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FEATURE ARTICLE

Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*

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ABSTRACT: *Batrachochytrium dendrobatidis* is a fungus belonging to the Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales, and is the highly infectious aetiological agent responsible for a potentially fatal disease, chytridiomycosis, which is currently decimating many of the world's amphibian populations. The fungus infects 2 amphibian orders (Anura and Caudata), 14 families and at least 200 species and is responsible for at least 1 species extinction. Whilst the origin of the agent and routes of transmission are being debated, it has been recognised that successful management of the disease will require effective sampling regimes and detection assays. We have developed a range of unique sampling protocols together with diagnostic assays for the detection of *B. dendrobatidis* in both living and deceased tadpoles and adults. Here, we formally present our data and discuss them in respect to assay sensitivity, specificity, repeatability and reproducibility. We suggest that compliance with the recommended protocols will avoid the generation of spurious results, thereby providing the international scientific and regulatory community with a set of validated procedures which will assist in the successful management of chytridiomycosis in the future.

KEY WORDS: Chytridiomycosis · *Batrachochytrium dendrobatidis* · Amphibian declines · Diagnostic assays · Sampling protocols

Chytridiomycosis, which is caused by the fungus *Batrachochytrium dendrobatidis*, is the most infectious and deadly disease ever documented in amphibians. It affects 93 species on all continents except Asia. Alex Hyatt and co-workers have developed validated diagnostic assays and sampling protocols for the specific identification of the fungus. This will greatly facilitate research aimed at decreasing its devastating effects.

Photo: Veronica Olsen

INTRODUCTION

Batrachochytrium dendrobatidis is a fungus belonging to the Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales and is the highly infectious aetiological agent responsible for a potentially

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fatal amphibian disease, chytridiomycosis. The fungus is responsible for many amphibian deaths and population declines in Australia, New Zealand, USA, Central America, South America, Spain and Germany (Berger et al. 1998, Lips 1999, Pessier et al. 1999, Mutschmann et al. 2000, Bosch et al. 2001, Bradley et al. 2002, Green et al. 2002, Ron et al. 2003, Weldon et al. 2004). The disease is one of the processes referred to by Stuart et al. (2004) threatening 48% of the rapidly declining amphibian species with extinction. Chytridiomycosis has been recognised by the Office International des Epizooties (OIE) ad hoc Group on Amphibian Diseases as one of the 2 pathogens of particular importance (the other one being viruses belonging to the family Iridoviridae and genus Ranavirus) in international trade in amphibians (www.oie.int/aac/eng/FDC%20reports/Oct%202006%20report%20(English).pdf). While a decision on whether the OIE should develop standards for international trade in amphibians is pending, chytridiomycosis would be one of the primary candidates for an amphibian disease to be listed by the OIE (E. M. Bernoth, Canberra, pers. comm.). In Australia the disease has been listed as a 'key threatening process' under the Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (EBPC Act). Consequential to this listing an Australian federal 'Threat Abatement Plan' ('a plan that provides research, management and other actions necessary to reduce the key threatening process concerned to an acceptable level in order to maximise the chances of the long-term survival in nature of native species and ecological communities affected by the process') has been prepared to provide a national strategy to manage the impacts of the disease on the environment (www.deh.gov.au/biodiversity/invasive/diseases/).

Effective management of chytridiomycosis will depend on different countries and regions recognising the disease as a 'threatening process' (a disease that threatens, or may threaten the survival, abundance, or evolutionary development of a native species or ecological community) and implementing strategies for its control. Briefly, managerial strategies will ultimately involve the detection of infected populations of both laboratory-housed and free-ranging animals, identification of infected geographical areas and control of human-mediated movement of animals from one location to another. To accomplish these demanding objectives, strategies must be underpinned with the use of validated sampling protocols and sensitive and specific diagnostic assays.

To date a range of assays exists, including histopathology (Berger et al. 1998, Briggs & Burgin 2003), histochemistry (Berger et al. 2002, Van Ells et al. 2003, Olsen et al. 2004), electron microscopy (Berger et al. 2002) and real-time PCR TaqMan assay (Boyle et al. 2004). This study assesses the above diagnostic assays and sampling protocols (developed in our laboratory) in terms of sensitivity, specificity, repeatability and reproducibility. Quantitation of these performance criteria will enable the assays and sampling protocols to be used in a range of functions, whereby the performance limitations of the above and the interpretative power of the resultant data are known. Such functions include (1) identification of *Batrachochytrium dendrobatidis* in adults and tadpoles from both captive and 'free-ranging' animals, (2) estimation of prevalence of infection of 'free-ranging' and captive populations, (3) identification of infected animals or groups towards implementing disease control measures, (4) demonstration of disease-free zones via surveying and (5) demonstration of eradication of infection from individuals undergoing treatment. These purposes are, in the main, analogous to those defined by the OIE (www.oie.int/VCDA/eng/en_background_vcda.htm) for assays intended for the detection and for minimising the translocation of pathogens via the international movement of livestock and associated commodities.

We also describe the comparative advantages and disadvantages of the diagnostic assays (histopathology, histochemistry and a quantitative real-time PCR TaqMan assay) and the different sampling protocols (toe clipping, water baths and filters, and swabs) per se and in relation to interpretation of data relating to individuals and populations. Finally, we outline the correct implementation of the diagnostic and sampling procedures, which will enable their successful repetition and reproduction in most laboratories, thereby minimising the possibility of generating spurious results.

MATERIALS AND METHODS

Husbandry. Frogs were housed, fed and euthanized as described previously (Berger et al. 2004) in accordance with procedures approved by the CSIRO (Australian Animal Health Laboratory) Animal Ethics Committee (AEC No. 990 and AEC No. 1033). Frogs were acclimatized at 23°C for 2 wk prior to infection. All animals used in these studies were known to be susceptible to the *Batrachochytrium dendrobatidis* isolates detailed below (Berger 2001).

Cultivation of *Batrachochytrium dendrobatidis*. *B. dendrobatidis* isolates used in these studies were AAHL 98 1810/3 from a sick, wild adult lacelid *Nictimyces dayi* and AAHL 00 545 from a wild-caught captive metamorph of *Litoria lesueuri*. Zoospore suspensions for infection of frogs were prepared by harvesting from 4 d agar plate cultures using a weak salt solution (DS) and counting in a haemocytometer as described by Boyle et al. (2004).

Animal experiments. ***Expt 1 — Histology vs. TaqMan PCR, and toe clipping versus bathing:*** The 4 to 7 mo old captive-bred great barred frogs *Mixophyes fasciolatus* (n = 30) were infected with 10^4 zoospores from *Batrachochytrium dendrobatidis* isolate AAHL 98 1810/3 by bath inoculation for 20 h. Uninfected controls (n = 12) were similarly bathed in DS solution. Frogs were sampled weekly by placing them in a container of 10 ml of DS water (bathing) for either 15, 30, or 60 min, followed by toe clipping (×2) for TaqMan assays and histological examination (see following subsection). All animals were housed in individual containers.

Expt 2 — Comparison of bathing, toe clipping and swabbing: Due to difficulties in sourcing animals, the species used in Expt 1 were substituted with 5 mo old captive-bred green tree frogs *Litoria caerulae.* A total of 21 frogs were infected with 10^4 zoospores from *Batrachochytrium dendrobatidis* isolate AAHL 00 545 by bath inoculation for 4 h. Uninfected controls were similarly bathed in DS solution. Frogs were sampled at Days 4, 7, 10, 14, 17, 21, 24 and 35 by toe clipping and either bathing (30 min) in 10 ml DS solution or swabbing the frog using fine-tip swabs (Medical Wire & Equipment Co. MW 100–100). Various swabs including wooden toothpicks and sponge and cotton wool-based applicators were previously tested for efficiency of DNA recovery. The best swabs were found to be the fine-tip swabs mentioned above (data not shown). The underside of the legs, feet and drink patch were comprehensively swabbed (3 to 5 times), and the swab was then broken off into a 1.5 ml Eppendorf tube. The bath water from half the samples was immediately filtered through a 0.45 µm filter (Sartorius Minisart No. 16555).

Expt 3 — Comparison of excised tadpole oral discs and swabs: Free-ranging *Litoria ewingi* and *L. tasmaniensis* tadpoles collected in Tasmania were swabbed as described below, and oral discs were excised and air dried on filter paper. The animals were 'free-ranging' and came from non-infected areas (D. Obendorf pers. comm.). All procedures described in this paper which involved cutting/excising incorporated the single usage of new (single use) scalpel blades and took place on disposable Petri dishes.

Sampling protocols: Toe clips for use in real-time TaqMan PCR were harvested into 1.5 ml tubes and stored at –80°C prior to DNA extraction. Toe clips to be processed for histology were fixed in 10% neutral buffered formalin.

Toe clips, oral discs and swabs: In the field, tadpole mouths were swabbed using fine-tip swabs; then the oral discs were dissected and air dried onto filter paper. In the laboratory, the air-dried oral disc was excised from the filter paper and extracted in 50 µl PrepMan Ultra (Applied Biosystems).

When swabbing frogs, the underside of the legs, feet and drink patch were comprehensively swabbed (3 to 5 times), and the swab was then broken off into a 1.5 ml tube. The mouthparts of tadpoles were swabbed by inserting the swab into the mouth and twirling the swab several times. Swabs were extracted in 50 µl PrepMan Ultra.

Water bath and filters: DNA was extracted from the bath water by pelleting the *Batrachochytrium dendrobatidis* in a bench centrifuge for 15 min at 1700 × *g*. The supernatant was decanted, and the pellet was resuspended by vortexing in the remaining liquid before transferring it to a 1.5 ml tube and microfuging for 3 min. The supernatant was removed, and the resulting pellet was resuspended in 25 µl PrepMan Ultra. Analyses of samples spiked with zoospores showed that the majority were recovered from such pellets (data not shown). Alternatively, bath water was immediately filtered through a 0.45 µm filter, the filter was removed, and DNA was extracted in 200 µl PrepMan Ultra.

Control experiments (not detailed in this paper) showed that failure to centrifuge the bath water within 8 h resulted in poor zoospore recovery. To investigate the fate of the zoospores in solution, zoospore numbers were counted in a BD FACSCalibur flow cytometer (BD Biosciences) over 31 h at room temperature and over 72 h at 4°C. TruCount tubes (BD Biosciences No. 340334) were used for quantification, and CellquestPro was used for analysis.

Expt 4 — Pooling versus individual swab assays: Costs of testing environmental samples using the TaqMan assay can be reduced by pooling swabs. However, this may compromise the sensitivity of the assay to unacceptable levels. We tested the reduction in sensitivity by spiking 1 swab with 1, 10, or 100 zoospores and then combining 1 spiked swab with 4 or 9 clean swabs. An increased volume of PrepMan Ultra was used for extraction of DNA — 200 µl for 5 swabs and 400 µl for 10 swabs. Each test was performed in triplicate and repeated 10 times.

Expt 5 — Storage: Inevitably there is a delay between sampling in the field and assaying the swab in the laboratory. We studied the long-term stability of zoospores on swabs by spiking with 5000 zoospores and storing at room temperature, –4 and –20°C for 1, 3, 6 and 18 mo before DNA extraction of the swab with PrepMan Ultra as described below. Each test was performed in triplicate and repeated twice.

We also examined the effect of storage of swabs in ethanol on zoospore recovery. Swabs were spiked with 1, 10, or 100 zoospores and stored in 1 ml of 70% ethanol for 14 d in 1.5 ml tubes. Before extraction, the tubes were vortexed for 15 s; the swab was removed and allowed to air dry before extraction

with PrepMan Ultra. The ethanol solution was pelleted for 1 min in a microfuge, the ethanol was removed, and the pellet was allowed to air dry before extraction in 50 µl PrepMan Ultra. Alternatively, the swab was removed from the ethanol and soaked in DS solution overnight before extraction of the swab and DS solution as described above. Each test was performed in triplicate and repeated 10 times.

Protocols for diagnostic assays. ***Histopathology and immunohistochemistry:*** Toe clips were collected at 6 time points from each of 42 animals (Expt 1). Of these animals, 12 were uninfected. The samples were processed and stained with either routine Lillie–Mayer haematoxylin and eosin (H&E), or by using a polyclonal antibody generated against *Batrachochytrium dendrobatidis* and immunoperoxidase (IPX) staining (Berger et al. 2002). The method was modified by deleting the digestion with trypsin and using a DAKO Envision+ kit instead of a DAKO LSAB kit. Single sections of H&E and IPX 'stained' slides were examined and scored by a skilled diagnostician (Lee Berger) for the number of sporangia present within the sections.

Extraction of DNA: DNA was prepared from toe clips, swabs, filters, or desiccated oral discs by extraction with PrepMan Ultra. Briefly, 50 µl of PrepMan Ultra (200 µl for filters) was added to each sample along with 30 to 40 mg of zirconium/silica beads (0.5 mm diameter, Biospec Products). The sample was homogenised for 45 s in a Mini Beadbeater 8 (Biospec Products). After brief centrifugation (30 s at 13 000 × *g* in a microfuge) to settle all material to the bottom of the tube, homogenisation and centrifugation was repeated. The homogenised sample was immersed in a boiling water bath for 10 min, cooled for 2 min, then centrifuged at 13 000 × *g* for 3 min in a microfuge; 20 µl of supernatant was recovered and stored at –80°C.

Real-time TaqMan PCR assay: *General procedure:* Quantitative real-time TaqMan PCR assays were performed using an Applied Biosystems Prism 7700 Sequence Detection System as described by Boyle et al. (2004).

Internal controls: During experimentation it was found that inhibitors of the TaqMan assay, such as soil on the swabs, may be present after the extraction process, resulting in false negatives. The presence of inhibitors can be detected by using an internal control. Applied Biosystems produces a synthetic amplicon from a plasmid source whose sequence is not known to occur in nature. This is VICTM labelled and primer limited for use in multiplex assays—TaqMan Exogenous Internal Positive Control Reagents (VICTM dye, Applied Biosystems No. 4308323). In later experiments, 1 µl 10× Exo IPC mix and 0.5 µl 50× Exo IPC DNA were included in the master mix of 1 well of the 3 triplicates. The Ct values (Boyle et al. 2004) in the VICTM layer should be comparable for controls and test samples. If the Ct value of the test sample in the VICTM layer was significantly higher than the negative control, the sample was diluted 1/100 or more, instead of 1/10. Samples that were highly positive for *Batrachochytrium dendrobatidis* in the FAM layer could be negative in the VICTM layer. It was not necessary to re-test these samples.

Quantitation of standards: Standards for quantitation of *Batrachochytrium dendrobatidis* were prepared as described in Boyle et al. (2004). Zoospore numbers obtained by counting in a haemocytometer were further confirmed by counting in a BD FACSCalibur flow cytometer (BD Biosciences) using TruCount tubes (BD Biosciences No. 340334) for quantification. CellquestPro was used for analysis.

Validation of diagnostic assays. ***Sensitivity:*** The main performance characteristics for an assay are accuracy and precision. The OIE defines accuracy as the level of agreement between a test value and the expected value for a reference standard of known activity or titre. Precision is defined as the degree of dispersion of results for a repeatedly tested sample. Accuracy therefore is determined by test parameters such as sensitivity and specificity, and precision is determined by repeatability and reproducibility. Sensitivity is defined as the proportion of known infected reference animals that test positive in the assay, and specificity is defined as the proportion of known uninfected reference animals that test negative in the assay. The OIE distinguishes between (1) analytical sensitivity (the smallest detectable amount of an analyte, e.g. antigen, antibody, nucleic acid, or zoospore equivalent), (2) diagnostic sensitivity (DSe), e.g. proportion of known infected reference animals that test positive in the assay, and (3) relative sensitivity, e.g. proportion of reference animals that, defined as positive by one or a combination of test methods, also test positive in the assay being compared. The OIE defines repeatability as the level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory (intra- and interassay variation), and reproducibility as the ability of a test to provide consistent results when applied to aliquots of the same sample at different laboratories.

Results were evaluated to assess and compare the capacity of the different tests and protocols to: (1) detect infection at least once during the experiment in each animal after being tested 5 times at weekly intervals (herd sensitivity); (2) detect infection each time when a sample from an infected frog was tested (individual sensitivity); (3) determine the 'dynamics' of the test response, e.g. rise and fall of zoospore equivalents

as infection progresses; (4) determine the 'diagnostic window', e.g. at what point in time the first infected animal is detected, or when more than 25, 50, 75 and 100% of the infected animals are positive in the test, or at what time post-infection the maximum percentage of animals give positive test results; and (5) determine the reliability of the test to identify an infected animal as positive after it has been diagnosed as positive for the first time. Data from Expt 1 were used to determine the sensitivity of the real-time TaqMan PCR assay using different sampling protocols (e.g. toe clip vs. 'wash'), compared with other diagnostic assays, namely histopathology (H&E staining) and histochemistry (IPX). Analytical sensitivity of the assay has been determined previously (Boyle et al. 2004).

Specificity: Specificity testing, in addition to that described by Boyle et al. (2004), was undertaken using 21 Australian chytridiomycetes from the orders Chytridiales, Blastocladiales and Spizellomycetales (Table 1). Fungi were grown in PYG broth (3 g glucose, 1.24 g yeast extract, 1.24 g peptone, 1000 ml distilled water). Then, 1 ml of culture was pelleted in a microfuge, the supernatant was discarded, and DNA was extracted in 50 µl PrepMan Ultra. Fungi were tested at 1/10 dilution in the TaqMan assay. An internal control, which was VICTM-labelled, primer-limited (Applied Biosystems No. 4310893E) for 18sDNA, was included in the master mix to ensure DNA was present and in comparable quantities to the *Batrachochytrium dendrobatidis* standards.

Table 1. Australian chytridiomycetes used in specificity testing of real-time TaqMan assay for *Batrachochytrium dendrobatidis*. None of these fungi were detected by the TaqMan assay

Designation	Fungus name	Order
Allo Mar CW16	*Allomyces arbuscula*	Blastocladiales
Poly Ad 2-0	*Catenaria* sp.	Blastocladiales
CC 4-10Z	*Catenophlyctis* sp.	Blastocladiales
Mar Ad 2-0	*Spizellomyces* sp.	Spizellomycetales
Mar C/C2	*Spizellomyces* sp.	Spizellomycetales
CC 4-10F	*Spizellomyces* sp.	Spizellomycetales
AUS 2	*Rhizophydium* sp.	Chytridiales
AUS 3	*Rhizophydium* sp.	Chytridiales
AUS 6	*Rhizophydium* sp.	Chytridiales
AUS 7	*Rhizophydium* sp.	Chytridiales
AUS 8	*Rhizophydium* sp.	Chytridiales
AUS 9	*Rhizophydium* sp.	Chytridiales
AUS 11	*Cladochytrium* sp.	Chytridiales
AUS 12	*Rhizophydium* sp.	Chytridiales
AUS 13	*Rhizophlyctis rosea*	Spizellomycetales
AUS 14	*Chytriomyces hyalinus*	Chytridiales
AUS 15	*Rhizophydium* sp.	Chytridiales
AUS 16	*Rhizophlyctis* sp.	Spizellomycetales
AUS 17	*Powellomyces* sp.	Spizellomycetales
Mar R2	*Rhizophydium* sp.	Chytridiales
Mar Ad 14	*Rhizophydium* sp.	Chytridiales

Repeatability and reproducibility: Precision is defined as the degree of dispersion of results for a repeatedly tested sample and determined by repeatability and reproducibility.

Repeatability and reproducibility were assessed using replicates of 4 internal standards, 0.1, 1, 10 and 100 zoospores, at the Australian Animal Health Laboratory (AAHL), James Cook University (JCU) and Griffith University (GU). Each run contained 3 replicates, and mean Ct values were calculated, as were coefficients of variation (CV). Data were assessed from 151 assays at AAHL, 50 assays at JCU and 17 at GU.

Field samples. A total of 104 samples were collected from animals (*Mixophyes iterates* and *M. fleayi*) from North Queensland, from a known *Batrachochytrium dendrobatidis*-infected population. Samples were collected by swabbing and toe clips. The swabs were analysed by quantitative real-time TaqMan PCR, and histology (H&E) was used for analyses of the toe clips. Single sections of H&E stained slides were examined and scored by an un-skilled diagnostician (i.e. someone not formally trained in interpretation of H&E stained slides) for the presence or absence of sporangia.

Statistics. Validation data were assessed using Excel basic statistics (mean, SD, CV). The bars used in graphs to show intra-assay variation are 2 SD over the mean. Sensitivity was calculated by dividing the number of animals with positive test results with the number of infected animals, and specificity was calculated by dividing the number of negative test results with the number of non-infected animals.

The statistical analysis used in this study was a repeated-measures GLM (general linear model, SPSS 2003, Version 12.0.1). The dependent variable in these analyses, zoospore equivalents (no. of zoospores + 1), was log transformed to meet assumptions of homogeneity of variance. The model included 3 factors: (1) sampling method (toe vs. wash), (2) wash time (15, 30 and 60 min) and (3) changes in zoospore numbers over 5 time periods (repeated measures on the same individuals, Days 7, 14, 21, 35 and 42).

RESULTS

TaqMan versus histology

Comparison of the 2 assay protocols in Expt 1, i.e. quantitative real-time TaqMan PCR (hereafter referred to as TaqMan assay) and histology (H&E and IPX), demonstrates a much higher diagnostic sensitivity for the TaqMan assay. H&E and IPX did not detect *Batrachochytrium dendrobatidis* until 14 d post-infection (p.i.) (Table 2, Fig. 1). The TaqMan assay, however, detected *B. dendrobatidis* at Day 7 p.i., with a DSe of 57%

Table 2. *Batrachochytrium dendrobatidis* infecting *Mixophyes fasciolatus*. Data from Expt 1. Sensitivity of conventional histology (H&E: haematoxylin and eosin) and histochemistry (IPX: immunoperoxidase) compared to TaqMan PCR of washes and toe clips; 2 sampling protocols were included, as it was not known at the time which procedure was more sensitive. Note: at the time of the experiment the swabbing technique had not been developed. Numbers shown for TaqMan PCR are means of 3 replicates per sample and represent zoospore equivalents; numbers for H&E and IPX: number of sporangia; NA: sections not suitable for examination. All control animals were negative for all assays at all time points. Infected animals were negative for all assays at Day 0

Animal no.	Day 7				Day 14				Day 21				Day 35				Day 42			
	TaqMan PCR		H&E	IPX	TaqMan PCR		H&E	IPX	TaqMan PCR		H&E	IPX	TaqMan PCR		H&E	IPX	TaqMan PCR		H&E	IPX
	Wash	Toe clip			Wash	Toe clip			Wash	Toe clip			Wash	Toe clip			Wash	Toe clip		
15 min wash																				
5	0	0	0	0	0	469	0	0	0	0	0	0	1	1 460	0	NA	4	198	3	1
7	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	2	0	0
8	0	0	0	0	4	0	0	0	2	0	0	0	2944	51	0	0	90	453	1	0
12	0	0	0	0	4	0	0	0	156	1890	0	0	545000	19600	38	64	44438	14300	17	17
14	15	0	0	0	33	300	0	0	713	45940	0	0	15938	751	1	2	15	27	0	0
20	0	0	0	0	0	0	0	0	66	0	NA	NA	4063	9640	39	43	906	3640	97	43
23	2[a]	0	0	0	0	0	0	0	0	0	0	0	96	0	0	0	117	1430	0	0
25	0	0	0	0	0	0	0	0	22	1190	5	5	719	358	0	4	61	2620	15	16
30	2[a]	0	0	0	0	0	0	0	9	0	0	0	1156	1	0	13	475	926	28	12
42	0	0	0	0	1	0	0	0	181	6	3	15	1469	5 400	63	59	288	698	8	7
30 min wash																				
4	5	0	0	0	0	31	0	0	106	14	0	0	1075	16700	2	3	38	49	0	2
11	2	0	0	0	18	318	0	0	68	0	0	0	4563	3740	21	32	37375	5430	30	25
19	0	0	0	0	0	260	0	0	1[a]	0	0	0	123	578	10	18	41	278	0	0
21	14	125	0	0	24	2	0	0	406	174	4	3	844	613	3	0	351	1 230	0	1
24	0	0	0	0	2[a]	5	0	0	0	0	0	0	0	94	3	1	0	0	0	0
26	13	0	0	0	68	237	0	0	391	5280	14	12	9125	20600	228	195	5650	51100	425	440
27	0	0	0	0	42	0	0	0	18	25	1	10	90	279	0	1	0	0	0	0
32	1[a]	0	0	0	11	0	0	0	4	0	0	0	101	889	20	15	9	277	0	0
33	1[a]	0	0	0	3[a]	0	0	0	169	0	0	2	25938	13300	102	98	1350	4910	90	62
41	14	0	0	0	204	421	0	0	12	1	0	0	2513	2040	2	18	5	369	4	5
60 min wash																				
1	0	0	0	0	0	85	0	0	2	190	0	0	2463	519	0	0	53	133	0	0
3	8	0	0	0	19	0	0	0	13	1430	0	0	353	0	0	0	54	2370	6	6
13	0	0	0	0	0	0	0	0	4	0	0	0	8	0	12	7	12	0	0	0
15	41	178	0	0	15	618	0	0	1463	5700	17	14	96	255	2	7	0	0	6	2
16	22	0	0	NA	108	43	0	0	156	0	0	0	663	452	9	12	4	59	0	0
18	0	0	0	0	0	296	0	0	4	0	4	4	33	65	0	0	18	515	0	2
29	0	29	0	0	17	1050	6	22	2025	56	0	0	1400	1770	12	11	2	66	0	0
36	86	2	0	0	421	321	15	1	3381	2440	26	25	4025	5940	50	50	105000	10100	7	18
37	1[a]	0	0	0	0	46	0	0	28	0	0	0	1200	4030	15	2	249	555	0	0
39	13	0	0	0	0	0	0	0	28	2	0	0	894	451	0	0	1 044	650	10	9

[a]<3 replicates returned a positive value

for the washing protocol and 13% for toe clips. At Day 14 p.i., the DSe values were 53 and 57% compared to 7% for the histological assays. At advanced stages of infection (e.g. Day 42), the TaqMan assay had a DSe of 87%, irrespective of the sampling protocol, compared to a DSe of 57% for IPX and 50% for H&E (Table 3).

Histopathology (H&E) versus histochemistry (IPX)

Fig. 1 and Tables 3 to 5 summarise the data from Expt 1. All histological sections prepared from uninfected *Mixophyes fasciolatus* were negative for *Batrachochytrium dendrobatidis.* Of the 150 samples from infected animals, 43 co-scored positive by both H&E and IPX. There were a total of 49 positive samples by IPX (DSe of 33.3%), of which 7 returned a negative result by H&E, and 44 positive samples by H&E (DSe of 29.5%), of which 1 returned a negative by IPX.

H&E test

Herd sensitivity: Of the 30 infected animals, the H&E test did not detect infection at any time in 3 of the experimentally infected animals (Nos. 1, 7 and 23) (Table 2). This resulted in a herd sensitivity of 90% after testing each animal weekly (5 times over 42 d) (Table 4); Day 0 was not included.

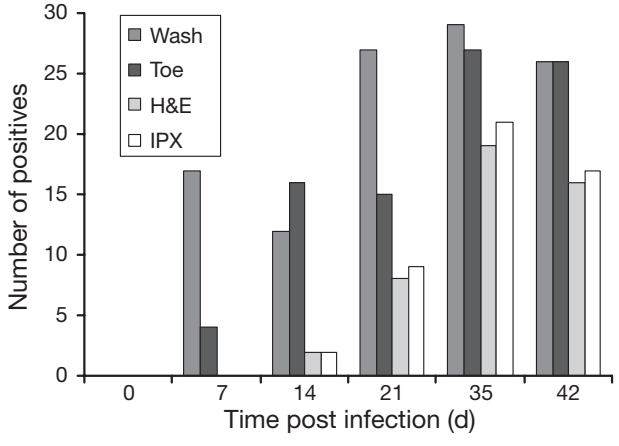

Fig. 1. *Batrachochytrium dendrobatidis* infecting *Mixophyes fasciolatus*. Comparison of histology (H&E and IPX) and TaqMan PCR (wash and toe) from Expt 1. Data relating to wash samples are pooled (i.e. contain 15, 30 and 60 min). Total numbers of animals were 30

Table 3. *Batrachochytrium dendrobatidis* infecting *Mixophyes fasciolatus*. Individual diagnostic sensitivity and confidence intervals (95% CI) of different diagnostic techniques and sampling protocols during animal experiments 0 to 42 d post-infection (Expt 1); 150 samples were taken (30 frogs at 5 time periods); Day 0 sampling is excluded from evaluation to assess sensitivity. DSe: diagnostic sensitivity; NA: not applicable

Day p.i.	Test/sampling protocol	No. of results Positive	Infected	DSe	Limit at 95% CI Lower	Upper
0	TaqMan PCR/Wash	0	0	NA	NA	NA
	TaqMan PCR/Toe clip	0	0	NA	NA	NA
	H&E	0	0	NA	NA	NA
	IPX	0	0	NA	NA	NA
7	TaqMan PCR/Wash	17	30	57	0.4	0.7
	TaqMan PCR/Toe clip	4	30	13	0.0	0.3
	H&E	0	30	0	0.0	0.1
	IPX	0	30	0	0.0	0.1
14	TaqMan PCR/Wash	17	30	57	0.4	0.7
	TaqMan PCR/Toe clip	16	30	53	0.4	0.7
	H&E	2	30	7	0.0	0.2
	IPX	2	30	7	0.0	0.2
21	TaqMan PCR/Wash	26	30	87	0.7	1.0
	TaqMan PCR/Toe clip	15	30	50	0.3	0.7
	H&E	8	29	28	0.1	0.5
	IPX	9	29	31	0.2	0.5
35	TaqMan PCR/Wash	29	30	97	0.8	1.0
	TaqMan PCR/Toe clip	26	30	87	0.7	1.0
	H&E	19	30	63	0.4	0.8
	IPX	21	29	72	0.5	0.9
42	TaqMan PCR/Wash	26	30	87	0.7	1.0
	TaqMan PCR/Toe clip	26	30	87	0.7	1.0
	H&E	15	30	50	0.3	0.7
	IPX	17	30	57	0.4	0.7

Table 4. *Batrachochytrium dendrobatidis* infecting *Mixophyes fasciolatus*. Herd versus individual sensitivity (%) observed during experimental infection (Expt 1)

	TaqMan PCR Wash	Toe clip	Histology H&E	IPX
Herd sensitivity[a]	100	97	90	87
Individual sensitivity[b]	76.7	58.0	29.5	33.3

[a]Ability to detect infection at least once (n = 30 frogs)
[b]Ability to detect infection each time (30 frogs × 5 samples = 150). Day 0 was excluded

Table 5. *Batrachochytrium dendrobatidis* infecting *Mixophyes fasciolatus*. Diagnostic window or capability to detect infection (Expt 1)

Infected frogs detected	TaqMan PCR Wash	Toe clip	Histology H&E	IPX
>25%	Day 7	–	Day 21	Day 21
>50%	Day 7	Day 14	Day 35	Day 35
>75%	Day 21	Day 35	–	–
100%				

Individual sensitivity: The number of 'positives' as determined by examination via H&E (Table 3) were considerably lower than those detected with the TaqMan protocol. The individual sensitivity was 29.5% (44/149) (Table 4).

Dynamics of test response: There was a steady increase in number of sporangia and number of positive samples in relation to days p.i. (Fig. 1, Table 2).

Diagnostic window: The time of the first positive result (Tables 2 & 5, Fig. 1) occurred late, in comparison with that in the TaqMan assay, e.g. the first positive results started at Day 14 p.i. At Day 21 p.i. >25% of the infected animals had been detected, and >50% of the infected animals had been detected after 35 d. H&E gave the lowest values for all tests in this category.

Reliability: After initial recovery, the test failed to detect sporangia in 13 of 30 animals (43%) at least once during the infection (Table 2). Most of these negative results occurred in the last phase of the experiment, suggesting lower sensitivity than in the TaqMan assay.

IPX test

Herd sensitivity: The IPX test did not at any stage detect infection in 4 experimentally infected animals (Nos. 1, 7, 8 and 23) during the course of the experiment (Table 2). This resulted in a sensitivity of 87% after testing each animal weekly (5 times over 42 d) (Table 4); Day 0 was not included.

Individual sensitivity: Mean values for positive reactions were considerably lower than with the TaqMan protocol, e.g. average positive numbers were all <25 for all days p.i., resulting in a Se of 33.3% (49/147 [3 not done]). The Day 0 group was excluded from the positive group. The results were similar to those with H&E (Table 3).

Dynamics of test response: There was a steady increase in number of sporangia and number of positive samples in relation to the days p.i. (Table 2).

Diagnostic window: The timing of positive reactions occurred late in comparison with that in the TaqMan assay, at Day 14 p.i. (Table 2, Fig. 1); >25% of the infected animals were detected after 21 d, >50% after 35 d (Table 5).

Reliability: After initial recovery, the test failed to detect sporangia in 11 out of 30 animals (37%) at least once during the infection (Table 2). Most of these negative results occurred in the later phase of the experiment, suggesting lower sensitivity than in the TaqMan assay.

TaqMan assay: wash versus toe clips

Data from Expt 1 were evaluated for diagnostic sensitivity and confidence intervals (Table 3). Results designated 'wash' refer to analyses conducted from pellets spun from the DS 'bath' solution. As the optimal time period for bathing animals was not known at this time, all bath times are included in the table. Specific data pertaining to different bath times can be found in the next subsection.

At Day 7 p.i., wash samples generated 17 positives (DSe of 57%) compared to 4 (DSe of 13%) from toe clips. All animals that returned positives via toe clips returned positive results via washing. Comparison of number of zoospores detected in toe clips versus washes is presented in the next subsection.

At Day 14 p.i., wash samples generated 17 positives (DSe of 57%) compared to 16 (DSe of 53%) from toe clips. However, at this time, only 10/30 of these animals co-scored positive via both sampling protocols. At Day 21 p.i., 26 animals tested positive for *Batrachochytrium dendrobatidis* by wash protocol (DSe of 87%) compared to 15 via toe clipping (DSe of 50%); all toe clip positive samples were positive via the washing protocol. At Days 35 and 42 p.i., approximately equal numbers of samples tested positive irrespective of the sampling protocol and the majority of those testing positive via toe clipping tested positive via the washing protocol (Table 2, Fig. 1). Within this experiment, data indicate that analyses of the centrifuged wash medium in which the frogs were immersed is the most sensitive protocol for detecting *B. dendrobatidis* early (Day 7 p.i.) in infection. As infection progresses over time, there is little difference between the 2 sampling methods.

Bath

Analysis of the data in Table 2 comparing wash time and method, i.e. toe clip versus wash, shows that there was no significant relationship between the number of zoospores and wash time ($F = 0.614$, $p = 0.545$). Multivariate tests found a significant interaction between day p.i. and the method of sampling (Pillai trace, $F = 3.818$, $p = 0.009$). That is, there was a difference between methods when compared at Days 7 and 21, but no difference when compared at Days 14, 35 and 42 p.i. The number of zoospores increased from Day 1 to 35; however, it decreased from Day 35 to 42.

Herd sensitivity: Herd sensitivity of 100% infection was detected in all animals tested 5 times in weekly intervals at least once during the experiment (30 animals positive/30 infected × 100) (Table 4).

Individual sensitivity: The average individual DSe during the experiment was 77% (115/150) (Table 4). The Day 0 group was excluded from the positive group. In comparison with the other tests and protocols, the TaqMan assay following the wash protocol identified the highest number of positive animals over the entire

period. Only at Day 42 did the TaqMan PCR/toe clip give similar results (DSe of 87% for wash and toe clip protocols). At Day 35 p.i., the TaqMan wash protocol scored the highest sensitivity of all tests in this experiment and detected 29 out of 30 infected frogs (DSe 97%) (Table 3). The confidence intervals were relatively wide even when good test values for DSe were obtained, e.g. DSe 97%. This was due to the small number of animals tested in this experiment (n = 30).

Diagnostic window: The TaqMan assay following the wash protocol was the most efficient assay, as this technique detected infection in >50% of the infected animals as early as 7 d p.i. More than 25, 50 and 75% of infected animals were detected at Days 7, 7 and 21, respectively (Table 5). Following the wash protocol, this test achieved the highest sensitivity, i.e. 97%, at Day 35 p.i. in this experiment (Table 3).

Dynamics of test response: The data show an overall increase in the number of positive animals and zoospore equivalents throughout the course of infection. At Day 7 p.i., 17/30 animals returned positive values, with up to 86 zoospore equivalents (14 ± 20), whereas at Day 42 p.i., 26/30 animals returned positive values, with numbers of zoospores ranging up to 105 000 (7602 ± 22 742) (Table 2).

Reliability: After initial recovery, the test failed to detect zoospore equivalents in 9 of 30 animals (30%) at least once during the infection, and, at a later stage, again gave positive results.

Toe clip

Herd sensitivity: The test did not identify 1 experimentally infected animal (No. 13) during the course of the experiment (Table 2). This resulted in a herd sensitivity of 97% after testing each animal weekly (5 times over 42 d; Table 4).

Individual sensitivity: The average individual DSe during the experiment was 58% (87/150) (Table 3). The Day 0 group was excluded from the positive group. There was an inconsistent detection of zoospore equivalents, with higher mean values for positive reactions at earlier stages until Day 35, but in total fewer animals were identified as positive in comparison with the wash protocol.

Diagnostic window: Following the toe-clip protocol, the diagnostic window to detect infection opens after 4 d (Expt 2; Tables 6 & 7). No other test was able to identify infection as early. Toe clipping detected infection in >50% of the infected animals after 14 d p.i. and in 75% of the infected animals after 35 d (Expt 1; Table 5).

Dynamics of test response: There was a steady increase in recovery of zoospore equivalents until Days 35 and 42 (Expt 1), where the numbers were approximately equal (3652 and 3412, respectively).

Table 6. *Batrachochytrium dendrobatidis* infecting *Litoria caerulae*. Diagnostic sensitivity (DSe) and confidence limits (95% CI) for different sampling protocols (Expt 2). Group 1: samples derived from Animals 1 to 7; Group 2: samples derived from Animals 8 to 14; Group 3: samples derived from Animals 15 to 21. The number of infected animals for each group was 7. All samples were negative at Day 0

Day p.i.	Sampling protocol	Group no.	No. positive	DSe	Limit at 95% CI Lower	Upper
4	Toe	1	1	29	0.1	0.7
	Wash		0	0	0.0	0.4
	Toe	2	1	14	0.0	0.6
	Filter		0	0	0.0	0.4
	Toe	3	4	57	0.2	0.9
	Swab		0	0	0.0	0.4
7	Toe	1	0	0	0.0	0.4
	Wash		2	29	0.1	0.7
	Toe	2	1	14	0.0	0.6
	Filter		0	0	0.0	0.4
	Toe	3	1	14	0.0	0.6
	Swab		2	29	0.1	0.7
10	Toe	1	2	29	0.1	0.7
	Wash		2	29	0.1	0.7
	Toe	2	0	0	0.0	0.4
	Filter		0	0	0.0	0.4
	Toe	3	1	14	0.0	0.6
	Swab		2	29	0.1	0.7
14	Toe	1	1	14	0.0	0.6
	Wash		3	43	0.1	0.8
	Toe	2	1	14	0.0	0.6
	Filter		1	14	0.0	0.6
	Toe	3	0	0	0.0	0.4
	Swab		3	43	0.1	0.8
17	Toe	1	3	43	0.1	0.8
	Wash		0	0	0.0	0.4
	Toe	2	0	0	0.0	0.4
	Filter		1	14	0.0	0.6
	Toe	3	1	14	0.0	0.6
	Swab		6	86	0.4	1.0
21	Toe	1	3	43	0.1	0.8
	Wash		1	14	0.0	0.7
	Toe	2	2	29	0.1	0.6
	Filter		3	43	0.1	0.8
	Toe	3	3	43	0.1	0.8
	Swab		3	43	0.1	0.8
24	Toe	1	4	57	0.2	0.9
	Wash		2	29	0.1	0.6
	Toe	2	3	43	0.1	0.8
	Filter		2	29	0.1	0.7
	Toe	3	4	57	0.2	0.9
	Swab		6	86	0.4	1.0
31	Toe	1	4	57	0.2	0.9
	Wash		3	43	0.1	0.8
	Toe	2	3	43	0.1	0.8
	Filter		3	43	0.1	0.8
	Toe	3	6	86	0.4	1.0
	Swab		7	100	0.6	1.0

Table 7. *Batrachochytrium dendrobatidis* infecting *Litoria caerulae*. Diagnostic window for TaqMan PCR using different sampling protocols (Expt 2)

Infected frogs detected	Toe clip	Bath	Filter	Swab
>25%	Day 4	Day 7	Day 21	Day 7
>50%	Day 24			Day 17
>75%	Day 31			Day 17
100%				Day 31

Table 8. *Batrachochytrium dendrobatidis*. Repeatability of TaqMan PCR after 151 runs at the Australian Animal Health Laboratory using 4 internal controls. Ct refers to the cycle threshold of the reaction and is related to how much PCR product is present; low values represent higher numbers of zoospore equivalents than higher Ct values

Ct	Internal standard (no. of zoospores)			
	100	10	1	0.1
Mean	27.3	30.8	34.3	38.2
SE	0.0	0.0	0.01	0.1
Median	27.2	30.7	34.3	38.0
Mode	27.3	30.6	34.0	37.2
SD	0.7	0.7	1.7	1.3
Sample variance	0.4	0.5	3.0	1.7
Kurtosis	–0.1	2.6	235.1	3.6
Skewness	0.1	0.6	–13.3	1.1
Range	3.5	6.0	33.4	10.6
Minimum	25.5	2848	3.0	34.4
Maximum	29.0	34.3	36.4	45.0
CV	2.4	2.3	5.1	3.5
Count	151	151	151	151

Reliability: After initial recovery the test failed to detect zoospore equivalents in 10 of 30 (33%) animals at least once during the infection, and, at a later stage, again gave positive results (Table 2).

Robustness of TaqMan assay

Sensitivity: The analytical sensitivity of the TaqMan assay was reported to be 0.1 zoospore equivalents (Boyle et al. 2004); this sensitivity relates to the number of zoospore equivalents within the reaction volume. The gold standard was used to assess diagnostic sensitivity in the infected animals in this experiment (30 infected frogs; Tables 2 & 3). Assuming that all animals were infected during the entire period of the experiment, the TaqMan assay following the wash and toe-clip protocols has an overall diagnostic sensitivity of 77 and 58%, respectively. Conventional histology (H&E and IPX) had a diagnostic sensitivity of 29.5 and 33.3%, respectively (Table 4).

Specificity: Diagnostic specificity is defined as the ability of an assay to distinguish the target agent from other infectious agents. The TaqMan assay did not return a positive result with any of the Australian Chytridiomycetes listed in Table 1, nor with any of the non-infected control groups in Expt 1 (data not shown). The diagnostic specificity of the assay was 100%. These results corroborate the findings from Boyle et al. (2004).

Repeatability: Repeatability and reproducibility (intra- and interassay variation) were measured by comparison of replicates (3×) of 4 internal standards (100, 10, 1 and 0.1 zoospores) at 3 different laboratories: Australian Animal Health Laboratory (AAHL), Griffith University (GU) and James Cook University (JCU). Being the developer of the test, AAHL produced the internal standards. Relevant statistical parameters indicate good intra- and interassay repeatability (Table 8).

Reproducibility: Reproducibility was assessed by comparison of the same 4 internal standards used in 3 different laboratories. Fig. 2 shows that mean Ct values for 0.1, 1, 10 and 100 zoospores for AAHL and GU were highly reproducible. Consistently higher values were obtained for all internal standards at JCU, where a Corbet Rotorgen instrument was used instead of an Applied Biosystems Prism 7700 Sequence Detection System. The CV was <8 in all laboratories, indicating good reproducibility (Table 9).

Table 9. *Batrachochytrium dendrobatidis*. Reproducibility of TaqMan PCR based on CV of 4 internal controls in 3 laboratories. JCU: James Cook University; GU: Griffith University; AAHL: Australian Animal Health Laboratory

	Internal standard (no. of zoospores)			
	100	10	1	0.1
JCU	3.3	3.4	3.0	3.2
GU	2.7	4.3	8.0	4.6
AAHL	2.4	2.3	5.1	3.5

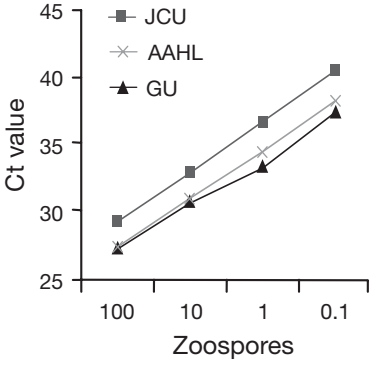

Fig. 2. *Batrachochytrium dendrobatidis*. Mean Ct values for 0.1, 1, 10 and 100 zoospores for James Cook University (JCU), Australian Animal Health Laboratory (AAHL), and Griffith University (GU)

Sampling protocols used in TaqMan assay

Toe clipping, bath, bath/filter and swabs

During the course of these experiments it became apparent that the longer the bath solution was left to stand, irrespective of temperature, the lower the number of positives and the lower the number of zoospore equivalents detected (data not included). These observations were confirmed by counting zoospores via flow cytometry over time (Fig. 3). At room temperature (23°C), the number of zoospores decreased from 2.1 to 0.62 million, or 70% over a 31 h time period. When stored at 4°C, numbers decreased from 2.14 to 1.66 million, or 22% over a similar time period, and 31% over 72 h. The implications of these data for collection of field samples, where centrifugation is not always possible within a short time span, led us to consider the filtration of bath water and the swabbing of frogs as feasible sampling protocols in comparison to the standard toe-clipping method.

Tables 6 & 7 detail comparative DSe for the TaqMan assay and the different sampling protocols derived from Expt 2. Data from this experiment indicate that toe clipping is the only collection protocol that can detect *Batrachochytrium dendrobatidis* infection as early as Day 4 p.i. However, at Day 31 p.i., the swabbing protocol was the most effective sampling method, with a DSe of 100%. At all other time points, except Day 4, the DSe of swabbing is consistently as good as or better than the corresponding toe clips, washes, or filters. The data differ compared to those derived from Expt 1, in that at Day 31 p.i. the wash method gave a DSe of 43% compared with 97% in Expt 1. Other sampling protocols assessed in Expt 2 were: filter, which scored 43% (Animals 8 to 14, termed Group 2), and toe clips, which scored 57% (Animals 1 to 7, Group 1), 43% (Group 2) and 86% (Animals 15 to 21, Group 3). The variation is discussed later.

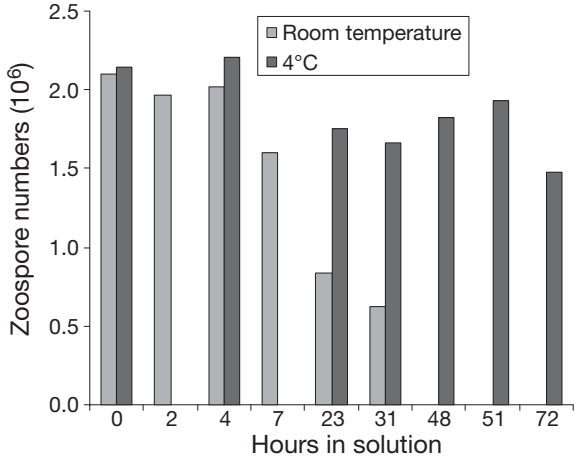

Fig. 3. *Batrachochytrium dendrobatidis*. FACS (flow cytometry) counts of zoospore numbers in solution over time

Oral discs

Swabs were also used to assess whether *Batrachochytrium dendrobatidis* could be detected on the mouth parts of tadpoles in the field. The results are shown in Table 10. There was generally an excellent correlation between a positive result for the excised oral disc and a positive swab, except when zoospore equivalent numbers were low (<400) for the oral disc. At these low levels of infection, the swabs returned a negative result. One sample (29) gave a negative result

Table 10. *Batrachochytrium dendrobatidis* infecting *Litoria ewingii* and *L. tasmaniensis*. Comparison of TaqMan data from excised mouth parts (oral discs) and swabbed mouth parts of tadpoles. Data shown in the table are the means of 3 replicates per sample and are representative of zoospore equivalents

	Sample	Oral discs	Swabs
L. ewingi	1	52988	82
	2	22141	33
	3	0	0
	4	10068	64
	5	9119	67
	6	0	0
	7	18082	346
	8	31914	114
	9	19313	241
	10	18661	591
	11	10352	19
	12	22352	111
	13	16413	389
	14	0	0
	15	0	0
	16	0	0
	17	652	5
	18	16486	81
	19	0	0
	20	0	0
	21	0	0
	22	0	0
	23	18104	2
	24	5	0
	25	9179	19
	26	11960	52
	27	1990	16
	28	0	0
	29	61237	0
	30	292	0
	31	1425	262
	32	17113	80[a]
	33	3545	58
L. tasmaniensis	34	10831	94
	35	52076	198
	36	369	0
	37	32255	36
	38	0	0

[a]<3 replicates returned a positive value

for the swab despite a high oral disc result. This assay was performed prior to the inclusion of a positive internal control in the assay so it is possible that the swab contained PCR inhibitors. Alternatively, the swabbing technique may not have been as comprehensive. The generally good correlation indicates that swabbing of live tadpole mouth parts is an adequate protocol for the detection of *B. dendrobatidis.*

Pooling of swabs

Table 11 shows the effect of pooling swabs. maximum number of swabs that can be pooled without lowering the sensitivity of the TaqMan assay is 5. This statement is based upon the assumption that an equivocal result (2 or fewer wells from a total of 3 return a positive value) can be regarded as positive. However, when a pool of 5 individual field swabs which returned a negative result were reanalysed individually, 1 low level (11 zoospore equivalents) positive was detected (data not shown).

Storage of samples

Storage of swabs in alcohol: The effect of storing swabs in 70% alcohol was assessed (Fig. 4). More zoospores were recovered from the swab than from the ethanol solution. When swabs were rinsed in DS water, following storage in ethanol, recovery of zoospores decreased compared to the protocol when zoospores were extracted directly from the swab.

Long-term storage of dry swabs: Swabs were spiked with zoospores and stored at room temperature (23°C), 4°C and –20°C for 18 mo. Fig. 5 shows data at different time periods for swabs spiked with 5000 zoospore equivalents and stored over an 18 mo time period. No decrease in zoospore recovery was observed over the 18 mo period.

Field samples

TaqMan assay: The TaqMan assay was used to test all swabs. Of the 104 samples, 81 tested negative and

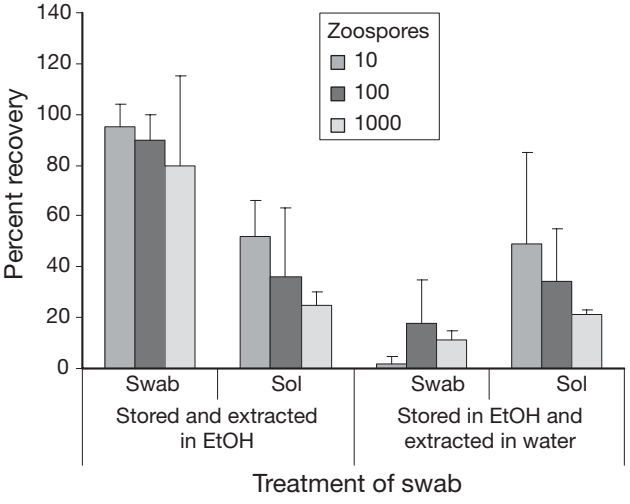

Fig. 4. *Batrachochytrium dendrobatidis*. Percent recovery of zoospores from swabs that have been stored in ethanol (EtOH) and extracted in either ethanol or in water. Swabs were spiked with 10, 100, or 1000 zoospores. Percent recovery is compared to recovery from dry swabs (Sol: the ethanol solution in which the swabs were placed)

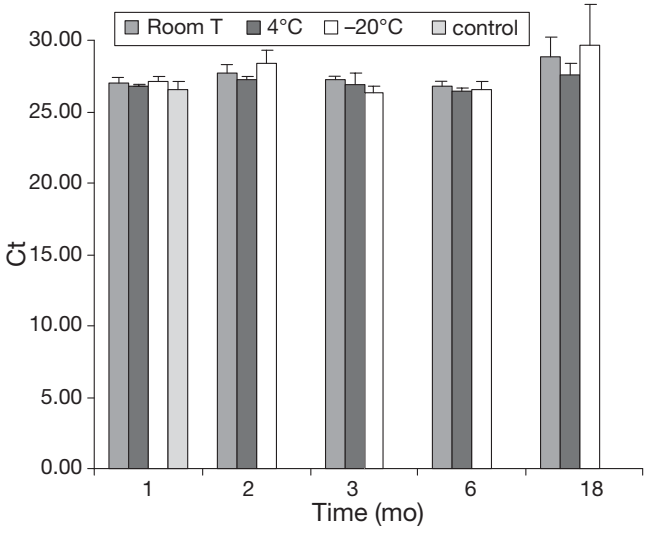

Fig. 5. *Batrachochytrium dendrobatidis*. Effect of storing swabs over time. Swabs were stored at room temperature (RT, 23°C), 4°C and –20°C. Bars represent Ct + SD. 'Ct' is defined in Boyle et al. (2004)

Table 11. *Batrachochytrium dendrobatidis*. Effect of pooling swabs on sensitivity of TaqMan assay (percentages). Each swab was spiked with a known number of zoospores. Pos.: positive; Eq.: equivocal; Neg.: negative

Zoospore	1 swab			5 swabs			10 swabs		
(n)	Pos.	Eq.	Neg.	Pos.	Eq.	Neg.	Pos.	Eq.	Neg.
1	83	17	0	46	54	0	10	60	30
10	100	0	0	100	0	0	0	50	50
100	100	0	0	75	25	0	83	17	0

Table 12. *Batrachochytrium dendrobatidis*. Analyses of swabs from field samples (no. of samples)

	TaqMan PCR	Histology (H&E)
Negative	81	97
Positive	16	1
Equivocal	7	6

16 tested positive for *Batrachochytrium dendrobatidis* (Table 12), resulting in a prevalence of 15 or 22%, depending on whether the 7 equivocal results were considered positives. Only 1 sample, which was positive by the TaqMan assay (2 zoospore equivalents, Animal 1040), was equivocal by histology. All other PCR positive samples were negative by histology.

Histology (H&E): Histological examination of the same 104 samples (Table 12) revealed a prevalence of 1% (or 7%, equivocal plus positive). One sample tested positive by both the TaqMan assay and H&E. Of the 6 equivocal histology samples, 5 tested negative and 1 tested positive by TaqMan assay.

DISCUSSION

Chytridiomycosis is a major disease among amphibians and is responsible for population declines and extinctions (Daszak et al. 1999, 2000, Rachowicz et al. 2005). The causative agent is *Batrachochytrium dendrobatidis*, which has been detected in amphibians in Australia, New Zealand, Europe, Africa, and South, Central and North America (Berger et al. 1998, Mutschmann et al. 2000, Bosch et al. 2001, Fellers et al. 2001, Bradley et al. 2002, Weldon et al. 2004). A critical facet to understanding the epidemiology and eventually to the successful control of the disease is the development and implementation of validated diagnostic assays. To date 2 main types of diagnostic assays have been published, namely those involving histochemistry (Berger et al. 2002, Olsen et al. 2004) and molecular diagnostic assays that amplify ITS-1 and ITS-2 regions of *B. dendrobatidis*. The molecular diagnostic assays include a conventional PCR assay (Annis et al. 2004) and a real-time TaqMan assay (Boyle et al. 2004). Real-time PCR differs from standard PCR in that the amplified products are detected directly during the exponential phase of the amplification cycle using fluorescent hybridisation probes, whereas standard PCR measures end-point products. This means that real-time PCR is more sensitive than conventional PCR where agarose gel resolution is poor, about 10-fold, compared with real-time PCR, which can detect 2-fold differences. Automation of real-time PCR leads to faster results. Results are objective rather than subjective, so the inclusion of standards of known numbers allows an accurate quantitation of unknowns. As the assay is performed in a closed system, i.e. no opening of tubes following PCR amplification, it is less prone to contamination, which can produce spurious results (Heid et al. 1996, Lie & Petropoulos 1998). The present paper assesses the 'fitness of purpose' of the above diagnostic assays and sampling protocols developed at AAHL by defining the sensitivity (individual and herd), diagnostic window, specificity, test dynamics, repeatability and reproducibility. It should be noted that the assay detects *B. dendrobatidis*, not the disease chytridiomycosis.

Sampling procedures

The requirements for effective sampling regimes are: they must (1) be easy to use in the laboratory and field, (2) be efficient in collecting *Batrachochytrium dendrobatidis*, (3) maintain a viable sample over time and (4) be non-destructive, to minimise harm and stress to the targeted animal. Four sampling protocols were developed and tested: toe clipping, bathing of animals, filtering bath water from washes through sterile disposable filters, and swabbing. Papers describing the clinical signs and pathological features of the disease (Berger et al. 1998, 2005a, Pessier et al. 1999) report a sloughing of skin from the epidermal surface. The sloughed skin is frequently derived from ventral surfaces of the abdomen, limbs and feet and is usually characterised under light and electron microscopy (Pessier et al. 1999, Berger et al. 2000, 2005a,b) by hyperkeratosis and the presence of zoosporangia. Collection of skin at these sites is an obvious way to maximise the chances of detecting *B. dendrobatidis*. All sampling protocols developed and used in this study exploit this characteristic feature of chytridiomycosis.

Toe clipping

Toe clipping is an established technique used for a variety of research activities, including the detection of *Batrachochytrium dendrobatidis*. Toe clips (analysed by the TaqMan assay) in general detect a higher number of zoospore equivalents than other protocols early in infection (e.g. Day 4 p.i.). The ability to detect zoospores at a higher frequency early in infection can be explained by the life cycle of the fungus (Berger et al. 2005a). Early in infection, *B. dendrobatidis* invades cells of the epidermis; the process by which this occurs is not known. Once within the cells of the *stratum granulosum* and *stratum corneum*, zoosporangia are formed. At this stage of infection, hyperkeratosis and

sloughing are not evident and the likelihood of detecting the fungus by bath or swabbing is unlikely. As infection progresses, hyperkerotosis increases, as does the associated sloughing and release of zoospores to previously uninfected regions of the skin (Berger et al. 2005b). The progressive nature of chytridiomycosis explains the increased number of zoospore equivalents over the course of infection and also why toe clips return higher zoospore counts.

An obvious advantage of toe clips is their use in histology and histochemistry. However, if such samples are to be analysed by TaqMan assay, as well as histology, the samples must be placed in alcohol (70%) and not in formalin. Unpublished data (A. D. Hyatt & D. G. Boyle) have indicated that, in general, DNA for the TaqMan assay cannot be extracted from samples stored in formalin. Whilst toe clipping is still popular, ethical issues are involved, including subjecting the animals to unnecessary levels of pain, stress and disease (May 2004, McCarthy & Parris 2004). Due to these ethical issues and the failure to demonstrate toe clipping as a significantly superior method for collecting *Batrachochytrium dendrobatidis*, we do not recommend it as a long-term sampling practice.

Bathing and filtering of bath water

Sampling methods involving the immersion of adults in a bath (such as DS water) are also effective. Bath times (15, 30 and 60 min) did not influence the number of positives or the number of detected zoospore equivalents. A major disadvantage of this technique is the decrease in zoospore numbers (up to 70%) upon storage of the bath water. Unpublished work from AAHL has shown that zoospore DNA can be recovered from the surface of the containers, thus indicating that the decrease is due to 'attachment' of the zoospores to container surfaces. Bath water must therefore be centrifuged within 8 h of collection if stored at room temperature, and the pellet must be stored at 4°C. This negative aspect can, however, be countered by filtering the medium and analysing the membrane. This method is unwieldy, as the membrane is difficult to extract from disposable filters or subject to cross-contamination if using re-usable filters. Although both of these methods possess similar sensitivities (i.e. detect equivalent number of positive samples), they are not suitable for field use, as excessive amounts of equipment, including containers, filters and even cooling equipment (e.g. car fridges or dry ice) must be carried into the field. The major advantage of the method is that zoospores can be collected from the entire surface of an animal, and it may, therefore, represent the only valid approach to reliably determine the zoospore load on an animal.

Swabbing

The fourth method developed for the detection of *Batrachochytrium dendrobatidis* is swabbing. With the exception of Day 4 p.i., this sampling protocol has the highest sensitivity of all protocols. This may be attributable to the sampling of all ventral surfaces. The inability to detect infection at Day 4 p.i. has been discussed. Increased detection efficiency may be achieved by employing a concentrated swabbing strategy to and between the digits.

Swabbing is a non-invasive method that can be performed in both the laboratory (with the sensitivity of sampling peaking at Day 35 p.i.) and field on both frogs and tadpoles. There are, however, several procedures that must be followed if representative results are to be obtained. For frogs, the swabbing should be directed to the ventral surfaces, and the swabs should be stored within the supplied containers. For tadpoles, swabs should be of the kind described in this paper, to avoid unnecessary physical damage to the animals. The swabs should be applied to the keratinized structures in the oral discs.

At present there is no evidence that storage in alcohol will improve sensitivity. If swabs are to be stored in alcohol, the swab, not the alcohol, should be analysed for *Batrachochytrium dendrobatidis.* Unpublished data from AAHL indicate that storage in alcohol is unnecessary even in climates where microbial contamination of the swabs may occur. We found that swabs spiked with micro-organisms from amphibian skin and cultured on the swab do not reduce the sensitivity of the assay. Our data indicate that swabs containing *B. dendrobatidis* are stable for at least 18 mo over a range of temperatures, and we therefore recommend that swabs be stored dry until analysis. We have not, however, tested longevity over the elevated temperatures that may be experienced in the field.

Recommended sampling protocol

Swabbing is the recommended sampling procedure for the detection of *Batrachochytrium dendrobatidis.* A range of swabs was evaluated during this study (data not included); the swab described was selected because it provides the best DNA recovery and ensures minimal risk of sample contamination and effective logistics within the laboratory. For example, it uses minimal assay-related reagents (i.e. absorption characteristics), is supplied with its own storage container, and the absorptive tip can be easily snapped off into the assay tube. The protocol also satisfies all selection criteria detailed above and has the distinct advantage of being easy to use in the field.

Diagnostic assays

Assays evaluated in this study include conventional H&E, IPX and quantitative real-time TaqMan assay.

Histology and immuno-histochemistry

All histological procedures are significantly less sensitive than TaqMan PCR. Published and unpublished results, using polyclonal (Berger et al. 2002) and monoclonal antibodies against *Batrachochytrium dendrobatidis* (A. D. Hyatt, D. G. Boyle, V. Olsen & J. White unpubl. data), indicate that monoclonal antibodies are also non-specific. It should, however, be noted that *B. dendrobatidis* is the only member of the Chytridiales that infects vertebrates, and other fungi such as *Mucor amphibiorium* (Order Mucorales, Family Mucoraceae), which infect amphibians, do not react with the antibodies (Berger et al. 1998, Berger 2001). Histopathology and associated immuno-histochemistry can therefore be used to identify *B. dendrobatidis*. The limitations of the technique are related to sensitivity. The probability of fungus being present in the tissue examined is related to the stage/time of infection and whether the infected skin is attached to the excised tissue. Advances in the immuno-histochemical protocol (Olsen et al. 2004) address one aspect of this potential problem by including a step that stains the outer keratinised epidermis. The absence of the keratinised layer signifies that the animal has shed its skin and the section may not be relevant for diagnosis. The problem relating to the stage of infection is associated with the difficulties of searching for low numbers of zoosporangia. The data from this study indicate that zoospore numbers increase over the course of infection, but may vary from one time point to another, and some toes may be infected, while others are not (refer to the preceding discussion). Berger et al. (2005b) also shows *B. dendrobatidis* to be preferentially located over ventral surfaces. Therefore, if histopathology or immunohistochemistry are to be used, then multiple sections (from 1 sample) may have to be examined and/or multiple sections (from >1 sample — same animal, but over time) may be required. Also, histopathology and histochemistry can be used to detect *B. dendrobatidis* in museum samples (Weldon et al. 2004). In brief, the disadvantages of these techniques include the inability to detect *B. dendrobatidis* early in infection (lack of sensitivity), low specificity, the requirement of experience and expertise, sampling statistics and the use of toe clipping. The advantages include the potential to screen for other infectious agents and the generation of archival material, which may be used in other, non-related investigations.

TaqMan assay

We validated the real-time TaqMan PCR assay with respect to sensitivity, specificity, repeatability and reproducibility. Validation was undertaken for laboratory-infected animals, and the results were compared to field data. Under field conditions, the TaqMan PCR assay was superior to H&E, which is consistent with data derived from laboratory experimental infection studies and the work of Kriger et al. (2006), who also used our assays and sampling protocols.

The use of the TaqMan assay in relation to different sampling protocols was also assessed. Under laboratory conditions, the diagnostic assay can be used to detect the presence of *Batrachochytrium dendrobatidis* as early as Day 4 p.i. and can be applied to a range of samples including sloughed skin, toe clips, bath pellets, filters and swabs. The success of this assay depends upon it being performed with the appropriate standards and internal controls. With respect to the 'appropriateness' of the sample, we have demonstrated that if samples are collected with an appropriate tool (e.g. swab) then reliable results can be generated. Inappropriate sampling such as swabbing the dorsal surface (Berger et al. 2005b) and storage of swabs in an inappropriate storage medium (e.g. alcohol or formalin) will decrease the chances of detecting *B. dendrobatidis*. Repeatability and reproducibility results from 3 laboratories suggest that this assay with the recommended sampling protocols is robust and can be transferred easily to a new laboratory. Finally, the real-time TaqMan assay must always be conducted with the described controls. If Ct values are outside the confidence limits, then the assay is not performing to its required 'standard' and all its aspects must be investigated.

Interpretation of data

The molecular assay validated in this paper can generate 3 types of results: negative, equivocal and positive. The interpretations of the data depend on the questions being asked.

Negative results

What constitutes a bona fide negative result? A valid negative is derived when all wells return negative values and (1) genetic material is present in all wells, (2) no inhibitors to the assay are present and (3) internal standards have been included. Two types of 'false negatives' can be identified:

(1) A false negative may result from the failure to include *Batrachochytrium dendrobatidis* within the sample volume. Although the analytical sensitivity of the real-time TaqMan assay is 0.1 zoospore equivalents (Boyle et al. 2004), a negative result may occur at early stages of infection due to low zoospore numbers. Also, an animal can be positive at one time point and negative at another. Possible reasons for this (e.g. shedding of skin) have been discussed above. If the data belong to a single animal, then that animal should be re-sampled if it was thought to be infected. For example, the data from this study indicate that if a negative result is obtained from an infected animal, then swabbing the animal 3 times over the following 14 d will return a positive result. If this is used as a guide, then a similar sampling regime should increase the probability of obtaining a positive result, if indeed the animal is infected. If the data relate to a population study where the prevalence of disease is known and a statistically relevant number of animals are being sampled, then re-sampling would not be required, as the false negatives would be accounted for in the sampling size.

(2) A false negative may result due to the presence of an inhibitor. The inclusion of a TaqMan exogenous internal positive control in the TaqMan master mix will distinguish false negatives due to real-time PCR inhibition. For example, a negative result for the target and a positive result for the internal positive control DNA indicate no target sequence is present, whilst a negative result for each suggests PCR inhibition. When the latter occurs it may be possible to remove the inhibitory effect by dilution. Unpublished data from our study suggest that the cleanliness of the swab is associated with the inhibition of a TaqMan reaction. In 30 submissions comprising 1436 swabs, 25% showed complete inhibition of the internal control. Large variation existed between submissions, ranging from 0 to 90%. The inhibition was usually, but not always, related to cleanliness of the swab, e.g. presence of foreign material such as dirt or detritus. Inhibitors may also originate from specific amphibian surfaces and/or life stages; further investigations are required to elucidate the origin of the inhibitors and to minimise their effect.

Equivocal results

Equivocal results for a single sample relate to those where only 1 or 2 wells in 3 in the TaqMan assay return a positive result. This could be evidence of contamination or of small numbers of *Batrachochytrium dendrobatidis* in the original sample. Contamination should be considered if the positive wells have high values and/or if there is evidence of aberrant values within the controls. A more likely explanation relates to sampling statistics. By this we infer that the original load of *B. dendrobatidis* was low. If there is a low number of initial zoospores/nucleic acid, then the probability of some of it being aliquoted into the final test volume must be low. Where equivocal data are involved, it may be necessary (depending on application of the test) to re-test the original sample and/or re-sample the animal(s).

Positive results

The number of zoospores increases during infection. Low numbers may represent an earlier stage of infection, but shedding of skin may also contribute to low counts. A frequently asked question is: 'What is a positive?' A positive result is one where specific amplification of the target nucleic acid occurs. For this reason it is important to run the reaction in triplicate; this assists in detecting *Batrachochytrium dendrobatidis* at low numbers. How to interpret low values and data where 1 or 2 wells from a total of 3 return a positive result are discussed above ('Equivocal results').

SUMMARY AND RECOMMENDATIONS

This paper has defined the sensitivity, specificity, repeatability and reproducibility of the real-time TaqMan diagnostic assay for the detection of *Batrachochytrium dendrobatidis.* Comparisons between this molecular assay and conventional histological and immuno-histochemical assays have also been performed; the use of the latter techniques in relation to their advantages and limitations has been defined. Different sampling protocols for the collection of *B. dendrobatidis* from amphibian skin have also been described and compared. A summary of the recommended use of these techniques, together with the interpretation of the associated data, can be found in Table 13. With the use of validated assays and sampling protocols for the detection of *B. dendrobatidis*, it is now possible to undertake validated testing and surveys within both individual animals and populations. This can include studies involving the pathogenesis of chytridiomycosis, assessing the absence of *B. dendrobatidis* in captive breeding programs and in endangered species, in addition to the screening of the environment for the fungus by swabbing endemic amphibians, other organisms and inanimate objects.

Finally, it is now possible to recommend to the OIE an internationally recognised set of protocols that can be used to detect *Batrachochytrium dendrobatidis* in amphibians. This will be important in classifying animals and regions with or without of chytridiomycosis,

Table 13. Summary of recommendations

Protocol		Recommendations
Sampling		
Field animals (swabs)		Swabs from Medical Wire & Equipment Co. MW 100–100 (1) Adults: Swab ventral surfaces and digits (2) Tadpoles: Swab mouth parts
Laboratory animals (range of sampling protocols can be used)		(1) Swabs (as above) (2) Bath and/or filter. If bath is used (a) As little as 15 min can be used. Sampling medium must be centrifuged immediately and the pellet stored at 4°C (b) Bath water can be filtered and filters stored at any temperature; negates requirement for centrifugation of bath water
Batching of samples (swabs)		(1) Maximum of 5 samples can be pooled (although low positives may be missed) (2) Only use for population studies where presence/absence data is sought
Storage of swabs		Store dry at any temperature up to 23°C. The impact of higher temperatures has not been assessed
Real-time TaqMan PCR		
Assay (performance)		(1) Assay must use 'quantitation standards' described in this paper (2) Ct values must be established against such standards (3) Performance of assay should be monitored and standard derived Ct values should be within confidence limits (4) Assay should always use internal controls to monitor inhibitor presence
Interpretation of data	Positive	All wells (3) return a positive value
	Equivocal	When <3 wells return a positive value (1) If sample is part of a population study where presence/absence data are sought, then animal re-sampling is not required (2) If sample is important (e.g. study involves individual animals), then (a) re-test sample and/or (b) re-test animal at least 3 times over a 14 d period
	Negative	When no wells return a positive value. If the sample is important (e.g. study involves individual animals), then re-test animal at least 3 times over a 14 d period

and to provide an accepted set of protocols for monitoring the spreading of *B. dendrobatidis* across regional, national and international borders. In addition, the diagnostic and sampling assays will continue to be important in studying host–pathogen interactions and in generating data on the prevalence of infection loads within morbid individuals and amphibian populations in general (MaCallum 2005). Such data are important for studies investigating the novel and endemic pathogen hypotheses by to which researchers are trying to determine whether *B. dendrobatidis* originated from one geographic area and is currently spreading to others, or whether it is ubiquitous and has recently increased in host range and pathogenicity due to some as yet unknown environmental event(s) (MaCallum 2005, Rachowicz et al. 2005).

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