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NOTE

Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians

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ABSTRACT: Chytridiomycosis, an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is associated with amphibian population declines worldwide. Investigation of the origin and spread of the pathogen requires examination of archived museum specimens of amphibians. Examination for *Bd* infection is usually done using histological techniques, which are often too destructive for valuable museum material. Three alternative methods for *Bd* detection (skin swabbing, brushing and scraping) were evaluated for ability to yield *Bd* DNA and destructiveness to specimens. Archived amphibians known to be *Bd* positive and which had been preserved in either formalin or ethanol for many years were used. Samples were analysed using a *Bd*-specific quantitative real-time Taqman PCR (qPCR) assay. There was no difference in the ability of each of the techniques to detect *Bd* infection, with the pathogen being detected in 75 to 81% of the 16 ethanol-fixed frogs examined. Visible evidence of sampling was left by scraping, but not by swabbing or brushing. The brush-qPCR technique detected higher counts of genomic equivalents than the other 2 sampling methods, although differences were not statistically significant. The qPCR assay did not detect *Bd* from any of the 6 formalin-fixed frogs examined, regardless of the sampling method. Nondestructive sampling techniques enable qPCR analysis of ethanol-preserved museum specimens for *Bd*. Recently, the incorporation of DNA cleanup steps allowed the detection of *Bd* in destructively sampled tissues from formalin preserved specimens. Further studies using nondestructive sampling incorporating DNA cleanup steps for the detection of *Bd* in formalin preserved specimens are warranted.

KEY WORDS: *Batrachochytrium dendrobatidis* · Amphibian chytridiomycosis · Amphibian declines · Museum specimens · Emerging infectious disease

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INTRODUCTION

Chytridiomycosis is a recently described emerging disease of amphibians caused by the nonhyphal zoosporic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998, Longcore et al. 1999). It has been implicated in epizootic amphibian mortality with resultant population declines on a global scale (Skerratt et al. 2007) and amphibian extinctions in Australia and Central and South America (Ron et al. 2003, La Marca et al. 2005, Alan Pounds et al. 2006, Schloegel et al. 2006). This highly pathogenic, virulent and readily transmissible emerging disease has no precedent in historical times (Skerratt et al. 2007). It has been described as the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and has a propensity to drive species to extinction (Gascon et al. 2007).

A range of diagnostic assays exists for the detection of *Bd* in live larval, post-metamorphic and adult amphibians (Hyatt et al. 2007). These include histopathology (Berger et al. 1998), immunohistochemistry (Berger et al. 2002, Van Ells et al. 2003, Olsen et al. 2004), electron microscopy (Berger et al. 2002), conventional poly-

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merase chain reaction (PCR) assay (Annis et al. 2004) and quantitative real-time Taqman PCR (qPCR) assay (Boyle et al. 2004). In order to determine the time and location of the emergence or introduction of this pathogen in different regions worldwide, it is important to examine archived specimens housed in museums and research institutions. Histopathology and immunohistochemistry are usually carried out to diagnose *Bd* infection from archived amphibians (e.g. Weldon et al. 2004, Ouellet et al. 2005), although recently, Walker et al. (2008) used qPCR to detect *Bd* infection in skin samples removed from formalin-fixed amphibian specimens. For each of these techniques, however, destructive sampling of specimens is necessary as toe clips or excised skin from the pelvic patch, hind limbs or the interdigital webbing are required (Green & Kagarise Sherman 2001, Lips et al. 2003, Puschendorf 2003, Weldon et al. 2004, Ouellet et al. 2005, Speare & Berger 2005, Walker et al. 2008), rendering these techniques unsuitable for the study of valuable museum specimens. When compared with histopathology and immunohistochemistry, qPCR has been demonstrated to be faster and to have higher sensitivity, repeatability and reproducibility, mainly because of its ability to detect the chytrid fungus at lower concentrations and at earlier stages of infection (Boyle et al. 2004, Kriger et al. 2006, Hyatt et al. 2007). Also, when compared to conventional PCR, qPCR is faster, more sensitive and able to give objective, accurate quantification of unknowns (Annis et al. 2004, Boyle et al. 2004, Kriger et al. 2006).

In the present study, we investigated the use of 3 alternative, non-invasive methods to sample archived amphibians for the detection of *Bd* using qPCR assay. We compared the ability of each method to detect *Bd* infection in known-positive ethanol- and formalin-fixed specimens and assessed the apparent destructiveness of each sampling technique, and hence the likely acceptability of each method to collection curators.

MATERIALS AND METHODS

Ethanol-fixed amphibians. We examined 14 adult midwife toads *Alytes obstetricans* and 2 adult fire salamanders *Salamandra salamandra*, which had been found dead in the wild in Spain between 2003 and 2005 and which had been fixed and preserved in 70% ethanol since collection. All specimens were sampled for *Bd* detection prior to preservation and all had been diagnosed as being *Bd* positive using either qPCR testing of unfixed skin or by histopathological examination of formalin-fixed skin (removed and fixed in formalin at the time of carcass collection) (S. F. Walker et al. unpubl. data). These specimens had been stored in ethanol for 2 to 4 yr prior to testing.

Formalin-fixed amphibians. We also examined 4 adult Mallorcan midwife toads *Alytes muletensis* and 2 adult Cape clawed frogs *Xenopus gilli* which had died with *Bd* infection in a captive collection. These specimens were originally diagnosed as being infected with *Bd* using histopathological examination or by qPCR. The *A. muletensis* specimens had been stored in 10% neutral buffered formalin for between 12 and 19 yr, while the *X. gilli* had been stored in formalin for 16 yr prior to testing.

Sampling techniques. Each of the preserved amphibians was subjected to each of the 3 sampling methodologies in the following order: (1) swabbing the skin with an MW100 sterile cotton-tipped swab (Medical Wire & Equipment); (2) brushing the skin with an interdental refill tapered brush (3.2 to 6.0 mm; Oral B Laboratories); and (3) scraping the skin with a No. 20 sterile scalpel blade (Swann-Morton). Specimens were firmly sampled with 4 strokes each along their ventral abdomen and pelvis, each ventral hind limb and the plantar surface of each hind foot. In order to minimize the possibility of false positives, each specimen was individually sampled so as to prevent possible cross contamination and a new pair of disposable gloves was used for each specimen. Visual assessment of damage to each specimen was made following each sampling procedure.

DNA extraction and real-time PCR assay. After sampling, the cotton tip of the swab, the whole interdental brush or the scraped material obtained were deposited separately in 1.5 ml Eppendorf tubes containing 50 µl of PrepMan Ultra (Applied Biosystems) and between 30 to 40 mg of zirconium/silica beads of 0.5 mm diameter (Biospec Products). DNA was extracted from each sample using the method described by Boyle et al. (2004) and, for each sample, a 1 in 10 aqueous dilution of DNA was stored at −80°C until processing. The diluted nucleic acid extracts were subsequently analysed using a *Bd*-specific qPCR assay of the ITS-1/5.8S ribosomal DNA region of *Bd*, using a Prism 7700 Sequence Detection System (Applied Biosystems) (Boyle et al. 2004, Hyatt et al. 2007). For each sample, the diagnostic assay was performed in duplicate, and standards of known *Bd* zoospore concentrations and negative controls were included with each PCR plate. A sample was considered to be positive when: (1) amplification (i.e. a clearly sigmoid curve) occurred in both replicated PCR reactions, and (2) values >0.1 genomic equivalents (GE) were obtained from both replicated reactions.

Statistical analysis. The GE values obtained for each of the 3 sampling techniques for the ethanol-fixed specimens were compared using the Friedman nonparametric 2-way ANOVA (SPSS 13.0). Zero GE values for the ethanol-fixed specimens were included in the analysis.

RESULTS

Thirteen of the 16 ethanol-fixed specimens were found to be *Bd* positive using the skin-swabbing technique; both skin-brushing and skin-scraping detected *Bd* infection in 12 of the ethanol-fixed amphibians, but *Bd* was not detected in any of the samples taken from the formalin-fixed amphibians. The qPCR results with GE values for each of the 3 sampling techniques used on the ethanol-fixed amphibians are presented in Table 1. The brush-qPCR technique detected higher counts of GE (30 998 ± 27 239 CI (95 %)) than either the swab-qPCR (7892 ± 13 805 CI) or the scrape-qPCR (23 783 ± 30 417 CI) methods, but this difference was not statistically significant (Friedman test; $p = 0.29$). Neither the skin-swabbing nor the skin-brushing technique caused visible damage to any specimen, but the skin-scraping technique caused visible loss of epithelium, which would be considered unacceptable by many museum curators.

DISCUSSION

Each of the 3 non-invasive sampling techniques used on known *Bd* positive, ethanol-fixed amphibians provided samples in which *Bd* DNA could be detected using the qPCR assay. Fourteen of the 16 ethanol-fixed specimens were positive to at least 2 sampling protocols (Table 1). Only specimen AU05117 was negative for all 3 different sampling methods. In 2 specimens, *Bd* was not detected using the skin swab, but it was detected either by both the interdental brush and the skin scrape methods (AU05120) or by the skin scrape method alone (CN05023). This might have been due to the more abrasive nature of the last 2 sampling techniques, possibly detecting intracellular zoosporangia located deeper in the skin layers.

Table 1. *Alytes obstetricans* and *Salamandra salamandra*. qPCR results including genomic equivalents (GE) obtained from 14 midwife toads and 2 fire salamanders preserved in 70 % ethanol. Samples were obtained by swabbing, brushing or scraping the skin and assayed using *Bd*-specific qPCR

Species Specimen	Skin swab Result	 GE	Skin brush Result	 GE	Skin scrape Result	 GE
A. obstetricans						
PY03069	+	174	+	2154	+	14
PY03070	+	243	+	510	–	0
PY03071	+	291	+	93	–	0
CN05015	+	349	+	696	+	5
CN05021	+	7	+	58	+	20
CN05023	–	0	–	0	+	144
CN05029	+	19	+	3808	+	1036
CN05030	+	303	+	16387	–	2.1×10^{-3}
CN05031	+	10101	+	78398	+	58845
CN05032	+	357	–	1.7×10^{-2}	+	1343
AU05117	–	0	–	0	–	0
AU05120	–	0	+	150581	+	227608
AU05121	+	113138	+	99669	+	91273
AU05122	+	1037	+	143599	+	9
S. salamandra						
PEN05189	+	14	–	0	+	6
PEN05191	+	234	+	17	+	220

In 2 specimens, *Bd* was detected using the skin swab and by the skin scrape, but not using the interdental brush, while in 3 specimens the skin scrape was the only technique which failed to detect *Bd*. The specimens were sampled using a swab first, followed by an interdental brush and finally via a skin scrape. It is possible that this sequence of sampling may have influenced the outcomes to some degree.

Regardless of the method used to sample the *Bd*-infected frogs, real-time PCR failed to detect infection in formalin-fixed frogs. Formaldehyde causes DNA degradation and cross-linking, inhibiting the PCR reaction, and these changes occur to an increasing degree the longer the period of fixation (Miething et al. 2006). This apparent incapacity of the qPCR assay prevents the widespread use of this diagnostic method for museum amphibians that have been stored in formalin. Nevertheless, new DNA extraction protocols from formaldehyde-fixed tissue have been developed, including one for the PCR detection of amphibian ranaviruses (Kattenbelt et al. 2000). Recently, *Bd* DNA from formalin-fixed amphibians (*Alytes nuletensis* and *Xenopus gilli*) was amplified from skin samples using the qPCR assay applied in the current study (Walker et al. 2008). Walker et al. (2008) purified skin samples using the Qiagen DNeasy blood and tissue DNA extraction kit (Qiagen) prior to DNA amplification (see supplementary information in Walker et al. 2008). Based on these findings, a protocol for PCR-based detection of *Bd* should include a DNA-cleanup stage prior to qPCR amplification. This modification might increase the usefulness of qPCR for the detection of *Bd* infection in archived amphibians.

Sampling archived amphibians in museums for *Bd* infection can be problematic, partly because of an understandable reluctance of institutions to permit the destructive sampling of their specimens. Neither the skin swabbing nor the skin brushing techniques used in the current study left visible signs of sampling. To date, the swab-qPCR technique is the preferred method to diagnose the chytrid fungus from live amphibians (Kriger et al. 2006). Although no statistically significant difference in the GE values was detected by the sampling methods used, the interdental brush technique is a modification of the swabbing technique which appears to have the ability to detect a higher GE than either the skin-swab or the skin-scraping methods. The skin-brushing technique, therefore,

might be more likely to detect *Bd* in specimens with low levels of infection, particularly when compared to the other nondestructive (skin-swabbing) technique, which gave the lowest GE values. It is possible that the apparent increased harvesting of *Bd* by skin brushing would become statistically significant with a larger sample size. Also, the brush-qPCR method has been used to successfully detect *Bd* DNA infection of the oral discs of an archived, ethanol-fixed *Xenopus laevis* tadpole (C. Soto-Azat unpubl. obs.). We therefore recommend the skin-brushing technique for the nondestructive sampling of fixed amphibian specimens, including museum reference specimens. Interpretation and use of the results would, however, need to consider the collection, preservation and storage history of the specimens tested. For example, fixative might be changed from formalin to ethanol over time, or a mixture of *Bd* positive and *Bd* negative specimens might have been stored together possibly resulting in cross-contamination. The amplification of *Bd* DNA from the skin of formalin-fixed amphibians has been demonstrated by Walker et al. (2008) with the incorporation of DNA cleanup steps. It is possible that the nondestructive skin-brushing technique would also be successful for formalin-fixed specimens if DNA cleanup steps are adopted. The interdental brush method described here would also be likely suitable for the nondestructive sampling of museum specimens for host or pathogen DNA for other purposes.

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