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Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians

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ABSTRACT: Polyclonal antibodies were produced for diagnosing chytridiomycosis in amphibians. Two sheep and 4 rabbits were inoculated with homogenized whole culture of *Batrachochytrium dendrobatidis* in Freund's complete adjuvant or triple adjuvant. Antisera from all animals reacted strongly with all stages of *B. dendrobatidis* and stained the walls, cytoplasm, rhizoids and zoospores in an indirect immunoperoxidase test. Significant cross-reactivity occurred only with some fungi in the Chytridiomycota, and there are no members of this phylum besides *B. dendrobatidis* that infect frogs. The immunoperoxidase stain is a useful screening test when combined with recognition of the morphology and infection site of *B. dendrobatidis*.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · Fungus · Amphibians · Immunoperoxidase · Polyclonal antibodies · Diagnosis

INTRODUCTION

Chytridiomycosis is a fatal disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis*, the only member of the Chytridiomycota that causes disease in vertebrates (Berger et al. 1999). This fungus has a broad amphibian host range and occurs worldwide. A total of 94 amphibian species from 15 families have been found infected with *B. dendrobatidis*, from Australia, South America, Central America, North America, Europe, New Zealand and Africa (Speare et al. 2001). Chytridiomycosis is the most common disease of Australian frogs (Berger et al. 1999). Although amphibian population declines are often due to habitat modification and multi-factorial causes (Alford & Richards 1999), the suspected introduction of chytridiomycosis to wild amphibians is the most likely cause of many declines in protected areas (Berger et al. 1999).

Chytridiomycosis is a highly infectious disease that appears to have spread nationally and internationally, possibly by movement of infected animals. Diagnosis of infected amphibians will be an important aspect of quarantine regulations aimed at preventing the introduction of disease to wild or captive populations. Current diagnostic tests rely on knowledge of the morphology of the fungus for identification by histology or examination of wet mounts of skin scrapings. Most sick frogs with chytridiomycosis have heavy infections with *Batrachochytrium dendrobatidis* that are easily recognized by standard histopathological techniques (Pessier et al. 1999, Berger et al. 2000). However histological

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diagnosis is insensitive when dealing with light infections in healthy animals or autolysed samples, or when tests are performed by inexperienced workers.

Diagnostic methods with improved sensitivity and ease of testing are needed. The production of polyclonal antibodies and the introduction of an immunoperoxidase (IPX) stain are the first steps in the development of more sophisticated tests for the detection of antigen.

MATERIALS AND METHODS

Antigen for immunization. A culture of *Batrachochytrium dendrobatidis* (isolate A98 1810/3) was obtained from a sick, wild adult Australian lacelid (*Nyctimystes dayi*). The culture was maintained on tryptone, gelatin hydrolysate agar (Longcore et al. 1999) for 5 mo before use. Sporangia were harvested by lightly scraping a 10 d old culture. Thirty milligrams of culture was mixed with 1 ml distilled water and left for 24 h at room temperature, then manually homogenized in a sterile petri dish and frozen at –80°C. After defrosting, the mixture was diluted with phosphate buffered saline (calcium- and magnesium-free) (PBSA) to a final concentration of 3.25 mg ml^{-1}. The protein concentration was determined using a Pyr Unicam PU8800 UV/VIS spectrophotometer at 280 nm, by interpolation of its absorbance from a standard curve calculated from known concentrations of bovine serum albumin (0.25, 0.5, 1.0 and 5.0 mg ml^{-1}). Freund's complete adjuvant and triple adjuvant (Quil A, DEAE-dextran, Montanide 888 oil) (Prowse 2000) were prepared using a Sorvall Omnimixer for emulsification. The final protein concentration of antigen in both preparations was 0.5 mg ml^{-1} antigen.

Immunization. Two rabbits (666 and 667) and 1 sheep (322) were inoculated with triple adjuvant intradermally with boosters at 7 and 11 wk post inoculation (pi). Two rabbits (668 and 669) and 1 sheep (386) were inoculated with Freund's complete adjuvant subcutaneously and boosted with Freund's incomplete adjuvant at 7 and 11 wk pi. At each inoculation, the rabbits received 0.5 mg fungus in 1 ml adjuvant and the sheep received 1 mg fungus in 2 ml adjuvant.

At 13 wk pi, serum from all animals were strongly stained for *Batrachochytrium dendrobatidis* in the IPX test (refer below), and at 15 wk pi animals were bled out under anesthesia (Australian Animal Health Laboratory, animal ethics approval no. 97-797). Blood was collected into SST gel and clot activator vacutainers. It was stored at 4°C for up to 2 d before centrifuging at 3000 rpm (Beckman J-6 M centrifuge) for 3 min. Serum was stored in 10 ml sterile Starstedt tubes at –80°C.

Fungal isolates for cross reactivity testing. Specimens of *Batrachochytrium dendrobatidis* from Australia, Ecuador, New Zealand and Germany were tested with the antisera. Ten other chytridiomycetes and 18 fungi from other phyla causing animal infections (including fungi from frogs, reptiles and fish) were obtained from various collections (Table 1). Most fungi were in agar cultures, but some identifiable fungi within animal tissues were also used. Two protozoans in amphibian tissue were also tested: a myxozoan and a coccidian. Samples of agar or tissue were fixed in 10% neutral buffered formalin, processed into paraffin blocks and sectioned for IPX staining.

IPX test. For indirect IPX staining, paraffin sections were dewaxed and incubated for 20 min with 0.1% trypsin in 0.1% aqueous $CaCl_2$ at 37°C for antigen unmasking. Slides were rinsed in distilled water and PBSA, loaded into Sequenza cassettes (Shandon, UK) and incubated with 200 µl of the anti-chytrid antisera at various dilutions in 0.1% skim milk powder/PBSA for 1 h at 37°C. After a 5 min rinse with PBSA, slides were incubated with biotinylated anti-rabbit, anti-goat immunoglobulin (Dako large volume DAKO LSAB kit, DAKO Corp, Carpinteria, CA, USA) for 20 min at 37°C. Slides were rinsed with PBSA then incubated with 3% H_2O_2 in distilled water for 20 min at room temperature (22°C) to block endogenous peroxidase activity. After rinsing in PBSA, slides were incubated with streptavidin peroxidase conjugated (DAKO LSAB kit) for 20 min at 37°C, rinsed again, then removed from the Sequenza cassettes. The antigenic complex was visualized using a 3-amino-9-ethyl carbasole (AEC) chromogen system (Sigma). The substrate and chromogen (freshly made AEC solution [2 mg AEC powder in 200 µl dimethyl formamide] added to 10 ml 0.05 M acetate buffer with 5 µl 30% hydrogen peroxide) were added and incubated at room temperature for 5 min. After washing, slides were counterstained in Lillie's modified hemalum, blued in Scott's tap water, rinsed in tap water and mounted in an aqueous mounting medium.

Negative controls consisted of test slides incubated with normal sera, pre-bleed sera or 1% skim milk instead of primary antibody. Sections of *Batrachochytrium dendrobatidis* culture (A98 1810/3) and sections of infected skin from a green tree frog (*Litoria caerulea*) (A99 1385/1) were used as positive control slides.

The IPX test was used to indicate the titer of antibodies from each animal (rabbits and sheep) and to characterize the cross-reactivity of antibodies with other fungi. These fungi are listed in Table 1.

Evaluation of the IPX test for diagnosis. Preliminary evaluation of the IPX as a diagnostic assay was conducted on 55 lightly infected and 15 control toe-clip samples from an experimental infection using juveniles of *Litoria caerulea*. The toe-clips were collected

Table 1. List of fungal species used, their strain identification numbers, the order and phylum they belong to, and their source. A: from Lee Berger, Australian Animal Health Laboratory, Australia; E: from Andrés Merino-Viteri, Museo de Zoologia, Quito, Ecuador; G: from Frank Mutschmann, Tierarztpraxis, Berlin, Germany; JEL: from the chytrid collection of Joyce Longcore, University of Maine, Maine, USA; N: from Bruce Waldman and Richard Norman, Massey University, New Zealand; T: from Kaye Humphreys and Karrie Rose, Taronga Zoo, Australia

Species	Strain identification	Order	Phylum	Source
Batrachochytrium dendrobatidis	A 98 1810/3	Chytridiales	Chytridiomycota	Australian lacelid (*Nyctimystes dayi*)
Chytriomyces angularis	JEL 45	Chytridiales	Chytridiomycota	Pollen bait
Diplochytridium lagenarium	JEL 72	Chytridiales	Chytridiomycota	*Oedogonium* sp. (algae)
Karlingiomyces sp.	JEL 93	Chytridiales	Chytridiomycota	Snakeskin bait
Powellomyces sp.	JEL 95	Spizellomycetales	Chytridiomycota	Pollen bait
Rhizophydium sp.	JEL 136	Chytridiales	Chytridiomycota	Pollen bait
Multiple axes	JEL 142	Chytridiales	Chytridiomycota	Onion skin bait
Rhizophydium sp.	JEL 151	Chytridiales	Chytridiomycota	*Lyngbya* sp. (algae)
Gonapodya sp.	JEL 183	Monoblepharidales	Chytridiomycota	*Lemna* sp.
Asterophlyctis sarcoptoides	JEL 186	Chytridiales	Chytridiomycota	Shrimp chitin bait
Allomyces macrogynus	JEL 204	Blastocladiales	Chytridiomycota	Snakeskin bait
Fusarium culmorum	T 801/99	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Phaeoacremonium parasiticum	T 819/99	Moniliales	Ascomycota	Bearded dragon (*Pogona barbata*)
Fusarium oxysporum (1)	T	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Fusarium oxysporum (2)	T 801/99	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Trichophyton terrestre	T 1067/00	Onygenales	Ascomycota	Blue-tongued lizard (*Tiliqua scincoides*)
Paecilomyces lilacinus	T 1618/1	Eurotiales	Ascomycota	Yellow anaconda (*Eunectes notaeus*)
Penicillium sp.	T 1440.1	Eurotiales	Ascomycota	Green and gold bell frog (*Litoria aurea*)
Candida sake	T 1103.1	Saccharomycetales	Ascomycota	Green and gold bell frog (*Litoria aurea*)
Fusarium sp.	T 1670.1	Hypocreales	Ascomycota	Gouldian finch (*Erythrura gouldiae*)
Gliocladium sp.	T 1212	Hypocreales	Ascomycota	Murray cod (*Maccullochella peelii*)
Verticillium sp	T 1212	Moniliales	Ascomycota	Murray cod (*Maccullochella peelii*)
Chrysosporium sp.	T 1333	Onygenales	Ascomycota	Hosmer's skink (*Egernia hosmeri*)
Acremonium sp.	T 1697.1	Hypocreales	Ascomycota	Diamond python (*Morelia spilota*)
Scopulariopsis brevicaulis	T 1258	Microascales	Ascomycota	Black-headed python (*Aspidites melanocephalus*)
Trichoderma viride	T 1344	Hypocreales	Ascomycota	Freshwater crocodile (*Crocodylus johnstoni*)
Monochaetia sp.	T 1226	Melancoliales	Ascomycota	Little penguin (*Eudyptula minor*)
Mucor amphibiorum (tissue)	A 96 1429/3	Mucorales	Zygomycota	Cane toad (*Bufo marinus*)
Aphanomyces sp. (tissue)	A 95 517	Saprolegniales	Oomycota	Cane toad (*Bufo marinus*)
B. dendrobatidis (tissue)	N 30916-00B	Chytridiales	Chytridiomycota	Southern bell frog (*Litoria raniformis*)
B. dendrobatidis (tissue)	E QCAZ 3691	Chytridiales	Chytridiomycota	*Atelopus* sp.
B. dendrobatidis (tissue)	G 99 1743/3	Chytridiales	Chytridiomycota	Yellow-lined poison dart frog (*Phyllobates lugubris*)
Myxidium immersum (tissue)	A 96 570/1	Bivalvulida	Myxozoa	Stony creek frog (*Litoria lesueuri*)
Goussia-like coccidian (tissue)	A 01 95/4	Eimeriidae	Apicomplexa	Great barred frog (*Mixophyes fasciolatus*)

19 d after frogs were exposed to 50 000 zoospores. All infected frogs eventually died from chytridiomycosis between 24 and 67 d after exposure. Sections of toe-clips were stained using antiserum from rabbit 667 diluted 1:1000. The results from the IPX test were compared with results from hematoxylin and eosin (H&E) staining. Sections of a naturally infected wild tadpole of *Mixophyes fasciolatus* were tested with antisera from all animals.

Fluorescence staining. For direct fluorescence staining, cultured *Batrachochytrium dendrobatidis* grown on glass slides were incubated with rabbit 667 antiserum diluted 1:100 in 0.1% bovine serum albumin/PBSA for 30 min at 37°C. After washing in PBSA, slides were incubated with 1:100 diluted anti rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (Silenus, Boronia, Australia) for 30 min at 37°C. They were then washed and mounted in glycerol. Slides were examined with a Reichert-Jung (Leica) Polyvar microscope with a tungsten lamp and a filter for fluorescence.

Electron microscopy. Both cultures of *Batrachochytrium dendrobatidis* were pelleted in 1.5% low gelling temperature agarose (Type VII) (Sigma) in 0.1 M cacodylate buffer (pH 7.2, 300 mOsm kg^{-1}) and fixed in 0.25% (v/v) buffered glutaraldehyde for 40 min. The samples were then processed as described by Hyatt (1991) and embedded in LR White. Ultra-thin sections were cut with a Reichart-Jung (Leica) Ultracut E and immuno-gold labeled (Hyatt 1991) with rabbit (666) anti *B. dendrobatidis* (1:1000). Sections were then double- stained with uranyl acetate and lead citrate before examination with a Philips CM 120 transmission electron microscope at 100 kV.

RESULTS

Antisera from all animals reacted strongly with isolates of *Batrachochytrium dendrobatidis* when used at dilutions between 1:100 and 1:1600 in the indirect IPX test on sections of culture and of infected skin. All stages of *B. dendrobatidis* were stained, and the walls, cytoplasm, septa, rhizoids and internal zoospores were highlighted (Figs 1 to 3). There was little or no background staining of amphibian skin.

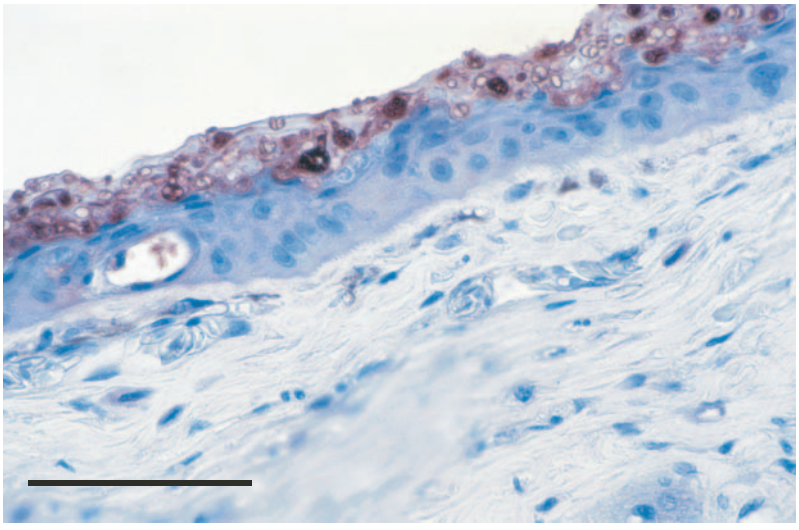

Fig. 1. Immunoperoxidase stain on skin of a *Litoria caerulea* with a heavy infection of *Batrachachytrium dendrobatidis*. There is strong staining of fungal walls, cytoplasm, zoospores and septa. Antiserum from rabbit 667 was diluted to 1:1000. Scale bar = 80 µm

When titrated at doubling dilutions from 1:100, serum reactivity began falling at 1:1600. Antisera from rabbit 669 and sheep 386 were not stained at 1:12 800, while antisera from the other animals still stained weakly at 1:25 600 (Table 2). The sheep and 1 of the 2 rabbits inoculated with Freund's adjuvant had lower titers of antibody than the 3 animals given triple adjuvant.

In sera collected from rabbits before vaccination there was some distinct, although minimal, staining of

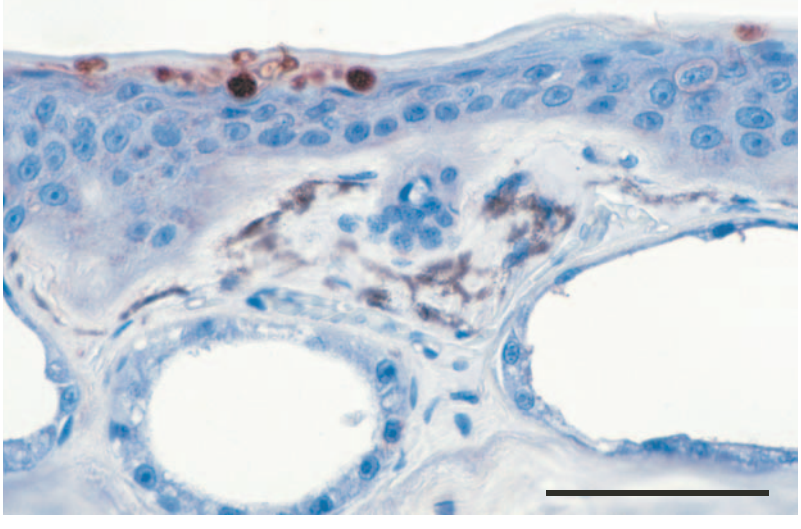

Fig. 2. Immunoperoxidase stain on skin of a *Litoria caerulea* with a light infection of *Batrachachytrium dendrobatidis*, showing the sensitivity of the test in highlighting a few sporangia. Scale bar = 80 µm

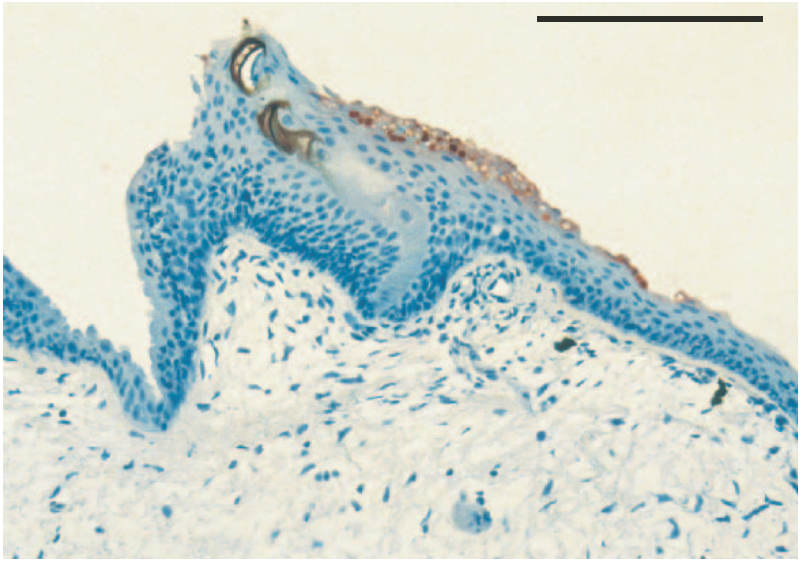

Fig. 3. Immunoperoxidase stain on the mouth parts of a tadpole of *Mixophyes fasciolatus* infected with *Batrachachytrium dendrobatidis*. Scale bar = 200 µm

cultured *Batrachochytrium dendrobatidis* when used at dilutions of 1:100 but not of 1:500. Sera collected from sheep before vaccination showed some staining at 1:100 and 1:500 but not at 1:1000. It was not determined whether this staining was non-specific or due to pre-existing antibodies in the animals.

There was cross-reactivity between antisera and a large number of fungi (Table 3). Significant staining is represented by >2+ in the table. The strongest staining occurred with fungi in the Chytridiomycota, but reactions also occurred with fungi in other phyla. Staining of other fungal species was sometimes limited to 1 or 2 distinct structures, e.g. zoospores, rhizoids, cytoplasm or cell walls, showing that these fungi had different antigens in common with *Batrachochytrium dendrobatidis*. When used at 1:100, antisera cross-reacted with between 6 out of 10 and 9 out of 10 other chytridiomycetes. Most of this cross-reactivity disappeared when the antisera were used at 1:1000, although a *Karlingiomyces* sp. (no. 93) and an undescribed isolate known as 'multiple axes' (no. 142) were still stained by rabbit antisera. Less cross-reactivity occurred with fungi from other phyla; antisera from rabbits and sheep reacted with between 3 out of 18 and 8 out of 18 fungi when used at 1:100, but staining was negligible at 1:1000. The 2 protozoans were not stained. Fungi that cross-reacted at 1:1000 were also incubated with antisera diluted out to 1:2000, but they were still stained (data not shown).

Table 2. Intensity of staining in the immunoperoxidase test using various concentrations of antisera against *Batrachochytrium dendrobatidis*. Animals 666, 667 and 322 were inoculated with triple adjuvant, and animals 668, 669 and 386 were inoculated with Freund's adjuvant. At higher dilutions, cell walls faded before zoospores

Dilution	Rabbit 666	Rabbit 667	Rabbit 668	Rabbit 669	Sheep 322	Sheep 386
1:400	+++++	+++++	+++++	+++++	+++++	+++++
1:800	+++++	+++++	+++++	++++	+++++	++++
1:1600	++++	++++	++++	+++	+++++	++++
1:3200	+++	+++	++++	++	++++	++
1:6400	+++	+++	+++	++	+++	+
1:12800	+	++	++	–	++	–
1:25600	+	+	+	–	+	–

Evaluation of the stain on toe-clips from experimentally infected frogs showed that the IPX test was more sensitive than H&E staining for diagnosis of chytridiomycosis. With the IPX test, 34 of 55 (62%) frogs tested positive, compared with 29 of 55 (53%) positives and 3 suspicious positives with H&E staining. Fifteen controls were negative by both methods. On re-examination of 4 negative or suspicious H&E sections that were positive by immunostaining, a few sporangia were recognized in the sections—these were mostly solid, immature stages within viable epidermal cells and were difficult to differentiate from cell nuclei. The stain was also effective in highlighting infection in the mouth of a tadpole (Fig. 3).

Fluorescence staining of cultured fungi resulted in strong staining of the surface of sporangia, and rhizoids were clearly outlined. Transmission electron microscopy of ultra-thin sections showed sporangia and zoospores to be gold labeled (Fig. 4). Labeling was strongly associated with the inner aspect of the sporangial wall, the limiting membrane of the zoospores, flagella and the contents of the zoospore vacuoles.

DISCUSSION

The antisera that were produced in this study will be valuable in improving diagnostic screening assays for the detection of *Batrachochytrium dendrobatidis* in amphibians. Although there was some cross-reactivity with other fungi, the antisera did not cross-react with other fungi known to infect amphibians. When the rabbit antisera were diluted out to 1:1000, apart from 2 chytridiomycetes, only *B. dendrobatidis* remained strongly stained. As structures of other fungi were still stained at the higher dilution the antibodies cannot be

Table 3. Reactions of antisera with fungi in the immunoperoxidase test. 1+: ill-defined pale staining; 2+: minimal staining of some elements; 3+: moderate staining of some or all elements; 4+: strong staining of some elements; 5+: strong staining all elements; ND: not done

Species	Rabbit 666			Rabbit 667			Rabbit 668			Rabbit 669			Sheep 322			Sheep 386		
	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K
Batrachochytrium dendrobatidis	+++++	+++++	+++++	+++++	+++++	+++++	++++	+++++	++++	+++++	++++	++++	+++++	+++++	++++	++++	++++	+++++
Chytriomyces angularis	+++	++	–	+	–	ND	+++	+++	–	++++	–	ND	+++	++	–	+++	+	–
Diplochytridium lagenanum	++++	+++	++	++++	+	–	+++	+	+	++++	+++	++	+++	++	–	++++	+++	+
Karlingiomyces sp.	++++	++++	+++	++++	++++	+++	+++++	+++	+++	++++	+++	+++	++++	++	++	++++	++	++
Powellomyces sp.	+	+	–	++	–	–	+++	–	–	++	–	–	++	+	+	++	+++	+++
Rhizophydium sp.	+++	–	–	++	–	–	+++	+	+	++	–	–	++	++	++	++	+	–
Multiple axes	+++++	++++	+++	+++++	++++	++++	++++	++++	++++	+++++	++++	+++	+++++	+++++	++++	+++	++++	+++
Rhizophydium sp.	+++	++	++	+++	–	–	++++	+	+	+++	–	–	++++	+++	++	+++	+++	++
Gonapodya sp.	+++++	++++	++	++++	+++	++	+++++	++++	++	++++	++	++	++++	++++	++++	++++	+++	+
Asterophlyctis sarcoptoides	+	+	–	++	+	–	++++	+++	+	+++	–	–	+++	–	++	+++	++	+
Allomyces macrogynus	++	–	ND	+++	–	ND	++	++	+	++	–	ND	++	+	ND	+++	++	ND
Subtotal of staining >++	7	4	2	6	3	2	9	5	2	7	3	2	7	3	2	8	5	2
Fusarium culmorum	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	+	–	ND	+	–	ND
Phaeoacremonium parasiticum	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	+	–	ND
Fusarium oxysporum (1)	++	++	+	+	+	ND	+	–	ND	+	+	ND	+	+	ND	++	++	+
Fusarium oxysporum (2)	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	+	–	ND	+	–	ND
Trichophyton terrestre	+++	+	+	+++	–	ND	+++	++	–	+	–	ND	++	–	ND	++++	–	ND
Paecilomyces lilacinus	+++	+	–	++++	++	+	++	–	ND	+++	–	ND	++	–	ND	+++	++	–
Candida sake	+	+	–	++	–	ND	++	+	ND	++	+	–	–	–	ND	++	+++	+
Penicillium sp.	+++	–	ND	++	–	ND	+++	–	ND	+	–	ND	++	–	ND	++	–	ND
Fusarium sp.	+	–	ND	–	–	ND	–	ND	ND	+++	–	ND	++	–	ND	+++	–	ND
Gliocladium sp.	+	–	ND	+	–	ND	++	–	ND	–	ND	ND	++	+	ND	++++	++	ND
Verticillium sp	+++	+	–	+++	++	+	+++	++	+	+++	++	+	+++	++	+	++++	+	+
Chrysosporium sp.	++	+	+	++	++	–	+++	++	+	++	+	–	+++	+++	+++	+	+	–
Acremonium sp.	+	–	ND	++	–	ND	+	–	ND	–	ND	ND	–	ND	ND	++	–	ND
Scopulariopsis sp.	+++	+	–	++	–	ND	++	–	ND	++	+	–	–	ND	ND	+++	–	ND
Trichoderma sp.	++++	++	–	++++	–	ND	++	–	ND	++++	++	–	–	ND	ND	++++	+	+
Monochaetia sp.	++	–	ND	+	–	ND	++	–	ND	++	–	ND	–	ND	ND	++++	+	–
Aphanomyces sp.	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND
Mucor amphibiorum	+	–	–	+	–	–	+	–	–	++	–	–	+++	–	–	+	–	–
Goussia sp.	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	+	ND	ND	–	ND	ND
Myxidium immersum	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND
Subtotal of staining >++	6	0	0	4	0	0	4	0	0	4	0	0	3	1	1	8	1	0
Total staining >++	13	4	2	10	3	2	13	5	2	11	3	2	10	4	3	16	6	2

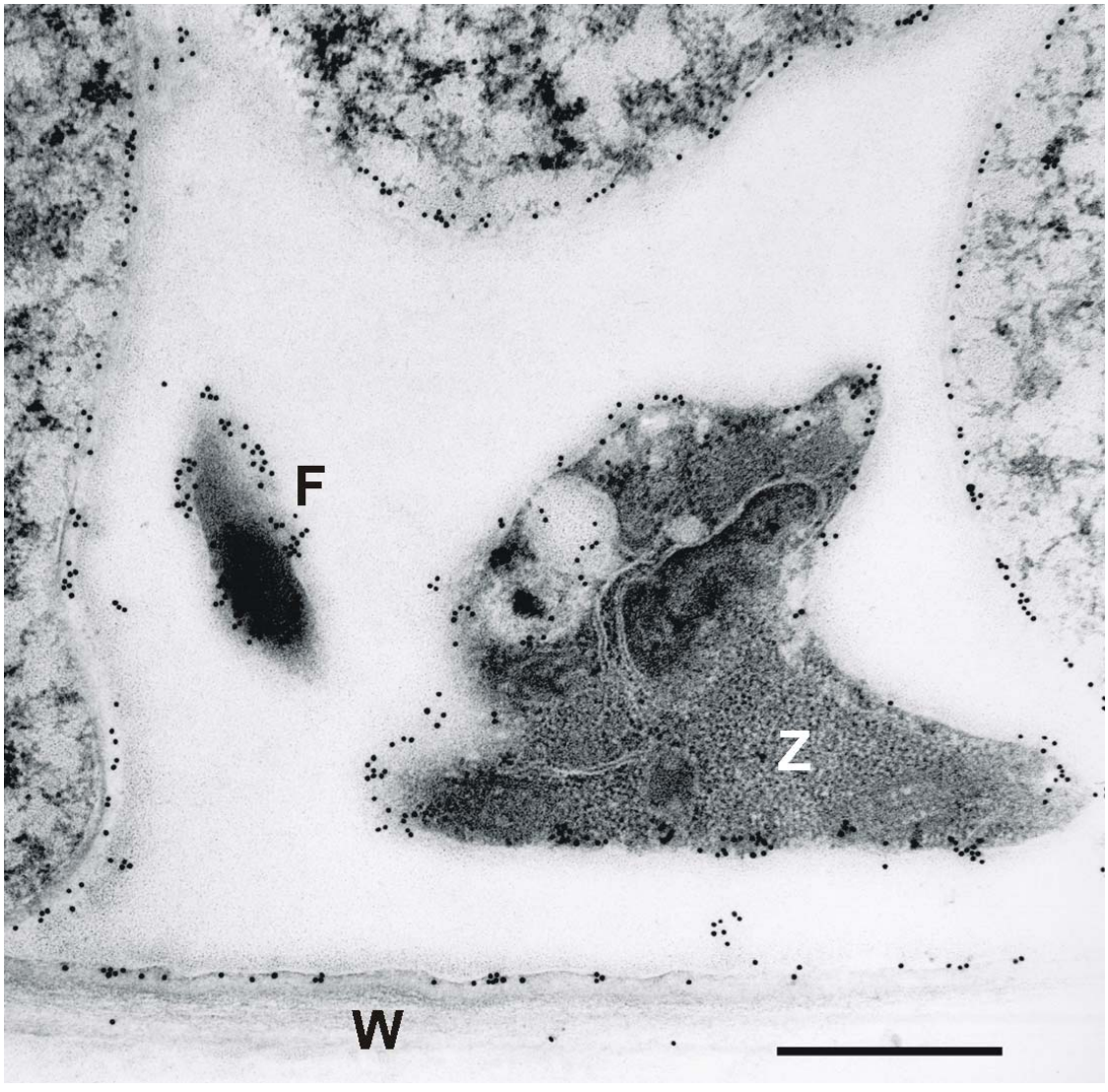

Fig. 4. Electron micrograph of a gold-labeled cultured zoosporangium, using polyclonal antiserum from rabbit 666. Note dense gold particles adhering to the inner edge of the wall (W), the membrane of the zoospore (Z), and the surface of the flagellum (F). Scale bar = 500 nm

considered to be species specific. The only other spherical fungus reported to infect amphibian skin in Australia is *Mucor amphibiorum* (Speare et al. 1997) and this fungus was not stained. The 2 fungi that cross-reacted strongly are chytridiomycetes, none of which are known to infect amphibians. One of these was the undescribed chytrid labeled 'multiple axes' (JEL 42), which groups most closely with *B. dendrobatidis* in some 18S rDNA phylogenetic analyses (James et al. 2000). The other strongly stained species, *Karlingiomyces* sp. (JEL 93), is phylogenetically distant in analyses of 18S rDNA sequence data.

Polyclonal antibodies are usually cross-reactive between fungal species, and even monoclonal antibodies are often not specific (Fenelon et al. 1999). Carbohydrates and glycoconjugates on cell walls may be highly antigenic and have epitopes common to a range of species (Gabor et al. 1993). Electron microscopy of gold-labeled sections showed that our polyclonal antibodies were directed mainly to the sporangial wall and zoospore membrane. Preparations containing only internal structures have been found to generate monoclonal antibodies that are more species specific (Gabor et al. 1993).

The polyclonal antibodies we produced are a basis for further work and the IPX test may be useful for detecting or confirming infection with *Batrachochytrium dendrobatidis* in frog skin. Histological meth-

ods may be less sensitive than other methods (e.g. enzyme-linked immunosorbent assays) as only small skin samples, such as toe-clips, can be collected ante mortem. Furthermore, sections contain just a thin strip of skin and may miss any sporangia. The IPX test can be used instead of, or as an adjunct to, H&E staining when increased sensitivity of testing is required, such as for importation of amphibians. It is useful for screening toe-clip samples from healthy frogs where only a few sporangia may be present, and these can be easily seen even at low magnification. The stain is also useful for testing necrotic or autolyzed samples from sick frogs that may contain few distinctive sporangia. In addition, the ease of interpretation of the assay permits scientists other than specialists (e.g. pathologists and mycologists) to diagnose infection. The benefits of increased sensitivity and quicker examination of slides stained by the IPX test must be weighed against the greater complexity of the staining method, which takes about 4 h.

It is recommended that the rabbit antisera be diluted to 1:1000 for immunostaining and that interpretation of results include examination of stained structures for the characteristic appearance of *Batrachochytrium dendrobatidis* (Berger et al. 1998, Pessier et al. 1999).

The data reported in this paper are part of a research effort into improving the diagnostic capability for the detection of chytridiomycosis (Hyatt et al. 2000). The immunostaining protocol is the first reported assay for the detection of *Batrachochytrium dendrobatidis* antigens. The polyclonal antibodies are being used in the development of an indirect enzyme-linked immunosorbent assay. Monoclonal antibodies to *B. dendrobatidis* are being evaluated for use in more specific tests. We do not know which type of antibody or test will prove to be the most useful for detection of *B. dendrobatidis* in amphibians, but the IPX test described here using polyclonal antibodies can now be used for improved diagnosis.

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