Methods in Ecology and Evolution

British Ecological Society

Methods in Ecology and Evolution 2013, **4,** 162–166 doi: 10.1111/j.2041-210x.2012.00263.x

Utilization of fast qPCR techniques to detect the amphibian chytrid fungus: a cheaper and more efficient alternative method

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Summary

1. A pathogen of great significance to amphibian populations is the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). It has been demonstrated as causing recent epizootics in wild populations and is also widely found in captive animals. It is listed as a notifiable disease within the pet, bait and food trade because of its risk of introduction into wild populations. Due to this status, there has been much emphasis on reliably identifying and quantifying the pathogens in amphibians. Quantitative polymerase chain reaction (qPCR) has served as the recent standard for identifying this pathogen's presence. Newer technologies have greatly improved these reactions enabling researchers to use smaller volumes and run the reactions in less time. These 'fast' qPCR chemistries are gaining popularity because the reduced volumes required to run the reactions can save funding resources and reduce the time to data acquisition.

2. In this study, we compare the results from differing reaction methodologies using the same DNA extracts from pathogens collected from wild sampled amphibians. In addition to comparing the standard methodology and fast methodology for both pathogens, we also conducted a reduced volume methodology using the standard TaqMan chemistry for *Bd*. Estimated pathogen loads from 114 field swab samples were compared among methodologies.

3. We found that for *Bd*, all three methodologies produced similar results for prevalence (presence/absence) estimates. In terms of estimating pathogen loads in the samples, both the standard and fast methodologies produced comparable estimates but the reduced volume methodology exhibited significantly lower values.

4. Therefore, it appears that the fast methodology is adequate for use with *Bd*, and potentially several other wildlife pathogens, in estimating both prevalence and quantity, but the reduced volume methodology is inadequate and not recommended for use in quantifying samples.

Key-words: amphibian conservation, chytridiomycosis, diagnosis, quantitative PCR

Introduction

Amphibians are currently experiencing the fastest extinction rates among all classes of vertebrates (Voyles *et al*. 2007). Nearly, one-third of all amphibian species are threatened with extinction (Stuart *et al*. 2004). The deadly infectious amphibian disease, chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is responsible for many of these drastic worldwide declines (Kilpatrick, Briggs & Daszak 2010). On a small scale, declines produce rapid reductions in population density and species richness (Lips *et al*. 2006), leading to rapid extinction events on a global scale (Smith, Lips & Chase 2009).

Knowing where Bd occurs is necessary for monitoring its spread among amphibian populations. A wide range of diagnostic tools have been developed including histology, PCR, standard qPCR, and a newly developed 'fast' qPCR. Histological examinations of the toes and/or skin by conventional stains can only pick up the presence of infection and are far less sensitive than PCR (Berger *et al*. 1998; Hyatt *et al*. 2007; Skerratt *et al*. 2011). PCR is sensitive to as few as 10 zoospores, but the presence of infection is determined qualitatively, resulting in a less precise test (Annis *et al*. 2004). Quantitative PCR is a more expensive and reliable method that is sensitive to a single zoospore (Kriger *et al*. 2006; Hyatt *et al*. 2007). It is highly specific to *Bd* and provides a reliable estimate of the pathogen load (Boyle *et al*. 2004). Noninvasive methods of sample collection include epidermal swabs, in which spores are picked up on cotton or nylon swabs. Invasive methods can be more accurate, but require the mouthparts to be cut from the tadpoles, or skin to be removed from adults. The analysis of swabs via qPCR is the most reliably used methodology for determining *Bd* presence and load in wild populations (Retallick *et al*. 2006).

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Continued advances in qPCR chemistry have led to the recent development of 'fast' qPCR methodologies. These reactions utilize the same primers and probes as the standard qPCR techniques but utilize a different master mix. Fast reactions require lower volumes that aid in reducing the time required. The ability to run these faster reactions is quickly becoming a standard feature on new qPCR machines, thereby increasing the chemistry's wide acceptance. One concern with using a fast methodology is its reliability to detect low pathogen loads. To our knowledge, only two papers have been published comparing fast and standard qPCR methodologies and neither examined *Bd*. One study on HIV (Yoder & Fishel 2008) found comparable results between the two methodologies while another study on Epstein-Barr virus (Hilscher, Vahrson & Dittmer 2005) exhibited a significantly lower performance when utilizing fast qPCR. A recent study has shown that fast qPCR can detect *Bd* (Ruthig & DeRidder 2012), but they did not compare the results directly to the standard method. Therefore, there is a strong need to examine the fast qPCR technique for its effectiveness at quantifying *Bd*.

This study aims to measure the differences in results between standard qPCR vs. fast qPCR. As not all laboratories are equipped with the new qPCR machines that are able to run fast qPCR, this study also tests if a low volume standard qPCR (hereafter low volume qPCR) provides similar results to either the fast or standard protocols. This alternate method alters only the volume of the reaction, but not the chemistry, thereby reducing costs without reducing the reaction time. Three treatments are used to compare these methodologies: Fast qPCR (10 μL volume), standard qPCR (25 μL volume) and low volume qPCR (10 μL volume). Specifically, we investigate the comparative outcomes of both the frequency of detection and the estimated zoospore load between the three different methods.

Materials and methods

BD SAMPLES

To determine positive or negative samples, we obtained pathogen loads (quantitative estimates of zoospore number per swab) from 114 samples. The samples were collected as part of a study investigating seasonal variation in infection of Costa Rican amphibians (Whitfield *et al*. 2012), where *Bd* has been present since at least 1986 (Puschendorf, Bolaños & Chaves 2006). Amphibians were sampled for *Bd* at La Selva Biological Station, a lowland Neotropics site in north-eastern Costa Rica known to have undergone massive amphibian declines (Whitfield *et al*. 2007).

We utilized a noninvasive and widely used swabbing technique (Boyle *et al*. 2004). Frogs were captured in the field by hand using either latex gloves or unused plastic bags. The body surfaces of all captured frogs were swabbed five times on the body surface where high loads of *Bd* are typically found (venter, hands and feet). Swabs were then placed in 70% ethanol to store for later DNA extraction.

PATHOGEN QUANTIFICATION METHODOLOGIES

Quantitative PCR can detect very low zoospore loads and provide a measure of this pathogen load calculated from a series of known standard dilutions. All methodologies utilized DNA extracted from swabs using the Qiagen DNeasy kit following the manufacturer's procedures eluted into 100 μL of buffer. This extraction technique is known to be reliable for extracting both *Bd* and amphibian DNA from the skin (Cheng *et al*. 2011; Prunier *et al*. 2012).

We utilized three different methodologies of qPCR to compare sensitivity and reliability: a well-documented standard qPCR, a low volume qPCR and a fast qPCR. For standard qPCR (Boyle *et al*. 2004), we used 25 μL reactions containing 5 μL of extracted DNA template, 900 nmol forward primer, 900 nmol reverse primer, 250 nmol MGB probe, and Taqman Universal Master Mix (Applied Biosystems, Foster City, CA, USA). Plates were run on a StepOnePlus qPCR machine (Applied Biosystems) at an activation stage of 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 60 s. For low volume qPCR, we utilized the same procedure as earlier but instead, reduced the total reaction volume to 10 μL containing 3 μL of template DNA. These same volumes were used for fast qPCR, also utilizing the same concentrations of primers and probe as above. Fast qPCR reactions require a different Taqman master mix to function properly at faster run speeds. We used 2X Taqman Fast Universal Master Mix (Applied Biosystems). Plates were run on the same qPCR machine but using a faster cycling method with an activation stage of 95 °C at 20 s, followed by 50 cycles of 95 °C for 3 s and 60 °C for 30 s.

For all three methodologies, samples had to be run over several plates. Reaction chemicals and DNA extract were kept frozen until initial use. DNA and qPCR chemicals were then kept refrigerated between plates to reduce any degradation that can occur either due to excessive heat or to repeated freezing and thawing. Each plate included a negative control (extracted uninfected swabs) and standard curve from 0·06–60 zoospore equivalents for lower volume methodologies and from 0·10–100 zoospore equivalents for the standard qPCR. This difference is due to the difference in template volume per reaction and has no impact on the final estimates or on qPCR efficiency. Standards were acquired from CSIRO, Livestock Industries in Australia and are the same standards used in the methods described by Boyle *et al*. 2004. All samples were run in triplicate. In all three methods, the sample was designated negative if there was no detection of *Bd* in any of the triplicate wells and deemed positive if detected in either two or all three wells. The sample was rerun if *Bd* was positive in only one of the three triplicate wells. If *Bd* was detected in the subsequent run, it was designated as positive and a quantity was determined from the 2–4 positive estimates. We used StepOne software v2.1 (Applied Biosystems) to quantify *Bd* loads from the qPCR results. To be conservative, we designated very low zoospore estimates as positive that might diagnostically be considered negative because the value of where to draw the line can vary. The re-examination of these data points as 0 s actually only serves to strengthen the relationships presented, and therefore, we have left the low values in as a more conservative approach.

The three methodologies were statistically compared via both prevalence (proportion of swabs with detectable *Bd*) and load (quantitative estimates of zoospores per swab). We compared the counts of positive and negative sample detections using a chi-square test. A one-way ANOVA was used to compare pathogen load values of all positive samples (but no negative samples) between methodology treatment groups. Data were log transformed to meet assumptions of the model. As methodologies utilized the same DNA samples, we then used paired t-tests to examine quantitative differences comparing results of positive pairs for each of the three methodologies. All statistics were performed using the R statistical computing environment, version 2.11.1 (Vienna, Austria).

Results

PREVALENCE COMPARISON

All three methodologies successfully detected chytrid fungus DNA from field samples. Across all samples, there were 58 total positive detections with no particular method exhibiting superior detection ability. Of these, 15 samples did not correspond across all three methodologies (Table 1). When observed from any particular method, though, the results are strongly concurrent. Comparison of prevalence was nearly identical between the three methodologies (Fast 53/114, Standard 51/114, Low 52/114; $\chi^2 = 0{\cdot}0715$, d.f. = 2, *P*-value = 0·9649). Of the 53 total positive detections in the fast method, 44 were detected across all three methodologies (85% correspondence). Of the 61 negative samples in the fast method, six appeared positive in other methodologies, although all had estimated loads of less than 1 zoospore. Interestingly, of the 53 positive fast samples, seven appeared negative under the standard methodology. One of these was estimated at 10 zoospores, but the remaining 6 registered less than 3 zoospores.

Table 1. Comparison of zoospore pathogen load estimates among all three methodologies without complete concordance of detection

Standard	Low	Fast
0·00	0·80	10·33
0·56	0·00	5·15
0·00	0·00	2·80
0·00	0·00	2·29
0·00	0·04	1·75
0·00	0·12	0·81
0·18	0·00	0·11
0·00	0·00	0·06
0·00	0·00	0·02
0·00	0·01	0·00
0·04	0·00	0·00
0·51	0·10	0·00
0·19	0·00	0·00
0·00	0·01	0·00
0·03	0·00	0·00

QUANTITATIVE COMPARISON

A one-way ANOVA compared across test type exhibited a significant difference in pathogen load estimates between methodologies ($F_{2,153} = 12{\cdot}61$, *P*-value < 0·0001; Fig. 1). Paired t-tests between test type using the same DNA samples revealed consistent significantly lower estimates from the low volume method when compared to both fast ($t = 10{\cdot}6445$, d.f. = 46, *P*-value < 0·0001) and standard ($t = 13{\cdot}8115$, d.f. = 45, *P*-value < 0·0001) methods. There was no significant difference between fast and standard values ($t = 1{\cdot}5043$, d.f. = 45, *P*-value = 0·1395). The average difference between these two methods was 0·74 ± 0·96 zoospores. The overall range of log values of zoospore estimates was from −2·8327 to 3·1058.

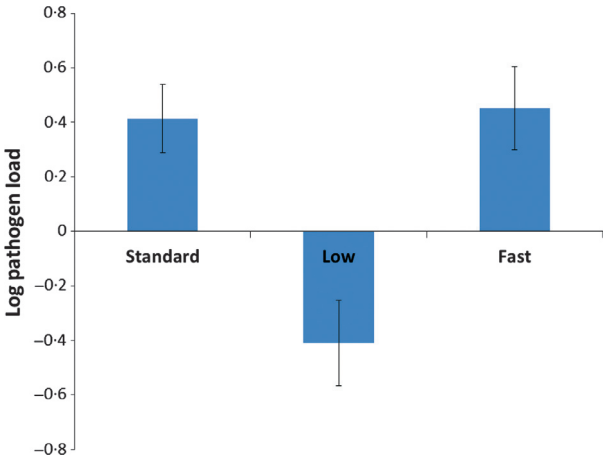

Fig. 1. Comparison of average log values of estimated zoospore load among test types. Standard, low, and fast designate the qPCR method used. Error bars represent ±SE.

Discussion

This study demonstrates that fast qPCR can be as effective as the standard methodology of qPCR for detecting *Bd*. All three qPCR methodologies similarly estimated prevalence of chytrid fungus from field samples, but only the fast method was able to correspond with the standard method to adequately estimate pathogen load (Fig. 1). These results indicate that the use of fast qPCR chemistry for detection of *Bd* field samples collected on swabs can be a reliable substitute for the traditional method. As tissue samples are likely to capture even higher pathogen loads, the fast methodology will likely be an adequate substitute for other sample collection methods as well.

PREVALENCE

In terms of prevalence, there was no significant difference in detection ability between the standard and fast qPCR methodologies. Where the two do not match up in terms of detection, the discrepancies between the tests are typically at very low zoospore counts (<1). One would think that the fast qPCR method would suffer more here because its reaction volume is low, but our data suggest that even the standard qPCR can miss positive results. These minor discrepancies are more indicative of the chemistry working at small amounts of DNA, rather than how well the two methodologies compare. As the sensitivity of the test is listed at a single zoospore equivalent (Boyle *et al*. 2004), this fits with why both methods are inconsistent at very low estimates.

Of the 53 samples that were positive for *Bd* (using any of the methods), 44 samples were detected positive across all three; showing an 85% correspondence between methodologies. Interestingly, even the low volume methodology fared well in estimating overall prevalence. No single method appeared to be superior in estimating prevalence, though there were false negatives that appeared in all three of the methods, including the standard method. The miss rate is very low though (2–3

samples per method) and should be of no surprise given the minute levels of DNA the diagnostic test is examining in those samples. When examining samples with more than three zoospore equivalents detected, the correspondence of positive detection was very high (24/25) between all the methods.

PATHOGEN LOAD

Previous work has shown that fast qPCR can be used to detect *Bd,* but no sample for sample comparison had yet been done (Ruthig & DeRidder 2012). This study shows a close approximation of zoospore counts between fast and standard methods (Fig. 1), where range in loads is from 0–1500 zoospores μL^{-1}. This is evidenced by the small average difference of less than a single zoospore. All estimates of paired samples were within one order of magnitude difference between the two methods. The variation between methods is similar to variation our laboratory can see upon rerunning the same samples using the same methodology.

This study also reveals that a low volume qPCR does not provide similar results as the other two methods for estimating load. Although the low volume qPCR was effective in detecting the presence of the pathogen, it consistently underestimated the overall load of zoospores (Fig. 1). This result is not surprising and is clearly the reason that higher volumes are needed for standard qPCR quantification of *Bd*. It is of clear importance that one must utilize fast chemistry to run low volume reactions effectively.

CAVEAT

While utilizing fast chemistry should be generalizable to other pathogen detections, there is variation in the ability of it to operate effectively. A separate laboratory that we know of attempted to utilize fast qPCR methodology using a different type of fast chemistry and exhibited poor correspondence (Richards-Hrdlicka 2012). These differences might be due to differences in the model of qPCR machine used, the chemistry utilized, or to slight differences in technique or pathogen load. It has been noted that fast qPCR Taqman can become unstable if left at room temperature for extended periods of time. Our cold and quick preparation of samples might be a key aspect of utilizing fast qPCR with *Bd* and other pathogens. Our methodology of keeping all the reagents cool until loading the plate onto the qPCR machine could be a key factor in fast chemistry performance. It should be noted that an advanced fast TaqMan has been released that is able to stay stable at longer periods of time at room temperatures. We have utilized this chemistry in noncomparative diagnostic tests and it appears to function similarly well as the previous fast chemistry.

Conclusion

In summary, where equipment is available, fast qPCR is an acceptable and potentially preferred methodology for quantifying *Bd*. Using fast qPCR can easily double the number of samples previously able to be analysed for the same cost. This is of utmost importance given the limited funding available for the detection of *Bd*. This ability to run more samples could have important implications *Bd* research, as sample collection is cheap compared with the cost to analyse samples. As with many other fields, this technological development should greatly enhance our abilities to better do diagnostic disease science.

Future work is needed to determine whether fast qPCR is as effective as standard qPCR in other nonamphibian pathogens. Previous studies, (Yoder & Fishel 2008) and (Hilscher, Vahrson & Dittmer 2005), also address the issue of reliability of the newly developed fast qPCR chemistry, in human diseases but exhibit opposing results. Ultimately, fast qPCR can provide significant savings on both time and cost of analysis, but caution should be used when examining particular pathogens at particularly low loads with new techniques.

Acknowledgements

We would like to thank several funding agencies that made this work possible. The samples were run on a qPCR machine provided via NSF MRI-0923419. Support for students on this work was provided by the Department of Biology at the University of South Dakota and by the Game, Fish, and Parks Department of the State of South Dakota. We would also like to thank those that were involved in sample preparation and reviewing earlier drafts of this manuscript: M. Noeprokroeff, H. Britten, K. Richards-Hrdlicka, S. Nash, and T. Snyders.

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Received 30 July 2012; accepted 16 September 2012
Handling Editor: Robert Freckleton