First report of *Mucor circinelloides* occurring on yellow catfish (*Pelteobagrus fulvidraco*) from China

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**Abstract**

Infected yellow catfish (*Pelteobagrus fulvidraco*) were sent from Niushan Lake Fishery, Hubei Province, China, to our laboratory for diagnosis. Macroscopic daffodil yellow mold was observed on the heads and fins of the fish and one *Mucor* species was isolated. Based on the morphological and molecular analysis, the species was identified as *Mucor circinelloides*. Its optimum growth temperature was 30°C and it could not grow at 40°C. The infectivity results showed wound infection could cause 100% cumulative mortalities at all experimental CFU (10^6, 10^7 and 10^8). The cumulative mortalities of the intraperitoneal infection increased along with the sporangiospore concentrations; the highest mortality was 90% with 10^8 CFU. Histopathological studies showed *M. circinelloides* could cause a series of pathological changes in the host tissues and they disseminated in different viscera, perhaps by the blood. This is the first report of *M. circinelloides* infection in yellow catfish.

**Introduction**

*Mucor* are opportunistic fungi belonging to the family *Mucoraceae* of the class *Zygomycetes*. They are ubiquitous in the environment and have been reported to be pathogenic in birds, animals and humans (Lie & Njo-Injo, 1956; Sugar, 1992, 2005). Mucormycosis is usually associated with immunosuppression, trauma and subsequent surgery in the human host (Lehrer *et al.*, 1980; Sugar, 1992, 2005; Kontoyiannis *et al.*, 2000, 2005; Gonzalez *et al.*, 2002; Almyroudis *et al.*, 2006) and generally causes localized cutaneous infection with high morbidity and even high mortality when disseminated (Ribes *et al.*, 2000; Lenane *et al.*, 2003; Almyroudis *et al.*, 2006). It is characterized by the formation of sexual spores (zygospores) and vegetative mycelium that lack septa, except to delimit old or injured hyphae or reproductive structures in *Mucorales*. Asexual reproduction occurs most commonly by the formation of nonmotile, unicelled sporangiospores in uni- or multisporous sporangia or merosporangia. Although the infectivity and nosogenesis involved with human mucormycosis are well documented, the only report to date describing it as a pathogen for fish is that of Yang *et al.* (2006), who isolated a *Mucor* sp. from *Takifugu obscurus* in Jiangsu province, China. However, their identification results were inconclusive because they identified it by phenotype only to genus level. If a case report of mucormycosis does not identify the species, it may be difficult to associate a disease specifically with a species (Kontoyiannis *et al.*, 2005), and accurate and quick treatment for the fungal pathogens may therefore be delayed. In the present study, we use integrated approaches including phenotype and molecular analysis of the internal transcribed spacer (ITS) rRNA gene to identify the first isolation of *Mucor circinelloides* from diseased yellow catfish of China. The effect of the infectivity and different infection routes on the outcome of the fungal infection was tested and the corresponding histopathological changes were also analyzed.

**Materials and methods**

**Microscopic examination, fungus isolation and identification**

In November 2007, a group of diseased yellow catfish (5–6-cm long) were captured from Niushan Lake Fishery...
Mucor circinelloides occurring on yellow catfish

(30°19′N; 114°31′E), Hubei province, China, and transported alive to our laboratory for diagnosis. The most conspicuous clinical symptoms were macroscopic daffodil yellow mold on the head and fins. The mycelium, necrotic tissue, gill, heart, liver, kidney, and spleen and intestines were aseptically checked by 20% KOH and Gram-stained. Mycelium or necrotic tissue material from the head of diseased fish were inoculated on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (50 mg L⁻¹) at 25 °C. After isolation and purification (Ke et al., 2009), one Mucor fungi strain FM07 was obtained.

The pure strain was cultured on SDA at 15, 20, 25, 30, 35 and 40 °C, respectively. Its morphological characteristics were studied carefully by slide culture technique (Souheil et al., 1999) and scanning electron microscopy (Quanta 200 SEM, Holland). The ITS rRNA gene molecular methods described as Ke et al. (2009) were applied to supplement the morphological identification, and the sequence of ITS region from FM07 has been deposited in the GenBank. The strain was identified as M. circinelloides.

**Infectivity experiment**

Approximately 200 yellow catfish (total length 3–4 cm) were obtained from a commercial fish farm. On arrival at the facilities of the Institute of Hydrobiology, all the fish were disinfected with potassium permanganate (20 mg L⁻¹). Mycelium or necrotic tissue material from the head of diseased fish were aseptically checked by 20% KOH and Gram-stained. These fish were divided into 12 groups (20 fish in each group) and kept in tanks under similar conditions (water volume 50 L; temperature 24–25 °C). They were fed twice a day with commercial feed and feces were removed daily. These fish had no history of disease or abnormality and were acclimatized for half a month before challenge.

Inocula were prepared from cultures of the strains on potato dextrose agar slants for 7 days at 28 °C to obtain sufficient sporulation. Spores were harvested by washing the agar surface with sterile 0.68% NaCl containing 0.05% Tween 80. Suspensions of spores were filtered through a nylon filter (pore size, 11 μm), counted in a hemacytometer, and adjusted to the desired concentration. Viability determination was performed by plating 10-fold dilutions prepared in 0.68% NaCl with 0.05% Tween 80. Plates were incubated at 28 °C, and CFU were counted after 18 h.

**Experiment 1: intraperitoneal infection**

Three groups of yellow catfish were injected with 200 μL FM07 suspension containing 10⁶, 10⁷ and 10⁸ CFU mL⁻¹, respectively, and one group of fish used as control was injected with 200 μL sterile 0.68% NaCl containing 0.05% Tween 80.

**Experiment 2: wound infection**

The fish in this experiment were artificially grazed by one end of a wire netting in advance (this process was done by the same person). Three groups of these wounded yellow catfish were then immersed in FM07 suspension containing 10⁶, 10⁷ and 10⁸ CFU mL⁻¹, respectively, for 2 days and one group of fish used as control was immersed in oxygenated water.

**Experiment 3: immersion infection**

Three groups of yellow catfish were immersed in FM07 suspension containing 10⁶, 10⁷ and 10⁸ CFU mL⁻¹, respectively, and one group of fish used as control was immersed in oxygenated water. All the fish were healthy and no wounds were found.

These experimental infections lasted 8 weeks. Mortalities were recorded during the experimental infections. All fish were examined for gross pathological changes. Any dead or moribund fish were checked for the presence of the fungal pathogen.

**Histopathology**

Live and moribund fish were killed with an overdose of tricaine methanesulphonate (MS-222). The tissues of the diseased fish from Niushan Lake and those with artificial infection were excised and fixed in Bouin’s fluid. Samples from healthy yellow catfish were also fixed in Bouin’s fluid as control. Part of the musculature was decalcified with formic acid–sodium citrate solution. All tissues were dehydrated with ethanol, embedded in paraffin wax, and blocks sectioned at 6 μm with a rotary microtome. Slides were stained with Harris’ hematoxylin and eosin. The stained sections on the slide were covered with Canada balsam and photographed under a microscope (Zeiss Axioplan 2 imaging and Axiopt 2).

**Results**

**Microscopic examination, fungus isolation and identification**

The hyphae in the necrotic tissue were nonseptate, broad and branched. The color of the pure culture was white initially and soon became grayish brown. Microscopic examination revealed globose sporangia, measuring 30–78 μm in diameter (Fig. 1a). Columellae were subglobose to obovoid and collarettes were conspicuous (Fig. 1b). Sporangioles were either long and branched sympodially or shorter with slightly recurved lateral branches. Sporangiospores were hyaline, ellipsoidal to slightly asymmetrical or obovoidal and measured 5.0–7.0 μm in length and...
3.2–5.5 μm in width. The sporangiospore walls were finely ornamented (Fig. 1c). Chlamydospores were produced in the basal mycelium, which were thick-walled, subglobose, oval or irregularly shaped, measuring 45 μm in length and 30 μm in width (Fig. 1d). Rhizoids and stolons were absent. The optimum growth temperature was 30°C and there was no growth at 40°C. There were no zygospores produced in test mating and this procedure was repeated with the same results.

A 638-bp ITS rRNA gene fragment was amplified from the fungi and was deposited in GenBank under the accession number GQ415044. Compared with the sequences of ITS rRNA gene available in GenBank, the amplified nucleotide sequence showed 100% homology with the ITS rRNA gene sequence of *M. circinelloides* (accession number EF583641) (Fig. 2). Based on the morphological and molecular evidence, the species was identified as *M. circinelloides*.

**Infectivity experiment**

Before fungal challenge, no fish died during the acclimatization period. The cumulative mortality and time of first death are shown in Table 1. The first dead fish was observed on the 15th day in the high-concentration (10⁸) wound infection group, and this group reached its 100% cumulative mortality on the 30th day. The 100% cumulative mortalities of medium- (10⁷) and low-concentration (10⁶) groups appeared on days 39 and 45, respectively. The fish from this group showed similar clinical symptoms with those infected naturally. The pathogen isolated from the fish (including dead and moribund fish) of these groups was identified as *M. circinelloides*.

In the intraperitoneal infection group, cumulative mortalities increased along with the concentrations of sporangiospore suspension. A 30% cumulative mortality occurred after 8 weeks in the low-concentration group. Cumulative mortalities of 45% and 90% appeared in the medium- and high-concentration groups, respectively. The clinical symptoms of this route of infection were celictasia, pyoperitoneum and large swollen liver. *Mucor circinelloides* was isolated from the cavum abdominis of the dead or moribund fish.

During the entire experimental time, there were no dead fish in the immersion infection and control groups, although *M. circinelloides* was obtained from mucus in a small number of immersion-treated fish.

**Histology**

A series of histopathological changes could be found in the ulcer granulation tissue, subcutaneous tissue, musculature and blood vessels. Inflammatory reaction, tissue necrosis...
and circulatory disturbance were the main symptoms. Many of the nonseptate, broad and branched hyphae were observed in ulcer granulation tissue and the cells near the hyphae were degenerate (Fig. 3a and b). The liver and kidney demonstrated different degrees of histopathological changes. Many erythrocytes were observed in the hepatic tissue section. Part of the hepatic tissue was necrotic. The profiles of liver cells were faint and the nucleus was dissolved (Fig. 3c). Hepatic tissue vessels were congested (Fig. 3d). Part of the connective tissue in kidney was proliferated and many hemosiderin granules were found. The renal tubule walls were incrassated and part of the renal tubules were atrophied. Serious inflammatory cell infiltration was present (Fig. 3e and f). No obvious histopathological changes were found in heart or intestine. The tissue sections from control groups were normal.

Fig. 2. Comparison of ITS rRNA gene sequence of strain FM07 with that of Mucor circinelloides (accession number EF583641). *, Conserved site; ., bp numbers of the ITS rRNA gene sequence.

Table 1. Cumulative mortalities and the time of first death of yellow catfish after infection by FM07 in three different experiments

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mode of infection</th>
<th>C*</th>
<th>Yellow catfish (n)</th>
<th>Day1</th>
<th>Mortality rate (%)</th>
<th>Day2</th>
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<tr>
<td>FM07</td>
<td>Intraperitoneal infection (Experiment 1)</td>
<td>10⁶</td>
<td>20</td>
<td>45</td>
<td>30</td>
<td>56</td>
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<td></td>
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<td>10⁷</td>
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<td></td>
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<td>10⁸</td>
<td>20</td>
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<td>90</td>
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<td>Wound infection (Experiment 2)</td>
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<td>Immersion infection (Experiment 3)</td>
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* Concentration of sporangiospore suspensions from strain FM07.
1 The day of first fish death.
2 The time when maximum cumulative mortality was obtained.
Discussion

Yellow catfish (*Pelteobagrus fulvidraco*) have great market potential and have been cultured widely in China in recent years. Many parasites and bacteria have been found and isolated from the yellow catfish. However, this is the first report of the isolation and characterization of *M. circinelloides* from yellow catfish. Infections caused by fungi have increased in recent years. Accurate identification of fungal pathogens is important for appropriate treatment. Phenotypic methods have traditionally been used to identify clinically important *Mucor* spp. (Wang et al., 1990; Finger-oth et al., 1994; Chandra & Woodgyer, 2002). However, the fact that most published reports refer only to the genus *Mucor* underlines the difficulties in species identification (Ribes et al., 2000). Although observation of zygospores enhanced the identification of heterothallic *Zygomycetes* (Weitzman et al., 1995; Iwen et al., 2005), maintaining a library of tester strains is not easy for many laboratories and mating tests do not always yield a positive result (Schipper, 1976; Sigler et al., 2002). The *Mucor* isolate FM07 in yellow catfish was more like oomycete species or some other filamentous fungi by gross examination. Under the microscope, uniform nonseptate, broad and right-angled...
branchied hyphae, globose sporangia and sporangiophores could be seen. Based on the morphological characteristics, the strain FM07 was identified as *Mucor circinelloides*. Interestingly, the ITS rRNA gene fragment of FM07 showed 100% similarity to both *M. circinelloides* (EF583641) and *Rhizomucor variabilis* (DQ118990). Voigt et al. (1999) found *R. variabilis* was phylogenetically very close to *Mucor* spp. However, *R. variabilis* has rhizoids and stolons and can grow well above 40 °C. These characteristics are very different from those of *Mucor* spp. and were not found in strain FM07. The results identified strain FM07 as *M. circinelloides*.

Infection trials showed that strain FM07 was pathogenic for yellow catfish by intraperitoneal and wound infection. However, the trials also revealed some differences between the two routes of infection (cf. results in Table 1). When the concentrations of sporangiospore suspension were increased, the cumulative mortality from different concentration groups went up correspondingly (30%, 45% and 90%) and the time to death of fish was reduced (45, 28 and 19 days) in intraperitoneal infection. In wound infection, the beginning time of death of fish from different concentration groups was similar to that in the intraperitoneal infection group, but the cumulative mortality was 100% in all wounded groups. In both experiments, when the concentration of sporangiospore suspension was increased the infected fish died more quickly. In immersion infection, there were no fish dead, although the strain FM07 was isolated from the mucus of some fish. These results suggest *M. circinelloides* is pathogenic to yellow catfish if a portal of entry is provided. Their infection may be associated with some primary pathogenic factor, for example trauma such as wound infection or poor environmental conditions. This phenomenon was consistent with the disease caused by *M. circinelloides* in humans (Chandra & Woodgyer, 2002; Iwen et al., 2007). In these cases, although *M. circinelloides* was reported as primary cutaneous zygomycosis, the patients all were known or suspected to have been exposed to trauma in different parts of body.

In this study, we found *M. circinelloides* could cause a series of pathological changes in host tissues and was disseminated in different organs. Vessel thrombosis and tissue necrosis are two major hallmarks of mucormycosis in human infection (Chkhotua et al., 2001). We found tissue necrosis and vessel congestion were present in tissue sections from infected fish. Moreover, Wang et al. (2005) and Yang et al. (2006) previously reported Mucor mycelium could invade blood vessels and cause embolism in humans and fish, respectively.

From the immersion infection and analysis of the human literature (Henderson et al., 2001; Lenane et al., 2003; Brown, 2005), we speculated that infection of healthy fish with *M. circinelloides* would be difficult. Results suggest that *M. circinelloides* may first attach to the surface of healthy fish and upon injury or stress invade the hypodermis. Broad hyphae or spores can penetrate the mucous membrane to invade ground substance and cause focal necrosis. Fungal incursions into the vessels result in circulatory disturbance and infarction. According to Yang et al. (2006), these acidic necrotic tissues accelerate the *Mucor* infection. The infected fish may perhaps have consequently died from dyspnea. New studies are expected to further the understanding of the pathogenic mechanism of mucormycosis in fish.

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**References**


