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Short communication

Experimental infection in gerbils by *Conidiobolus lamprauges*

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ABSTRACT

Conidiobolomycosis is an emerging entomophthoramycosis caused by fungi *Conidiobolus* spp. Animal models are essential for the study of infectious disease in various areas such as pathogenesis, diagnostic methods, treatment and prevention. There is not currently an animal model for conidiobolomycosis. The aim of this study was to create an experimental infection protocol for *Conidiobolus lamprauges* in gerbils (*Meriones unguiculatus*). The study animals were randomly divided into four groups of four animals: immunosuppressed with cyclophosphamide (CPA) and infected with *C. lamprauges* (G1), immunocompetent and infected with *C. lamprauges* (G2), immunosuppressed with CPA (G3), and an immunocompetent control group (G4). Clinical signs were observed only in G1 animals, where the mortality rate reached 75% by day 7 after infection (AI) with a median survival of 2 days. *C. lamprauges* was detected only in G1, both by PCR and by isolation. Necropsies of the G1 animals showed lesions in the nasal cavity and lung tissue. These lesions were characterized by polymorphonuclear infiltrate cells and by the presence of hyphal structures under silver staining. This animal model will be useful for further investigation of diseases caused by *C. lamprauges*, particularly of those associated with immunosuppression factors in naturally occurring animal infections.

1. Introduction

Conidiobolomycosis is an emerging entomophthoramycosis caused by *Conidiobolus coronatus*, *Conidiobolus incongruus,* and *Conidiobolus lamprauges*. It is described in immunocompetent individuals, but it has also been reported in patients with HIV [1], renal transplant patients [2], malignant lymphoma [3], and patients with leukemia [4,5]. In immunocompromised patients, unlike in healthy patients, the infection is usually invasive and can disseminate to multiple organ systems.

C. lamprauges has been implicated as causative agent of conidiobolomycosis outbreaks in sheep [6–8]. It is common in tropical regions and causes high mortality rates. Little is known about the behavior of this fungus in the host, or the host's response to fungal challenge.

Animal models are essential for the study of an infection's physiopathology, allowing researchers to determine diagnostic methods and to treat and prevent infectious diseases [9]. They are the best tools to clarify interactions between the host and pathogen, such as those between host organs and cell types involved in the infection [10].

Experimental studies of species belonging to subphylum Entomophthoromycotina have been reported using different hosts, such as studies of *Basidiobolus* spp., and *C. coronatus* in rodents; *Basidiobolus ranarum* in Canadian toads; and *Conidiobolus obscurus* in insects [11–15] However, little is known about the dynamics of infection caused by *C. lamprauges*, including pathogenicity, host defense mechanisms, early diagnostic signs, and treatment. To date, an animal model, which is indispensable for studying disease, has not been constructed for this infection. The aim of this study was to create an experimental infection protocol for *C. lamprauges* in gerbils.

Abbreviations: AI, After Infection; CPA, Cyclophosphamide; GMS, Grocott's Methenamine Silver; PCR, Polymerase Chain Reaction; SDA, Sabouraud Dextrose Agar.

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2. Materials and methods

2.1. Experimental design

Thirty-two gerbils (*Meriones unguiculatus*), aged between 4 and 8 weeks and weighing between 20 and 50 g, were used for all experiments. The animals were housed in a temperature- and humidity-controlled environment and had free access to feed and water. All procedures and animal care were conducted in accordance with guidelines provided by the Brazilian College of Animal Experimentation (COBEA). The study was approved by the Ethics Committee on Animal Use (CEUA, protocol number 23108.050885/13-8) of the Federal University of Mato Grosso (UFMT).

Gerbils were randomly divided into four groups of eight animals: immunosuppressed and infected (G1), immunocompetent and infected (G2), immunosuppressed (G3), and the control group (G4). The groups were kept in separate cages.

2.2. Fungal inoculum

An isolate of *C. lamprauges* (INCQS 40316) obtained from a natural infection in a sheep host was characterized and maintained at Veterinary Microbiology Laboratory at UFMT. For the inoculum, the isolate was grown on Sabouraud Dextrose Agar (SDA) plates at 37 °C for five days. Spores were harvested with a Drigalski spatula by washing the plates with sterile 0.01% Tween 80, followed by centrifugation at 3.354*g* for 17 min and a wash with sterile ultrapure water. The spores were subsequently suspended in sterile saline and counted with a hemocytometer. Samples of the spore suspension were diluted to 1:10, 1:100, and 1:1000, and 0.1 mL of each dilution was inoculated on SDA plates to determine the number of viable spores present.

2.3. Immunosuppression

Based on preliminary experiments, cyclophosphamide (CPA; Sigma-Aldrich, St Louis, MO, USA) was chosen as an immune inhibitor to immunosuppress gerbils before nasal fungal inoculation. Three days before administration of *C. lamprauges*, animals in G1 and G3 were given an intraperitoneal injection of CPA at a dose of 150 mg/kg and the animals in G2 and G4 were given an intraperitoneal injection of sterile saline.

2.4. Infection

Before infection, about 0.1 mL of blood was taken from the cranial vena cava [16] of each gerbil and the serum was stored at −80 °C. Three days following the CPA administration, gerbils were anesthetized intramuscularly with a mixture of ketamine (40 mg/kg) and midazolam (1 mg/kg). The animals were placed in a supine position with the head tilted up at approximately 60° in order to facilitate the inoculum inhalation into the lower respiratory tract. Each of the animals in G1 and G2 was given an intranasal inoculation of 40 μl of *C. lamprauges* spores (2.5×10^6 spores/mL), while the animals in G3 and G4 were given sterile saline.

The animals were observed daily for 20 days. Necropsies were performed on all dead or euthanized animals, and fragments of nasal tissue, lung, liver, kidneys, spleen, and brain were collected.

2.5. Laboratory analyses

For fungal isolation, the fragments were washed in sterile saline with ampicillin (50 mg/L), macerated, and placed on SDA with 0.05 g/L of chloramphenicol. These fungal cultures were incubated at 30 °C and 37 °C for 15 days and checked daily for growth.

Fungal infections and suspected colonies were confirmed by PCR. The DNA of isolated colonies and tissue was extracted as described by Sambrook and Russell [17], followed by PCR using oligonucleotides encoding a partial sequence of the 18S rDNA gene for *C. lamprauges* [18].

For histopathology, tissue fragments were fixed in 10% neutral buffered formalin, processed routinely, and stained using hematoxylin-eosin, and Grocott's methenamine silver (GMS).

2.6. Statistical analyses

Statistical analyses were performed using R software. Survival curves were obtained using the Kaplan-Meier method. A death was considered an event and the live animals at the end of experiment were censored. The groups were compared using the log-rank test. Differences were considered significant when $p < 0.05$.

3. Results

Sick animals were observed only in G1, with a mortality rate of 75% at day 3 after infection (AI) and a median survival of 2 days (Fig. 1). There was a significant difference between the survival curve of G1 and other groups ($p < 0.05$). Twenty-four hours after infection, the animals in G1 showed anorexia, apathy, lethargy, weight loss, severe breathing difficulty, sneezing, nasal itching, and serous or mucohemorrhagic nasal discharge. At day 2 AI, one animal in this group showed regression of clinical signs and was euthanized at the end of the experiment.

The necropsy of G1 animals showed nasal congestion and hyperemic areas in the lung cranial lobe. Microscopically, nasal tissue lesions showed multifocal areas of necrosis, diffuse congestion, and fibrin-suppurative rhinitis, all associated to hyphae inside and near blood vessels. The GMS staining showed hyphae that appeared thin with irregular cell walls. The hyphae were sparsely septate, ramified, and sometimes demonstrated a bulbous dilatation at the ends (Fig. 2).

Two animals showed severe diffuse congestion and moderate multifocal fibrin-suppurative pneumonia associated with hyphae inside and near blood vessels (Table 1). No macroscopic or microscopic alterations were observed in other organs. The fungus was re-isolated from the nasal fragments of the three infected animals, which were also positive for *C. lamprauges* using PCR.

The gerbils in G2, G3, and G4 showed no visible clinical signs of infection, with moderate nasal irritation one day after infection that stopped the next day. These individuals and one animal from G1

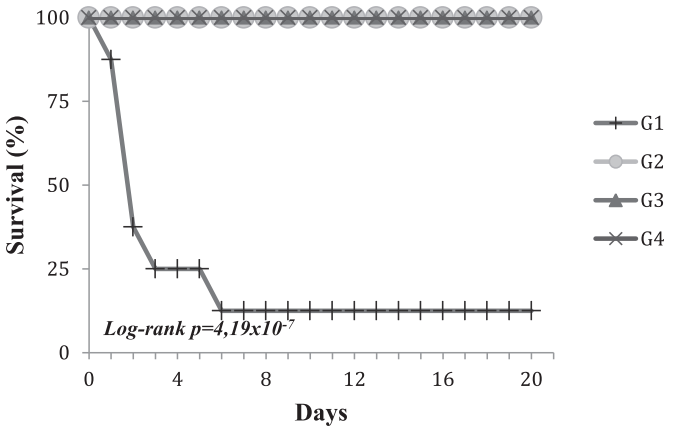

Fig. 1. Survival curves of the gerbils infected by *Conidiobolus lamprauges*.