ORIGINAL ARTICLE

Using environmental DNA for detection of *Batrachochytrium salamandrivorans* in natural water

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Abstract

Rapid, early, and reliable detection of invasive pathogenic microorganisms is essential in order to either predict or delineate an outbreak, and monitor appropriate mitigation measures. The chytrid fungus *Batrachochytrium salamandrivorans* is expanding in Europe, and infection with this fungus may cause massive mortality in urodelans (salamanders and newts). In this study, we designed and validated species-specific primers and a probe for detection of *B. salamandrivorans* in water. In a garden pond in close proximity to the *B. salamandrivorans* index site in the Netherlands, *B. salamandrivorans*-infected newts had been detected in 2015 and have been monitored since. In 2016 and 2017, no *B. salamandrivorans* was detected at this site, but in 2018 *B. salamandrivorans* flared up in this isolated pond which allowed validation of the technique in situ. We here present the development of an environmental DNA technique that successfully detects *B. salamandrivorans* DNA in natural waterbodies even at low concentrations. This technique may be further validated to play a role in *B. salamandrivorans* range delineation and surveillance in both natural waterbodies and in captive collections.

KEYWORDS

amphibians, *Batrachochytrium salamandrivorans*, early detection, emerging infectious diseases, environmental DNA

1 | INTRODUCTION

The emerging pathogen *Batrachochytrium salamandrivorans* (Bsal) causes mass mortality events of fire salamanders (*Salamandra salamandra*) in Europe. Effective disease management depends on data availability (e.g., data on pathogen distribution), and during all epidemic phases (Langwig et al., 2015), active and passive surveillance are key elements in determining infection status and the required conservation actions (Lawson, Petrovan, & Cunningham, 2015; Spitzen-van der Sluijs, 2018). The current scattered distribution of Bsal in Europe (Dalbeck et al., 2018; Spitzen-van der Sluijs et al., 2016), the poor detectability of sick and deceased individuals (unpubl. data) and the variable prevalence in wild salamander and newt populations (Dalbeck et al., 2018; Spitzen-van der Sluijs et al., 2016; 2018; Yuan et al., 2018), is factors that make active surveillance for Bsal cost-ineffective, while Bsal has the potential to further expand

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(Beukema et al., 2018) either via anthropogenic or natural pathways (e.g., Fitzpatrick, Pasmans, Martel, & Cunningham, 2018; Stegen et al., 2017). Active surveillance for Bsal is performed by collecting skin swabs from the amphibian host (Hyatt et al., 2007), and then analyzing these swabs for the presence of Bsal DNA using quantitative PCR (qPCR) techniques (Blooi et al., 2015a; 2015b). The reliable detection of Bsal at low prevalence requires sampling a large number of individuals (DiGiacomo & Koepsell, 1986) and is therefore expensive. Passive surveillance for infection in newts during their aquatic phase is unreliable, as hosts can carry infections asymptomatically (Stegen et al., 2017), and dead newts quickly decompose in water.

The analysis of environmental DNA (eDNA) (i.e., DNA obtained from environmental samples without the prior isolation of any target organism; Tab erlet, Coissac, Hajibabaei, & Rieseberg, 2012) is now widely used as an alternative tool for species detection (Tsuji, Takahara, Doi, Shibata, & Yamanaka, 2019), especially for macroorganisms. This approach has been proven to have a higher detection capability and cost-effectiveness compared with traditional methods, for example, (Biggs et al., 2015). eDNA techniques have been demonstrated to be a reliable tool for the detection of *Batrachochytrium dendrobatidis* (Bd), and ranavirus in the environment even at early stage before any amphibian mortality has occurred (e.g., Kamoroff & Goldberg, 2017; Miaud, Arnal, Poulain, Valentini, & Dejean, 2019).

We here present the development of an eDNA technique, validated in aquaria and in a natural waterbody, that successfully detects the presence/absence of Bsal, even at low zoospore densities. This technique can be applied to study the expansion or presence, but not prevalence, of Bsal over larger areas in a reliable, cost-effective, and efficient way.

2 | METHODS

2.1 | Primer and probe validation

Species-specific primers and a probe were designed in this study for the amplification of a short Bsal DNA fragment of 54 bp by PCR. A 788 bp genomic sequence containing internal transcribed spacer 1 (partial), 5.8S ribosomal RNA (complete), and internal transcribed spacer 2 (partial) was used for the primer design (GenBank accession number KC762295). The PrimerQuest program (IDT, Coralville, USA; retrieved December 12, 2012 from http://www.idtdna.com/Scitools) was used to design the primers and probes that amplified a short fragment of 54 bp (primers excluded). The primers amplified the same internal region which is used for the detection of Bsal in skin samples by PCR (Martel et al., 2013). To test primer specificity, they were first validated in silico using the ecoPCR program (Bellemain et al., 2010; Ficetola et al., 2010) on the EMBL-Bank release 123, allowing three mismatches per primer. Then, an *in vitro* validation was undertaken using real-time PCR on one DNA sample from Bsal (183 GE) and *B. dendrobatidis* (Bd, 100 GE). The real-time qPCR was carried out using a final volume of 25 μl, containing 3 μl

of template DNA, 12.5 μl of TaqMan Environmental Master Mix 2.0 (Life Technologies), 6.5 μl of ddH$_2$O, 1 μl of forward primer (Bsal_F: CACATTGCACTCTACTTT, 10 μM), 1 μL of reverse primer (Bsal_R: AAGACAAGGAAATGAATTAAA, 10 μM), and 1 μl of probe (Bsal_Pr: 6-FAM- TGATTCTCAAACAGGCATACTCTAC -BHQ-1, 2.5 μM) using thermal cycling at 50°C for 5 min and 95°C for 10 min, followed by 50 cycles at 95°C for 30 s and 53.3°C for 1 min. To test the sensitivity of primer and probes, the limit of detection (LOD, i.e., the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, i.e., the lowest amount of target DNA that yields an acceptable level of precision and accuracy) were calculated by running a dilution series of a known amount of total Bsal DNA, ranging from 100 ng/μl (1.83 x 10^4 GE/μl) to 10^{-10} ng/μl (1.83 x 10^{-8} GE/μl) with 12 qPCR replicates per concentration below 10^{-3} ng/μl (1.83 x 10^{-1} GE/μl). Total Bsal DNA was extracted from a 5-day-old culture containing both sporangia and zoospores using Prepman Ultra reagent (Applied Biosystems), and the final DNA concentration was measured using a NanoDrop spectrophotometer. LOD and LOQ were calculated using the method proposed in Klymus et al. (2019).

The final validation was performed by spiking two liters of distilled water with a known amount of Bsal zoospores. Bsal zoospores in distilled water were collected from the Bsal type strain AMFP1, grown in TGhL broth as described before (Martel et al., 2013). The water samples were spiked with one, 10, 100, 1,000, or 10,000 Bsal spores, and three samples replicates were made per spore concentration. Bsal spores were collected in H$_2$O from a full-grown culture containing mature sporangia. Once the zoospores were released, the water containing the zoospores was collected and passed over a sterile mesh filter with pore size 10 μm (Pluristrainer, PluriSelect). The flow-through was used as the zoospore fraction (> 90% purity). In total 15 samples of 2L of water were obtained and then filtered through VigiDNA® 0.45-μM cross-flow filtration capsules (SPYGEN) using a sterile 100 ml syringe. The filter was filled with 80 ml of CL1 Conservation buffer (SPYGEN, le Bourget du Lac, France) and stored at room temperature before the DNA extraction. DNA extraction was performed following the protocol described in Miaud et al. (2019) in a dedicated room for water DNA sample extraction, equipped with positive air pressure, UV treatment, and frequent air renewal. Before entering this extraction room, personnel changed into full protective clothing comprising disposable body suit with hood, mask, laboratory shoes, overshoes, and gloves in a connecting zone. All benches were decontaminated with 10% commercial bleach before and after each manipulation. For DNA extraction, each filtration capsule, containing the CL1 buffer, was agitated for 15 min on an S50 shaker (cat Ingenieurbüro™) at 800 rpm and then the buffer was emptied into a 50-ml tube before being centrifuged for 15 min at 15,000 × g. The supernatant was removed with a sterile pipette, leaving 15 ml of liquid at the bottom of the tube. Subsequently, 33 ml of ethanol and 1.5 ml of 3M sodium acetate were added to each 50-ml tube and stored for at least one night at −20°C. The tubes were centrifuged at 15,000 *g* for 15 min at 6°C, and the supernatants were discarded. After this step, 720 μl of ATL buffer from the DNeasy

TABLE 1 The sampling events for *Batrachochytrium salamandrivorans* prevalence and infection intensity by eDNA samples and by swab samples in one garden pond in 2015, 2016, and 2018

Year/month	date (DD/MM/YY)	eDNA[a]	species (# P/# total)	GE (range)	
2015	April	01-04-15		Ia[b] (0/1); Lv[c] (0/5)	NA
	May	27-05-15		Ia (0/5); Lv (4/24)	193-2140 GE
	June	18-06-15		Ia (0/3); Lv (0/14)	NA
2016	March	31-03-16		Ia (0/4)	NA
	April	14-04-16		Ia (0/2); Lv (0/4)	NA
		19-04-16	N (0/12)	NA	NA
		22-04-16		Ia (0/1)	NA
		27-04-16		Ia (0/7); Lv (0/2)	NA
	May	11-05-16		Ia (0/6); Lv (0/4)	NA
	June	12-06-16		Ia (0/2); Lv (0/7)	NA
2018	April	05-04-18		Ia (0/9); Lv (1/4)	200 GE
		12-04-18	P (2/12) and P (1/12)	Ia (0/18); Lv (4/12)	22–44GE
		21-04-18	P (8/12) and P (7/12)	Ia (0/13); Lv (0/7)	NA
	May	23-05-18	N (0/12)	Ia (0/21); Lv (0/44)	NA
	June	12-06-18	N (0/12)	Ia (0/1); Lv (0/13)	NA
	August	13-08-18	N (0/12)	NA	
	September	14-09-18	N (0/12)	NA	

[a]N (no Bsal detected); P (Bsal detected) (nr positive replicates/nr. total replicates).

[b]*Ichthyosaura alpestris.*

[c]*Lissotriton vulgaris.*

Blood & Tissue Extraction Kit (Qiagen) was added. The tubes were then vortexed, and the supernatants were transferred to 2-ml tubes containing 20 μl of Proteinase K. The tubes were finally incubated at 56°C for two hours. Subsequently, DNA extraction was performed using NucleoSpin® Soil (MACHEREY-NAGEL GmbH & Co.) starting from step 6 and following the manufacturer's instructions. The elution was performed by adding 100 μl of SE buffer twice. After the DNA extraction, the samples were tested for inhibition by qPCR following the protocol described in Biggs et al., 2015. If the sample was considered inhibited, it was diluted fivefold before the amplification. The real-time amplification was carried out following the protocol described in the in vitro section with 12 qPCR replicates per sample. To detect potential contamination, one DNA extraction control and a qPCR negative control (with 12 replicates) were performed in parallel. A dilution series of DNA ranging from 1.83×10^3 GE/μl to 18.3 GE/μl was used as the qPCR standard. Samples were run on a BIO-RAD CFX96 Touch real-time PCR detection system in a room dedicated to amplified DNA analysis with negative air pressure and physically separated from the DNA extraction room.

2.2 | Study site

We sampled a garden pond (water surface 58 m²) situated in the Bsal index site in the Netherlands, the Bunderbos (Martel et al., 2013; site 3 in Spitzen-van der Sluijs et al., 2016). This pond is part of a large-scale Bsal survey and as such it has been, and still is, frequently sampled from 2015 onwards, by swab sampling of individual animals, as described in Blooi et al. (2013). In the pond, reproduction of smooth newts (*Lissotriton vulgaris*), alpine newts (*Ichthyosaura alpestris*), and common frogs (*Rana temporaria*) occur annually. Other present, but not reproducing species are common toad (*Bufo bufo*) and water frogs (*Pelophylax* sp.). These anuran species are considered to be resistant to a Bsal infection (Martel et al., 2014). There is no larval deposition of fire salamanders, but this latter species has been seen in the garden.

2.3 | Sampling protocol eDNA

Bsal produces motile zoospores and environmentally resistant, encysted spores that float on the water surface (Stegen et al., 2017). Therefore, water samples were collected only from the top 1 cm of the water in 2018, but prior to the Stegen et al. (2017) publication, water samples were also collected from 5–10 cm depth. The eDNA sampling was conducted before the swab sampling of the individual animals (Table 1) on the same days. An exception on this was the first sampling opportunity. On April 5, 2018, Bsal was sampled via swabs from individual newts. Upon the confirmation of Bsal presence, we subsequently collected eDNA and collected swabs from individual animals seven days later (April 12, 2018).

The field sampling for eDNA was performed following the protocol described in (Miaud et al., 2019). The sampling kits were composed of a sterile water sampling ladle, a self-supporting sterile Whirl-Pak® bag, a sterile syringe, gloves to minimize

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contamination, a VigiDNA® 0.45-µM cross-flow filtration capsule (SPYGEN), and a bottle containing 80 ml of CL1 Conservation buffer (SPYGEN). Using the ladle, subsamples of 100 ml water were collected around the pond margin to create a pooled water sample of approximately 2 L in the sterile self-supporting plastic bag. Samples were collected while the surveyor stood only on the pond bank or on muddy pond edges without entering the water to avoid possible contamination from the surveyors' boots, or by stirring up sediment. The water sample was homogenized by gently shaking the bag to ensure that eDNA was evenly mixed through the sample, and then the 2 L of sampled water was filtered through the VigiDNA 0.45 µm filter using a sterile 100 ml syringe, directly in the field. Subsequently, 80 ml of CL1 conservative buffer was added to the filter and the filters were stored at room temperature for a maximum period of six weeks before the DNA extraction. The DNA extraction and amplification were performed as described above in the primer and probe validation section.

2.4 | Sampling individual animals using swabs

Smooth newts and alpine newts were captured using two to six amphibian fykes (type: Laar M2, rectangular 30 x 30 x 50 cm, mesh size 4 mm, distribution: Laar Technology & Consulting Ltd.), that were placed at the edges of the pond for max 12 – 16hr and were left overnight. The owners did not want dip nets to be used and search intensity varied per field visit. During each sampling event, the garden (60 by 20 m) was searched for newts in their terrestrial phase as well. Ventral skin swabs were taken from postmetamorphic newts, using aluminium sterile cotton-tipped dry swabs (rayon-dacron, COPAN, UNSPSC CODE 41,104,116) following the procedure and biosecurity measures described in Hyatt et al. (2007) and Van Rooij et al. (2011). All samples were kept frozen at − 20°C until further analysis for the presence of Bsal DNA through real-time PCR, as described by Blooi et al. (2013). Skin histopathology as described in Martel et al. (2013) was performed to detect Bsal infection on dead newts. The aim during each site visit was to collect and sample at least 30 and maximally 60 specimens/visit.

3 | RESULTS

3.1 | Primer and probe validation

The primer pair and the probe showed 100% specificity both in silico and in vitro. The LOQ in this study was 4 GE/µl, and the LOD was 2.93e-06 GE/µl. The threshold cycles observed in the spiked water sample were below the LOQ, and thus it was not possible to correlate the number of spores with the quantity of DNA retrieved. For this reason, we used the number of replicates amplified in a sample for relative quantification, rather than reporting the amount of eDNA detected quantitatively, as suggested in Biggs et al. (2015). The number of replicates was positively correlated with the number

of spores spiked in the water sample (ANOVA p-value = 0.00245, Figure 1).
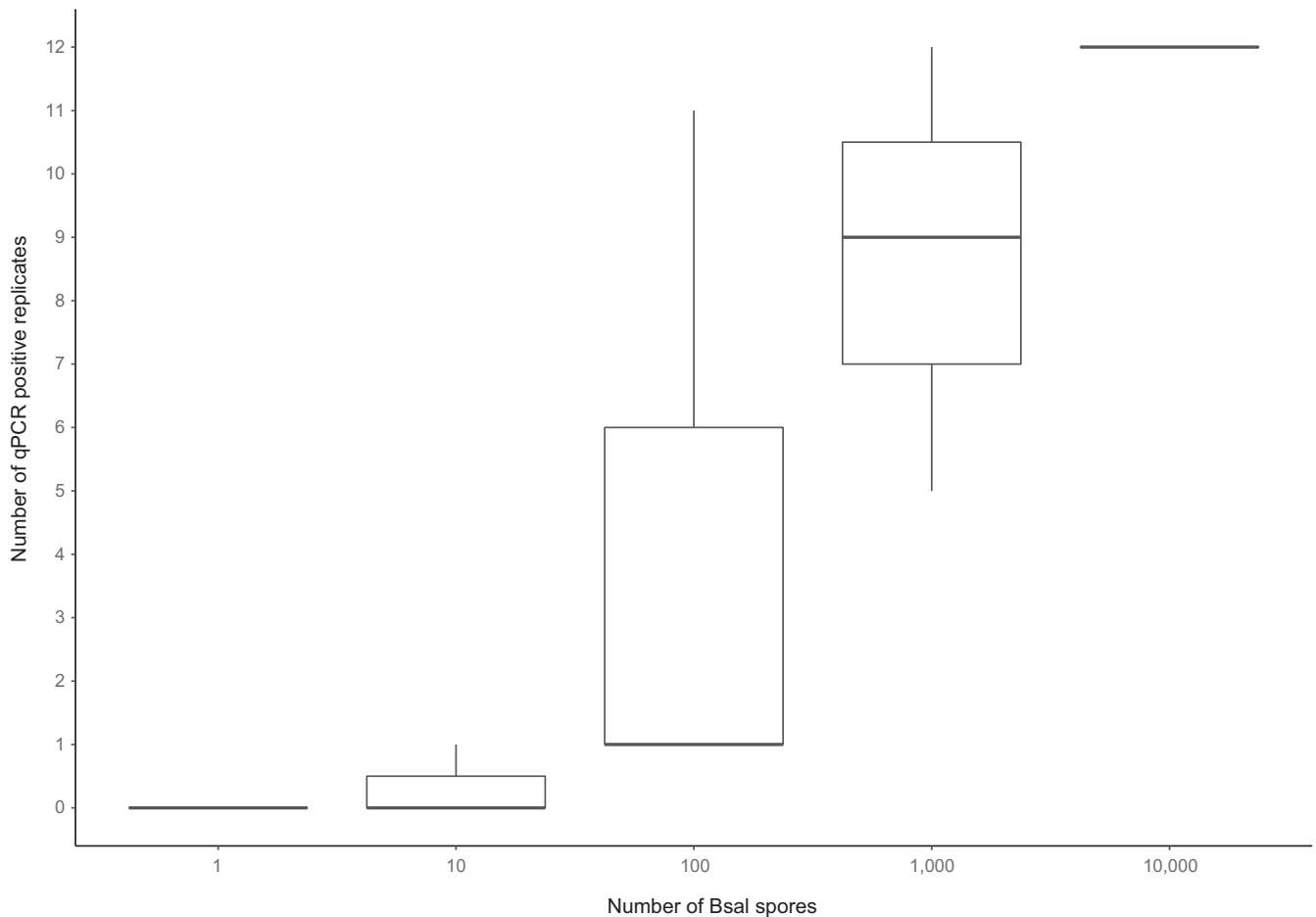
3.2 | Field sampling (swabs and eDNA)

We were able to validate the eDNA technique for Bsal in situ (Table 1). In the garden pond, Bsal DNA was detected on April 5, 2018 in skin swabs (a Bsal-positive smooth newt (200 GE)). The next sampling (12 April 2018) using both eDNA (two samples) and the traditional method (skin swabs) revealed Bsal in the eDNA samples (1 and 2 out of 12 qPCR replicates) and in the swabs (4/30), albeit with low infection loads (Table 1). Remarkably, during the third sampling occasion (April 21, 2018), the collected eDNA samples returned positive with a high number of positive PCR replicates (7 and 8 out of 12 qPCR replicates), but the collected 20 swabs tested negative for Bsal. At later sampling events, Bsal was no longer detected, neither in eDNA samples nor in the swabs. In August and September 2018, metamorphosed newts could not be found despite extensive search effort in the water and on land, presumably because of the extremely dry weather conditions. In June 2016, a dead alpine newt and four dead smooth newts were found, and in June 2018 one dead alpine newt was found. None of these animals tested positive for Bsal.

4 | DISCUSSION

The aim of this study was to test the potential of eDNA sampling for Bsal range delineation, and we have shown that this technique can be applied to detect Bsal in stagnant water.

A caveat of this study was the single study site, as well as the limited number of swabs and eDNA samples, especially in 2016. Due to the landscape configuration, not many ponds are present in the Bunderbos. This garden pond is the only pond in the Bunderbos where we could find Bsal-infected aquatic newts (in 2015 and again in 2018). Since its discovery in 2013, Bsal has continuously been present in the Bunderbos, but Bsal prevalence is low and in the other years, Bsal-infections in newts and salamanders have only been detected in terrestrial individuals (Spitzen-van der Sluijs et al., 2018; unpublished data). Budget restrictions did not allow for the collection of more samples in this study.

To detect Bsal, we used one filter per sampling event (collected with an integrative sampling to avoid the patchiness of eDNA in lentic environments (Harper et al., 2018) and 12 qPCR replicates, which is a similar replication level as suggested by (Schmidt, Kéry, Ursenbacher, Hyman, & Collins, 2013) and higher than suggested by Ficetola, Tab erlet, & Coissac (2016) and Mosher et al. (2017). At the site, no differences were observed in the two field replicates collected in parallel during two sampling sessions. The validation using water samples spiked with zoospores of Bsal gave similar results as were obtained in the experiment of Mosher et al. (2017) for Bd. These results confirm the reliability of the analysis for Bsal detection.

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FIGURE 1 Positive relationship between the number of spores spiked in the water sample (*x*-axis) and the number of qPCR positive replicates (*y*-axis)

A noticeable outcome is the detection of Bsal via eDNA but not via the swabs (Table 1), which could be explained by a low prevalence of the fungus. To detect a pathogen that occurs at low prevalence, sampling of a large number of individuals is required (DiGiacomo & Koepsell, 1986). At the time of our sampling (April 21, 2018) only 20 swabs could be collected (no more individuals could be caught). To detect Bsal with this limited sample size, the Bsal prevalence should range, depending on the level of confidence, between 15% and 20% (DiGiacomo & Koepsell, 1986). This prevalence is rather high for endemic situations and has so far only been observed during Bsal outbreaks in fire salamander populations and in Asian urodelan populations (Dalbeck et al., 2018; Laking, Ngo, Pasmans, Martel, & Nguyen, 2017; Spitzen-van der Sluijs et al., 2016; Stegen et al., 2017; Yuan et al., 2018). The objective to minimally sample 30 individuals/ visit was only reached twice during the entire study, and this unpredictability is inherent to fieldwork.

The fire salamander is a mainly terrestrial species, and as such the here described eDNA technique is less suitable to set the range of Bsal in terrestrial environments and in fire salamanders or other terrestrial species, yet the technique will be useful to demarcate the distribution of Bsal by Bsal-vectoring and susceptible newt species in their aquatic phase. These results highlight the high detection

capability and advances of our eDNA technique. It also shows it is a useful detection method complementary to the collection of skin swab samples. Collecting swabs remains necessary in active pathogen surveillance for detecting prevalence and infection intensity. Equally, passive surveillance for dead or moribund animals remains indispensable.

We have shown that eDNA sampling can be used to detect Bsal in water and as such the technique may be further validated to play a role in Bsal range delineation and surveillance in both natural situations and in collections. eDNA-based methods allow for rapid, reliable, and cost-effective screening for Bsal and can therefore be applied to monitor pathogen presence or absence in high-risk invasion areas or in collections. Also, eDNA-based methods will help to delineate the outbreak and allow for the evaluation of the effectiveness of management measures.

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