Histopathological Characteristics of Experimental *Candida tropicalis* Induced Acute Systemic Candidiasis in BALB/c Mice

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ABSTRACT

Systemic candidiasis caused by *Candida tropicalis* is potentially fatal in human but studies relating the histological characteristics with *C. tropicalis* induced acute systemic candidiasis are few in number. Hence, this study was undertaken to establish and to characterize the distinctive histopathological features of acute systemic candidiasis by varying the inoculum size of *C. tropicalis* injected in mice. Thirty BALB/c mice were divided into three groups and were injected with either 1×10⁶ or 1×10⁷ *C. tropicalis* cells. On observation, the histopathological findings and the fungal burden load following intravenous injection were similar to the previously reported mouse model for *Candida albicans*. Viable yeast cells in the kidneys reached approximately 22.5 log₁₀ CFU g⁻¹ at day 7 post-infection. Compared with the control group, the infected mice group developed acute pyelonephritis characterized by infiltration of large masses of neutrophils within the infected nephrons. Similarly, foci of acute renal inflammation within the kidney were more pronounced when the *C. tropicalis* inoculum was increased. Interestingly, we also observed infection of mice with a higher inoculum of *C. tropicalis* resulted in more severe invasiveness. In conclusion, these results suggest that the BALB/c mouse is highly susceptible to *C. tropicalis* dissemination and represents a significant model system of acute systemic candidiasis.

Key words: *Candida tropicalis*, murine, candidiasis, histopathology

INTRODUCTION

*Candida* species is part of the normal commensal flora that present in gastrointestinal tract, mucocutaneous surfaces of oral cavity and vagina of many mammals (Meiller et al., 2009). However, the primary factor that determines whether *Candida* species can remain as
commensal rather than opportunistic pathogens depends on the integrity of the host immune system (Hube, 2004). A number of factors could contribute to this, for example the increased prevalence of immunodeficient patients resulted from impaired cellular immunity, diabetes mellitus, usage of broad-spectrum antibiotics and corticosteroids and AIDS (Antoniewicz et al., 2009). Unfortunately, fatal Candida infection continues to be alarmingly frequent due to reduced efficacy of the antifungal drugs, appearance of drug resistance in Candida and tedious diagnostic procedures (Fernandez-Arenas et al., 2007).

To date, research efforts have shown that C. tropicalis is highly responsible for most of the invasive infection in leukemic or neutropenic patients (Pedraz et al., 2006) and the second most frequent pathogenic Candida species (Zeugg et al., 2001). Interestingly, C. tropicalis had been also reported be more successful than C. albicans or C. glabrata in invading mucosal surfaces or in colonizing intravascular catheters (Yang et al., 2003). In addition, a high correlation of systemic infection in blood infections had been associated with C. tropicalis infection and this might be attributed by a greater virulence in C. tropicalis compared to other Candida species (Ng, 1999). While systemic candidiasis in mice have been described previously (Ashman et al., 2004; Mavor et al., 2005; Tuite et al., 2005), the histopathological characteristics of C. tropicalis induced systemic infection are still largely unknown. For these reasons, we attempted to establish and further characterize an acute systemic candidiasis model of C. tropicalis infection in the BALB/c mouse. Using two different inoculum sizes of C. tropicalis, we compared the degree of the histological damage and severity of the invasiveness between these groups. Such model is essential in understanding the histological differences of the transition from asymptomatic colonization to symptomatic Candida infection in humans.

MATERIALS AND METHODS

Strain and culture condition: A virulent clinical isolate of C. tropicalis clinical strain CT6338, (clinical isolate from a neutropenic patient with candidemia) was used in this study. Prior to inoculation, the yeast was grown for 24 h on Sabouraud Dextrose Broth 37°C. Yeast cells were harvested by centrifugation and suspended in Phosphate Buffer Saline (PBS).

Experimental animals: Thirty female BALB/c mice weighing approximately 20 g were used in the experiment. The study was conducted under the approval from Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM/FP/ES/PADS/BR/03/001993). The mice were maintained in a conventional animal facility with 12 h light-dark cycle and allowed tap water and commercial-pelleted feed ad libitum.

Infection of animals: The experimental acute systemic candidiasis model of mice was prepared as described previously (Romani, 2001). Briefly, mice were allowed to acclimatize for 1 week prior to infection challenge. To induce systemic candidiasis, mice were randomly divided into three groups of ten each and were infected via tail vein with appropriate inoculum of C. tropicalis cells: Group I was infected with a lower (1×10⁶) C. tropicalis inoculum and Group II with higher (1×10⁷) C. tropicalis inoculum. Another group (n = 10) i.e., the normal control group was injected with PBS instead of the yeast suspension. Challenged mice were observed closely over a 7-day period for the development of symptoms. At the end of the 7-day observation period, mice were humanely
euthanized by cervical dislocation and kidneys were removed for fungal enumeration, gross morphological analysis and any evidence of abnormality was noted.

**Enumeration of viable *C. tropicalis* in kidney:** The number of viable *C. tropicalis* present in kidney was determined. Kidney was removed aseptically and homogenized in 5.0 mL sterile ice-cold PBS. Yeast cells were enumerated after plating of 0.10 mL tissue homogenate (1: 10 serial dilutions in Sabouraud Dextrose Broth) onto Sabouraud Dextrose Agar plate containing 50 μg mL⁻¹ chloramphenicol in duplicate and incubating for 24, 48 h at 37°C. Total colony-forming units (CFU) were determined by using automatic colony counting (Alpha Innotech, CA, USA) and counts were expressed as the log₁₀ CFU per organ and log₁₀ CFU per gram tissue. Growth of more than 10 CFU per 0.1 mL tissue homogenate is considered as indicative of systemic infection (Romani, 2001). An enrichment procedure was also performed by inoculating 1.0 mL tissue homogenate into a culture tube containing Sabouraud Dextrose Broth with chloramphenicol to detect low-grade colonization and to ensure the validity of homogenate plating.

**Molecular confirmation of viable *C. tropicalis* in kidney:** Viable *Candida* cells from kidney homogenate were grown in Sabouraud Dextrose Broth at 37°C for 24 h. DNA extraction was performed using Wizard Genomic DNA Isolation System (Promega, USA) and carried out according to manufacturer’s instruction. *C. tropicalis* specific primers were used to confirm the identity of the *Candida* species isolated from the kidney homogenate (Bougnoux et al., 1999). The PCR reactions contained 1 x PCR Buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 10 μM each forward (5'-ATTTCTTTGCTGGCGGAGG-3') and reverse primers (5'-GGCCACTAGCAYATATGCG-3') and 0.1 U Taq Polymerase (Promega, USA). A negative-DNA control was carried out by adding 1.0 μL of sterile ultrapure water. The PCR amplification was carried out using gradient thermal cycler (Eppendorf, Germany) at 95°C for 5 min followed by 29 cycles of 95°C for 20 sec, 57°C for 15 sec, 72°C for 65 sec and finally extension at 72°C for 5 min. Amplification products (10 μL) were electrophoresed through a 1.5% (w/v) agarose gel containing 0.4 μg mL⁻¹ ethidium bromide in 1 X TAE buffer and documented using gel documentation system (Alpha Innotech, CA, USA). Expected amplicon size for *C. tropicalis* specific primers is 373 bp.

**Histopathological studies:** To access the presence of candidiasis, mouse kidneys were fixed in 10% neutral buffered formalin and were processed by standard tissue processing techniques. Paraffin-embedded specimens were cut into thin sections (5 μm) and were stained with haematoxylin and eosin for histopathological analysis. In addition, selected sections were also stained by periodic acid-Schiff reagent to visualize fungi, and counterstained with light green for characterization of host cells.

**C. tropicalis invasiveness scoring:** Invasiveness scoring was performed as described previously (Balish, 2009). Briefly, three sections for each tissue harvested from at least three mice were ranked as follows: 0, no hyphal penetration of mucosal surfaces per high-power field (HPF x400); 1+, 1-10 organisms per HPF; 2+, 10-50 organisms per HPF; 3+, abundant yeast and hyphae but infection is not confluent (50-100 organisms per HPF) and 4+, confluent invasion of mucosal surfaces with yeast and hyphae (>100 organisms per HPF). Presence of yeast cells and hyphae in blood vessels, glomeruli, tubuli and papillae and presence of necrosis were also noted.
Statistical analysis: The data were presented as the Mean±standard error of mean (SEM). Normally distributed data were analyzed using parametric tests, i.e., Student’s paired t-test for comparisons between two groups.

RESULTS
Fungal burden load: Cultures of specimens from kidney homogenate at day 7 post-infection are shown in Table 1. We observed that Group I mice which had lower C. tropicalis inoculation (1×10⁶) had lesser viable CFU count as compared to Group II mice (1×10⁷). It was established that, all of the challenged mice demonstrated presence of viable C. tropicalis in both plating and enrichment procedure but was not detected in the control group. Macroscopically, there was extensive gross pathology in the kidneys of the infected mice especially in Group II, characterized by a tan, mottled in color which grossly apparent on the irregular renal surface. Small lesions were also seen in the surface of the kidney with the presence of abscesses (Fig. 1). No gross pathology was evident in the kidneys of control group. A picture of agarose gel electrophoresis with amplicon size approximately 370 bp has been shown in Fig. 2.

Molecular confirmation of viable C. tropicalis in kidney: PCR amplification using genomic DNA from the viable Candida cells (grown from kidney homogenate) with C. tropicalis specific primers resulted in specific amplification of a single amplicon of approximately 370 bp. Hence, we confirmed that the identity of the post-infection strain was similar to the original strain.

Histopathological analysis of C. tropicalis-infected kidney: Haematoxylin- and eosin-stained kidney sections in mice showed presence of lesions in the C. tropicalis infected mice and revealed severe acute pyelonephritis in Group II mice. We observed some abscess formation within the renal parenchyma (Fig. 3a-f) with large masses of neutrophils within infected nephrons into the collecting ducts. Within 7 days post-infection, the C. tropicalis cells were apparent in the renal glomerular tuft, perivascular and periglomerular foci with infiltration of mixed macrophages and polymorphs in the renal cortex. In addition, the renal papillae which infrequently contained cells were penetrated with Candida cells, both yeast and pseudohyphae with severe mononuclear cell inflammation. Abscesses, composed primarily of leukocytes dominated the inflammatory infiltrate in Group II but were less obvious in the renal lesions in Group I mice. In addition, the infected kidney in Group II also demonstrated a substantial amount of pseudohyphae form (Fig. 4a-d) in comparison with yeast form which is not observed in either the control group or Group I. Low level magnification examination in Group II also revealed pronounced inflammation with a granulomatous appearance in both cortex and medulla (Fig. 3). Moreover, one of the mice from

<table>
<thead>
<tr>
<th>Group</th>
<th>Log₁₀ CFU/organ (Kidney)</th>
<th>Log₁₀ CFU/g (Kidney)</th>
<th>Invasiveness scoring**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group I (1×10⁷ cells)</td>
<td>1.52±0.08*</td>
<td>10.65±0.55*</td>
<td>0.833</td>
</tr>
<tr>
<td>Group II (1×10⁷ cells)</td>
<td>3.79±0.20*</td>
<td>22.54±1.41*</td>
<td>3.167</td>
</tr>
</tbody>
</table>

** Invasiveness was estimated by histopathology scores of candidiasis in kidney tissues at day-7 after acute systemic candidiasis with C. tropicalis C70338. 0 = No infected area; 1 = 1-10 organisms per HPP; 2 = 10-50 organisms per HPP; 3 = Abundant yeast and hyphae but infection is not confluent; 4 = Confluent invasion of mucosal surfaces with yeast and hyphae
Fig. 1: Gross morphology of mouse kidney at day 7 (a) Mice kidney from Group II. Note the presence of yellowish abscesses and lesion indicated by arrow, (b) Group II, (c) Control group, (d) Group I. Note the irregular and swollen kidney surface and E) Group II

Fig. 2: Representative picture of agarose gel electrophoresis with amplicon size approximately 370 bp Lane 1-2: Candida species from fungal burden load experiment; Lane 3: Positive control C. tropicalis ATCC 750, Lane (-): Negative control and Ladder: 100 bp Fermentas Genuler

Group II also showed necrotizing papillitis which depicted by ischemic and suppurrative necrosis of the tips of the renal papillae. Microscopically, the renal papillary tips showed coagulative necrosis with adjacent neutrophilic infiltration and associated with loss of cellular detail (Fig. 3). Control mice showed normal characteristic kidney histology without any sign of abnormal features.

Invasiveness scoring: For invasion severity, there was an obvious difference between Group I and Group II as shown in Table 1. Higher invasiveness scoring was observed for Group II which
Fig. 3(a-f): Histological structures of mouse kidney sections (a-b) Normal kidney sections of control group mice, (c) Kidney section of Group I. *C. tropicalis* cells are not visible, (d) *Candida* cells in the perivascular and periglomerular region with a cortical focus of chronic inflammation Group I, (e) *Candida* cells were present in the papillae region with severe neutrophilic infiltration and mild coagulative necrosis, Group II, (f) *Candida tropicalis* pseudohyphae, destruction of parenchyma and a granulomatous inflammation, Group II. Hematoxylin and eosin staining at x 100 magnification; Bar = 200 µm
Fig. 4(a-d): Periodic Acid Schiff stained kidney section from mice challenged with *C. tropicalis* by intravenous injection (a) Group I (200x magnification), (b) Group I (400x magnification). Evidence of multifocal *C. tropicalis* infection and numerous yeast form within renal cortex. (c) Group II (200x magnification), (b) Group II (400x magnification). Evidence of *C. tropicalis* infiltration within the kidney parenchyma, predominantly in pseudohyphae form.

Fig. 5: Influence of *C. tropicalis* inoculum size on viable CFU from mice kidney (n = 10) after post-infection and invasiveness scoring. Results showed higher *C. tropicalis* inoculum size on increases presence of viable CFU from mice kidney (n = 10) after post-infection and invasiveness scoring.
had received higher \textit{C. tropicalis} inoculation with the presence of frequent pseudohyphae in comparison to yeast cells. In contrast, Group I showed a higher amount of yeast cells than pseudohyphae with lesser tissue damage. No invasive candidiasis was evident for the control group. In summary, our results show that during acute systemic candidiasis, higher \textit{C. tropicalis} inoculum will cause higher invasion ability as compared to lower inoculum size.

\section*{Discussion}

Invasive candidiasis can range in severity from simple bloodstream infections to fatal deep seated infections which involved colonization of major organs (Caston-Osorio \textit{et al.}, 2008; Pfaller and Diekema, 2007). Bloodstream infection or candidemia usually occurs in hospitalized patients with underlying risk factors such as neutropenia, cancer chemotherapy, antimicrobial agents and usage of catheters. Thus, \textit{Candida} cells can enter the bloodstream via direct penetration from epithelial tissues, due to damage of barriers in the body caused by surgery or drug treatment, or may spread from biofilms produced on medical devices. As a consequence, candidemia has the highest crude mortality and morbidity of all bloodstream infections where it accounts for 30 to 70\% cases among cancer patients and 26 to 75\% among non-cancer patients (Bouza and Munoz, 2008; Mavor \textit{et al.}, 2005). Fortunately, murine model of acute systemic candidiasis has been a standard for investigating mechanisms of candidal virulence, host defense and evaluation of new antifungal agents (Spellberg \textit{et al.}, 2005). The importance of this rodent \textit{in vivo} model for the study acute systemic candidiasis is further highlighted by the fact that models in higher primates are yet to be established and the feasibility in tissue samples procurement. Moreover, the nature and distribution of lesions generated by intravenous administration of \textit{Candida} in systemic candidiasis into susceptible mice closely resemble those found in humans in terms of the target organs for colonization, active replication and the ensuing pathology characteristics (Ashman \textit{et al.}, 1996).

Previous studies of \textit{C. albicans} infection have demonstrated that the highest fungal burden load can be found 1-2 days post-infection in the kidney of infected mice and might be facilitated by enhanced formation of germ tubes in the hypertonic renal medulla besides absence of host leukocytes in the renal tubules (Odds, 1988). Rapid clearance of \textit{Candida} cells by the host immune response cells has been proposed to be one of the important features determining candidiasis. Further, as the balance of commensalism and infection is tightly associated with host immunity, this clearly explains the higher prevalence of candidiasis in immune compromised host. However, when a large inoculum size is used, the remaining viable yeast cells will able to multiply and subsequently cause disease manifestation (Ashman \textit{et al.}, 1996). Consistent with previous research, our study has shown that the increase of inoculum size (Fig. 5) for acute systemic candidiasis produces more viable CFU from the kidney homogenate (Arendrup \textit{et al.}, 2002; Spellberg \textit{et al.}, 2005; Yordanov \textit{et al.}, 2005). These observations are compatible with the histopathological changes observed in the infected mice compared to control group. The degree of invasiveness could be attributed to higher amount of \textit{Candida} cells present within the target organ and the subsequent effects on the increased tissue damage. This could be accounted as \textit{C. albicans} enolase had been reported to bind to host plasminogen and resulted in enhanced invasion of human brain microvascular endothelial cells (Jong \textit{et al.}, 2003). Therefore, it can be deduced that \textit{C. tropicalis} could possibly utilize the advantages of enolase in invasion of the host cells. Nevertheless, this exploitation had been described in \textit{Salmonella} sp. and \textit{E. coli} where both species increased their expression of plasminogen receptors to enhance their invasive capability (Chapman, 1997; Korhonen \textit{et al.}, 1997). Hence, higher number of \textit{C. tropicalis} cells would translate into higher
efficiency of plasminogen binding by enolase and cause localized proteolysis of host extracellular matrix proteins. As a consequence, such ability could allow fungal hyphae to traverse the vascular endothelium and eventually increasing the dissemination and colonization of target organs in host. We must point out that, although the host immunity is a crucial factor in determining outcome of pathogen dissemination, C. tropicalis virulence related factors are also important and have to be taken very carefully when considering degree of invasiveness. This is clearly ascertained by the fact that virulence factors of Candida species which comprises of adhesins, transitional growth, proteolytic and lipolytic enzymes and phenotypic switching are able to facilitate in the pathogenesis (Meiller et al., 2009). Some other works have suggested that have that C. tropicalis possesses tropiase, a novel proteinase which could hydrolyze keratin, casein, fibrinogen and collagen (Okumura et al., 2007). Hence, this improves C. tropicalis ability in capillary permeability and hemolysis.

Histological examination also revealed the presence of pseudohyphae in kidney tissues of C. tropicalis-infected mice in Group II and was associated with a pronounced inflammatory response, characterized by neutrophilic and mononuclear cells infiltration and occasionally necrosis of the papillae. Conversely, inoculation with approximately 100-fold-lower C. tropicalis showed lesser amount of pseudohyphae but apparent yeast form in both renal cortex and medulla. This illustrates the biological distinction in the activation of host innate immune responses towards different Candida inoculum size and was also in agreement with previous studies which have shown that formation of pseudohyphae might promote neutrophil infiltration (Ashman et al., 1996; Brieland et al., 2001). The involvement of neutrophils in the initial stages of systemic candidiasis corresponds to the significance of neutrophils in human infections and thus validates the mouse as a physiologically relevant animal model. Particularly convincing evident linking the host defense mechanism and Candida infection comes from studies on immunosuppressive mice upon Candida challenge where the authors found cyclophosphamide administration after inoculation increased stomach CFU 40- to 370-fold and intestinal CFU 30- to 80-fold (De Repentigny et al., 1992). In addition, we also observed the presence of C. tropicalis in the periglomerular cortical tissue rather than in the glomerular mesangium. This suggests that the glomerular mesangium has some innate candidial ability than does the surrounding cortical vasculature (Baghian and Lee, 1991)

Moreover, the existence of mesangial cells which is able to phagocytose and degrade particulate matter from blood might aid in its candidial activity and preventing colonization in the glomeruli (Young et al., 2000). The most significant histopathological finding was the presence necrotizing papillitis in one of the mouse from Group II, an infrequent form of acute pyelonephritis while majority of the infected mice had severe to mild acute pyelonephritis. Proliferation of C. tropicalis in the renal tubules with consequent necrotizing papillitis would cause obstruction of the renal pelvis and hydronephrosis (Karlovecz, 2003). To the best of our knowledge, no past studies had reported the post-infection strain confirmation by molecular technique. Majority of the studies disregard the importance of confirming the Candida species obtained from the fungal burden load as the indigenous yeast C. pintolopesii in young mice and can be clearly distinguished from C. albicans by a longer incubation period. However, this confirmation step is critical to exclude the possibility of the involvement of C. pintolopesii in the acute systemic candidiasis due to the ability of Candida species to switch from commensalism to pathogenic modes.

In conclusion, the present study demonstrated that C. tropicalis induced acute systemic candidiasis in mouse model is associated with pathological evidence of systemic infection and was parallel with other previous publications. Our study highlights that different C. tropicalis inoculum
size exhibit histological difference, suggesting that host innate responses played a vital role in fungal clearance. Overall, we believe the colonization and invasiveness data from this study will be extremely useful in future experiments particularly in studying the host-pathogen interaction besides the mechanisms involved in Candida species pathogenicity.

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