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Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR

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ABSTRACT: Chytridiomycosis is a lethal disease of amphibians associated with mass mortalities and population declines worldwide. An accurate, non-invasive technique for detecting chytridiomycosis is urgently needed to determine the current geographical distribution of the disease, and its prevalence in wild amphibian populations. Herein we evaluate a recently devised, rapid, non-invasive, swab-PCR assay. We sampled 101 wild juvenile *Mixophyes iteratus* by both a skin swab for use in PCR analysis, and a toe-clip for examination by histological methods. The swab-PCR assay detected chytridiomycosis infection in a minimum of 14.9% of frogs, whereas histology detected infection in no more than 6.9% of frogs. We conclude that the swab-PCR technique is the more reliable means of detecting chytridiomycosis in wild amphibians, and that it precludes the need for toe-clipping as a means of sampling for the presence of the disease in future surveys. Further, we document a significant negative relationship between a juvenile frog's snout-vent length and its likelihood of being infected with the disease.

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

INTRODUCTION

Chytridiomycosis is a lethal disease of amphibians associated with mass mortalities and population declines worldwide (Berger et al. 1998, Lips 1999, Bosch et al. 2001, Green et al. 2002, Ron et al. 2003, Weldon & du Preez 2004). This emerging infectious disease is caused by *Batrachochytrium dendrobatidis*, a recently identified chytrid fungus (Berger et al. 1998) that infects the keratinized cells of the amphibian epidermis (Longcore et al. 1999). Due to the low degree of genetic variability among strains collected on different continents (Morehouse et al. 2003) and its identification in the international amphibian pet trade (Aplin & Kirkpatrick 1999), laboratory trade (Reed et al. 2000, Parker et al. 2002), food trade (Mazzoni et al. 2003) and zoo trade (Pessier et al. 1999), the fungus is thought to have been disseminated throughout the world in recent decades by anthropogenic means (Cunningham et al. 2003). *B. dendrobatidis* has been detected in over 200 amphibian species from 14 families and 2 orders (Anura and Caudata) (Speare & Berger 2004, updated with recently published accounts), and as it has an extremely wide host range, is likely to be found in more species as searching continues.

Knowledge of the prevalence of chytridiomycosis in wild amphibian populations is required for the proper design of disease monitoring protocols (DiGiacomo & Koepsell 1986), the successful implementation of captive-breeding and re-introduction programs (Vig-

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gers et al. 1993), and the determination of differential effects the pathogen may have on various amphibian populations. However, disease surveys to date have been severely limited by the shortcomings of available diagnostic techniques, which until very recently have been: (1) insensitive, yielding many false-negatives (Berger et al. 2002, Boyle et al. 2004); (2) non-specific, leaving open the possibility for false-positives and observer bias; (3) invasive, requiring a skin sample such as webbing (Weldon et al. 2004) or toes (Berger et al. 2002, Lips et al. 2003, Boyle et al. 2004); and (4) time-consuming. While it has been suggested that chytridiomycosis in severely infected postmetamorphic individuals can be easily diagnosed by the presence of abnormal epidermal sloughing, reddening of the ventral surfaces, and behavioral changes such as lethargy and loss of righting reflex (Berger et al. 1999a), animals at these later stages of infection are rarely found in the wild, due both to the short time span over which they are likely to survive, and the high rate at which they are scavenged (Green et al. 2002). Testing of wild amphibians is therefore dependent upon laboratory analysis of samples taken from apparently healthy individuals that do not exhibit clinical signs of disease.

Diagnosis of chytridiomycosis to date has relied largely on histological examination of skin tissue stained with haemotoxylin & eosin (H&E) (Berger et al. 1998, 1999b, Aplin & Kirkpatrick 2000, Waldman 2001, Bonaccorso et al. 2003, Hopkins & Channing 2003, McDonald et al. 2005). Sensitivity and specificity of this technique has been improved slightly by the use of polyclonal antibodies and immunoperoxidase (IPX) staining (Berger et al. 2002), but even this method may fail to detect a large proportion of infected animals (Boyle et al. 2004). More recently, a co-localisation technique (Olsen et al. 2004) has been designed to stain both *Batrachochytrium dendrobatidis* and the keratinized epidermis that the fungus infects, but no experiments have been undertaken to quantify the sensitivity of this technique.

Fig. 1. Sampling for *Batrachochytrium dendrobatidis*: a cotton swab is firmly run over the skin of *Litoria wilcoxii* (Photo: D. Hall)

The recent development of a real-time Taqman PCR assay (Boyle et al. 2004) allows for the rapid, quantitative detection of *Batrachochytrium dendrobatidis* zoospores recovered from toe-clips of infected animals. This assay has been shown to greatly improve both the sensitivity and the specificity of chytridiomycosis diagnosis. Boyle et al. (2004) demonstrated that the Taqman PCR assay could detect chytrid zoospores on newly infected individuals 7 to 14 d prior to histological methods, and was nearly twice as likely as histological methods to detect the fungus.

The toe-clip PCR technique (Boyle et al. 2004), however, is not without problems: a toe-clip is required, which in light of new evidence relating toe-clipping to decreased survivorship (McCarthy & Parris 2004), raises ethical concerns and may also result in difficulties acquiring animal ethics permits; furthermore, the analysis of a single toe may not be sufficient for the detection of the fungus, which does not necessarily infect every toe (Boyle et al. 2004). To overcome these issues, a non-invasive sampling technique has been developed in which a cotton swab is firmly run over the skin of the amphibian (Fig. 1). As chytridiomycosis is a cutaneous infection, the swab removes sloughing skin which may contain chytrid zoosporangia and zoospores, and the swab then replaces the toe-clip in the real-time Taqman PCR assay. A. D. Hyatt & D. G. Boyle (unpubl. data) confirm that in experimentally infected individuals, the swab-PCR technique is at least as sensitive as the toe-clip PCR technique described by Boyle et al. (2004).

However, the efficacy of the swab technique on wild-caught animals has never been evaluated, and thus it is unknown if it is acceptable for use in field situations. Wild amphibians are likely not exposed for long periods of time to the high number of zoospores (10^3 to 10^7) used to infect animals in laboratory trials (Berger et al. 1998, 2002, Lamirande & Nichols 2002, Boyle et al. 2004, Daszak et al. 2004, Parris 2004, Rachowicz & Vredenburg 2004), and are thus likely to carry relatively light infections. Furthermore, swabs taken off wild individuals are likely to be covered in dirt and microorganisms, and subject to the high heat and humidity of field conditions, which may degrade the fungal DNA, resulting in false-negatives.

The aim of our study was to compare the sensitivity of the swab-PCR technique with that of the toe-clip histology technique, in a field situation. The toe-clip histology technique (Berger et al. 1999b) has been the most commonly used method of diagnosis in past field surveys for chytridiomycosis, and thus a comparison of the 2 techniques would allow for an assessment of the accuracy of past surveys: high numbers of false-negatives (as determined by negative histology results from frogs that yielded positive PCR results) would imply an underestimation of chytridiomycosis prevalence in past surveys that have used histological examination of toe-clips. Furthermore, demonstrating a significant improvement in sensitivity over the histological technique would validate the swab-PCR technique as not only an acceptable method of sampling wild amphibians for chytridiomycosis, but also as the preferred method.

MATERIALS AND METHODS

Study population. We sampled wild *Mixophyes iteratus* (giant barred frog) at London Creek (26.83° S, 152.89° E), a low altitude (150 m) site near the town of Peachester in southeast Queensland, Australia. This population was chosen for several reasons: (1) it was known from past sampling to be chytridiomycosis-positive (H. B. Hines unpubl. data), (2) the frogs were already being used in a mark-recapture study, so no extra toe-clipping would be necessary to undertake this project, (3) due to the non-declining status of the population and the fact that morbid frogs had not been sighted at the creek in several years (H. B. Hines pers. obs.), infected individuals within the population were expected to harbor light infections. The examination of individuals with light infections by an insensitive diagnostic technique is likely to pronounce the inadequacies of that technique, as the low numbers of zoosporangia present are likely to go undetected and thus yield many false-negatives (Van Ells et al. 2003). Conversely, severe infections would likely be detected even by an insensitive diagnostic technique, possibly leading to the erroneous conclusion that there is no difference in the sensitivities of the techniques being compared, when one does indeed exist.

Field collection. On 4 sampling dates between 3 November 2003 and 22 March 2004, a total of 101 juvenile *Mixophyes iteratus* were caught using clean, unused plastic bags. Each frog was both toe-clipped (for use in histological examination) and swabbed (for use in the Taqman PCR analysis). Toe-clipping consisted of removing a portion of between 1 and 3 toes (mean = 2.3), the exact number of toes used depending on the identifier number (Hero 1989) being used to individually mark that animal for a concurrent mark-recapture population study that was taking place. Toes were placed in 70% ethanol immediately after clipping. The swab technique was performed by firmly running a cotton swab (Medical Wire & Equipment, MW 100-100) once over the frog's dorsal surface; once over one of the frog's sides, from groin to armpit; once on the ventral surface; once on the underside of one thigh; and once on the webbing of one foot. Swabs were then replaced in their original container, and were frozen at –20°C upon return from the field (within 10 h of sampling). Swabs remained frozen until PCR analysis could take place (range = 62 to 470 d; mean = 255.4 d). Snout-vent length (SVL) of all frogs was measured to the nearest 0.1 mm using vernier calipers.

Laboratory analysis. Diagnosis of toe-clips for the presence of *Batrachochytrium dendrobatidis* was by histological examination (Culling 1963, Berger et al. 1999b). All toe-clips were decalcified for 48 h in 10% formic acid, histologically processed in a Shandon Hypercenter XP tissue processor, embedded in paraffin wax, longitudinally sectioned at 5 µm, and stained using H&E. The H&E stain was chosen over other staining procedures because, as mentioned previously, it has been the most commonly used method in past surveys, and we wanted to be able to assess the accuracy of those surveys. The stratum corneum and stratum granulosum of 5 consecutive serial sections from the middle of each sample were examined under a compound microscope at 200× and 400× magnification. Approximately 35 fields of view at 200× magnification were examined per toe. Samples were considered positive for chytridiomycosis if damaged epidermis was present and chytrid zoosporangia clearly evident. Due to the inherently low specificity of the diagnostic technique, diagnosis of infection by *B. dendrobatidis* on some samples could not always be unequivocally determined, a problem also noted in previous studies (Retallick et al. 2004, Ouellet et al. 2005). These samples were labeled 'suspicious', and were characterized by minimal or no sloughing of the epidermis and one to a few structures which resembled chytrid zoosporangia.

Swabs were prepared for real-time Taqman PCR analysis following procedure outlined by Boyle et al. (2004), except that nucleic acids were extracted using 50 µl PrepMan Ultra and the tip of the swab was used instead of a toe. To ensure the integrity of results, a negative control (H_2O) was run in triplicate on every 96-well PCR plate. Samples in which *Batrachochytrium dendrobatidis* was detected in all 3 wells of the triplicate analysis were considered positive for chytridiomycosis; samples in which *B. dendrobatidis* was detected in 1 or 2 wells, but not all 3, were considered

to have yielded equivocal results and were labeled 'suspicious', and samples in which no *B. dendrobatidis* was detected were considered negative for chytridiomycosis.

Data analysis. Chi-squared tests were performed to determine if there was a significant difference in the sensitivities of the 2 diagnostic techniques, and odds ratios produced. Logistic regression was performed to determine if there was a significant relationship between the length of time a swab was frozen, and the PCR result (positive/negative) obtained for that swab. The mean SVL of frogs yielding positive PCR results was compared to that of frogs yielding negative PCR results using an independent *t*-test. The relationship between SVL and the number of zoospores (mean value of triplicate assay, log + 1 transformed) detected was assessed using linear regression. All analyses were performed in STATISTICA 6.0 (StatSoft).

RESULTS

Both diagnostic techniques examined were able to detect *Batrachochytrium dendrobatidis*, but the techniques varied significantly in their detection capabilities (Table 1). Depending on whether the suspicious histological results were considered positive, negative, or were excluded from the analysis, histology detected the fungus on 6.9, 0.99 or 1.1% of the frogs examined, respectively. The Taqman PCR assay detected *B. dendrobatidis* on 22.8, 14.9, or 16.1% of the frogs, these figures again depending on inclusion of the suspicious results. The swab-PCR technique was significantly more sensitive than was toe-clip histology, regardless of whether suspicious results were considered positive ($\chi^2_{1,0.05} = 10.02$; $p = 0.002$), negative ($\chi^2_{1,0.05} = 13.3$; $p = 0.0003$), or were excluded from the analysis ($\chi^2_{1,0.05} = 13.7$; $p = 0.0002$), and the odds of detecting *B. dendrobatidis* were 4.0, 17.4, and 18.1 times higher using the swab-PCR technique than using toe-clip histology, respectively.

Table 1. *Batrachochytrium dendrobatidis* infecting *Mixophyes iteratus*. Infection status of 101 juvenile frogs as determined by 2 diagnostic techniques used to detect chytridiomycosis. Numbers in parentheses: percentage of total rogs sampled that yielded the specified result

Diagnostic technique	No. positive	No. suspicious	No. negative
Toe-clip histology	1 (1.0)	6 (5.9)	94 (93.1)
Swab-PCR	15 (14.9)	8 (7.9)	78 (77.2)

All frogs with suspicious PCR results were deemed negative by histology (Table 2). Only 1 frog that was considered suspicious by histology yielded a positive PCR result (a lightly infected frog whose swab bore 2 zoospore equivalents). The single positive histological result was from a frog determined by PCR to be negative. The mean number of zoospore equivalents on swabs from infected frogs was 88.9 ± 56.4 (SE).

Frogs yielding positive PCR results were caught on only 2 of the 4 sampling dates (Fig. 2), and the 22 March sampling session yielded a significantly smaller proportion of positive PCR results than did the 4 February sampling session ($\chi^2_{1,0.05} = 4.48$; $p = 0.034$). *Batrachochytrium dendrobatidis* was detected on multiple swabs that had remained frozen for 377 d, and there was no correlation between a frog's PCR result (positive/negative) and the length of time that had expired

Table 2. *Batrachochytrium dendrobatidis* infecting *Mixophyes iteratus*. Comparison of results of all frogs that yielded a non-negative result by either Taqman PCR or histology, and quantitation of *B. dendrobatidis* zoospores on swabs taken from infected frogs (determined by Taqman PCR assay, expressed as triplicate mean). –: negative histological result

Taqman PCR result	Histology result
Positive	
924	–
109	–
80	–
61	–
45	–
29	–
28	–
24	–
18	–
4	–
4	–
3	–
2	Suspicious
2	–
0.4	–
Suspicious	
4	–
1	–
1	–
1	–
0.4	–
0.3	–
0.2	–
0.1	–
Negative	
0	Positive
0	Suspicious
0	Suspicious
0	Suspicious
0	Suspicious
0	Suspicious

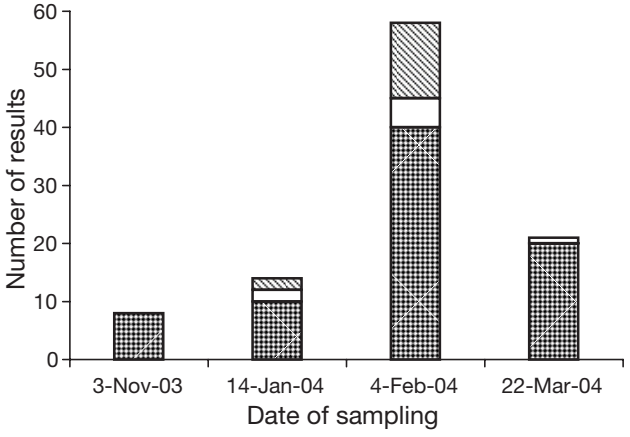

Fig. 2. *Batrachochytrium dendrobatidis*. Temporal distribution of sampling, and number of positive, suspicious and negative PCR results obtained on each sampling date (diagonally hatched: positive; white: suspicious; cross-hatched: negative)

between sampling and PCR analysis (logistic regression: Wald = 1.08; p = 0.30; Fig. 3). Thus, it is unlikely that significant degradation of the fungal DNA took place while swabs were frozen.

Frogs that yielded negative PCR results had a significantly larger SVL (mean = 42.1 mm) than frogs yielding positive PCR results (mean = 38.0 mm) (t-value = –3.16; df = 91; p = 0.002; Fig. 4). This relationship was significant even when the results of frogs sampled during only 1 sampling session were analyzed (4 February 2004: t-value = –2.59; df = 51; p = 0.013) and thus it is unlikely to be an artefact of any potential sampling bias that may have occurred (whereby a disproportionate number of small frogs may have unwittingly been sampled during a period of increased chytrid levels). There was no relationship between SVL and the number of zoospores found on infected (positive) frogs (n = 15; r^2 = 0.194; p = 0.10), but the sample size may have been too small to determine an effect, if one did indeed exist. When suspicious frogs (n = 8) were included in the analysis, there was a significant negative relationship between SVL and zoospores (n = 23; r^2 = 0.196; p = 0.034).

DISCUSSION

We have shown that the swab technique in conjunction with the Taqman PCR assay can be at least twice as likely as histology to detect chytridiomycosis in wild frogs, a result consistent with the findings of Boyle et al. (2004). As the swab-PCR technique yields quantitative data, allows for more rapid analysis of samples, provides higher specificity than does histology, and is less harmful to the frog, we recommend it as the preferred technique for the sampling of wild amphibians for chytridiomycosis in future surveys.

The increased sensitivity of the swab-PCR technique described herein is likely due to the much greater proportion of the amphibian's body being sampled than in any previously described diagnostic technique. *Batrachochytrium dendrobatidis* does not evenly distribute over the amphibian skin (Pessier et al. 1999) and, therefore, the thin strips of skin examined in histological diagnoses may not harbor chytrid zoosporangia, even on severely infected individuals (Boyle et al. 2004). As the specificity of the Taqman PCR assay was confirmed by the failure of the assay to detect any of 5 closely related species of *Chytridiomycetes* fungi (Boyle et al. 2004), it is unlikely that the higher number of positive diagnoses by the swab-PCR technique was due to false positives.

Conservation programs will benefit greatly from the improved sensitivity of this technique over those used in past surveys, as false-negatives compromise the quality and usefulness of disease prevalence data and obscure the relationships under investigation. The high sensitivity of the swab-PCR technique will allow researchers to achieve a more thorough understanding

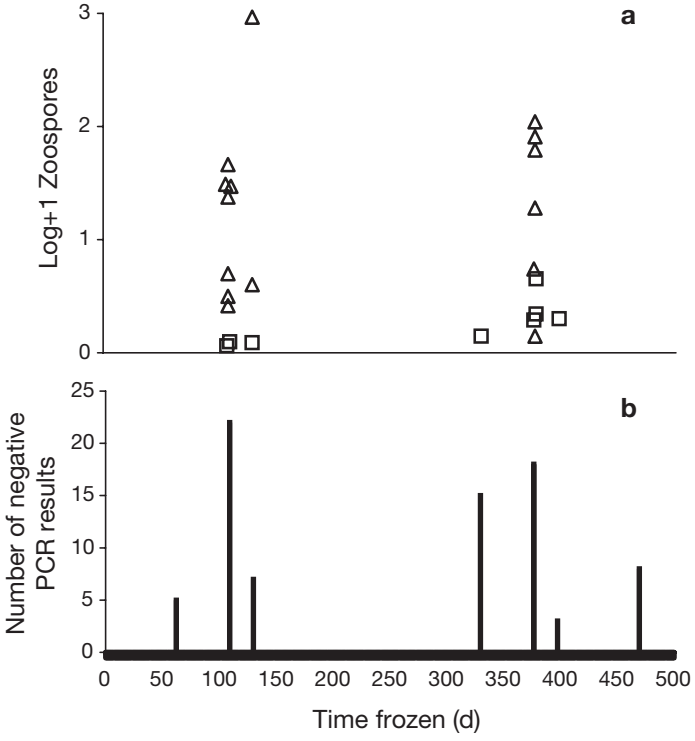

Fig. 3. *Batrachochytrium dendrobatidis*. Effect of storage time on PCR result (a) Quantitation of positive and suspicious results based on mean number of *B. dendrobatidis* zoospores detected in triplicate assay (Δ: positive PCR results, n = 15; □: suspicious PCR results, n = 8). (b) Number of negative results (n = 78)

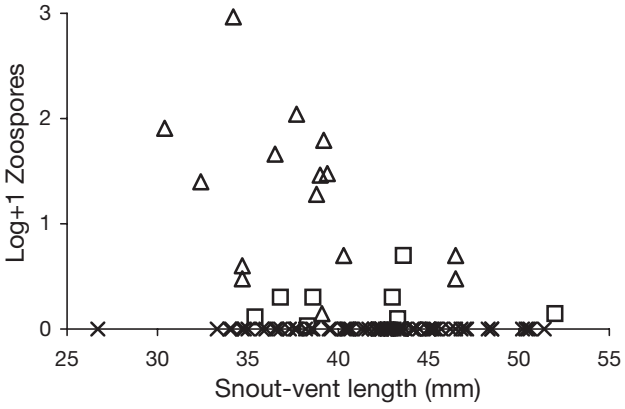

Fig. 4. *Batrachochytrium dendrobatidis* infecting *Mixophyes iteratus*. Relationship between snout-vent length (SVL) and number of *B. dendrobatidis* zoospores found on positive (△, n = 15), suspicious (□, n = 8) and negative (×, n = 78) individuals, as determined by Taqman PCR assay

of the differential effects that chytridiomycosis may have on populations and species living at various altitudes and latitudes, and in different breeding habitats. This will allow for prioritization of disease monitoring in locations where the disease status of populations is unknown, and will increase our ability to predict which populations and species are most susceptible to population decline.

The non-invasive nature of the swab-PCR technique will allow for the sampling of threatened amphibian species without the ethical issues surrounding toe-clipping (May 2004), or the associated possibility of decreasing survivorship inherent in surveys that use toe-clipping (McCarthy & Parris 2004). It will also facilitate investigation into the possibility that there are non-amphibian hosts/vectors of *Batrachochytrium dendrobatidis* (e.g. crayfish, fish, birds), and will assist researchers in determining the ways in which the fungus persists in areas where amphibians have disappeared, and the ways it may be transported into naïve amphibian populations.

The results of our study imply that past surveys have likely underestimated the prevalence of chytridiomycosis in wild amphibian populations. This has important ramifications for the design of disease monitoring protocols for surveys focused on determining the presence or absence of chytridiomycosis in specific locations. The proper design of these surveys relies on knowledge of the minimum disease prevalence that would be expected in the population to be sampled if indeed the disease were present (DiGiacomo & Koepsell 1986). This prevalence is used to determine the number of animals that need to be sampled (and yield negative results) before one can be 95% confident that the disease is absent from the population being sampled (Fig. 5). The design of protocols for chytridiomycosis surveys that will be undertaken using the swab-PCR technique described herein should therefore not be based on prevalence rates determined by field studies that have employed less sensitive diagnostic techniques.

Chytridiomycosis levels in juveniles of our study population decreased with increasing SVL (Fig. 4). There are at least 3 possible explanations for this relationship: (1) individuals experienced an ontogenetic shift in immune capacity, resulting in decreasing disease levels with increasing age (and therefore SVL); (2) the presence of *Batrachochytrium dendrobatidis* on infected individuals negatively influenced their developmental rates; (3) infected individuals experienced high mortality before achieving large body size, resulting in few captures of large, infected frogs. Further work is needed to resolve these issues, and to determine the generality of our results. We recommend that researchers report frogs' SVLs in future published studies.

The quantitative nature of Taqman PCR data opens up new avenues of chytridiomycosis research. There are currently no published studies which have quantified the degree to which the severity of frogs' chytrid infections varies among populations. This information is necessary, as populations with equal disease prevalence may differ significantly in the severity of their infections. Without the ability to detect and quantify this difference, a researcher may falsely conclude that neither population is more threatened than the other, and appropriate management action may not be implemented. We recommend that quantified results of the numbers of zoospores per sample accompany prevalence data in all published studies. This will provide baseline data that can be used to track future changes in chytridiomycosis levels in the same popula-

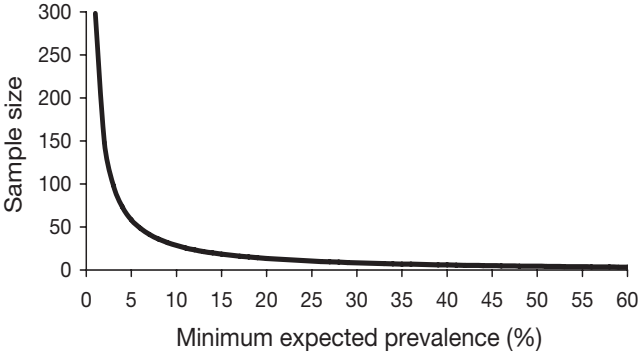

Fig. 5. Number of animals required for sampling (if all samples yield negative diagnoses) to be 95% confident that disease is absent from a population, based on minimum expected disease prevalence. Graph constructed from data in DiGiacomo & Koepsell (1986)

tion, and will also allow inferences to be drawn regarding the differing levels of the disease between populations. For quantified data to be comparable across studies, a standardized swab technique should be used by researchers worldwide.

We found no relationship between the length of time a swab remained frozen and its subsequent PCR result (Fig. 3), suggesting that only limited degradation of the fungal DNA took place while the swabs were frozen. It remains unknown, however, how much degradation occurs in the time between sampling and freezing the swabs (from 1 to 10 h in this study). As research expeditions may take place in remote areas and/or hot, humid regions where no form of refrigeration is available for prolonged periods of time, future research should focus on determining how long swabs can remain in field conditions without adverse effects.

It should be noted that the swab-PCR technique is not without error, as exhibited by: (1) the presence of equivocal results, and (2) the failure to detect *Batrachochytrium dendrobatidis* on the single frog that tested positive by histology. Equivocal results can conceivably arise from low-level contamination during laboratory analysis, in which 1 or 2, but not all 3 wells of the triplicate are exposed to *B. dendrobatidis* from an outside source (e.g. airborne zoospores or technician error). They can also be obtained if the actual number of zoospores present on the sample is very low (1 to a few). This is due to the fact that the sample must be diluted prior to PCR analysis, and only a small portion of the original sample is analyzed in each well of the triplicate. Thus it is possible that chytrid DNA may not end up in all 3 wells of the triplicate. The number of equivocal results we obtained through the PCR assay (n = 8) was roughly equal to the number of suspicious results we obtained through histology (n = 6), and is therefore no more of an issue than it was with the older techniques. However, equivocal results can be reduced in future studies by re-analysis of the supernatant that remains from the original DNA extraction process. The failure of the swab-PCR technique to detect *B. dendrobatidis* on the frog deemed positive by histology may be due to the swab not having been run over any part of the frog's body that was infected. The swab technique we used consisted of only 5 strokes of the swab over the frog's body, and therefore was not comprehensive. Increasing the number of strokes should reduce the likelihood of a false-negative.

Even with these potential shortfalls, the swab-PCR technique remains an excellent tool for the diagnosis of chytridiomycosis infection in wild amphibians, and should greatly improve our ability to acquire the data necessary to more thoroughly understand chytridiomycosis and to conserve remaining amphibian populations.

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