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Pathogenesis of Systemic *Candida glabrata* Infection in an Intravenous Challenge Murine Model

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The incidence of systemic infection caused by *C. glabrata* is increasing in immunocompromised patients and resulted in high mortality rate due to antifungal resistance. The pathogenesis underlying *C. glabrata* infection still remains elusive and requires extensive study on it. Hence, this study was aimed to elucidate the pathogenesis of a clinical *C. glabrata* isolate from a Malaysian patient in an intravenous challenged murine model. Mice were challenged intravenously with *C. glabrata* (1x10^8 organisms/mouse) via lateral tail vein and parameters such as quantitative yeast culture, red blood cells and haemoglobin counts, blood plate assay and histopathology were adopted to evaluate the pathogenesis of systemic *C. glabrata* infection. Transcript level of erythropoietin from blood at day 7 post infection was quantified via RT-qPCR. Kidneys of infected mice have highest fungal recovery rate as compared to other organs and there were yeast infiltration with mild inflammation seen in kidney and brain tissues. Red blood cells and haemoglobin counts were reduced throughout the infection period and this reduction which might be associated with the action of haemolysin enzyme of *C. glabrata* in conjunction with iron scavenging for the fungal growth. Erythropoietin mRNA level was found to be up-regulated in blood which indicated a possible role for erythropoietin in compensating the red blood cells loss throughout the infection period. This study reflected the core events during systemic *C. glabrata* infection and involvement of erythropoietin which could be of clinical relevance during systemic *C. glabrata* infection. However, further comprehensive *in vitro* and *in vivo* studies are warranted.

Key words: *C. glabrata*, Erythropoietin, Haemolysin, Red blood cells and haemoglobin counts, Histopathology, Quantitative yeast counts.

Candida glabrata is a commensal yeast that living in healthy mammalian host. However, it can cause mucosal and severe life threatening invasive infections when there is defect in host immune system. To date, *Candida albicans* is still the most common *Candida* species recovered from human infections. However, there is an increasing prevalence of infections caused by non- *Candida albicans Candida* (NAC) species, especially infection caused by *C. glabrata*[1]. *C. glabrata* is the second most common cause of candidemia in the United States, which accounts for approximately 20% of all *Candida* bloodstream isolates after *C. albicans*[2].

Rick factors to develop invasive *C. glabrata* infection include broad-spectrum antifungal therapy, indwelling vascular catheters,

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gastrointestinal surgery, cancer chemotherapy, organ transplant, leukemia and neutropenia[3, 4]. Infections caused by *C. glabrata* are particularly difficult to treat, due to its highly resistance to several antifungal drugs such as fluconazole [5] Hence, systemic *C. glabrata* infections often result in high morbidity and mortality in immunocompromised patients despite antimycotic treatment is given [,6 7].

Haemolysin is putative virulence factor for *Candida* to establish disseminated infection in host. *Candida* can secrete haemolysin enzyme to destroy red blood cells and acquire iron from host and subsequently establish disseminated infection in host[8, 9]. Erythropoietin (EPO) is a multifunctional cytokine which involved in modulating the immune response and stimulating erythropoeisis[10]. EPO ameliorates hemolytic anemia in malaria and acts as potent anti-inflammatory cytokine in infectious disease[11, 12]. However, there is no previous study which link erythropoietin to systemic *Candida* infection.

The susceptibility to *Candida* infection in animal models can be determined by tissue burdens of infection, mortality rate and histopathology. Although previous murine model of systemic *C. glabrata* has been developed, differences in the virulence of *C. glabrata* strains used and mice species differ in their susceptibility to systemic infection with the yeast. Hence, in this present study, we aimed to characterize the pathogenesis of a clinical *C. glabrata* isolate isolated from blood culture as well as investigate the involvement of erythropoietin during systemic C. glabrata infection in an immunocompetent BALB/C mice model.

METHODS AND MATERIALS

Ethic statement

All animal experiments were performed according to the guidelines and approved by Animal Care and Use Committee (IACUC), Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM/FPSK/PADS/BR/UUH/00486).

Generation of Systemic Candidiasis

Six-week-old female BALB/c mice (weighing 20-25 g) were used for all animal experiments. The animals were randomized, assigned to groups and were given food and water *ad libitum*. *C. glabrata* cell inocula were prepared from a 24 h culture (SDB at 37 °C), which had been washed twice and re-suspended in phosphate-buffered saline (PBS) at the required density by using Improved Neubauer haematocytometer (Camlab, UK). Female BALB/c mice were challenged intravenous through tail-vein injection of a 200 µl inoculum of *C. glabrata* (1x10^7 organisms/mouse). In the control group, 200 µl of PBS was used instead of the yeast suspension.

Quantitative yeast count

At days 1, 3, 7 and 14 post infection (p.i) with *C. glabrata*, three mice were euthanized, and target organs (kidney, brain, lung, spleen and liver) were excised for fungal burden determination. Organs were homogenized in 5 ml of sterile phosphate-buffered saline and tissue homogenates from individual mice were serially diluted and inoculated on SDA plates and incubated for 48 hr at 35°C prior to quantification of *C. glabrata*. The results were expressed as log $_{10}$ CFU per gram tissue.

Determination of haemolysin activity

Haemolysin activity of clinical *C. glabrata* isolate (Cg blood) was evaluated with a blood plate assay as described previously [21,23]. A loopful of an overnight yeast culture (approximately 10^8 cells/ml) was aseptically deposited onto the medium and the plate was then incubated at 37°C in 5% CO_2 for 48 hours. *Candida albicans* ATCC140154 strain was used as positive control in this study.

Red blood cells and haemoglobin counts

For blood profile, 0.2 ml of whole blood was drawn from mice at days 0, 3, 7 and 14 post infection via cardiac puncture. Blood samples were subjected to full blood analysis by using the automated hamatology analyzer (Sysmex KX-21, USA) and the changes in the red blood cells and haemoglobin counts were monitored and recorded.

Erythropoietin gene expression

Murine erythropoietin (*Epo*) mRNA expression from whole blood infected with *C. glabrata* at day 7 post infection was quantified by RT-qPCR. Blood from three mice infected with *C. glabrata* were drawn at day 7 post infection. Total RNA from blood was extracted using AquaPure RNA Blood Kit (Biorad, USA) according to manufacturer's directions and stored at -80°C prior to analysis. RNA integrity was checked by running normal agarose gel electrophoresis and RNA

concentration was quantified by NanoDrop® ND-1000A spectrophotometer (NanoDrop Technologies Inc, USA). Isolated RNA (500ng) was reversed transcribed to cDNA with iScript reverse transcription supermix (Biorad, USA). Samples were then subjected to two steps amplification of 40 cycles using Evagreen dye (Biorad, USA) with cycling condition: initial denaturation 95°C for 3min, followed by denaturation; 95°C for 15sec, annealing; 61°C for 3 sec in the CFX96 Miniopticon detection system (Biorad, USA) as specified by the manufacturer. Housekeeping genes, beta-actin and GAPDH were used and to allow normalization between samples. Water controls (non-template controls) were included to ensure specificity. The murine erythropoietin expression at day 7 post infection was compared with the uninfected groups and was analyzed by Mann-Whitney test. A $P<0.05$ value was considered as statistically significant.

Pathology

At day 7 post-infection, groups of three surviving mice were euthanized, and tissues (kidney, spleen, brain, lung and liver) were excised and fixed in 10% buffered formalin. Fixed tissues were subjected to tissue processing, embedded with paraffin, sectioned and stained with haematoxylin-eosin and periodic acid Schiff (PAS) stain. The inflammatory response and presence of yeast infiltration in target organs of mice infected systemically with *C. glabrata* were assessed by light microscopy.

RESULTS

Quantitative yeast count

As shown in the figure 1, *C. glabrata* was successfully recovered from all organs with different growth rates at day 1, 3, 7 and 14 days respectively post infection. Fungal loads in lungs and livers were rapidly decreased by nearly 2 logs and 1 log respectively from day 1 to day 14 post-infection. There were no significant changes in the fungal loads in spleen and brain within 14 days post infection and the fungal loads were around 3.5-4.0 log. Fungal loads in kidneys remain steady throughout 14 days post-infection, which were nearly 4 logs. Fungal loads in kidney remains the highest among all organs within the 14 days post-infection.

Histopathology

The histological results showed that there were masses of round to oval budding yeast cells (blastospores) in kidney, brain and lung tissues at day 7 post infection with *C. glabrata*. Few yeast cells were found in spleen and liver was found absence of yeast form of *C. glabrata*. The kidneys showed mild inflammation. Fungal elements were distributed much higher in the cortex, especially into the proximal and distal tubules than the medulla. In brain tissues, mild inflammation was observed in the brain parenchyma. There was no evidence of tissue pathology observed in spleen, liver and lung tissues as there were no architecture loss of the organs, no inflammation, hemorrhage, abscess/microabscess formation or necrosis was observed. Infiltrations of neutrophils were not observed in all the infected tissues (Figure 2).

In vitro haemolysin production

The extracellular haemolysin activity of the *C. glabrata* (Cg blood) is shown in Table 1 and Figure 3. *C. glabrata* (Cg blood) showed beta haemolysis with a translucent, well-defined, ring shaped formation was visible around the colony which indicated the presence of haemolysin activity.

Red blood cells and haemoglobin counts

As shown in the figure 4, the RBC and Hb counts of mice systemically infected with *C. glabrata* were consistently reduced within the 7 days of post-infection. The RBC counts was reduced approximately 9 % at day 3 post-infection and further reduction of 17.6% at day 7 post infection and reduced 2% at day 14 post infection as compared to day 0 of pre-infection. The Hb counts was reduced 9% at day 3 post infection and approximately 17.4% at day 7 post infection

Table 1. Haemolytic activity of *Candida glabrata* on sheep blood Sabouraud-dextrose agar (SDA); (-) indicates no activity

Candida isolate	Haemolysis index		
	Alpha	Beta	None (gamma)
Candida glabrata (cg blood)	_	2.16	_

and reduced 4.7% at day 14 post infection as compared to day 0 of pre-infection.

Erythropoietin mRNA expression

As shown in figure 5, the transcript level of erythropoietin in mice systemically infected with *C. glabrata* was significantly up-regulated for more than two fold from blood (p=0.035) as compared to uninfected control.

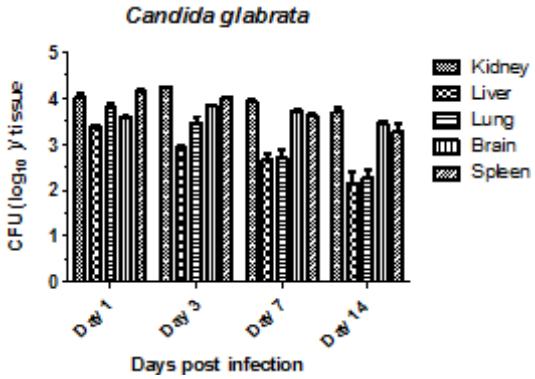

Fig 1. Growth of *C. glabrata* in tissues of immuncompetent BALB/C female mice. Mice were inoculated with *C. glabrata* (1x10^7 organisms/mouse). At specific time intervals post infection, mice were euthanized and the recovery of *C. glabrata* from livers, lungs, spleens, brains and kidneys were quantified by culturing the tissue homogenates on Sabouraud dextrose agar (SDA agar). Results represent the mean +SD of CFU (log_{10})/tissue of three mice per time intervals.

DISCUSSION

Despite the increased medical significance of systemic *C. glabrata* infections in humans, there is still relatively little information on the pathogenesis of this species as most of the research is focusing on *C. albicans*. Hence, it is imperative to understand the core events during systemic *C. glabrata* infections in an infection model to understand how the pathogen interacts with and disrupts host cell function in disease progression. Therefore, the present study elucidated the pathogenesis of systemic *C. glabrata* infection by a clinical isolate of *C. glabrata* isolated from blood culture in a immunocompetent intravenous challenged mice model.

The findings in this study demonstrated that there were differences in the colonization levels and clearance rates of *C. glabrata* from different tissues. Quantitative yeast count revealed significant yeast levels in the brain, kidney, lungs, liver and spleen where kidney was mostly colonized by *C. glabrata* even at day 14 post-

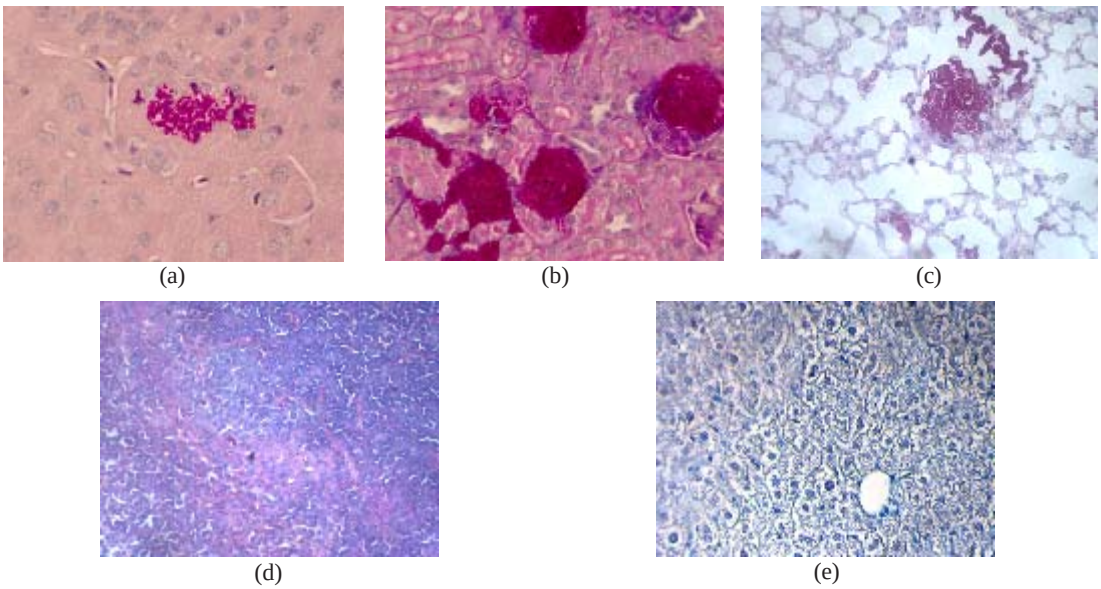

Fig 2. Histological sections of brain, kidneys, spleen, lungs and livers tissues at day 7 post infection with *C. glabrata*. (a) Brain tissues; PAS stain; magnification, x400. (b) kidney tissues; PAS stain; magnification, x400. (c) Lung tissues; PAS stain; magnification, x400. (d) Spleen tissues PAS stain; magnification, x400; (e) Liver tissues; PAS stain; magnification, x400; (Arrows indicates presence of the fungal bodies).

infection. This is in agreement with findings from [13] and [14], which demonstrated persistent recovery of *C. glabrata* from immunocompetent mice in infected organs. The pattern of tissue colonization of *C. glabrata* infection in mice follows the trend seen in most animal models of intravenous induced systemic candidiasis[15, 16]. Since kidney does not have large resident phagocytic cell population and has poor yeast traffic. Hence, clearing of *Candida* cells from kidney tissues are not as effective as other organs. Therefore, high yeast load in the kidneys are most likely due to yeast replication at this site and it is speculated that kidney is responsible for dissemination of yeast to other organs since kidney is the most affected organ throughout the infection period.

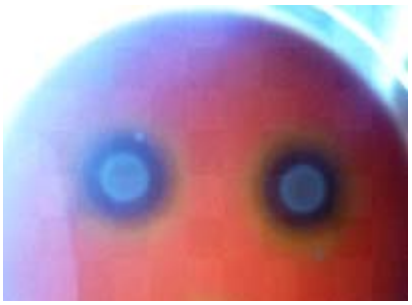

Fig 3. Haemolysin production of (a) *Candida glabrata* isolate (Cg blood) and (b) control *Candida albicans* (ATCC140154) on sheep blood agar. A translucent, well-defined, ring shaped formation was visible around the colony when there was presence of haemolysin activity.

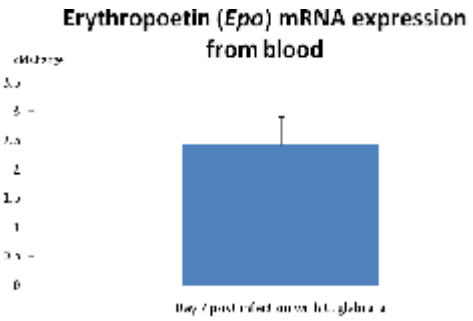

Fig 5. Temporal expression of erythropoietin mRNA from blood of *C. glabrata*-infected mice at day 7 post-infection. Transcript level for erythropoietin was quantified by RT-qPCR. Bar graph represents average expression of each gene normalized to the reference genes, â-actin and GAPDH, to attain a normalized gene expression ratio. Results were means of three biological replicates and two technical replicates for each biological replicate. Bars indicate standard error of mean and * revealed significant differences at the level of $P < 0.05$ as compared with uninfected group, respectively. The fold changes was calculated based on the normalization of the target gene with the average of housekeeping genes (Beta actin and GAPDH) using the $2^{\wedge\text{-average cq}}$ method in infected mice as compared to that in uninfected mice.

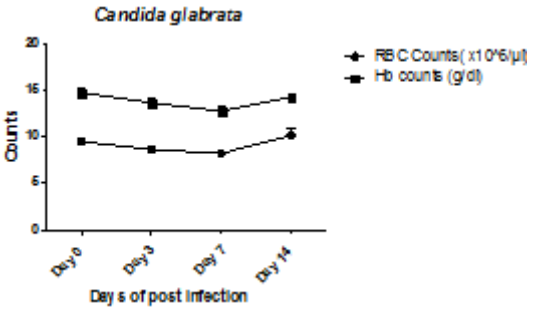

Fig 4. RBC and Hb Counts of mice systemically infected *C. glabrata* (1x10$^{\wedge 8}$organisms/mouse). At specific time intervals post infection, blood were drawn from mice and subjected to blood analysis. Results represent the mean + SD of of six mice per time intervals.

The histological findings showed that *C. glabrata* was monomorphic yeast, where there was only blastospore of *C. glabrata* was detected in the kidneys, spleen and lungs at day 7 post–infection. *C. glabrata* blastospore was presence in cluster in infected tissues. Although the presence of *C. glabrata* was detected, however, there was minimal of inflammatory response elicited in infected tissues. This was accompanied by the lack of evidence of neutrophil infiltration. Unlike in *C. albicans* infection, massive neutrophil infiltrations with moderate to severe inflammation were observed in infected tissues[17, 18]. The differences in histological findings explained the differences in virulence between *C. glabrata* and *C. albicans* in murine models as massive neutrophil infiltration contribute significantly to host tissue destruction which subsequently leads to host deterioration and sepsis in murine models.

C. glabrata was found to be presence in periglomerular cortical tissue rather than in glomerular mesangium, suggesting that the glomerular mesangium may possess a higher innate candidicidal/fungicidal potential than does the surrounding cortical vasculature[19]. Besides that,

C. glabrata was distributed frequently in gray matter than in white matter where gray matter has a higher density of capillaries and endothelial cells than does white matter[20] which would enable *C. glabrata* to acquire nutrients from capillaries and facilitate penetration of endothelial cells and subsequently establish infection in human host.

In this study, *in vitro* production of haemolysin by *C. glabrata* was demonstrated and *C. glabrata* displayed beta haemolysis on blood agar. It should be noted that haemolysin involved in the red blood cells destruction and iron acquisition activity in host[21, 8, 9]. Besides that, we demonstrated that mice infected systemically with *C. glabrata* results in the steady reduction of both the RBC count and haemoglobin level within 14 days of post-infection. Tsang *et al*[9] and Luo *et al*. [8] reported that *C. glabrata* was able to produce haemolytic factors *in vitro* to lyse erythrocytes and use haem as an iron source. This is supported by discovery of haemolysin-like protein gene (HLP) in *C. glabrata*[22, 23]. Hence, with the findings from both *in vitro* and *in vivo*, we speculate that the reduction of both RBC count and haemoglobin level *in vivo* is likely caused by the action of extracellular haemolysin enzyme of *C. glabrata* to destroy red blood cells (RBC) and extract the iron from haemoglobin for their survival in host in order to establish disseminated infection in host. Apart from that, the expression of HLP gene and haemolysin was associated with phenotypic switching in *C. glabrata* which may provide colonizing populations for rapid reponses to the changing physiology of the host[23] which may be an imporrtant virulence attribute of *C. glabrata* to cause disseminated infection in mammalian host.

Erythropoietin (EPO) is the main humoral stimulus of erythropoiesis[10]. Interestingly, our result showed that mice infected systemically with *C. glabrata* results in increment of erythropoietin formation from blood at day 7 post infection. The increment of erythropoietin expression might stimulate erythropoiesis process in host for compensation of red blood cell loss during systemic *C. glabrata* infection. Hence, with the up-regulation of erythropoietin and consistent clearing of *C. glabrata* cells by host immune response, the reduction of RBC count and haemoglobin level was reduced from day 7 to day 14 post infection, which was from 17.6% at day 7 post infection to 2% at day 14 post infection for RBC counts and 17.4% at day 7 post infection to 4.7% at day 14 post infection for haemoglobin level.

In some animal studies, administration of EPO protects kidney tissue from renal injury and improves renal function of ischemia-reperfusion of acute kidney infection[24-26]. Taken together, we speculate that beside a role of EPO in stimulating erythropoiesis, EPO might be involved in regulating systemic *C. glabrata* infection. However, there is no previous study which link erythropoietin and systemic *C. glabrata* infection. Hence, future study such as knockout mice can be used to study the relation between EPO and systemic *C. glabrata* infection.

This study has several limitations which include study of the pathogenesis of *C. glabrata* infection by using a single example of yeast strain and quantification of erythropoietin mRNA expression at a single time interval. Future study should look at erythropoietin protein production, quantification of erythropoietin mRNA expression at different time intervals and expansion of study to look at the local host response towards systemic *C. glabrata* infection.

In conclusion, this study demonstrated the pathogenesis of systemic *C. glabrata* infection in a mice model. Kidney has highest fungal loads and remains the most affected organ during systemic *C. glabrata* infection. There were minimal inflammatory responses elicited towards systemic *C. glabrata* infection. Red blood cells and haemoglobin counts were reduced and the reduction might be associated with the action of extracellular haemolysin enzyme of *C. glabrata*. This study also demonstrated the involvement of EPO during systemic *C. glabrata* infection. However, further comprehensive in vitro and in vivo studies are warranted to clearly elucidate its roles as beneficial, detrimental or redundant during systemic *C. glabrata* infections.

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