal cells from the bottom chamber for CFU determination.

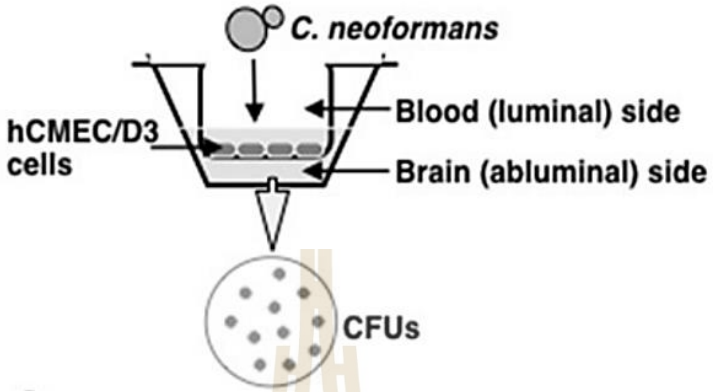

Figure 3.2 Blood-brain barrier model for transcytosis. *In vitro* monolayer of hCMEC/D3 cells are grown on the luminal (as blood capillary surface) and abluminal (as brains capillary surface). Cryptococci are added to the luminal side and collected from the abluminal side (Vu *et al.,* 2014).

3.2.1.1 Trans-endothelial electrical resistance measurement (TEERs)

The *in vitro* transcytosis assay was used to determine the transmigration of *Cryptococcus*. An endothelial volt/ohm meter for TEER-EVOM2 (World Precision Instruments, Sarasota, FL) was used to measure the TEER value. The measurements in endothelial monolayers cultured in the *in vitro* model were ranged between 30-50 Ω.cm² (Weksler *et al*., 2013). To obtain the sample resistance (R_{Cells}), the blank value of the membrane without cell (R_{Blank}), was subtracted from the total resistance (R_{Total}) of the sample;

$$R_{Tissue}\ (\Omega) = R_{Total} - R_{Blank}$$

For TEER values, the final unit area resistance (Ω.cm²) was calculated by multiplying the sample resistance (R_{cells}) by the membrane area (cm²);

$$\text{TEER}\ (\Omega.\text{cm}^2) = R_{cells}\ (\Omega) \times \text{Area of membrane}\ (\text{cm}^2)$$

The concentric pairs of electrodes on the upper and lower of the membrane caused a coincident current density flow across the membrane, and EVOM2 offered the transmembrane electrical resistance according to the current. All TEER

values were determined after subtracting the background and timing of insert membrane.

3.2.1.2 Permeability assays with FITC-dextran

The permeability assay was used to investigate the intact of BBB model in before and after transcytosis. In the *in vitro* model of the BBB was monitored by the passage of FITC-dextran (70-kDa). After the cells were infected with the wild-type and mutant strain of *Cryptococcus*, 1 mg/mL of FITC-labeled dextran was added to each well and incubated for 1 h at 37°C and 5% CO_2. Each sample from the upper and bottom chamber were read in 3 replicates on fluorescent plate (Black bottom, Corning) by the microplate reader with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Only the collagen coated on membrane without addition of D3 cells was present as a negative control. For positive control that was contained D3 cells but this model did not treat with cryptococcal strains.

$$\%\text{FITC rejection} = \left(1 - \frac{\text{FITC concentration in lower chamber}}{\text{Initial concentration of FITC}}\right) \times 100$$

3.2.2 *In vitro* growth kinetics of yeast and fungal cells

Cryptococcal strains were grown in YPD broth and incubated at 30°C and 37°C with shaking (200 rpm) for 24 h. The optical density of starting inoculum was used at 600 nm (OD_{600}) of 0.1. The OD_{600} measurements were taken every 3 h for 24 h and 6 h until 72 h using a spectrophotometer. The triplicates of the experiments were performed.

3.2.3 Virulence factor

3.2.3.1 Urease test

Each strain was cultured overnight (about 18-24 h) at 30°C in YPD broth medium then cryptococcal cells were centrifuged and resuspended in 1X sterile Phosphate Buffered Saline (PBS), pH 7.4. Equal numbers of cryptococcal cells (OD_{600} of 0.7) were spotted (5 µL) onto urea agar plate with substitution of 40% urea solution as

sole nitrogen source. The two strains of cryptococcal cells on the plates were incubated at 30°C and 37°C for 7 days. Each tube was observed during the incubation period.

Another urease activity assay was used RUH broth (Roberts *et al.*, 1978, Kwon-Chung *et al.*, 1987) to detect ureolytic activity in the wild-type and mutant yeast strains. After the adjusted the cell to 0.7, cell suspensions were inoculated in RUH broth and incubated with rotation 200 rpm at 30°C and 37°C for 10 h. The suspensions of each strain were collected in 3, 6, 9, and 10 h by centrifugation at 5,500 rpm for 5 min. After that the suspensions were measured at OD_{560nm} using spectrophotometer. The assay was performed in duplicates for each time interval.

3.2.3.2 Melanin production

To observe the production of melanin, the cryptococcal strains were cultured and maintained in 5 mL of YPD broth. The cryptococcal strains were incubated in the incubator shaker at 30°C overnight. After incubation, strains were centrifuged, washed twice, and resuspended with PBS. Then, the suspension was adjusted to OD_{600} of 0.7 and 5 µL of the suspension was seeded onto Niger seed agar (NSA). Each strain was incubated at 30°C and 37°C for 10-14 days. Melanization was examined qualitatively by the color of the colonies grown on the medium. The colonies were observed after 2 weeks of incubation. If a strain produced melanin, the colony and the media immediately surrounding the colony was turned a brown/black color and the amount of color produced was scored on a 0–5 scale with 0 being no color and 5 corresponding to when the colony was black (McClelland *et al*., 2016).

3.2.3.3 Phospholipase activity

The extracellular phospholipase activity of each strain was performed on Malt Egg Yolk agar and incubated at 30°C and 37°C for 10 days. The Phospholipase activity (Pz value) was determined by calculating the ratio of the diameter of the colony (DC) to the diameter of the colony plus the precipitation zone

(Pzd) for three colonies per strain using the equation below and was repeated for twice (McClelland *et al.*, 2016).

$$Pz = Dc/Dc + Pzd$$

Therefore, Pz = 1.00 indicated no activity (negative) strain; Pz < 1.00 and > 0.64 indicated median activity (positive) and Pz < 0.64 indicated a strong activity (strongly positive) for phospholipase activity (Cardoso *et al*., 2018).

3.2.3.4 Capsule formation

A single colony from YPD agar of each strain was cultured in YPD broth medium and incubated at 30°C for overnight. Then the suspension of each strain was washed twice and resuspend with 1X PBS, adjusted the concentration of yeast cells with OD_{600} of 0.7 and incubated in 2 mL of capsule induction medium (DMEM; Dulbecco's Modified Eagle's Medium plus 20% FBS; Fetal Bovine Serum) and was incubated at 30°C and 37°C for 48 h with shaking at 200 rpm. The cell suspension was washed, centrifuged at 5,500 rpm for 3 min, and resuspended in 500 µL of 1X PBS. To observe and measure the size of the capsule, the cell suspensions were mixed with an India ink, and then observed under a microscope using light microscope (40 objective lens) at least five different fields randomly chosen and photographed, and 30 cells were analyzed. To calculate the capsule ratio, the diameter of the whole cell and the cell body was measured and the capsule volume was defined as the difference between the volume of the whole cell (yeast cell and capsule) and the volume of the cell body (no capsule).

3.2.4 *In vitro* phenotypic assay

The extent of invasive growth of cryptococcal strains was determined under the nitrogen-limiting condition. Two strains of *Cn*, the wild-type (H99) and *amt2Δ* mutant strains were grown under nutrient-limiting condition on Yeast nitrogen base (YNB) media (without amino acids plus 10 µM, 30 µM, and 50 µM ammonium sulfate; $((NH_4)_2SO_4)$ at 30°C and 37°C for 1 week. Cells on the surface of agar were observed

under microscope to indicate the pseudohyphae growth. This experiment was repeated three times.

3.2.5 The sensitivity assay

The sensitivity to nitrogen source of *Cn* was identified on the YNB medium contained ammonium concentrations (10 μM, 30 μM, and 50 μM). Two strains of *Cn*, wild-type and *amt2Δ* mutant strains were cultured in YPD broth medium overnight at 30°C for 15 days. The cells were suspended and washed with sterile H_2O. Then, the serially diluted cells were spotted on YNB contained differently concentration of $(NH_4)_2SO_4$. The results were observed the growth on medium.

3.2.6 Statistical analysis

Data are shown as mean ± standard error of mean (SEM) from at least three independent experiments. Statistical significance was established at $*p < 0.05$ in the experiments between wild-type and *amt2Δ* mutant strain. The unpaired t-test was used for comparison of two groups in the transcytosis assay. Virulence factor assays were used Mann-Whitney U test for comparison (GraphPad Prism 9.5.1.)

CHAPTER IV
RESULTS AND DISCUSSION

4.1 The transmigration of *C. neoformans* across through the *in vitro* BBB model

In a previous study, the overall transcriptome of host response to *Cn* infection revealed that some genes were upregulated during *Cn* transmigration. The Ammonium transporters gene was upregulated to 1.98-fold change (Jamklang *et al*., 2018). Therefore, we were interested in this gene and studied the association of the gene with the transmigration process and the relevant with other important virulence factors.

To study the role of the ammonium transporter gene (*AMT2*) in the transmigration process of *Cn*, the mutant strain of Cn lacking *AMT2* gene (*amt2Δ*) was used for our study. To investigate the capability of *amt2Δ* mutant strain to cross the *in vitro* model of BBB (Figure 3.2. in chapter III) using human brain microvascular endothelial cells (hCMEC/D3) in which collagen was coated on the insert membrane and the hCMEC/D3 cell line was grown to confluency and allowed fully differentiated into intact barrier with appropriate concentration of media from 1 to 0.5 and 0.25 dilution. After that the integrity of model was measured using TEER investigation. Before the model was used to test with the wild-type and *amt2Δ* mutant strains, the TEER value for BBB model was 48.03 ± 4.89 Ω.cm². According to previous studies, the TEER value of the model of the endothelial monolayer cultured under static condition were around 30-50 Ω.cm² (Weksler *et al*., 2013).

The result of the permeability of the BBB was performed by FITC dextrans assay determined by calculating the percentage of FITC dextrans concentration that was rejected by monolayer membrane. The percentage of rejection in the wild-type and

amt2Δ mutant strains before transcytosis assay showed 96.84 ± 1.07% whereas, the negative control which is the membrane coated with collagen but there were no endothelial cells after inoculation grown on the membrane exhibited less than 20%. After incubation of *Cn* in the BBB model, the percentage of rejection showed a slight decrease to 91.94 ± 1.59% (Figure 4.1). From the result, the tight junction of the BBB model was still intact before and with *Cn* in the transmigration assay

In order to evaluate the *amt2Δ* mutant strain whether it has an ability to transmigrate through the endothelial cells by collected the suspension (yeast cells with EBM-2 media) in the abluminal side (lower chamber) and plated for counting colony forming units (CFUs) (Figure 4.2). Cryptococcal free cells of the wild-type strain was $8.36 \times 10^5 \pm 1.71$ CFU/mL whereas the *amt2Δ* mutant strain lost the ability to transmigrate through hCMEC/D3 monolayer that was significantly decreased to $2.26 \times 10^5 \pm 0.94$ CFU/mL (Figure 4.3), suggesting that the ability of *amt2Δ* was reduced in transmigration of *Cn* across the BBB.

This finding demonstrated that *AMT2* gene plays an important role in the transversal of BBB. This study was able to evaluate the mechanisms of *Cn* used for invasion and cause cryptococcal infection in the brain. This study concluded that *Cn* traverse across the BBB using transcellular mechanism because the integrity of the brain endothelial cells was not greatly disrupted after infected with cryptococcal strains. Therefore, the deletion of *AMT2* of *Cn* reduced the ability of *Cn* to transmigrate causing cryptococcal infection. These results might be useful for the research and development on drug development to disrupt specific molecules in the therapeutic mechanism for cryptococcal disease.

In the *in vivo* and *in vitro* model of BBB for cryptococcal transmigration, it was observed inside the endothelial cells of the brain, suggesting that the fungal cells were directly up taken by the endothelial cells, and *Cn* transmigrate through the cytoplasm to reach the brain (Chang *et al.*, 2004). Recent evidence of the responses of HBMEC to transcellular brain invasions of *Cn* include membrane rearrangements, activation of intracellular signaling pathways, and activation of the cytoskeletal system (Tseng *et al.*, 2015). The evidence that transcellular allows pathogens to cross the BBB by transcytosis through the human brain microvascular endothelial cells (BMECs) by taking advantage of cellular endocytic

processes was observed in several brain infection pathogens such as *Escherichia coli,* group B *Streptococcus, Listeria monocytogenes*, and *Candida albicans* (Shi *et al.*, 2010; Chang *et al.*, 2011; Kim, 2008). Regarding the mechanisms of transmigration process of *Cn* across the BBB, there were reports on the involvement of glycoprotein CD44 expressed by BMECs in enhancing the binding of *Cn* with hyaluronic acid, results in filamin and actin cytoskeleton rearrangement that correlated with *CPS1* gene (Jong *et al.*, 2008). In addition, a novel metalloprotease (*MPR1*), one class of metalloprotease is required for attachment and internalization brain endothelial cells without protein complex disruption (Vu *et al.*, 2014). Moreover, *PLB1*, *ITR1a*, *ITR3c, and RUUB1* are other genes that induce *Cn* to transverse through the brain via transcellular mechanism (Liu *et al.*, 2013; Fisher *et al.*, 2002; Tseng *et al.*, 2012).

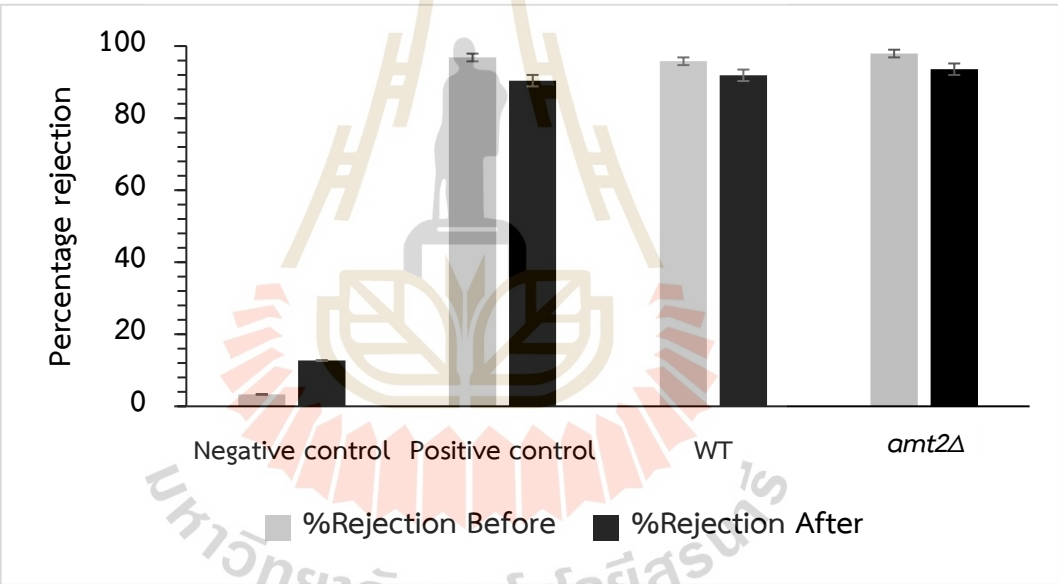

Figure 4.1 The permeability of BBB model using FIT-C dextrans before and after treated with *Cn*. The TEER values of the hCMEC/D3 monolayer are more than 90% that acceptable for transcytosis assay. Negative control without hCMEC/D3 monolayer showed lower percentage of rejection less than 20%

In conclusion, the cryptococcal strains across the BBB utilizing a transcellular pathway as the main mechanism that involved the use of some molecules to trap or disrupt the receptor on endothelial cells and then across through the brain. In addition, the lack of *AMT2* gene significantly reduced the transmigration of *Cn* in the *in vitro* BBB modal and also the absence expression of *AMT2* did not affect the integrity of barrier

membrane, suggesting that the transmigration of *Cn* depends on the functional expression of *AMT2* gene. However, the mechanisms are virtually unknown. Therefore, the transmigration process associated with *AMT2* gene of *Cn* is necessary to investigate the mechanism in the future direction.

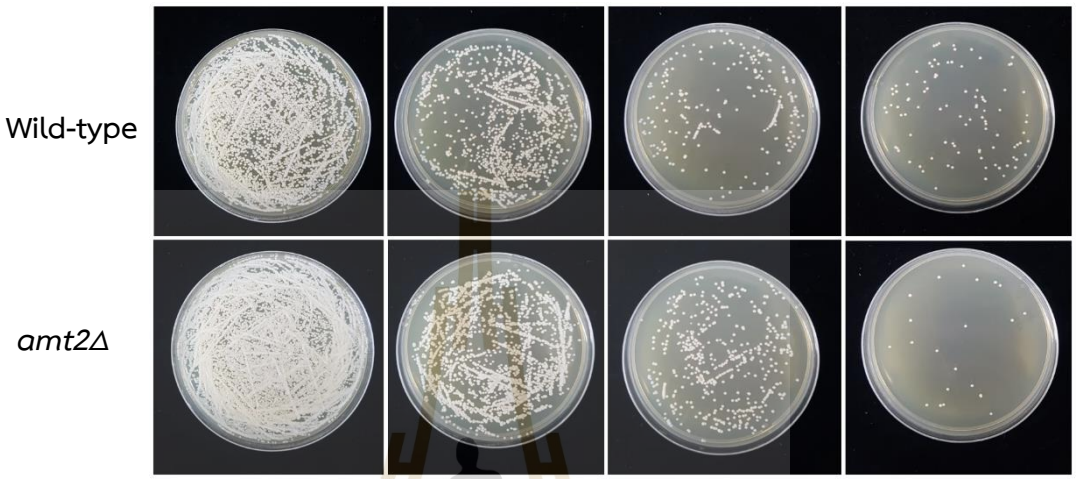

Figure 4.2 Comparison of colony forming units of wild-type and mutant strains in dilution plating (from left to right undiluted, 10^{-1}, 10^{-2}, 10^{-3}) on YPD agar.

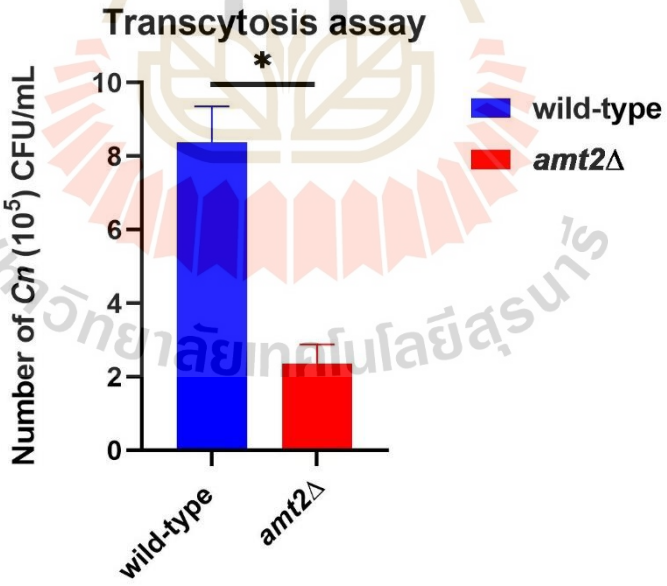

Figure 4.3 The CFU count determination of transcytosis assay of the wild-type and *amt2Δ* mutant strains in the in vitro BBB model. In comparison with the wild-type strain, the capacity of the *amt2Δ* mutant strain to pass through the brain is significantly reduced using unpaired t-test. (Mean ± SEM, $n = 3$, $^*p < 0.05$) (GraphPad Prism 9.5.1).

4.2 *In vitro* growth kinetics of yeast cells

Cryptococcus neoformans is an encapsulated environmental fungus that has likely acquired the requisite adaptive mechanisms through interactions in mammalian core body temperature. To demonstrate the growth kinetics, the knockout strain was cultured in the different temperature including 30°C (environment) and 37°C (mammalian host). The results showed that the growth exponential phase of the *amt2Δ* mutant strain was slightly slower than the wild-type strain when incubated at 21-46 h in both 30°C and 37°C but remained stable during 2 to 3 days of the *in vitro* method using YPD broth medium. Moreover, the ability for thermal growth tolerances at mammalian temperature between the two strains was not significantly decreased compared by the independent t-test with $p < 0.05$ (Figure 4.4).

The results of this study showed that the *amt2Δ* mutant did not have a defect to the kinetics growth curve and also *amt2Δ* mutant strain was capable to grow and proliferate at 37°C as comparable to the thermotolerant wild-type strain. This ability leads *Cn* to invade and disseminate in animal infection from the lung to the central nervous system causing deadly cryptococcal disease (Maziar and Perfect, 2016). Previous work has demonstrated the wild-type strain could survive and proliferate under body temperature of rabbit at 39.5°C in cerebrospinal fluid (CSF) that meant to closely mimic the human host situation in which patients usually present with fever and are often undergoing steroid treatment (Casadevall *et al.*, 2002). In addition, the mammalian temperature involved the enhancing in virulence factor such as the large capsule enlargement and cell body size as well as adaptive morphological characteristics (Zaragoza *et al.*, 2011). Interestingly, this study also presented the results that yeast cells were enveloped with the massive capsule size (Result from 4.5). Also, another virulence includes urease activity, melanization, phospholipase activity and phenotypic assay were confirmed the abilities at both 30°C and 37°C to determine the virulence factors.

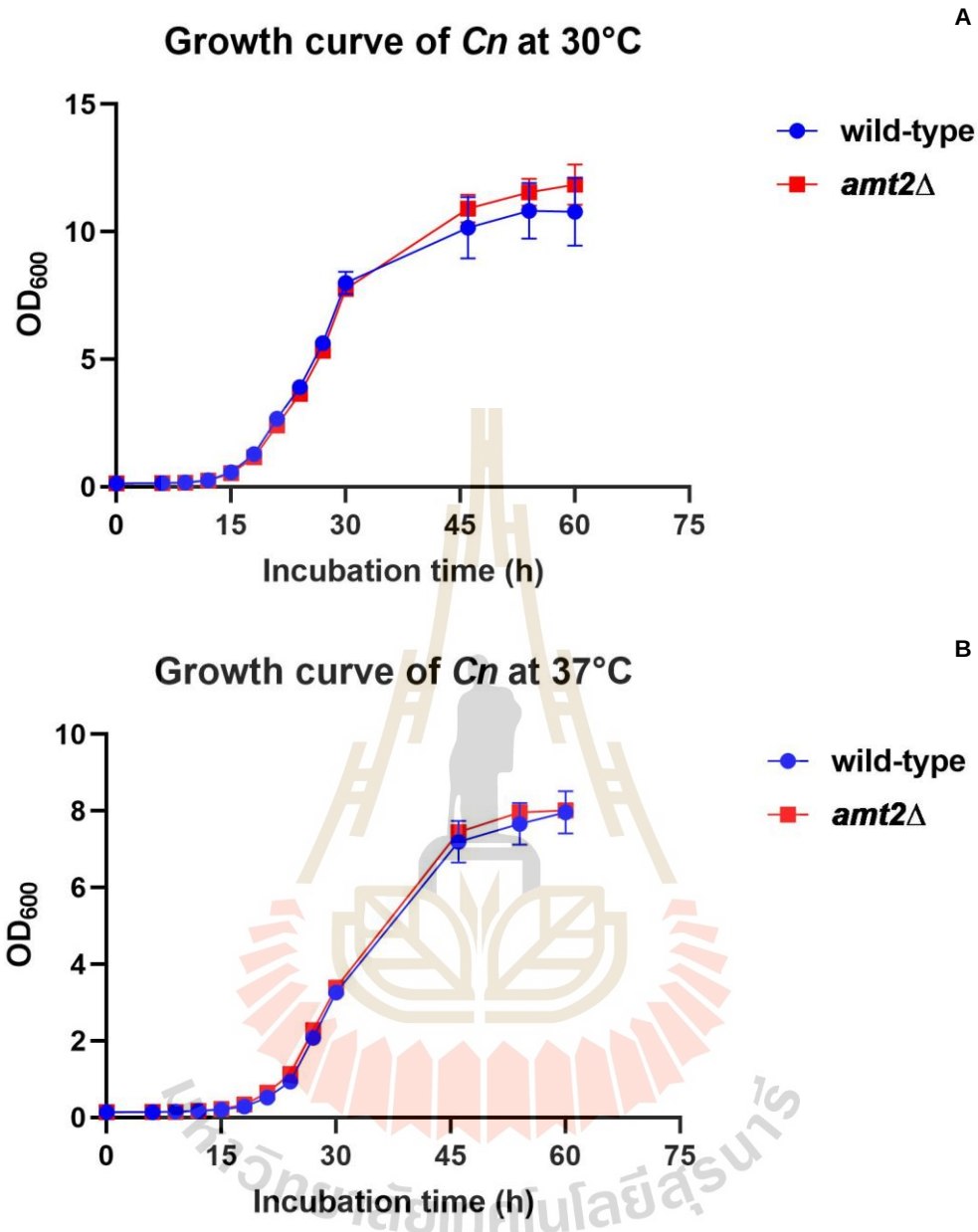

Figure 4.4 Growth kinetic of both wild-type and mutant strains in normal environment (30°C) and mammalian host (37°C). The growth rate of cryptococcal strains were not significantly different using Mann-Whitney U test (Mean ± SEM, *$p < 0.05$) (GraphPad Prism 9.5.1).

4.3 Virulence factor of *Cryptococcus neoformans*

4.3.1 Capsule formation

The polysaccharide capsule is a necessary virulence factor found only in the fungal pathogen, *C. neoformans*. The polysaccharide is secreted while they live in a stress environment and also host tissues during infection (Selvig *et al*., 2011). Several factors including high CO_2 levels, iron deficiency, and serum enhance the *in vitro* capsule growth (Zaragoza and Casadevall, 2004). In our research, capsule production was induced at 37°C cultures in DMEM with FBS. However, studies about the effect of serum on capsule size are scarce (Zaragoza *et al*., 2003).

This experiment revealed that capsule formation was observed after incubated in DMEM with 20% FBS for 48 h and the polysaccharide capsule size at 37°C was slightly larger than when incubated at 30°C. The capsule is larger in the stationary phase which is around 48 h than during exponential growth (Janbon *et al*., 2004). The capsule size of the mutant strain was significantly decrease when compared to the wild-type in both temperatures as shown in the Figure 4.5. The average ratio of capsular size of the wild-type and *amt2Δ* mutant strains at 30°C were 1.63 ± 0.12 μm and 1.45 ± 0.14 μm, respectively. At the human temperature, the wild-type strain demonstrated 1.845 ± 0.15 μm in size and the *amt2Δ* mutant showed 1.61 ± 0.13 μm.

These results indicate that the deletion of *AMT2* affects the size of polysaccharide capsule production. Nitrogen sources greatly induce capsule production of *Cn* that correlated the nitrogen metabolism causing signal for capsule induction (Frazzitta *et al*., 2013). From our study, the deletion of *AMT2* transceptor may be repressed the capsule expression because *AMT2* gene associated with the nitrogen transporting into the cryptococcal cells. If *Cn* lose ability to transport nitrogen source, it could be low signal for capsule production. This can be summarized that the decreasing of capsule size links to increase the phagocytosis from host immune response. There were reports on prolonged cell cycle progression resulted in cells with larger capsule which shields the yeast cell from a variety of host immune defenses and inhibits phagocytosis, leukocyte migration, and cytokine generation (García-Rodas *et al*., 2014; Crawford *et al*., 2020).

The genes directly associated with capsule biosynthesis were found to be capsule-associated genes (*CAP* genes) including *CAP10, CAP59, CAP60,* and *CAP64* (Janbon, 2004). Additionally, the cAMP/PKA pathway is crucial for controlling titan cell growth and cell surface modification which response to nutritional and host signals by glycolysis related gene (Kronstad and Caza, 2019).

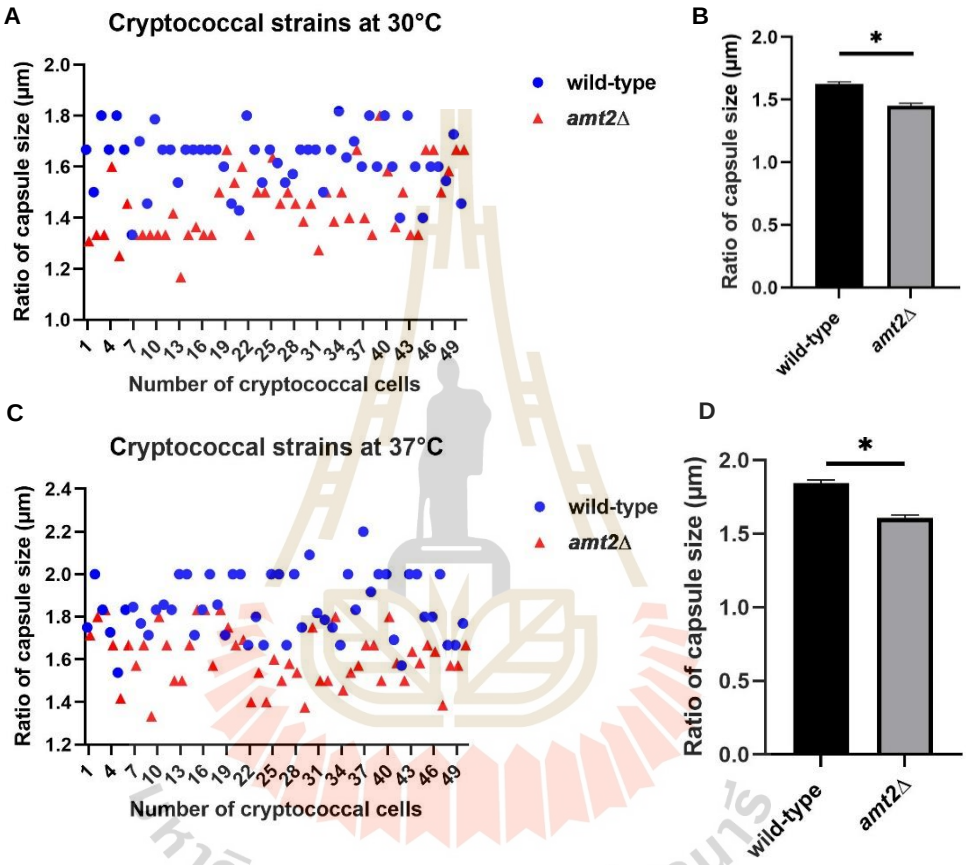

Figure 4.5 The ratio capsule size of cryptococcal strains showed both significantly different at 30°C (A-B) and 37°C (C-D). The *amt2Δ* mutant strain showed smaller polysaccharide capsule size than the wild-type that compared with Mann-Whitney U test (Mean ± SEM, $n = 50$, $^*p < 0.05$) (GraphPad Prism 9.5.1).

Our results are not correspondent with previous studies that have demonstrated that the pathogenicity of *Cn* was unaffected by the absence of ammonium permeases. In both the mouse and wax moth insect models of cryptococcosis, mutant cells lacking one or both *AMT* permeases (*AMT1* and *AMT2*) were just as pathogenic as wild-type cells (Rutherford *et al*., 2007). However, mutant strain still has ability to produce polysaccharide capsule.

4.3.2 Urease activity

Another crucial component of *Cn* is urease which catalyzes the hydrolysis of urea into carbon dioxide and ammonia called "ureolytic activity". The reality that urea is equally distributed throughout the human body raises the possibility that mammalian infections utilize it as a food source (Rutherford *et al.*, 2014). Urease was first implicated in the pathophysiology of cryptococcosis in a study on experimental mouse models of the disease (Cox *et al*., 2000). In our work, the ability of urease production was investigated in the *amt2Δ* mutant strain using urea agar and RUH medium.

In the urea agar plate, the *amt2Δ* mutant demonstrated the urease activity that broke down urea into ammonia and turned medium containing phenol red from light orange to pink color. The results revealed that *amt2Δ* mutant strain has ability to secrete urease at the incubation time of 3, 6, and 9 h but the reaction rate was slightly slower than that of the wild-type strain. The different temperatures (30°C and 37°C) effect on the growth rate of cryptococcal strains as colonies shown in Figure 4.6. For the quantitative data, ureolytic activity was determined by RUH broth and the results were shown in Figure 4.7. At longer incubation periods, both strains showed a greater capacity in urease releasing. The mutant and wild-type strain did not significantly differ in urease level at the same incubation time. Therefore, the mutant strain has ability to produce urease which is not only interacting with macrophage using alkalinization to increase the pH surrounding environment but also promotes *Cn* to exit from macrophages without destroying the host cells (Fu *et al.*, 2018). In addition, urease promotes *Cn* to across BBB by conversed urea to ammonia which may be migrated via paracellular penetration or induced some signal transduction such as morphological change (Tseng *et al.*, 2015 and Shi *et al.*, 2010).

The urease enzyme catalyzes the hydrolysis of urea, which results in the production of ammonia (NH_4) and carbonic acid (H_2CO_3). The *AMT1* and *AMT2* transporters import the ammonia molecules into intracellular for growth development. The gene carbonic anhydrases (*CAN2*) also produces a carbonic anhydrase of the ß-class (Perfect *et al.*, 2013) shown in Figure 4.8.

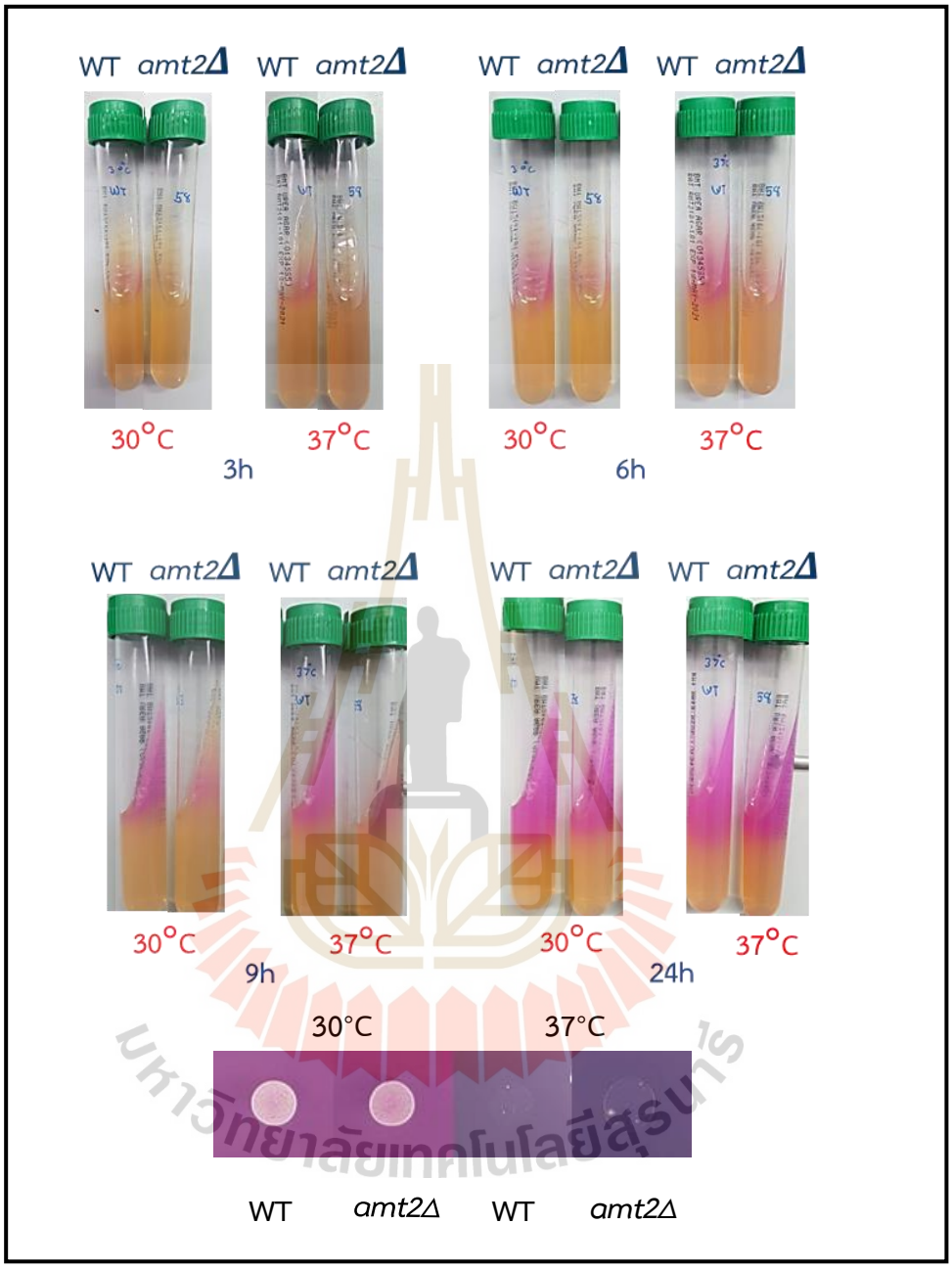

Figure 4.6 The urease activity of the wild-type and *amt2Δ* mutant strain at 30°C and 37°C for 3, 6, 9 and 24 h on urea agar. The character of colonies was observed after 48 h incubation that showed white colonies at 30°C and the growth was better than incubation at 37°C.

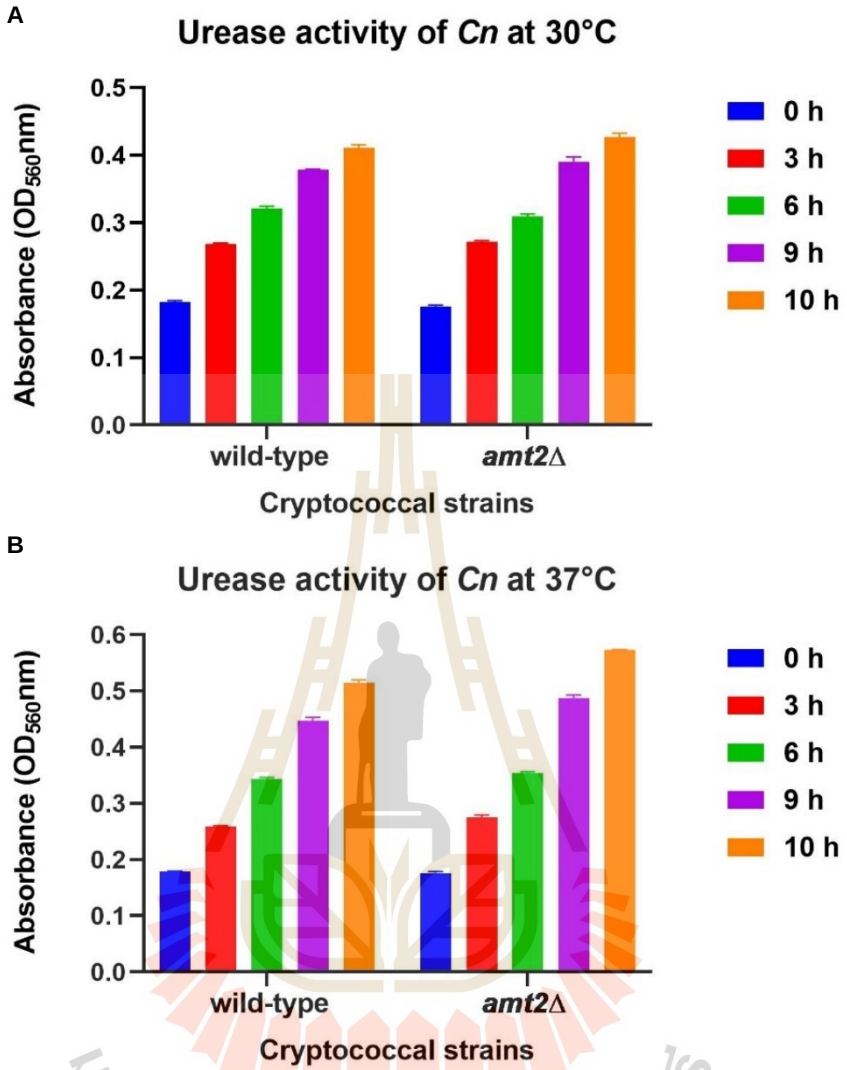

Figure 4.7 Ureolytic activity test with RUH broth incubated at 30°C (A) and 37°C (B) for 3, 6, 9 and 10 h. The wild-type and *amt2Δ* mutant strain were not significantly different in releasing urease activity and the longer incubation time induced more urease activity whereas the different temperature did not affect the urease activity. Using Mann-Whitney U test ($n = 3$, $p < 0.05$) (GraphPad Prism 9.5.1).

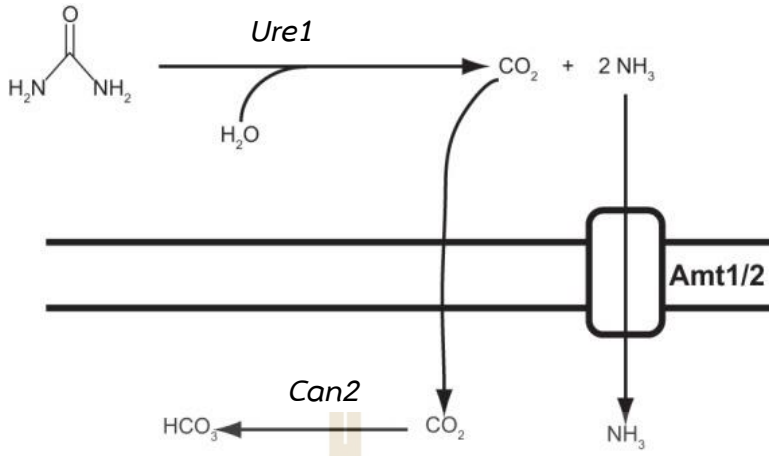

Figure 4.8 Schematic diagram of urease and urea metabolism of *Cn*. The *URE1* gene converses urea to product substates then were used as energy for yeast development (Perfect *et al.*, 2013).

According to these findings, the lack of *AMT2* or ammonium transporter gene had no effect on urease activity reflecting that the mutant has no defect on urease production. Previously studied suggested that ammonium transporter is the nutrient receptors that involves ammonium transporting and signaling that take up ammonia from extracellular via passive diffusion and regulate signal transduction for pseudohyphal growth, respectively (Khademi *et al.*, 2004; Dabas *et al.*, 2009). Therefore, *AMT2* gene may not be specifically correlated with urease producing of *Cn*.

Several research works have directly deleted the related genes, urease system such as *URE1* with three accessory proteins encoded by *URE4, URE6,* and *URE7* that were found to have the efficiency in the brain invasion (Singh *et al.*, 2013). The *URE1* deleted strains significantly reduced the infection rate in mice (Coelho *et al.*, 2018). In addition, the nutrient stress is another factor that affects the urease activity. Under nutrient-limited conditions, the wild-type strain of *Cn* (H99) releases the highest level of urease activity (Toplis *et al.*, 2020). The evidence from Olszewski *et al.* (2004), the direct injection of *URE1* deleted *Cn* with intracranial infection in mice indicated that the *URE1* deficient strain was significantly decreased in systemic dissemination.

In conclusion, the deletion of *AMT2* gene did not reduce the ureolytic activity because the role of *AMT2* gene or ammonium transporter is responsible for control of the level of ammonium between the cell and environment that induces processes to

promote survival during nutrient stress whereas the urease activity was related with urease gene (*URE1*) that promote fungal infections.

4.3.3 Melanin production

The melanin production is present in the fungal pathogen which synthesizes melanin to protect themselves from severe condition. In *Cn* cells, melanin is produced from phenolic compounds that occur in melanosomes, which are small vesicles that are transported and deposited into the cell wall (Casadevall *et al.*, 2009). To test malanization in our strains, we utilized Niger Seed Agar (NSA) containing caffeic acid as substate.

The results showed that both of the wild-type and *amt2Δ* mutant strain did not exhibit brown or black colonies after 10 days incubation at 30℃ and 37℃ that may be because both strains have lost the ability to release or express laccase enzymes to catalyze phenolic substates (Figure 4.9). Our experiments have compared *Cn* with the *Candida albicans* but this strain did not produce melanin pigment. These could be the effect of the culture medium not having the proper conditions to promote biosynthesis and also cryptococcal strains being maintained for an extended period of time at -80℃, which could damage or weaken some of capabilities of yeast cells. As the results in previous study from Menezes *et al.*, 2011 found that *C. albicans* did not produce dark pigmented melanin when incubated on NSA. In the environment and animal hosts, *Cn* can produce melanin pigments from a wide variety of phenolic compounds that serves as a rich source of precursors in the form of catecholamines, nitrogen-containing diphenolic compounds (Casadevall *et al.*, 2017). Additionally, some virulence factors can be lost by a single mutation, as demonstrated by laboratory-generated, temperature-sensitive which makes the explanation of a retained phenotype attractive (Robert and Casadevall, 2009). Several previous studies reported that melanin production in *Cn* commonly used L-DOPA as the precursor (Nosanchuk *et al.*, 2015). The results from Baker *et al.*, 2022 indicated that there are a lot of catecholamines found in brain tissue therefore the use of dopamine as polymerization precursor is capable of producing melanin pigment comparable to that produced during infection.

In general, the melanin producing in virulence was required for induction from laccase gene under nutrients starvation conditions (Zhu and Williamson, 2004).

The dominant laccase enzyme activity is *LAC1* gene which regulates transcription factors including *Bzp4, Usv101, Mbs1*, and *Hob1* as shown in Figure 4.10 (Dongpil *et al.*, 2019). Moreover, *LAC1* plays a role in the ability of *Cn* to disseminate to extrapulmonary organs and then infect intravenously as well as disseminate the CNS (Noverr *et al.*, 2004).

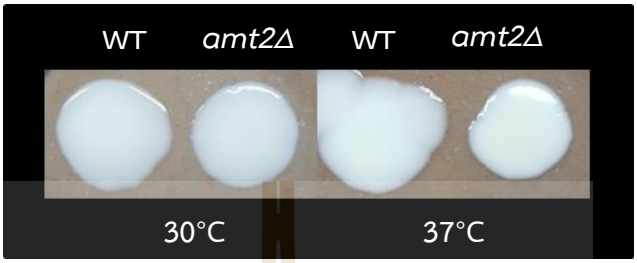

Figure 4.9 Melanization of the wild-type and *amt2Δ* mutant strain. Both of the wild-type and mutant did not turn to brown colonies.

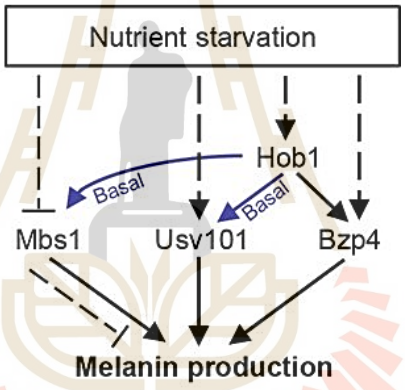

Figure 4.10 Nutrient starvation induces melanin pigment. The condition with limiting-nutrients induced *LAC1* gene by signaling to cascade proteins called *HOB1* gene then activated *BZP4, USV101* and *MBS1* gene that subsequently activated the melanin production

This experiment concluded that *amt2Δ* mutant strain cannot secrete enzyme for melanin pigment. We hypothesized that the gene responsible for melanin pigment production would probably not be related to the lack of *AMT2* gene, meanwhile, *LAC1* gene may be sustained in the yeast genome but did not express. To test this hypothesis, *LAC1* gene expression should be confirmed using PCR method or/and the levels of protein expression using a western blot analysis. Moreover, the *Cn* isolates from cryptococcosis patients which may have a stronger capacity to produce melanin pigment than the *Cn* in laboratory because the human host has the complex environment inside the host that is not fully reproduced in the laboratory (Renney *et al.*, 2021).

4.3.4 Phospholipase production

Phospholipases are a heterogeneous group of enzymes that are able to hydrolyze ester linkages in glycerophospholipids which are the major components of cells membrane (Djordjevic, 2010). This mechanism induces the integrity of cell membrane losing. In *Cn,* phospholipase B is a well-characterized virulence factor (Cox *et al.,* 2001; Noverr *et al.,* 2003; Djordjevic, 2010). Therefore, the *amt2Δ* mutant strain was investigated for phospholipase production using egg yolk agar.

Our results revealed that both the wild-type and *amt2Δ* mutant strain did not manifest the precipitation zone around the colonies after incubated at 30°C and 37°C for 14 days. For colony morphology, both the wild-type and mutant colonies were appeared as entire margin with white colonies at 30°C and 37°C. On the colony surface, they were grown with concentric ring-like pattern formed when incubated at 30°C but the precipitation zone was not formed. At 37°C, *Cn* showed the elevation of the colonies without opaque as shown in Figure 4.11 This result indicated that *Cn* was lost the ability to secrete or express phospholipase enzymes activity. This may be due to the unsuitable environment for growth. Moreover, several virulence factors can be lost by a single mutation, as demonstrated by laboratory modification or temperature sensitivity (Robert and Casadevall, 2009).

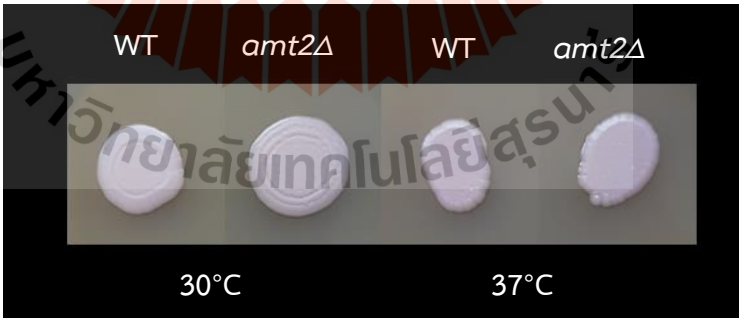

Figure 4.11 Phospholipase activity of the wild-type and *amt2Δ* mutant strain at 30°C and 37°C for 14 days. Both strains did not have the precipitation zone surrounding the colonies.

Phospholipase is regulated by *PLB1* gene which is a phospholipid-modifying enzyme with multiple enzymatic activities (Chen *et al.*, 1997). The optimal pH range for *PLB1* performance is 4.0 to 5.0 (conditions comparable to those of the phagosome), and it is active at 37°C (Chen *et al*., 2000). For this experiment, the tested strains were

cultured on egg yolk agar pH 7.6 ± 0.2 to determine the phospholipase activity. Most of the yeast grow very well between pH 4.5- 6.5 that are important in their metabolism (Yalcin *et al.*, 2008). Moreover, pH also alters gene expression, the *PLB* protein expressed with pH 4-5 on SDS/PAGE (Chen *et al.*, 2000).

These results showed that both the wild-type and *amt2Δ* mutant did not produce phospholipase. This can be the result of inappropriate testing conditions. Therefore, *LAC1* gene or protein expression in both strains should be confirmed using RT-PCR or a western blot technique, respectively.

4.4 Phenotypic assay

Cn can alter a variety of development patterns, including yeast and filamentous forms, depending on environment conditions. There is the evidence that *Cn* enable change morphological during direct transcytosis. The fungal cells penetrate the capillary endothelium which were stopped and trapped on mouse brain capillary without replication (Shi *et al.,* 2010). As a result, we looked into whether the ammonium permease gene, *AMT2*, can cause pseudohyphal development under nitrogen-limiting circumstances and whether AMT2 gene is sensitive to various nitrogen supply concentrations.

In this study, we found that both the wild-type and *amt2Δ* mutant strain did not form pseudohyphae after 3, 5 and 7 days of incubation on yeast nitrogen base (YNB) media containing 10 μM and 50 μM ammonium sulfate at both 30°C and 37°C as shown in Figure 4.12. The colony characteristics of the wild-type and the knockout strains exhibited that small white colonies, circular shape, flat with raised entire margin and mucoid texture. These results indicated that *AMT2* gene did not induce pseudohyphae growth under ammonium-limiting condition that might be the case because the concentration of ammonium entering the cell was insufficient to activate the ammonium transceptor. Ammonium is present in blood and cerebrospinal fluid (CSF) in concentrations of 29 M and 11.9 M, respectively (data from the Human Metabolome Database website). Previous studied demonstrated pseudohyphae growth of *Cn* using YNB medium that contained nitrogen source with 2% glucose (Heitman *et al.*, 2012 and Lee *et al.*, 2011). The carbon source is major energy source for fungal development

that induce a morphological switch from budding to pseudohyphal growth (Gagiano *et al*., 2002). The main carbon sources available in a host during fungal infection are glucose, lactate, and acetate (Ries *et al*., 2018). Although the downstream regulatory mechanism detail of glucose in *Cn* has not been thoroughly defined but the signaling cascades regulating via cAMP/protein kinase A (PKA) or mitogen-activated protein (MAP) kinase pathways (Lengeler *et al*., 2000; Kronstad *et al*., 2011; Wang *et al*., 2011). In addition, *AMT2* gene is required for the initiation of invasive growth of haploid to diploid cells (mating) under low-nitrogen conditions which is mediated by diffusible molecules as pheromones (Rutherford *et al.*, 2008 and Manney *et al.*, 1981). In the yeast model, *S. cerevisiae*, nitrogen starvation condition induces pseudohyphal growth of diploid, but not haploid (Gimeno *et al.,* 1992). The work by Lee and colleagues in 2012 studied in *amt1Δ* or *amt2Δ* single mutant strains, *Cn* pseudohyphae were observed to forming with wrinkled colonies whereas *amt1amt2* double mutants failed to generate pseudohyphae and showed a smooth colony under nitrogen-limiting conditions. Moreover, the unisexual mating of *Cn* exhibited pseudohyphal growth at the edge of the colonies using medium contained glucose, galactose, or sucrose as the fermentable carbon source. As one of the most energy source provides rapid metabolism for growth and colonization (Rutherford *et al.*, 2019). Therefore, the induction of pseudohyphae forming require the deprivation of either a carbon source or a nitrogen source that leads to diploid yeast and results in pseudohyphae forming.

In conclusion, the deletion of *AMT2* gene did not induce pseudohyphal growth in this work but the sensitivities of the mutant strain still exhibited as similar to the wild-type strain that may be because occurred from the absence of carbon source. Therefore, the medium for pseudohyphal growth should be supplemented with carbon source. According to the findings of this experiment, the phenotypic assay might be not suitable for comparison the morphological change of the wild-type and mutant strains. With this, it suggests that the morphological change phenotype should be tested in cryptococcal cells while exposed with the hCMEC/D3 cell.

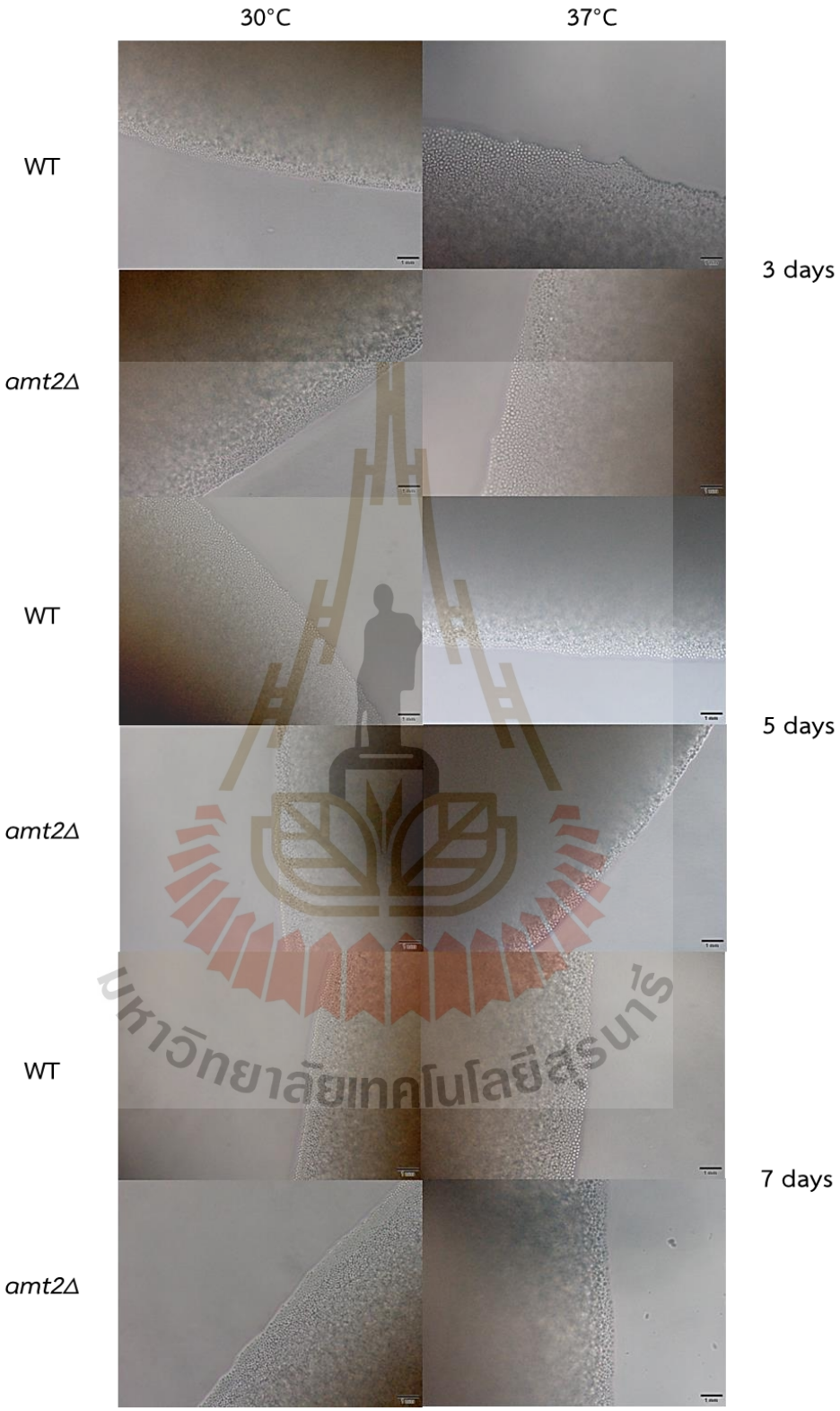

Figure 4.12 Phenotypic assay after incubated for 3, 5 and 7 days on YNB media under light microscope (20X objective lens).

4.5 The sensitivity of *Cn* growth under the limiting-nitrogen source

For this work, the sensitivity assay was used to identify growth rate on nitrogen source for *AMT2* activity as shown in Figure 4.13. The growth rate did not reveal any obvious difference between the wild-type and mutant strain on the medium contained ammonium concentrations (10 μM, 30 μM, and 50 μM). Moreover, the wild-type and mutant strains showed the same colonies morphology that appeared circular shape, convex elevation and small white colonies. These results suggest that the deficient *AMT2* gene did not affect the ability to utilize nitrogen source because *Cn* has two ammonium permease gene *AMT1* and *AMT2*. Although, *AMT2* encodes a high-affinity ammonium permease that is induced by nitrogen-limiting conditions whereas *AMT1* is a low-affinity ammonium permease but it is expressed constitutively (Rutherford *et al.*, 2008). In fact, the lack of either *AMT1* or *AMT2* genes did not affect the sensitivity of *Cn* that related with psedohyphal growth under low-ammonium condition. The double mutant strain of *amt1Δamt2Δ* became sensitive and lost its capacity to generate pseudohyphals.

In conclusion, the growth ability of wild-type and *amt2Δ* mutant strains were the same, suggested that *Cn* is able to produce or secrete some metabolites for growth which were associated with the virulence factor that actually facilitated *Cn* across through the brain causing cryptococcal infection.

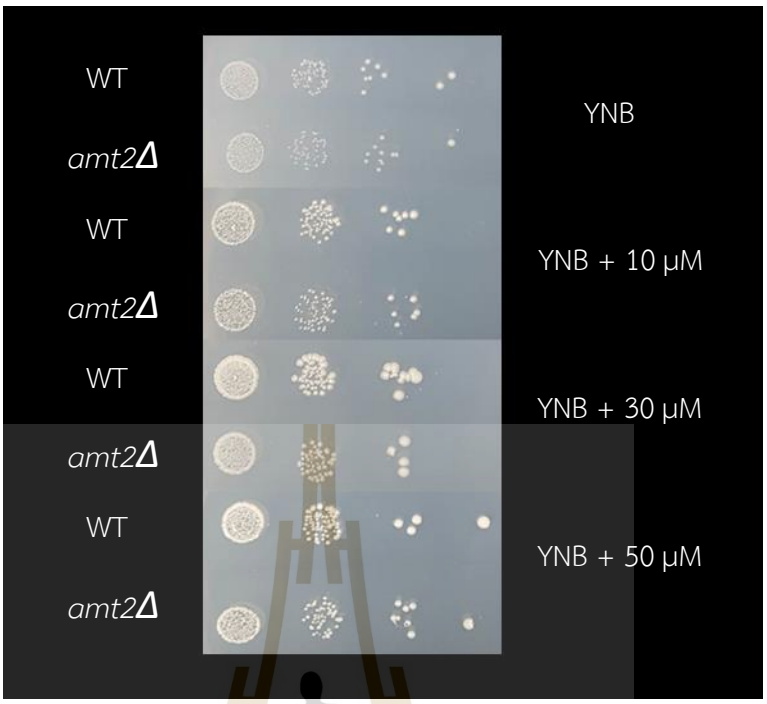

Figure 4.13 The sensitivity assay of *Cn*. The wild-type and *amt2* mutant strain were serially diluted and dropped on the medium contained ammonium concentrations (10 μM, 30 μM, and 50 μM) after incubated for 15 days at 30°C.

CHAPTER V
CONCLUSION

Cryptococcus neoformans is an encapsulated yeast that causes fetal infections, especially in immunocompromised individuals including the HIV-positive population via inhalation and dissemination from the lung enter the brain causing central nervous system (CNS) disease. Although, the brain has the blood brain barrier (BBB) to protect the harmful substance, *Cn* can transmigrate using paracellular, transcellular, and Trojan horse mechanism. Moreover, the morphological change is another event that helps *Cn* across through the brain. The Ammonium transporter gene, *AMT2* has been reported that it was associated with morphogenesis by generating pseudohyphal forming triggered by nitrogen-limiting conditions.

In this study, the ability of the *amt2*Δ mutant strain and wild-type strain to possess the virulence factors including urease, phospholipase, and melanin production were not substantially different, however the ability in capsule formation was lowered in the mutant strain. These results indicated that the loss of *AMT2* did not influence the majority of the pathways of the *Cn* virulence factors involved in producing cryptococcal infection. The phenotypic assay showed that the *AMT2* gene was unable to promote pseudohyphal development, possibly due to a lack of carbon sources, and that this assay may not be ideal for testing. Furthermore, because *Cn* continued to maintain the *AMT1* gene in the cells, the sensitivities of the mutant strain continued to display similarly to those of the wild-type strain. Both the wild-type strain and *amt2*Δ mutant strain used the transcellular mechanism in transverse of the BBB for the transmigration assay. In conclusion, the *AMT2* gene is critical for *Cn* transmigration through the BBB. The synthesis of the cryptococcal capsule was reduced as a result of *AMT2* gene deletion. *AMT2* gene, however, did not correlate with kinetic growth, other common virulence factors, or morphological changes under the limiting-nitrogen situation. Consequently, further research into the gene expression (at the level of the

situation. Consequently, further research into the gene expression (at the level of the entire genome) or pathways implicated in the downregulation of the capacity to generate transmigration in mutant relative to wild-type strains is necessary.

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APPENDIX

APPENDIX

EQUIPMENTS AND INSTRUMENTS

A. Equipment

Name	Source
Autoclave	Hirayama, Japan
Autopipettes (10, 100, 1000 ul.)	Satorius, Finland
Balance	Denver instrument, Germany
Cell culture flask (25 cm^2.)	Thermo scientific Nunc, China
Cell culture flask (75 cm^2.)	Thermo scientific Nunc, China
Cell Culture Insert 1.0 µm PET	Millicell, Germany
Centrifuge himac CF12RX	Hitachi, Japan
Class II Biohazard Safety Cabinet	Esco, Singapore
Conical tube (15, 50 ml.)	Thermo scientific, USA
Cryovial	Thermo scientific, USA
Cuvettes	Hellma, Germany
Pipet filter	Thermo scientific, USA
Forma Series II Water Jacked CO_2 Incubator	Thermo scientific, USA
Haemocytometer	Hausser scientific, USA
Hot Air Oven	Binder, Germany
Hotplate stirrer C-MAG HS 7	IKA, USA
Incubator shaker	New Brunswick Innova, USA
Light microscope Olympus DP20	Olympus, Japan
Light microscope ECLIPSE E200	Nikon, China
Media bottle (250, 500, 1000 ml.)	Duran, Germany
Petri dish	Smart Biotech, Thailand
pH meter	Mettler toledo, China
12-well cell culture plate	Thermo scientific Nunc, Denmark

Serological pipette (5ml,10ml.)	Thermo scientific Nunc, Korea
Sterile syringe filter (0.2 µm.)	MilliporeSigma, Germany
Syringe without needle (10, 50 ml.)	Nipro, Thailand
Tips (10, 100, 1000ul)	Thermo scientific, Mexico
T80+ UV/VIS spectrophotometer	PG Instruments, UK

CURRICULUM VITAE

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