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Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay

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ABSTRACT: *Batrachochytrium dendrobatidis* is a major pathogen of frogs worldwide, associated with declines in amphibian populations. Diagnosis of chytridiomycosis to date has largely relied upon histological and immunohistochemical examination of toe clips. This technique is invasive and insensitive particularly at early stages of infection when treatment may be possible. We have developed a real-time PCR Taqman assay that can accurately detect and quantify one zoospore in a diagnostic sample. This assay will assist the early detection of *B. dendrobatidis* in both captive and wild populations, with a high degree of sensitivity and specificity, thus facilitating treatment and protection of endangered populations, monitoring of pristine environments and preventing further global spread via amphibian trade.

KEY WORDS: Chytrids · *Batrachochytrium dendrobatidis* · Amphibian declines · Real-time PCR Taqman assay · Chytridiomycosis

INTRODUCTION

Chytridiomycosis is a serious disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (Berger et al. 1998, 1999, Pessier et al. 1999). It is now believed to be responsible for widespread global declines in amphibian populations (Daszak et al. 1999). First reported in Panama and Australia in wild frog populations undergoing rapid declines, and subsequently identified in Africa, South America, Central America, North America, Europe and New Zealand (Berger et al. 1998, 1999, Lips 1999, Mutschmann et al. 2000, Bosch et al. 2001, Waldman et al. 2001, Bradley et al. 2002, Weldon 2002), the infection has been found in both wild and captive populations. Two amphibian orders (Anuran and Caudata) encompassing 14 families and 93 species have been diagnosed with infections worldwide (see www.jcu.edu.au/school/phtm/phtm/frogs/ampdis.htm). Earliest records from archival material have been identified in North American *Rana pipiens* collected in 1974 (Carey et al. 1999). Chytridiomycosis has been described as an emerging infectious disease with available evidence providing clear links to worldwide amphibian declines (Daszak et al. 2003). Multilocus sequence typing of 35 strains of *B. dendrobatidis* from North America, Africa and Australia has shown low variability of the DNA sequences among 10 loci (5918 bp), providing support for this theory (Morehouse et al. 2003).

In post-metamorphic frogs, *Batrachochytrium dendrobatidis* infects the keratinised epidermal cells of the frog causing a hyperkeratotic and hyperplastic response of the stratum corneum and stratum granulosum. While reasons for death are unknown, postulated causes include disruption to osmoregulation and toxin release (Daszak et al. 2001). Non-fatal infections of keratinised tadpole mouthparts have been documented with subsequent infection of the frog following metamorphosis (Berger et al. 1999, Speare 2002). Diagnosis to date has largely relied upon histological

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examination via haematoxylin and eosin staining of toe clips or skin scrapings (Daszak et al. 1999, see also www.jcu.edu.au/school/phtm/phtm/frogs/histo/chhisto.htm). Immunoperoxidase staining with polyclonal antibodies to *B. dendrobatidis* increases the specificity and sensitivity of detection (Berger et al. 2002). More recently, a staining protocol has been described which enhances diagnosis by the co-localisation of *B. dendrobatidis* and keratin in the skin of amphibians (V. Olsen, D. Boyle, D. Mendez, A. D. Hyatt unpubl.). However, the drawbacks of histological testing include the degree of expertise needed for identification, the invasiveness of sampling technique—typically performed by toe-clipping of live animals—the length of time required, the low sensitivity of the test, and the variability of infection levels amongst toes. In the field, examination of oral disc abnormalities in tadpoles with a 10× hand lens has been recommended as a preliminary indication of chytridiomycosis (Fellers et al. 2001), although these abnormalities can also be caused by DDT intoxication. The fungus can be identified by isolation and culture (Longcore et al. 1999), but this requires a high degree of expertise and time.

Early detection of *Batrachochytrium dendrobatidis* is vital to the control of spread of the disease via global amphibian trade. *B. dendrobatidis* has been identified in animals imported for zoo collections (Pessier et al. 1999), in international pet trade (Mutschmann et al. 2000), in the food trade (Mazzoni et al. 2003) and in laboratory animal trade (Parker et al. 2002). Early detection of infection would allow possible curative treatment to be undertaken, at least in captive populations where formalin/malachite green solution has been shown to be effective on adult *Xenopus tropicalis* (Parker et al. 2002). Itraconazole baths (0.01%) were found to be effective in treating the terrestrial species *Dendrobates tinctorius* (Nichols et al. 2001), although whether this treatment is effective and safe for use in tadpoles is yet to be determined. An elevated temperature of 37°C has also been suggested as an effective treatment for captive *Litoria chloris* (Woodhams et al. 2003). Early detection would also allow treatment and protection of endangered populations, the potential for repopulation of species from captive bred populations, and the monitoring of environments where infections have not previously been reported.

Batrachochytrium dendrobatidis has also been shown to be infective for up to 7 wk in lake water (Johnson et al. 2003). If contamination of water, soil and animals could be sensitively detected, appropriate quarantine and disinfection strategies could be implemented to prevent further spread of disease.

A PCR-based test that would quickly identify *Batrachochytrium dendrobatidis* infection with a high degree of sensitivity and specificity, preferably using a non-invasive sampling method, is highly desirable. Ribosomal DNA occurs as multicopy genes in eukaryotes, typically 100 to 400 copies per haploid genome for fungi, with conserved regions for 5.8, 18 and 28S DNA separated by internal transcribed spacers (ITS-1 and ITS-2) and an intergenic spacer. Sequences of 5.8, 18 and 28S rRNA molecules are highly conserved, whereas the ITS region and intergenic spacer units evolve quickly (Long et al. 1980). This variability and high copy number facilitates the design of species-specific PCR-based tests of high sensitivity and specificity for molecular diagnostic tests such as identification of bacteria (Barry et al. 1991, Jensen et al. 1993) and fungi (Harmsen et al. 1999, Henry et al. 2000).

Recent advances in molecular technologies have seen the development of real-time Taqman assays (Livak et al. 1995). These are faster and more sensitive than conventional PCR and allow quantitation of the target DNA, making them ideal methods for diagnostic tests. In this paper we describe a rapid, sensitive and specific Taqman assay for the quantitative detection of as little as 1 zoospore of *Batrachochytrium dendrobatidis* in tissues from infected frogs. The test can detect *B. dendrobatidis* infection in frogs 7 to 14 (or more) d before infection can be detected by histological methods.

MATERIALS AND METHODS

Cultivation of *Batrachochytrium dendrobatidis* and related organisms. A zoospore suspension of *B. dendrobatidis* isolate AAHL (Australian Animal Health Laboratory) 98 1810/3 from *Nictimystes dayi* was prepared by seeding TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g agar, 1000 ml distilled water) with 2 ml of actively growing broth culture. The plates were allowed to dry until most of the solution had diffused into the agar at which time the plates were sealed, inverted and incubated at 23°C for 4 d. Zoospores were harvested by flooding the plate twice with 2 ml of DS solution (a weak salt solution resembling pond water comprising 10^{-3} M KH_2PO_4, 10^{-4} M $MgCl_2$ and 2×10^{-5} M $CaCl_2$). Zoospore numbers were counted in a haemocytometer. Other *B. dendrobatidis* isolates, AAHL 98 1469/10 from a captive-bred metamorph *Lymnodynastes dumerilli*, AAHL 00 545 from a wild-caught captive metamorph *Litoria lesueuri* and AAHL 99 1385/12 from wild adult *Litoria caerulea*, were similarly cultivated and prepared.

Five other Chytridiomycetes from the order Chytridiales: *Rhizophydium* sp. (JEL136), *Rhizophlyctis–Rhizophydium*-like (JEL142), *R. ?haynaldii* (JEL151); the order Monoblepharide: *Gonapodya* sp. (JEL183);

and the order Blastocladiales: *Allomyces macrogynus* (JEL204) (supplied by J. Longcore) were tested to verify the specificity of the Taqman assay. Cultures were grown in broth in 25 cm^2 flask containing either mPmTG (0.2 g peptonized milk, 0.2 g tryptone, 1.0 g glucose, 500 ml distilled water) for JEL136, JEL142, JEL151 and JEL183 or PmTG (0.5 g peptonised milk, 0.5 g tryptone, 2.5 g glucose, 500 ml distilled water) for JEL204. For JEL136, 142 and 151, sporangia were scraped off the flask and clumps disrupted by forcing through a 26 gauge needle 10 times. Sporangia numbers were estimated by counting in a haemocytometer. Samples of cultures (JEL142 10^4 zoosporangia, and JEL136 and JEL151 10^5 zoosporangia) were pelleted in a microfuge (Hettich, Micro 12-24) at a force of $13 \times 10^3 \times g$ for 1 min, resuspended in 200 µl of PrepMan Ultra (Applied Biosystems), and DNA was extracted as detailed below. JEL183 and JEL204 grow as hyphae so individual counts were not possible. Samples containing a similar amount of fungi to the other preparations were pelleted in a microfuge and DNA-extracted in the same manner.

Frog samples. Great Barred frogs (30) *Mixophyes fasciolatus* were experimentally infected with 10^4 zoospores from *Batrachochytrium dendrobatidis* isolate AAHL 98 1810/3 by bath inoculation for 20 h. Uninfected control frogs (12) were similarly bathed in DS solution. Live infected frogs were sampled by toe clipping, as this site has been shown by histology to have a high level of infection. (Berger et al. 1998a). Two toeclips were taken at 7 d intervals from Day 0 to 28. For use in real-time PCR, toes were harvested into sterile 1.5 ml tubes and stored at –80°C prior to DNA extraction. Toes to be processed for histology were fixed in 10% neutral buffered formalin.

During the course of test development, dead frogs collected in the field were submitted to the laboratory for diagnosis. Sick frogs from a breeding and research facility were also submitted. Toe clips from these frogs were processed for histology or DNA extracted as described below.

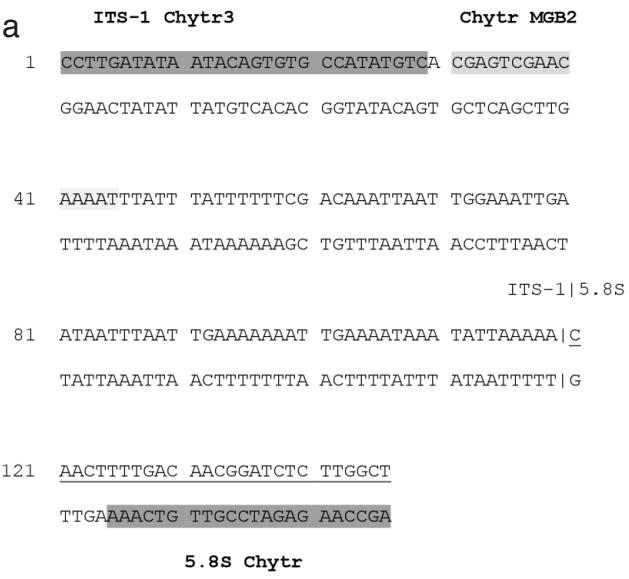
Histology. Toeclips were processed using an immunoperoxidase (IPX) staining method adapted from Berger et al. (2002). The digestion with trypsin was deleted as it was found to be unnecessary, and a DAKO Envision+ kit was used instead of the DAKO LSAB kit as it decreased incubation times.

Extraction of nucleic acid from samples. Nucleic acids were prepared from whole toes (approx. 1 to 3 mg wet weight) by extraction with PrepMan Ultra. Forty µl of PrepMan Ultra was added to each toe along with 30 to 40 mg of Zirconium/silica beads measuring 0.5 mm diameter (Biospec Products). The toe was homogenised for 45 s in a Mini Beadbeater 8 (Biospec Products). After brief centrifugation (30 s at $13 \times 10^3 \times g$ in a microfuge) to recover all material to the bottom of the tube, the homogenisation and centrifugation was repeated. The homogenised toe clip was immersed in a boiling water bath for 10 min, cooled for 2 min, then centrifuged at $13 \times 10^3 \times g$ for 3 min in a microfuge. Twenty µl of supernatant was recovered and stored at –80°C.

DNA sequencing. This was performed on the 4 *Batrachochytrium dendrobatidis* isolates. The target DNA was amplified using BD Biosciences Advantage 2PCR Kit with primers BOB5 and BOB6 in an Applied Biosystems Gene Amp PCR System 9700 thermocycler. Products were viewed on a 1% agarose gel and the 740 bp product was purified using a QIAquick PCR Purification kit (Qiagen). This was sequenced using an ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit run on an ABI 377XL automated sequencer with primers BOB3, 5′ GCG TTC TTC ATC GTT GCG AGA GCC 3′, BOB3.1, 5′ AGT GCA ATG TGC GTT CAA AGA 3′, BOB5, 5′ ATG CTT AAG TTC AGC GGG 3′ and BOB6 5′ CCG ATT GAA TGG CTT AGT GAG ACC 3′. These primers were designed from sequence data obtained from a large PCR product generated using generic primers targeting 18S and 28S rDNA (J. A. T. Morgan unpubl. data).

Real-time Taqman PCR assay. Real-time Taqman PCR assays were conducted using an Applied Biosystems Prism 7700 Sequence Detection System. Twenty-five µl reactions containing 12.5 µl 2 × Taqman Master Mix (Applied Biosystems), PCR primers at a concentration of 900 nM, the MGB probe at 250 nM and 5 µl of DNA (diluted 10^{-1} in water) were prepared in triplicate. Included in each 96-well assay plate were control reactions containing DNA from 100, 10, 1 and 0.1 *Batrachochytrium dendrobatidis* genome equivalents prepared as described below and controls with no DNA template. The default ABI Prism 7700 amplification conditions (2 min at 50°C, 10 min at 95°C), followed by 15 s at 95°C and 1 min at 60°C for 50 cycles were used. The AB 7700 instrument software calculates and plots the change in fluorescence signal from the fluorescent labelled probe (Δ Rn) during the PCR cycling reaction versus the cycle number. Δ Rn is calculated using the equation Δ Rn = (Rn+) – (Rn–), where Rn + is the fluorescence signal of the product at any given time and Rn– is the fluorescence signal of the baseline emission during early amplification cycles in which no fluorescence signal is detected — the instrument default setting is cycles 6 to 15; however, this can be adjusted if a signal is detectable before cycle 15 in any of the assays. An arbitrary threshold is then set at the midpoint of the log Δ Rn versus cycle number. The Ct number is defined as the cycle number at which the Δ Rn crosses this threshold. Using a Δ Rn of 0.10, the Ct values for each test and control reaction

a



b

Primer Sequences

ITS1-3 Chytr 29 bases

5'- CCTTGATATAATACAGTGTGCCATATGTC-3'

5.8S Chytr 22 bases

5'- AGCCAAGAGATCCGTTGTCAAA -3'

Minor groove binder probe sequence

Chytr MGB2 15 bases

5' - 6FAM CGAGTCGAACAAAAT MGBNFQ – 3'

Fig. 1. *Batrachochytrium dendrobatidis.* rDNA sequence of internal transcribed spacer (ITS-1) and 5.8S used for design of Taqman real-time PCR primers and probe. (a) rDNA sequence including ITS-1/5.8S junction with primer and probe locations. (b) Primer sequences and minor groove binder probe sequence. GenBank accession # AY598034

were determined. A standard curve was constructed from the control reactions containing 100, 10, 1 and 0.1 *B. dendrobatidis* zoospores and the concentration determined for the test samples expressed as the number of Zoospore equivalents.

Quantitation standards. Standards for quantitation of *Batrachochytrium dendrobatidis* were prepared by extraction of DNA from zoospores grown on TGhL plates as described. The zoospores were harvested from plates by washing, and counted 4 times using a haemocytometer. In all, 10^7 zoospores were pelleted in a microfuge ($13 \times 10^3 \times g$ for 1 min), the supernatant removed and the pellet resuspended in 200 µl of PrepMan Ultra. The suspension was boiled for 10 min, cooled for 2 min, microfuged for 3 min, and 150 µl supernatant removed. This DNA was diluted in distilled water to 2×10^5 ml^{-1} genome equivalents, and aliquots stored at –20°C.

RESULTS

rDNA region sequencing

rDNA sequencing was performed on 4 *Batrachochytrium dendrobatidis* isolates. Sequencing of the 18S, ITS-1 and 5.8S regions was completed for these isolates, although several regions proved difficult to sequence, including a very G-rich (~12 to 13 Gs), and an AT-rich stem/loop structure, in the ITS-1 (5′-TGTTTTTTCAAA AAACA-3′). The DNA sequence of the 4 strains was identical over the region examined. Initial attempts to design a SYBR Green and Taqman assay wholly contained within the ITS-1 were discontinued when it was found that the SYBR Green assay worked effectively but the designed Taqman assay failed, apparently due to the highly G-rich stretch and the apparent AT-rich stem/loop structure. The SYBR green assay did, however, confirm the feasibility of developing a highly sensitive test targeted to the rDNA sequences.

The final test was designed with the forward primer ITS-1 Chytr 3 and the Taqman probe Chytr MGB2 located wholly within the ITS-1 region, but excluding the G-rich stretch and the AT-rich stem/loop structure which had been identified as problematic. The reverse primer 5.8S Chytr was located within the 5.8S region immediately adjacent to the ITS-1/5.8S junction (Fig. 1). PCR amplification and sequencing of chytrid fungi rDNA sequences from skin samples and scrapings from infected frogs collected from numerous locations around Australia, Panama, USA and Ecuador showed general conservation of the sequences in the region used for the design of the assay. A total of 29 samples yielded sequence over this region; 26 of the 29 were identical to the sequence derived from the 4 *Batrachochytrium dendrobatidis* isolates (J.A.T. Morgan unpubl. obs.). Three of the samples had a 4 base pair deletion and 4 base pair changes in the 8 base pairs immediately adjacent to the deletion. Although located between the Taqman probe Chytr MGB2 and the 5.8S Chytr primer, this region was excluded during the primer and probe design to ensure that the assay would work on strains carrying this apparent deletion and base changes.

Blast sequence alignments of the ITS-1 and 5.8S rDNA sequences used for design of the Taqman assays showed that the ITS-1 (120 nucleotides) failed to align with existing sequences in the databases. The 5.8S rDNA (27 nucleotides) aligned exactly over the first 21

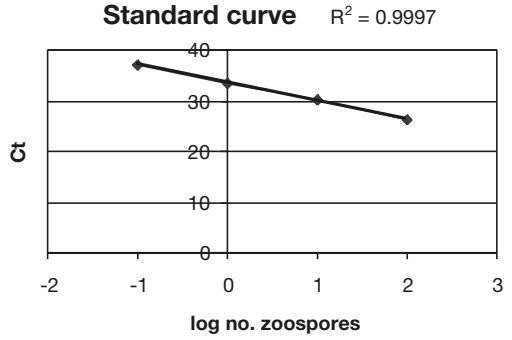


Fig. 2. *Batrachochytrium dendrobatidis.* Standard curve used for the determination of the pathogen in experimental samples. Plot of log no. zoospores against Ct value at Δ Rn of 0.1. Establishment of detection limits of standards

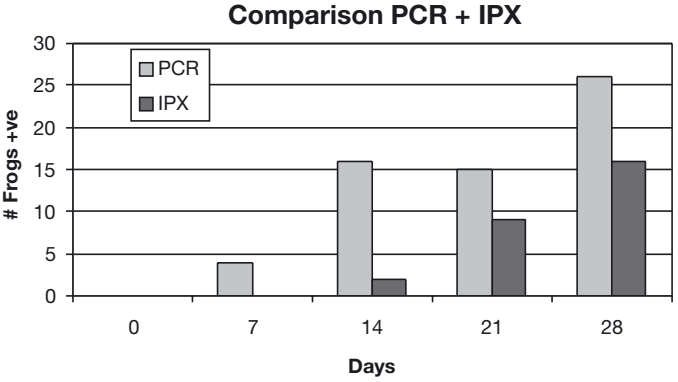


Fig. 3. *Batrachochytrium dendrobatidis.* Detection in frogs *Mixophyes fasciolatus* by PCR and IPX. Number of frogs positive by PCR or IPX at each sampling day. Number of frogs infected = 30. All controls were negative (data not shown)

nucleotides with *Entyloma* and *Tilletiales* species—both of which are classified within the Fungi Basidiomycota. This is not surprising given that 5.8S rDNA is highly conserved over many species. However, specificity for the test design was ensured by the location of the forward primer and probe within the unique sequences of the ITS-1 region.

Sensitivity and specificity of Taqman assay

Preliminary testing of Qiagen- and PrepMan Ultra-extracted zoospores showed that both methods were effective for the extraction of DNA. PrepMan Ultra was marginally better, with mean Ct values 1 to 2 units lower. For speed and ease of processing, the PrepMan Ultra protocol for DNA extraction was subsequently used in all test development.

The DNA standard was diluted to give 100, 10, 1 and 0.1 genome equivalents for use in the Taqman assay. These give reproducible results (Fig. 2), with an R^2 consistently close to 1. Dilutions of 0.01 genome equivalents could be detected, but the accuracy and reproducibility was not as great, so this dilution was not included for quantitation purposes. At these low dilutions, the assay detects *Batrachochytrium dendrobatidis*, but quantification is not accurate. For analysis of test samples, extracted DNA was diluted 10^{-1} in water, and 5 µl of this dilution used in a 25 ul PCR reaction, as higher concentrations of PrepMan Ultra reagent were found to be inhibitory (authors' unpubl. data).

Specificity was confirmed by use of 3 other isolates of *Batrachochytrium dendrobatidis* (AAHL 98 1469/10, AAHL 99 1385/12 and AAHL 00 545) and 5 other fungi from the Chytridiomycetes. The 3 *B. dendrobatidis* isolates were all detected by the assay, while none of the other fungi were amplified (Table 1).

Table 1. *Batrachochytrium dendrobatidis.* Establishment of specificity by testing on different isolates and related organisms. Ct of 50 indicates no DNA amplification. DNA dilution of JEL142 equivalent to 10 zoospores; JEL136 and JEL151 is equivalent to 100 zoospores

Order Fungus	Ct	No. zoospore equivalents
Chytridiales		
Batrachochytrium dendrobatidis Standard (AAHL 98 1810/3)	27.2	100
B. dendrobatidis AAHL 98 1469/10	25	455
B. dendrobatidis AAHL 00 545	24.5	617
B. dendrobatidis AAHL 99 1385/12	26.4	222
Rhizophydium sp. (JEL136)	>50	0
Rhizophlyctis–Rhizophydium-like (JEL142)	>50	0
R. ?haynaldii (JEL151)	>50	0
Monoblepharide		
Gonapodya sp. (JEL183)	>50	0
Blastocladiales		
Allomyces macrogynus (JEL204)	>50	0

Comparative evaluation of Taqman assay and histology

Experimental infection of frogs was used to compare the current histological method of diagnosis with the Taqman assay. Fig. 3 shows detection of *Batrachochytrium dendrobatidis* as early as 7 d post-infection using the Taqman assay. By 14 d, infection was detected in more than half the frogs using this assay, compared with infection in only 2 of 30 frogs detected by histology. For most frogs, *B. dendrobatidis* could

Table 2. *Batrachochytrium dendrobatidis* quantitation in frogs. Numbers for PCR are numbers of zoospore equivalents as determined in Taqman assay. Numbers for IPX (immunoperoxidase) are numbers of sporangia observed per toe clip. All controls were negative (data not shown). All frogs were negative by both methods at Day 0 (data not shown)

Frog no.	Day 7		Day 14		Day 21		Day 28	
	PCR	IPX	PCR	IPX	PCR	IPX	PCR	IPX
1	–	–	85	–	190	–	–	3
3	–	–	–	–	1430	–	7	–
4	–	–	31	–	14	–	1660	51
5	–	–	469	–	–	–	145	–
8	–	–	–	–	–	–	29	6
11	–	–	318	–	–	–	602	4
12	–	–	–	–	1890	–	1910	29
14	–	–	300	–	45940	–	1460	14
15	178	–	618	–	5700	14	439	38
16	–	–	43	–	–	–	1790	17
18	–	–	296	–	–	4	157	11
19	–	–	260	–	–	–	229	4
20	–	–	–	–	–	–	7	–
21	125	–	2	–	174	3	343	30
24	–	–	5	–	–	–	1550	–
25	–	–	–	–	1190	5	70	5
26	–	–	237	–	5280	12	1010	7
27	–	–	–	–	25	10	500	–
29	29	–	1050	22	56	–	128	–
30	–	–	–	–	–	–	380	–
32	–	–	–	–	–	–	19	–
33	–	–	–	–	–	2	16	–
36	2	–	321	1	2440	25	518	13
37	–	–	46	–	–	–	2	–
39	–	–	–	–	2	–	1260	–
41	–	–	421	–	1	–	5	–
42	–	–	–	–	6	15	2000	56

be detected using the Taqman assay 2 wk prior to detection by IPX (Table 2), although there were some anomalies (Frogs 18 and 33 at Day 21) where sporangia were detected at low levels by IPX, but not in the Taqman assay. This was not surprising given that the assays were conducted on different toe clips, and at low levels of infection some toes may be infected while others are not. High PCR results did not correlate to IPX detection in 30% of frogs; even at the highest detected numbers (Frog 14, Day 21), when it would be reasonable to expect a positive IPX result, none were observed. This, again, is probably a reflection of the sampling method, where one toe may be infected whereas another is not. Also, the toe clips were not serially sectioned through the whole toe, so some infections may not have been detected.

Analysis of diagnostic samples confirmed that the Taqman assay could sensitively detect *Batrachochytrium dendrobatidis* from field samples. These samples were in poor condition, being desiccated and covered in mud. At low levels of infection, the Taqman assay was more sensitive than IPX. In 3 of the field samples, *B. dendrobatidis* was detected by PCR but not IPX, and in the captive bred submissions 1 sample was similarly positive by PCR and not IPX. There was a good correlation in these samples between high PCR numbers and detection by IPX staining (Table 3).

DISCUSSION

Improvements to histological methods for diagnosis of *Batrachochytrium dendrobatidis* infection have been made using polyclonal antibodies and dual staining procedures to detect keratin as well as *B. dendrobatidis*. However, these methods are relatively slow, taking several days for diagnosis, and require an expert diagnostician to make the identification. Variability between levels of infection on toeclips, the need for serial sectioning and examination of many slides for detection of *B. dendrobatidis* infection (particularly at early stages of infection), and the desirability of non-invasive testing of frogs led to the development of a rapid, sensitive PCR-based assay. The high abundance (several hundred copies per genome) and the species-specificity of rDNA ITS in eukaryotic organisms pro-

Table 3. Frog species submitted for diagnosis with results of IPX and real-time PCR. IPX results are as follows: +, 1 to 10 sporangia; ++, 11 to 100 sporangia; +++, 100+ sporangia; ?+, ambiguous result. Numbers for PCR are numbers of zoospore equivalents, nd: not determined

Species		IPX results	Real-time PCR
Lymnodynastes dumerilii	Wild	++	910
Lymnodynastes dumerilii	Wild	+	25800
Lymnodynastes peroni	Wild	–	10
Crinia signifera	Wild	nd	12
Litoria reniformis	Wild	–	0
Lymnodynastes dumerilii	Wild	–	1052
Litoria tasmanionsis	Wild	+++	333579
Litoria tasmanionsis	Wild	+++	153361
Litoria infrafrenata	Captive-bred	–	–
Litoria infrafrenata	Captive-bred	–	–
Litoria caerulea	Captive-bred	–	–
Litoria infrafrenata	Captive-bred	–	–
Litoria infrafrenata	Captive-bred	–	–
Lymnodynastes peroni	Captive-bred	+	933
Lymnodynastes dumerilii	Captive-bred	?+	–
Mixophyes fasciolatus	Captive-bred	+	1450
Mixophyes fasciolatus	Captive-bred	–	10
Litoria ewingi	Captive-bred	++	88800
Litoria ewingi	Captive-bred	+	13
Litoria ewingi	Captive-bred	+	201

vided the opportunity to design a real-time Taqman assay with high specificity and sensitivity for *B. dendrobatidis*.

The specificity of our Taqman assay was confirmed by testing on 3 other *Batrachochytrium dendrobatidis* isolates and 5 other members of the Chytridiales order. The assay detected all of the 3 other isolates of *B. dendrobatidis*. Morehouse et al. (2003) found extremely low variability during multilocus sequence typing of 35 strains of *B. dendrobatidis* from North America, Africa and Australia. We therefore expect that this Taqman assay will detect all strains of *B. dendrobatidis* worldwide. High conservation of the rDNA sequence in the ITS-1 region used to design the Taqman assay was observed in 26 of 29 sequences obtained from frog samples from Australia and the Americas. None of the 5 other members of the Chytridiales order were detected in the assay. Although this is not a comprehensive analysis of all closely related fungi, phylogenetic analysis of 54 chytrids identified JEL142 and JEL151 as the most closely related to *B. dendrobatidis* (James et al. 2000). Neither of these fungi, nor the other 3 species tested, were detected in our assay. As many fungi may opportunistically infect the skin of sick frogs potentially producing false positives, the lack of detection of these closely related fungi gives confidence in the specificity of the assay.

The high copy number of the ITS-1 region facilitates the detection of low numbers of organisms, as seen by the ability to reliably detect a nominal amount of 0.1 genome equivalents of *Batrachochytrium dendrobatidis*. This allows the detection of infection at much lower levels than is possible with histology, where the chances of detecting 1 sporangium on a toe clip are quite small unless the whole toe is serially sectioned and examined. Additionally, there are up to 10 zoospores in each sporangia (Berger et al. 2000), giving several hundred targets in a DNA-based assay, compared with 1 sporangium by histology. This sensitivity has been demonstrated by the detection of infection in experimentally infected animals as early as 7 d post-infection and at least 2 wk prior to detection by histological methods. Additionally, the Taqman assay is rapid in comparison to histology. It is possible to extract and quantify up to 27 samples in less than 4 h, a process that would take several days by histology.

Variability of infection among toe clips remains a problem in the diagnosis of chytridiomycosis. This was exemplified in 6 individuals in our study by the detection by PCR of infection at Day 14, no detection at Day 21, and detection again at Day 28. This is probably a result of low levels of infection, where some toes of the same individual are infected whilst others are not. To overcome this problem, alternative sampling methods, such as baths and swabs, where the whole frog is sampled in a non-destructive manner, are being assessed to determine optimum sampling regimes. Preliminary data suggest these methods will be more sensitive than toe clipping, more reliable, as they sample the entire frog, and obviously less destructive to the animal.

The Taqman assay was shown to be applicable to field samples, even those in sub-optimal condition, and again was more sensitive than histology. Fungal DNA is stable, allowing detection even in degraded samples. The presence or absence of *Batrachochytrium dendrobatidis* was confirmed on captive-bred animals. The ability of an assay to detect low levels of organisms at an early stage of infection is vital to prevent the spread of infection between tanks in breeding facilities. It would also allow isolation and possible treatment of infected frogs.

When optimum sampling protocols and the Taqman assay are applied to screening frog populations, we will have powerful tools to help prevent the spread of *Batrachochytrium dendrobatidis*. Quarantine regimes applicable to movement of zoo animals, pets, laboratory animals and the food trade will be able to be implemented. Early detection will allow early treatment and protection of endangered species, and possible repopulation of species into depopulated habitats from captive breeding populations. Application of the assay to environmental samples may lead to a greater understanding of the process of infection and help prevent its spread, particularly to areas not already infected.

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