Mucormycosis in the platypus and amphibians caused by *Mucor amphibiorum*

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Mucormycosis in the platypus and the anuran (frogs and toads) is a serious fungal disease affecting these aquatic taxa. *Mucor amphibiorum* infection causes significant morbidity and mortality in free-living platypuses in Tasmania. Infection has also been reported in free-ranging cane toads and frogs from mainland Australia, but not confirmed in platypuses from the mainland. This paper reviews mucormycosis in the platypus and anuran, including consideration of the clinical, epidemiological, pathological and diagnostic features.

Mucor amphibiorum

Mucor amphibiorum is a dimorphic fungus in the Mucorales order of the Zygomycetes class of fungi. Its sporangiospores, when found in infected tissues, occur as the yeast form (spherule-like structures, containing 2–11 daughter spherules) or develop into the more usual non-septate hyphal form on culture media or in the environment[1,2]. Infections (Table 1) have been reported in a range of anurans (frogs, toads), and the platypus[1,3,4,6,8–10]. Transmission between captive anurans and salamanders has been documented; while experimentally infected reptiles remained clinically healthy with only small lesions at necropsy, and no lesions were reported in

Table 1. Hosts reported with *Mucor amphibiorum* infection globally.

Scientific Name	Common Name	Geographical origin and/or place held	Reference
Aparasphenodon sp.	Casque-headed frogs	South America (captive in Germany)	1
Bufo bufo	Common toad	Europe (captive in Germany)	1
Rhinella marina (Bufo marinus)	Cane toad	QLD & NT, Australia (free-living)	3
Dendrobates sp.	Poison arrow frog	South America (captive in Germany)	1
Limnodynastes peronii	Striped marsh frog	Australia (free-ranging)	4
Litoria adelaidensis	Slender Tree Frog	Australia (captive in Perth Zoo)	5
Litoria caerulea	Australian green tree frog	Australia (captive in Germany)	6
Litoria caerulea	Australian green tree frog	QLD, Australia (free-living)	4
Litoria infrafrenata	White-lipped tree frog	Australia (captive in Melbourne Zoo)	7
Litoria infrafrenata	White-lipped tree frog	Australia (captive in Perth Zoo)	5
Ornithorhynchus anatinus	Platypus	TAS, Australia (free-living)	8, 9, 10
Rana temporaria	European common brown frog	European (experimental infection)	6
Rana esculenta	Edible frog	Europe (experimental infection)	6
Salamandra salamandra	Fire salamander	Germany (Captive)	1
Trachycephalus sp.	Milk frog	South America (captive in Germany)	1

QLD, Queensland; NT, Northern Territory; TAS, Tasmania.

experimental infections of laboratory animals[1,6]. *Mucor amphibiorum* appears to be endemic in Australia, infecting free-living frogs and toads in Queensland, New South Wales and Northern Territory, with accidental introductions into captive frogs in Melbourne and Perth[5,7] and platypuses in Tasmania. It seems unlikely that it was introduced into Australia with cane toads in 1935, as Speare *et al* (1994)[3] was unable to isolate it from 41 cane toads sampled in Hawaii or Costa Rica.

Mucormycosis in anurans

Mucor amphibiorum was first reported from a German Zoo in 1972, where it resulted in disseminated disease in a common green tree frog (*Litoria caerulea*) imported from Australia, and subsequently in frogs, toads and salamanders in neighbouring exhibits[1,6].

In amphibians, mucormycosis caused a disseminated disease with multiple white nodules in liver, kidney, bladder and lung, the emaciated animal dying within 2–4 weeks. Skin involvement via lymphatic spread was observed in 42% of infected toads, but skin ulcers were rare[11]. Histologically, nodules consisted of granulomas and pyogranulomas containing thick-walled spherules (5–37 µm diameter, containing 0 to 10 daughter spherules). Of nine *M. amphibiorum* isolates from cane toads (*Rhinella marina*), five were positive mating strains and four were negative mating strains[11]. The route of entry of *M. amphibiorum* in the anuran is likely ingestion of soil contaminated by faeces excreted by infected animals. *M. amphibiorum* was isolated from 2/20 soil specimens from an endemic site in Townsville where resident cane toads had mucormycosis. Furthermore, the organism has been shown to grow and sporulate in soil[11].

Figure 1. Gross appearance of mucormycosis in the Tasmanian platypus. (*a*) Severe chronic ulceration of left hind leg, with granulation tissue encircling leg and spur. (*b*) Ulceration of the dorsal tail (60 x 43 mm), with thickened edges and central cavitation. (*c*) Chronic ulceration of right hind leg (100 x 40 mm), with serous exudate and bleeding. (*d*) Hairless raised nodules on tail, some full thickness and exuding pus (bar = 10 mm).

Mucormycosis in the platypus

Munday and Peel (1983)[8] first described four cases of ulcerative dermatitis in dead and debilitated Tasmanian platypuses from the Elizabeth River in Campbell Town, but the causative agent was not identified as *M. amphibiorum* until 1993[9]. *M. amphibiorum* causes a severe granulomatous and often ulcerative dermatitis in the platypus, which may progress to involve underlying muscle and occasionally disseminate to internal organs, particularly the lungs[9], leading to death. In the absence of the systemic spread of the organism, death can also result from secondary bacterial infections or impaired thermoregulation and mobility.

All 17 platypuses with mucormycosis captured during a 12 month Tasmanian study[10] were alert and displayed normal responses to capture and handling. Gross appearance of skin lesions varied from non-ulcerated, hairless nodules and abscesses, to ulcers with undermun or thickened margins, sinuses exuding pus, or exuberant granulation tissue (Figure 1). Some lesions appeared as discrete entities. Others coalesced to form plaques. Lesions were found on haired regions including the hind limbs (38%), forelimbs (6%), tail (19%), trunk (6%) and head (6%), and unhaired regions such as the webbing of the forelimbs (13%) or bill (6%). Some affected animals had lesions at more than one site. One platypus had a tail ulcer which reduced in size over a three month period. Of 13 isolates of *M. amphibiorum* from 17 diseased platypuses, all were of the positive mating strain[10]. One platypus *M. amphibiorum* isolate was tested showed susceptibility to amphotericin B, but resistance to both itraconazole and fluconazole[10]. In a pathogenicity study using cane toads, Stewart and Munday (2004)[12] found that the positive mating strain and platypus-derived isolates of *M. amphibiorum* were more pathogenic than negative mating strains or anuran-derived isolates. In a disinfectant trial, a positive mating strain of *M. amphibiorum* from a platypus was more resistant to disinfectants (Phytoclean®, Path-X®, F10sc®) than a negative strain from a frog[13]. *M. amphibiorum* was not isolated from 40 faecal or 8 healthy skin samples from platypuses or 14 environmental samples including soil, water, frog faeces, and *Ixodes ornithorhynchi* ticks. *Mucor circinelloides* was isolated from samples of soil, platypus and frog faeces; *Mucor hiemalis* was cultured from platypus faeces and *Mucor saturninus* from soil samples from the study site. *Mucor circinelloides* was reported from one platypus ulcer[14], but was later thought to be a contaminant as it was incapable of infecting cane toads[12].

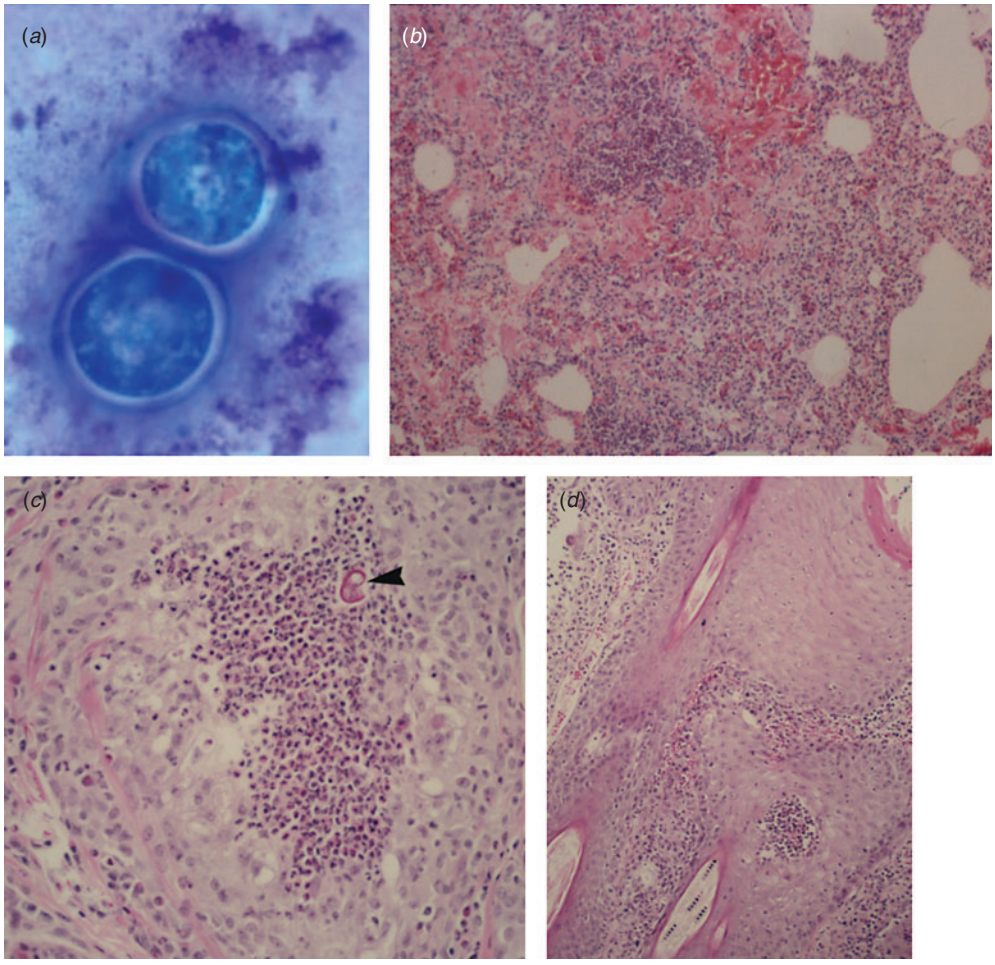

Figure 2. Cytological and histological features of mucormycosis in the platypus. (*a*) *Mucor amphibiorum* in a Diff Quik-stained smear from a case of platypus mucormycosis. (*b*) Granulomatous pneumonia in a platypus lung (H&E, x200). (*c*) Central neutrophils and a ruptured spherule (arrowhead) surrounded by macrophages, lymphocytes and plasma cells in a discrete granuloma (H&E, x280). (*d*) Pseudoepitheliomatous epidermal hyperplasia in a thigh lesion from a platypus (H&E, x140).

The sudden emergence of mucormycosis in Tasmanian platypuses in 1982 may have resulted from accidental introduction of this pathogenic fungus with 'banana box frogs' from Queensland[12] to a naïve Tasmanian platypus population (similar to the recent introduction of the chytrid fungus into Tasmania[15]). Alternately, an endemic Tasmanian strain of *M. amphibiorum* may have mutated to become pathogenic for platypuses[16,18]. Since the index cases of mucormycosis in the platypus in 1982[8], the distribution of the disease has slowly expanded but remained endemic to the catchments draining into the Tamar River. Spread of the agent could be via movement of platypuses and other aquatic hosts or fomites such as contaminated fishing gear and tyre treads. In 1994, mucormycosis prevalence in the platypus at Brumbys Creek was 33%[10]. By 2009, the prevalence of platypus mucormycosis across Tasmania appeared to be declining[17,18].

Diagnosis of mucormycosis in frogs, toads and the platypus

Diagnosis of mucormycosis is based on culturing *M. amphibiorum* from characteristic lesions. Aseptically collected representative specimens (including fine needle aspirates, swabs and punch biopsies) should be inoculated onto Sabouraud's dextrose agar with and without gentamicin (50 IU/mL) and incubated at 28°C. Single colonies can then subcultured onto plates containing Sabouraud's dextrose agar without antibiotics or potato dextrose agar for more detailed morphological studies and mating experiments[8]. Two mating strains, CBS 763.74 (positive type strain) and CBS 185.77 (negative reference strain) were used to assess zygospore production in aerial hyphae[2]. By definition, positive strains produce zygospores only in test matings with negative strains.

Clinical signs (Figure 1), the presence of spherules in cytology preparations or histological sections from lesions (Figure 2) further support a diagnosis of mucormycosis in anurans or platypus, but are less specific than culture. *Corynebacterium ulcerans* and an unidentified fungus were isolated from cutaneous lesions resembling mucormycosis in two platypuses[18]. Several environmental *Mucor* species other than *M. amphibiorum* display dimorphism including *M. circinelloides*, *M. hiemalis* and *M. saturninus*, and could potentially result in similar-appearing spherules in lesions. In the platypus, *M. amphibiorum*-specific serum immunoglobulin may be detected by ELISA[19]. To date, no PCR has been used to identify *M. amphibiorum* DNA from clinical (platypus and amphibian) or environmental samples, although panfungal PCR

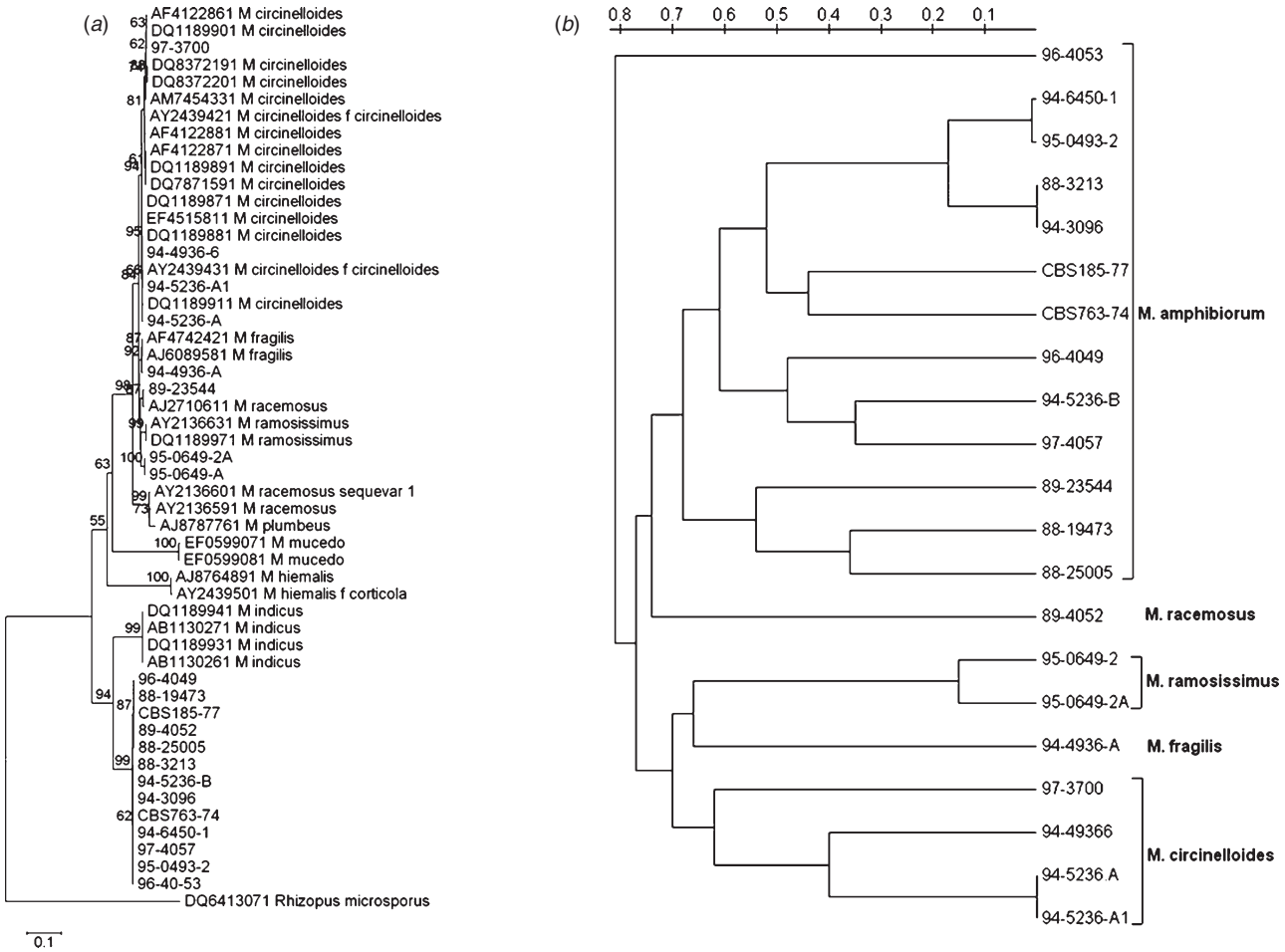

Figure 3. Genotypic analysis of *Mucor* spp. Isolates[20]. (*a*) Consensus Neighbour-joining tree generated from sequence alignments of the rDNA ITS regions of *Mucor* sp. isolated from platypus and species from the GenBank database. Bootstrap support values are indicated for each branch. (*b*) Dendrogram based on the genetic differences as determined by analysis of 135 amplified fragments generated from ISSR amplification.

assays have been used to detect a range of fungi in fresh and paraffin-embedded tissues[20,21] and the fungal microbiome of canine duodenal samples[22].

Molecular studies of *Mucor* spp. of platypus, anuruan and environmental origin

A collection of 21 *Mucor* isolates representing isolates from platypus, frogs, toads and environmental samples were obtained for genotypic analysis (Figure 3)[23]. Internal transcribed spacer (ITS) region sequencing and GenBank comparison confirmed the identity of most isolates. Platypus isolates formed a clade containing the reference isolates of *M. amphibiorum* from the CBS repository. The *M. amphibiorum* isolates showed close sequence identity with *Mucor indicus* and consisted of two haplotypes, differentiated by single nucleotide polymorphisms within ITS1 and ITS2 regions. Except for one, all isolates from platypuses were in one haplotype. Multi-locus fingerprinting via the use of intersimple sequence repeats (ISSR) PCR identified 19 genotypes. Two major clusters were evident: (1) *M. amphibiorum* and *Mucor racemosus*; and (2) *Mucor circinelloides*, *Mucor ramosissimus*, and *Mucor fragilis*. Seven *M. amphibiorum* isolates from platypuses were present in two subclusters, with one isolate appearing genetically distinct from all other isolates. Isolates classified as *M. circinelloides* by sequence analysis formed a separate subcluster, distinct from other *Mucor* spp. The combination of sequencing and multilocus fingerprinting has the potential to provide the tools for rapid identification of *M. amphibiorum*.

Future work should include the development and refinement of molecular tools to detect free-living forms of *M. amphibiorum* in the environment as well as infective forms in tissue lesions. The potential for other aquatic vectors for *M. amphibiorum* needs to be assessed. Such developments will likely lead to an improved understanding of the environmental niche of the fungus and how it is spread in Tasmania. This could lead to control measures to prevent further spread of this disease.

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Biography

Joanne Connolly teaches Veterinary Microbiology and is the Course Coordinator of the Captive Vertebrate Management Program at Charles Sturt University in Wagga Wagga. The major themes of Dr Connolly's research are veterinary microbiology, public health, as well as wildlife biology and disease. Research topics of interest include *Mucor amphibiorum*, *Cryptococcus neoformans*, *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* and *Chlamydophila* in animals and host-agent-environmental relationships.