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Evaluation of the pathogenicity of *Candida zeylanoides* in BALB/c mice

Ali Reza KHOSRAVI[1,*], Hojjatollah SHOKRI[2], Donya NIKAEIN[1], Ahmad ERFANMANESH[3], Mahnaz FATAHINIA[1], Jalal ASHRAFI HLAN[4]

[1]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[2]Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[3]Academic Center of Education, Culture and Research (ACECR), Tehran, Iran
[4]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

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Abstract: Systemic candidiasis is an opportunistic infection caused by *Candida* species in animals. The purpose of this study was to evaluate the pathogenicity of different doses of *Candida zeylanoides* in BALB/c mice. Thirty mice were selected in this study. Different doses of the yeast were intravenously inoculated to the animals. At first, clinical signs and survival time of infected mice were recorded. Then both mycological and histological examinations were performed for detection of *Candida* in different tissues. The results showed that the injection of 1×10^8 cells of *C. zeylanoides* caused high mortality (group 1). The mortalities occurred within 7–12 days (mean: 9.5 days) in group 2 (inoculated with 2×10^7 yeast) and 15–25 days (mean: 20 days) in group 3 (inoculated with 1×10^7 yeast cells) after infection. The kidneys had the highest burden of yeasts (6×10^4 CFU/g). The results of histological examination showed masses of round to oval budding yeast cells along with a few pseudohyphae in different tissues. These results provide evidence that the pathogenicity of *C. zeylanoides* is directly related to the inoculum size of the infecting species, representing the highest yeast burden in the kidneys of the infected BALB/c mice. In order to obtain the exact virulence factors in *C. zeylanoides*, this study should be continued in the future.

Key words: *Candida zeylanoides*, pathogenicity, virulence, mice

1. Introduction

The frequency of invasive fungal infections has increased significantly over the past 2 decades (1,2). This increase in infection is associated with excessive morbidity and mortality (3) and is directly related to the increasing numbers of patients who are at risk for the development of serious fungal infections, including patients undergoing blood and marrow transplantation, solid-organ transplantation, and major surgery (especially gastrointestinal surgery); patients with AIDS, neoplastic disease, and advanced age; patients receiving immunosuppressive therapy; and premature infants (4,5). Members of the genus *Candida* are natural inhabitants of the skin, gastrointestinal, genital, and upper respiratory tracts of humans and animals (6). *Candida* species were reported to be the fourth most common cause of bloodstream infections with high mortality rates (7). They were isolated as causative agents in 10%–15% of all nosocomial infections, 70%–80% of all nosocomial fungal infections, and 8%–10% of nosocomial bloodstream infections (8). *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* were the most frequently encountered causative agents in systemic candidiasis (9,10).

C. zeylanoides is relatively rare in humans and animals, but it is included in the *Atlas of Clinical Fungi* (11) and has been reported on skin, nails, and blood as an opportunistic yeast pathogen and also in a report of endocarditis in an HIV-positive patient (12–15). Hazen (14) described *C. zeylanoides* as a new and emerging pathogen and observed that this yeast could be isolated from blood cultures repeatedly, indicating a constant shedding into the bloodstream. In previous studies, this fungus has been reported from the skin of a bottlenose dolphin in Japan and the genital tract of Iranian female camels (16,17). The purpose of this study was to investigate the pathogenicity of different doses of *C. zeylanoides* in BALB/c mice. Since this study has proven this fungus can invade tissues in this exact condition, it seems it will be categorized as an emerging opportunistic fungus in the future.

2. Materials and methods

2.1. Organism and growth conditions

Candida zeylanoides CA534, originally isolated from the genital tract of female camels, was cultured on Sabouraud dextrose agar containing chloramphenicol (0.005%,

* Correspondence: khosravi@ut.ac.ir

Merck Co., Darmstadt, Germany) at 35 °C for 24 h. For re-identification of the yeast CHROM agar, β-glucosidase test, urease test, sugar fermentation, and assimilation tests by RAPID yeast plus system (Remel Inc., USA) were used as major taxonomic criteria. Yeast cells from at least 5 colonies 1 mm in diameter were suspended in 5 mL of sterile 0.85% saline, and cell concentrations were adjusted using a hemocytometer. The viable count was confirmed by serially diluting the yeast suspension 10-fold and plating each inoculum onto Sabouraud dextrose agar plates.

2.2. Experimental systemic candidiasis

Thirty female BALB/c mice, weighing 25 to 30 g, were purchased from the Razi Institute of Iran. The animals were kept under standard conditions in an animal house at the Faculty of Veterinary Medicine, Tehran, Iran. They were divided into 6 groups (5 mice [A, B, C, D, E] per each group) and infected as follows: group 1 was inoculated intravenously with 1×10^8 cells, group 2 was inoculated intravenously with 2×10^7 cells, group 3 was inoculated intravenously with 1×10^7 cells, group 4 was inoculated intravenously with 5×10^6 cells, group 5 was inoculated intravenously with 1×10^6 cells, and a control group in which the mice were inoculated intravenously with sterile normal saline. Clinical signs and survival time were evaluated for 4 weeks after yeast inoculation.

2.3 Tissue culture and pathology

All the euthanized mice and those that died spontaneously were subjected to detailed necropsy examinations under aseptic conditions.

For tissue culture, representative tissue samples from the brain, lungs, kidneys, liver, and spleen of the infected animals were removed, placed in glass vials containing 10 mL of sterile saline, and subsequently homogenized. One hundred milligrams of the homogenates was made in sterile normal saline and cultured on Sabouraud dextrose agar containing antibacterial antibiotics (20 IU penicillin and 40 µg/mL streptomycin). The plates were incubated at 35 °C for 14 days, and fungal colonies were counted and expressed as colony forming units (CFUs) per gram of tissue.

For histological examination, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5–7 µm, and stained with hematoxylin and eosin (HE). Selected sections were stained with periodic acid-Schiff (PAS) as well.

3. Results

Clinically early deaths occurred 2–4 days postinoculation (PI) in group 1, and neurological signs such as circling, ataxia, severe movements of the head, tilting of the head and neck towards one side, and somnolence in groups 2, 3, and 4 were monitored. These signs were observed more severely in group 3. The mortalities occurred within 7–12 days PI in group 2 and 15–25 days PI in group 3. Although no animals died in groups 4 and 5, in group 4 animals had mild clinical signs. The animals in groups 4, 5, and the control group were euthanized 30 days PI (Table 1).

The results of tissue cultures are given in Table 2 as log CFU/g. The highest burden of *C. zeylanoides* was found in the kidneys, followed by the brain, lungs, liver, and spleen in all treatment groups (Figure 1).

In the histological examinations, masses of round to oval budding yeast cells (blastospores) measuring 3–5 µm in diameter along with a few pseudohyphae in different tissues were observed. Focal necrosis with glial cell aggregation, perivascular cuffing, hemorrhage zones around vessels, focal gliosis, and vascular edema accompanying the yeast cells inside the macrophages were determined in the brain (Figure 2). Mostly, multifocal necrosis, hemorrhage, and mononuclear interstitial infiltrations were detected in the kidneys (Figure 3). Infiltration of polymorphonuclear cells (PMNs) and hyperemia were predominant in the lungs. Numerous aggregations of yeast cells were observed in

Table 1. Clinical signs and mean CFU/g of different doses of *C. zeylanoides* in treatment groups.

Groups	Yeast dose	Clinical signs	Mean survival time (days)	Tissue culture (mean CFU/g)				
				Brain	Kidneys	Liver	Spleen	Lungs
1	1×10^8	Early death	2	2×10^3	1×10^4	7×10^3	2×10^3	1.1×10^3
2	2×10^7	Slight neurological symptoms	9.5	3.5×10^3	1.4×10^4	2×10^3	1×10^3	1.4×10^4
3	1×10^7	Ataxia, circling, somnolence, anorexia	20	1.4×10^4	1.6×10^4	2×10^3	3×10^2	1.4×10^4
4	5×10^6	Neurological signs at first but recovered after 30 days	Euthanized	0	6×10^4	3×10^2	3×10^2	0
5	1×10^6	No clinical signs	Euthanized	0	0	0	0	0
Control	–	No clinical signs	Euthanized	0	0	0	0	0

Table 2. Log CFU/g in different tissues of treatment groups*.

Groups	Mice	Brain	Kidneys	Liver	Spleen	Lungs
Group 1 (Mice inoculated with 10^8 yeast cells)	A	3.23	3.96	3.74	3.00	2.95
	B	3.34	4.13	3.85	3.40	3.15
	C	3.43	3.99	3.88	3.18	3.00
	D	3.26	3.99	3.90	3.40	2.90
	E	3.30	3.91	3.85	3.48	3.11
	mean	3.32	4.00	3.85	3.32	3.03
	Mice	Brain	Kidneys	Liver	Spleen	Lungs
Group 2 (Mice inoculated with 2×10^7 yeast cells)	A	3.59	4.13	3.45	3.30	4.17
	B	3.48	4.23	3.23	3.04	4.10
	C	3.58	4.13	3.34	3.26	4.15
	D	3.59	4.07	3.26	3.46	4.12
	E	3.45	4.16	3.11	3.38	4.20
	mean	3.54	4.15	3.29	3.31	4.15
	Mice	Brain	Kidneys	Liver	Spleen	Lungs
Group 3 (Mice inoculated with 1×10^7 yeast cells)	A	4.11	4.32	3.48	2.32	4.19
	B	4.21	4.06	3.18	2.54	4.12
	C	4.23	4.18	3.26	2.43	4.16
	D	4.03	4.27	3.32	2.52	4.18
	E	4.17	4.14	3.28	2.58	4.08
	mean	4.16	4.20	3.31	2.49	4.15
	Mice	Brain	Kidneys	Liver	Spleen	Lungs
Group 4 (Mice inoculated with 5×10^6 yeast cells)	A	–	4.80	2.26	2.28	–
	B	–	4.76	2.43	2.45	–
	C	–	4.86	2.56	2.58	–
	D	–	4.68	2.60	2.61	–
	E	–	4.76	2.49	2.32	–
	mean	–	4.78	2.48	2.47	–
	Mice	Brain	Kidneys	Liver	Spleen	Lungs
Group 5 (Mice inoculated with 1×10^6 yeast cells)	A	–	–	–	–	–
	B	–	–	–	–	–
	C	–	–	–	–	–
	D	–	–	–	–	–
	E	–	–	–	–	–
	mean	–	–	–	–	–

*No clinical signs were observed in control group.

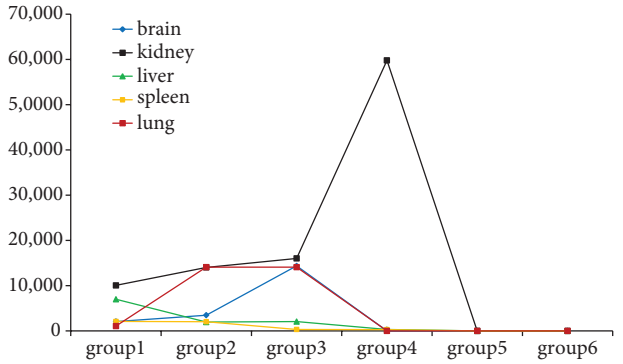

Figure 1. The mean CFU/g of yeast cells in different tissues of studied groups.

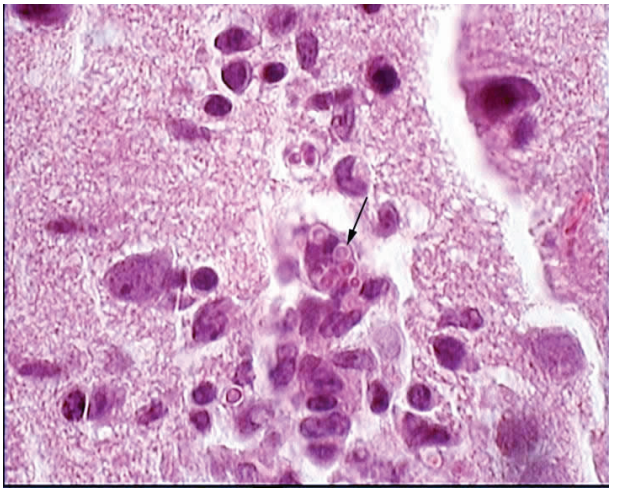

Figure 2. Mouse, group 3. Focal gliosis and yeast cells into the macrophages in the brain section of infected mice. HE, 1000×.

the brain (Figure 4), but single or few yeast cells with buds were determined in the other studied tissues.

4. Discussion

Candida infections are a growing problem among immunocompromised subjects (1). Although most episodes of candidiasis are still caused by *C. albicans*, opportunistic infections due to other *Candida* species have been reported with increasing frequency (18). There is little information regarding *C. zeylanoides* pathogenicity (5,9). The involvement of this yeast in fungemia, arthritis, endocarditis, and skin infections in humans was documented (13,14). In a study by Shokri et al. (16), *C. zeylanoides* was isolated as the predominant (20.3%) yeast flora in the genital tract of Iranian female camels. In the present study, a systemic candidiasis was induced in BALB/c mice following intravenous inoculation of *C. zeylanoides* and observations were recorded until death. Clinical signs observed in the infected mice were initially characterized by circling, ataxia, and neurological signs along with restlessness in the later stages. These findings can be attributed to severe stress due to systemic fungal infections, which are compatible with previous reports with experimental candidiasis in other animal models (19).

The results of this study showed that an injection of 1×10^8 yeast cells leads to early death in mice, with the average survival time of 2 days. In a previous study, by Riazipour et al. (20), the mean survival time in BALB/c mice infected with *C. albicans* (1×10^6 cells intravenously) was determined as approximately 12.4 days. In our study animals in group 5 that were inoculated with the same dose as *C. albicans* did not reveal any clinical signs during the 30 days. Interestingly, for the mice in group 2, which were injected with 2×10^7 yeast cells, the survival time was up to 9.5 days. It seems that the survival time in the above-mentioned dose is close to injection with 1×10^6 cells of *C. albicans*. In comparison to *C. albicans*, *C. zeylanoides* is less virulent and indicates a higher survival time in infected animals. In a previous study, the esterase activities of *C. albicans* and *C. zeylanoides* isolates were evaluated. It was revealed that enzymatic activity of *C. zeylanoides* was weak, whereas *C. albicans* showed strong enzymatic activity (21). It seems that esterase and other enzymes have important roles in *Candida* invasion.

In this study, the presence of fungal elements in different tissues, especially in the kidneys and brain, and perivascular invasion were the most predominant microscopic characteristics of systemic candidiasis. The histological findings were initially characterized by congestion and hemorrhages in different tissues along with focal areas of necrosis. These were followed by the development of multiple microabscesses in the kidneys, brain, lungs, liver, and spleen, in order of severity. Mean infection rate was significantly higher in the kidneys (6×10^4 CFU/g) than it was in the other organs. Brown et al. (22) reported that *Candida* organisms multiplied to a greater extent in the kidneys than in the liver, spleen, and lungs of rats and mice. As mentioned, discrepancy in *Candida* distribution is dependent on the animal species challenged, the growth conditions used for the challenge inoculum, differences between fungal strains, and the route of *Candida* species inoculation.

Many studies have reported kidney fungal burden as the primary endpoint when analyzing the virulence and/or the susceptibility of *Candida* species in mice with systemic candidiasis (23,24). Importantly, there are significant differences among the endothelial cells that line the vasculature of the different organs, as well as the immunologic milieu of these organs (25). These differences provide a compelling rationale to investigate the capacity of *Candida* species to traffic to and persist in

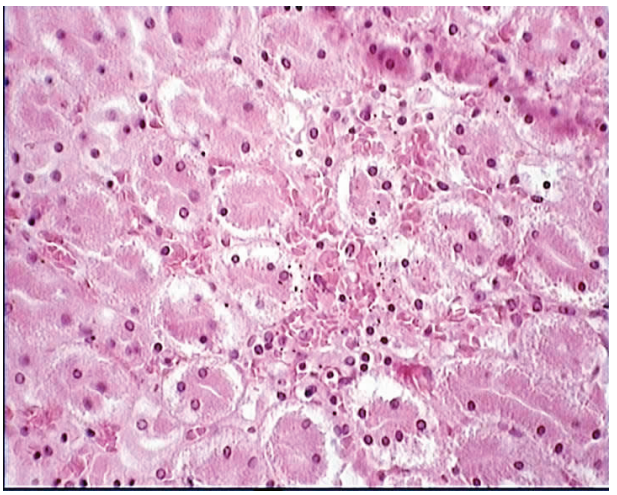

Figure 3. Hemorrhage zones in the kidneys with numerous yeast cells. HE, 400×.

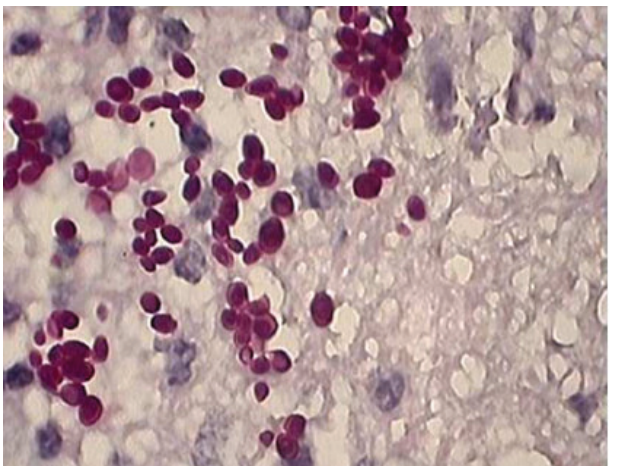

Figure 4. Mouse, group 3. Presence of different shapes of yeasts in the brain tissue of infected mice. PAS, 1000×.

organs other than the kidney. The brain is a particularly important target organ in subjects with hematogenously disseminated candidiasis (26). To invade the brain parenchyma, blood-borne *Candida* cells must adhere to and traverse the endothelial cell lining of the blood vessels within the central nervous system. Brain endothelial cells are significantly different from those lining systemic blood vessels. For example, they have tight junctions that are not present in the endothelial cells in other vascular beds (27).

In conclusion, an increase in inoculum size was associated with an increase in the mortality rate of the infected mice. The highest counts of *C. zeylanoides* were recovered from the kidneys, followed by the brain, lungs, liver, and spleen. We suggest that use of survival-standardized inocula provides a novel approach for future investigations to differentiate early- and late-stage host and pathogen factors involved in the infectious disease process with this fungus. Since the various components of the cell wall and cytoplasm of *Candida* species are important in the pathogenicity, this study should be continued to obtain the exact virulence factors in *C. zeylanoides*.

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