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Potential Concerns with Analytical Methods Used for the Detection of *Batrachochytrium salamandrivorans* from Archived DNA of Amphibian Swab Samples, Oregon, USA

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As amphibians are among the most threatened groups of vertebrates on the planet (Daszak et al. 2000; Wake and Vredenburg 2008; Hoffmann et al. 2010), rapid responses have been developed to characterize threats such as emerging infectious diseases (e.g., emergency management techniques, formulated research methods, and disease surveillance) (Olson et al. 2013; Alroy 2015; Yap et al. 2015). Chytridiomycosis is an amphibian disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) (Rosenblum et al. 2010) and the more recently described *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al. 2013). *Bsal* is implicated in salamander die-off events in Europe (Martel et al. 2013) and was found to be lethal to multiple Western Palearctic salamander species in a laboratory challenge experiment (Martel et al. 2014). Additionally, exposed frogs resisted infection in this study (N = 5) (Martel et al. 2014). Discovery that *Bsal* can be carried by at least three Asian salamander species commonly found in international trade (e.g., Japanese Fire-bellied Newt [*Cynops pyrrhogaster*], Chuxiong Fire-bellied Newt [*Cynops cyanurus*], and Tam Dao Salamander [*Paramesotriton deloustali*]) elevated concerns for inadvertent human-mediated range expansion and subsequent exposure to naïve amphibian hosts, i.e., those with no acquired immunity (Martel et al. 2014; Grant 2015; Gray et al. 2015). *Bsal* has been suggested to be of Asian origin (Martel et al. 2013; Martel et al. 2014), but has yet to be detected in large-scale surveys across China in wild and captive amphibians, or in museum specimens (Zhu 2014). Discovery of *Bsal* in a captive salamander collection in the United Kingdom, and associated mortality, further raised concerns for trade-mediated disease risk (Cunningham et al. 2015). The current distribution of *Bsal* is unknown; however, two risk models identified regions in North America as highly vulnerable to *Bsal*, including areas in the western USA (i.e., the Sierra Nevada and Pacific Northwest: (Yap et al. 2015; Richgels et al. 2016). *Bsal* has not been reported among North American salamanders to date but this could be due to a need for enhanced biosurveillance.

In 2015, we analyzed archived extracted DNA for the presence of *Bsal*: 385 samples from the order Caudata and 797 samples from the order Anura, (Table 1). Anuran samples were included because of their significance with *Bd* infection and transmission and uncertainty relative to *Bsal* infection, even though they have not been reported to be infected by *Bsal* during laboratory challenges (Martel et al. 2014). Herein we report that two samples had *Bsal*-positive results via qPCR and Sanger sequencing, from an adult Rough-Skinned Newt (*Taricha granulosa*) and American Bullfrog (*Lithobates catesbeianus*) larva, but we could not confirm the *Bsal*-positive finding using an additional analysis (end point PCR of 28S gene region). This leads us to believe that we do not have *Bsal* and the results could only be explained by contamination or an organism similar to *Bsal*. The objectives of this paper are to: 1) detail the methods and results of analyses of archived samples of extracted DNA for the presence of *Bsal*—our samples were derived from amphibian skin swabs originally collected for a *B. dendrobatidis* study in Oregon, USA (Chestnut 2015); and 2) discuss implications for future studies and monitoring projects. Our intent here is to document important lessons-learned for other similar *Bsal* investigations due to the high-priority of *Bsal* research as an emerging infectious disease in North America and elsewhere.

Methods

DNA extraction and quantification standards.—Genomic DNA (gDNA) was extracted from skin swabs collected at 16 randomly selected wetlands in western Oregon from December 2011 through December 2012 for a different study (Chestnut 2015). These swabs were originally placed in a sterile 2.0 microcentrifuge tube and stored on ice until they could be

TABLE 1. Species tested for *Batrachochytrium salamandrivorans* (*Bsal*) in Oregon, USA, including number of individuals sampled that were positive for *Batrachochytrium dendrobatidis* (*Bd*+) in 2012 (Chestnut 2015), the number of individuals that were positive for a *Batrachochytrium salamandrivorans*-like (*Bsal*-like+) organism via qPCR in 2015 and the total number of sites with either *Bd*+ or *Bsal*-like+ organisms via qPCR (out of 16 total sites). The phrasing "*Bsal*-like" is used because end-point PCR did not confirm our qPCR finding. NA = Not Applicable.

Species	Total no. animals sampled	No. *Bd*+ individuals	No. *Bsal*-like+ individuals	No. *Bd*+ sites	No. *Bsal*-like+ sites
Ambystoma gracile Northwestern Salamander	72	0	0	NA	NA
Ambystoma macrodactylum Long-Toed Salamander	91	0	0	NA	NA
Taricha granulosa Rough-Skinned Newt	222	2	1	1	1
Lithobates catesbeianus American Bullfrog	630	51	1	5	1
Pseudacris regilla Pacific Treefrog	156	4	0	2	NA
Rana aurora Northern Red-Legged Frog	11	0	0	NA	NA

stored at 4°C in the laboratory. Samples were extracted within four months of collection and analyzed for *Bd* by qPCR in 2012 (Chestnut 2015). All extracted DNA from these 2012 archived samples have been stored for three years at -20°C prior to *Bsal* analyses for this study in 2015.

A 161-base pair (bp) *Bsal* target sequence was synthesized based on the positions of the previously published TaqMan assay primer (Blooi et al. 2013) and the publicly available genomic database NCBI reference sequences NR_111867/KC762295. The synthetic DNA was amplified by PCR to create a double-stranded template, that was validated for purity and to be the predicted size using agarose gel electrophoresis. This synthetic DNA was then used as a positive control without an original isolated *Bsal* sample. PCR product was purified using the QIAquick PCR Purification Kit (Valencia, California), and quantified by Qubit dsDNA HS assay (ThermoFisher Scientific, Grand Island, New York). The number of amplicon copies present in the purified template solution was calculated as *number of copies (molecules) = (ng x 6.0221 x 10^{23} molecules/mole))/ (161 x 660 g/mole x 1 x 10^{9} ng/g)*. A tenfold serial dilution series containing 10^{9}–10^{1} copies/ µL of the *Bsal*-5.8S-ITS2 amplicon was prepared and used to create a standard curve for calibration of the qPCR assays.

Biological positive controls (gDNA extracted from *Bsal*) of the European *Bsal* isolate (KC762295) were kindly provided by An Martel and Mark Blooi (Ghent University, Belgium) and were included to verify qPCR and end-point PCR performance. All *Bsal* standards and positive controls were handled in an isolated clean room, and never opened nor stored around the Oregon samples. A non-template control, with water added instead of template, was run in triplicate for all qPCR assays. All primers, probe, and synthetic positive control oligonucleotides were obtained from IDTdna (Coralville, Iowa).

Real-time PCR (ITS qPCR).—DNA samples (N = 1182) were tested for the presence of *Bsal* using qPCR amplification of the 5.8S-ITS2 rDNA (Blooi et al. 2013). The TaqMan qPCR assay was performed on the ABI viiA™7 qPCR system using a 20 ul reaction containing 1ul DNA template, 1X Life Technologies TaqMan Environmental Master Mix 2.0 (Applied Biosystems®, Carlsbad, California), the previously described STerF and STerR primers (Martel et al. 2013) each at 300 nM, and a modified STerC *Bsal* probe (Blooi et al. 2013) at 250 nM. The STerC probe specific for the European *Bsal* was used (5' – 6-FAM/ACA AGA AAA TAC TAT TGA TTC TCA AAC AGG CA/3BHQ_2 – 3'). The amplification conditions consisted of: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min. Analysis of qPCR run quality was calculated using the manufacturer's software (ABI viiA™7 v1.2.1) and included the linear regression coefficient of determination (r^2) for standard dilution series and reaction efficiency calculations. Reaction efficiencies (*E)* were calculated from the slopes of the standard curve. Each sample was tested for the presence of *Bsal* and the number of copies present; samples containing ≥ 10 copies/ µL were scored as positive. The qPCR products of positive samples were cleaned with the Qiagen PCR purification kit (Valencia, California) prior to sequencing.

End-point 28S rDNA PCR.—DNA samples that were *Bsal*-positive with the qPCR assay (N = 2) and the *Bsal*-positive control were again tested for the presence of *Bsal* using end-point PCR amplification of 28S rRNA. Primers Bsal6Fwd (5'- ACG CTT GAA ACC AGT ATT GAG TG – 3') and Bsal6Rev (5' – TAC AGC TGC GTT CCT CAG TC – 3') were developed to target a unique 28S rRNA region in the European *Bsal* isolate. For PCR amplification, a 25-ul PCR cocktail containing 1 ul of sample and 1 µM of each primer was added to 1X Promega GoTaq® Green Master Mix (Madison, Wisconsin). Amplifications were run with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 35 s at 95°C, 90 s at 57°C and 45 s at 72°C. Products were subjected to a final extension at 72°C for 7 min. After amplification, 5 µL of the PCR product was validated visually by gel electrophoresis at 90V for 45 min on a 1.2% I.D.NA® agarose gel (FMC Bioproducts, Rockland, Maine). End-point PCR products were cleaned with the Qiagen PCR purification kit (Valencia, California) and stored at 4°C.

Sanger Cycle-Sequencing.—Confirmation of the qPCR products was performed via Sanger-cycle sequencing using the STerF and STerR primers. Direct sequencing reactions

were performed using BigDye® cycle sequencing kit (Applied Biosystems®, Carlsbad, California), and those reactions were cleaned with Agencourt CleanSEQ® (Beckman Coulter, Brea, California). The cleaned samples were sequenced in both directions using an ABI 3130XL genetic analyzer. Bsal6For and Bsal6Rev were used for sequencing the 28S rDNA region.

Results

Real-time PCR (qPCR) assay.—Two of 1182 (0.17%) samples detected *Bsal* via the TaqMan assay. Interestingly, these two samples were not run on the same plate, by the same technician, or on the same day. Oregon sample 1 had 99.08 ± 15.35 copies/µl and was DNA from an adult Rough-Skinned Newt from the central Willamette Valley. Oregon sample 2 detected *Bsal* in two of the three (triplicate) sample runs and had 8,075.58 ± 11,100.16 copies/µl of *Bsal*, and was DNA from a swab of an American Bullfrog larva sampled near Portland, Oregon. *Bd* was detected at these sites, but not in these samples, during previous analyses conducted in 2012 (Chestnut 2015) (Table 1).

Sanger cycle-sequencing.—To further confirm the sequence of TaqMan-positive samples, we performed Sanger cycle-sequencing of the two positive qPCR products. These 120-bp sequences were 100% identical to GenBank accession NR111867.1.

End-point PCR.—We developed a primer set for the 28S rDNA gene of *Bsal* to provide a second locus for confirmation of *Bsal*. This end-point PCR assay, however, did not amplify the target from either of the two 5.8S-ITS2 TaqMan-positive swab samples. Extracted DNA of the European *Bsal* isolate positive control was positive with these primers, with the amplification of the 207-bp product [accession #: KT805425].

Discussion

North America is home to approximately 50% of the world's salamander biodiversity, elevating concerns for the potential effects of emerging infectious diseases that affect multiple hosts such as *Bsal*, and development of rapid-response strategies (Gray et al. 2015; Grant et al. 2015). Native North American newts (*Notophthalmus viridescens, Taricha granulosa*) died from *Bsal* during laboratory challenge experiments conducted in Belgium (Martel et al. 2014), supporting the concern that amphibian populations across North America may be vulnerable to *Bsal* (Yap et al. 2015; Richgels et al. 2016). A 5.8S-ITS2 TaqMan qPCR assay was published by Blooi et al. (2013) for detection of *Bsal* that caused salamander die-offs in Europe. We detected *Bsal* DNA using this qPCR Bsal assay, and confirmed this result via sequencing of the qPCR product. In our laboratory, the assay amplified positive control DNA as well as two of 1182 Oregon samples, tested in triplicate, albeit with high variability in the one Oregon sample (#2) that only had two out of three (triplicate) samples wells test positive. The *Bsal* qPCR-detected swab samples were from an adult Rough-Skinned Newt and Bullfrog larva; to date, no infections of anurans have been reported (Martel et al. 2014). No additional DNA or archived material was available from the samples collected in 2011–2012, so no additional confirmation could be done on these samples.

Given the complex DNA pools present in environmental swab samples and the limited range of PCR conditions that can be tested, false positives or potential contamination are always a concern, particularly when an environmental assay is used in novel areas. We therefore sought confirmation by additional methods, especially as no *Bsal* sample had been isolated into culture from Oregon populations. Sanger sequencing of qPCR assay products confirmed the amplicon to be 100% identical to the expected *Bsal* product. Although we detected *Bsal* DNA using standard techniques (Hyatt et al. 2007; Blooi et al. 2013), additional end-point PCR did not verify the initial assay results. Therefore, we cannot be certain whether the source of this "*Bsal*-like" DNA is contamination, a novel *Bsal* strain, an undescribed *Batrachochytrium* species, or another microbe entirely. In the absence of a cultured isolate, we can only rely on sequence similarity to reference databases. As it stands, very little is known about *Bsal* and only five nucleotide sequences were present in GenBank (search date: 12 April 2016). There is a need for additional sequence data in the databases in order to design other assays and to learn more about the pathogenicity of *Bsal*. Although proper handling techniques were used, it is possible that the synthetic control aerosolized and contaminated the two positive samples. In future studies, only diagnostically synthetic oligonucleotides should be used as positive controls. Without this, any positive could be construed as potential contamination.

To help determine the presence of *Bsal* in the Oregon samples, we also developed an additional primer set that amplified a 28S ribosomal DNA region with the biological *Bsal* control. The product differentiated *Bsal* from other fungi in GenBank including *Bd*. Furthermore, no amplicon or synthetic template for this locus had ever been produced previously in our laboratory, reducing the potential for contamination. Reactions with these primers failed to amplify the expected product in the Oregon samples. Possible explanations for the negative result for this assay in the Oregon samples include variation in the 28S primer binding sites resulting in false negatives and that the original positives were in fact spurious amplifications, for which cross-contamination is a formal possibility. However, the pattern is not typical of contamination, as all replicates of the two samples were positive, negative controls were negative, and the two samples differed substantially and repeatedly in the amount of template detected by qPCR. Alternatively, a related but unknown chytrid may be present; *Bd* and *Bsal* are described species of the same genus because they are virulent pathogens with similar hosts and disease symptoms, but they are very distinct genetically and there may well be other cryptic species in the environment.

The result of this study emphasizes the need for further *Bsal* research in the Pacific Northwest and other regions of North America identified as being highly vulnerable to *Bsal*. It would also be advantageous to culture any isolate prior to identification to confirm the presence of a viable organism. Whereas microbial culture is possible with clinically diseased animals or field collections of specimens, the analysis of archived extracted DNA (as was used in this study) precludes this option. This study had multiple outcomes: 1) adoption in future work of a diagnostically synthetic control to assure no false positives; 2) development of US national protocols (USFWS 2016, FR Doc. 2016–00452); 3) informing several US national-scale procedures (e.g., *Bsal* Task Force organization and products: salamanderfungus.org); 4) development of *Bsal*-diagnostic primers for the 28S region; 5) detection of *Bsal*-like environmental sequences that future monitoring plans should be cognizant of when selecting genetic markers and interpreting the results.

At this time, we are taking a precautionary approach. The landowners of the sites where the *Bsal* qPCR-detections were

found have been notified, amphibian population monitoring is ongoing, and further work is underway to obtain more samples from Oregon amphibians. It is urged that laboratories with relevant sample archives test these samples for *Bsal*, ideally with a multilocus approach. Based on the patterns reported in the global *Bd* response system, it is not implausible that multiple strains of *Bsal* exist and that they differ from one another in their nuclear DNA content, ploidy of zoospores, and virulence towards various amphibian species. In summary, we have tested two loci for the presence of *Bsal* with inconsistent results, confirming the need for more rigorous testing and development of reliable diagnostic procedures in the event that rapid-response testing is needed. We suggest these tests include the diagnostically synthetic control (to ensure no false *Bsal*-positive results occur) along with a multilocus test that is broad range with longer amplicons to confirm the presence of *Bsal*.

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