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Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens

Isaac Standish[1,*], Eric Leis[1], Noel Schmitz[1], Jeena Credico[2], Sara Erickson[1], Jennifer Bailey[1], Jacob Kerby[3], Kenneth Phillips[1], Teresa Lewis[2]

[1]US Fish and Wildlife Service, Midwest Fisheries Center, La Crosse Fish Health Center, Onalaska, WI 54650, USA
[2]US Fish and Wildlife Service, Midwest Fisheries Center, Onalaska, WI 54650, USA
[3]Biology Department, University of South Dakota, Vermillion, SD 57069, USA

ABSTRACT: Amphibian populations worldwide are facing numerous threats, including the emergence and spread of infectious diseases. In the past 2 decades, *Batrachochytrium dendrobatidis* (*Bd*), a parasitic fungus, and a group of viruses comprising the genus *Ranavirus* have become widespread and resulted in mass mortality events and extirpations worldwide. In 2013, another novel fungus, *B. salamandrivorans* (*Bsal*), was attributed to dramatic declines in populations of fire salamander *Salamandra salamandra* in the Netherlands. Experimental infections demonstrated that *Bsal* is highly pathogenic to numerous salamander genera. In an effort to prevent the introduction of *Bsal* to North America, the US Fish and Wildlife Service (USFWS) listed 201 salamander species as injurious wildlife under the Lacey Act. To determine infection status and accurately assess amphibian health, the development of a sensitive and specific diagnostic assay was needed. We describe the optimization and validation of a multiplex quantitative polymerase chain reaction (qPCR) protocol for the simultaneous detection of *Bd*, *Bsal*, and frog virus 3-like ranaviruses. A synthetic genome template (gBlock®) containing the target genes from all 3 pathogens served as the positive control and allowed accurate quantification of pathogen genes. The assay was validated in the field using an established non-lethal swabbing technique to survey local amphibian populations throughout a range of habitats. This multiplex qPCR demonstrates high reproducibility, sensitivity, and was capable of detecting both *Bd* and ranavirus in numerous locations, species, and life stages. *Bsal* was not detected at any point during these sampling efforts.

KEY WORDS: Multiplex qPCR · *Batrachochytrium dendrobatidis* · Ranavirus · *Batrachochytrium salamandrivorans* · Amphibian pathogens · Wisconsin

INTRODUCTION

Amphibia as a taxonomic class is highly diverse, representing species that can serve as both indicator and 'flagship' species for ecological health (Caro & O'Doherty 1999, Sewell & Griffiths 2009). However, amphibian populations have been in steady decline since the 1970s (Stuart et al. 2004, Lips et al. 2005, Sodhi et al. 2008, James et al. 2009, Scheele et al. 2017). Initially, some declines were linked to habitat destruction (Gupta 1998), ultraviolet (UV) radiation (Blaustein et al. 2003), or other causes (Johnson et al. 1999, Hayes et al. 2010). Evidence of the role that viral and fungal pathogens, specifically ranaviruses and the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), contributed to these declines began to be reported in the 1990s (Cunningham et al. 1996, Berger et al. 1998, Bollinger et al. 1999, Daszak et al. 1999).

Ranaviruses are a group of large enveloped double-stranded DNA (dsDNA) viruses in the genus *Ranavirus*, within the family *Iridoviridae*, which can

*Corresponding author: sirisaac_standish@fws.gov

infect 3 classes of poikilothermic vertebrates (Chinchar 2002, Chinchar et al. 2009): bony fishes (Waltzek et al. 2014), amphibians (Miller et al. 2011), and reptiles (Allender et al. 2013, Sim et al. 2016). Recent phylogenetic analysis indicates fish were the historic ranavirus host (Chinchar et al. 2011), but some strains have recently evolved into multi-taxonomic group pathogens (Schock et al. 2008, Brenes et al. 2014a, Price et al. 2017). There is also evidence that the highly virulent and widespread nature of ranaviruses (Hoverman et al. 2012) can result in population-level effects (Cunningham et al. 1996, Green et al. 2002, Teacher et al. 2010). Multiple large-scale ranavirus mortality events have been documented in several amphibian species (Miller et al. 2011), most notably in tiger salamanders *Ambystoma tigrinum* (Jancovich et al. 1997, 2001, Bollinger et al. 1999), and common frogs *Rana temporaria* (Cunningham et al. 1996). To date, at least 72 amphibian species have been shown to be susceptible to ranavirus infection (Miller et al. 2011).

Large-scale mortalities caused by *Bd* have resulted in amphibian population declines throughout the montane rainforests of Australia (Laurance et al. 1996, Retallick et al. 2004, Schloegel et al. 2006), Central America (Berger et al. 1998, Lips et al. 2006), and the western United States (Padgett-Flohr & Hopkins 2009, Vredenburg et al. 2010). At this point, *Bd* is indeed panzootic with positive detections in 52 of 82 countries sampled and detection in 42% of all the sampled species (Olson et al. 2013). This finding is troubling, as *Bd* dispersal will only further increase the risk of population declines of recorded amphibian species currently threatened with extinction (Monastersky 2014). Moreover, the discovery that *Bd* may reside on or within invertebrate hosts such as crayfish (McMahon et al. 2013, Brannelly et al. 2015, Betancourt-Román et al. 2016), freshwater shrimp (Rowley et al. 2006), and nematodes (Shapard et al. 2012) further complicates the disease dynamics of this pathogen.

In addition to *Bd*, another emerging chytrid fungus pathogen of international concern is *Batrachochytrium salamandrivorans* (*Bsal*). *Bsal* was recently isolated and characterized by Martel et al. (2013) following dramatic declines of fire salamanders *Salamandra salamandra terrestris* in the Netherlands (Spitzen-van der Sluijs et al. 2013), subsequently in neighboring Belgium (Spitzen-van der Sluijs et al. 2016), as well as captive urodelans in the UK and Germany (Cunningham et al. 2015, Sabino-Pinto et al. 2015). Phylogenetic analyses in combination with experimental infections — examining the host range of *Bsal* — indicated a potential origin and reservoir in southeast Asian salamander and newt species (Martel et al. 2014). In these initial experiments, *Bsal* infection was limited to urodelans (Martel et al. 2014); however, it does appear that anurans may also serve as reservoirs (Stegen et al. 2017). The experimental evidence indicating that *Bsal* has a wide urodelan host range sparked increased concern for salamander populations worldwide.

Scientists and researchers have formed an international *Bsal* task force and held workshops to create a strategic plan for the prevention of *Bsal* introductions into North America (Gray et al. 2015, Grant et al. 2016). One of the top priorities of this strategic plan was the establishment and deployment of standardized diagnostic practices for *Bsal* detection (White et al. 2016). Molecular assays such as quantitative polymerase chain reaction (qPCR) have been developed and proven highly valuable for the detection of both *Bd* (Annis et al. 2004, Boyle et al. 2004) and FV3-like ranavirus (Allender et al. 2013, Sim et al. 2016). However, when the established *Bd* assay (Boyle et al. 2004) proved unsatisfactory for the detection of *Bsal,* a second primer set was developed. These 2 primer sets were coupled in a duplex assay capable of detecting both pathogens simultaneously (Blooi et al. 2013). In the present study, we describe the validation and subsequent modification of this duplex qPCR with the goal of incorporating FV3-like ranavirus detection capabilities into a multiplex assay capable of detecting all 3 of these amphibian pathogens simultaneously using non-lethal methods. Field application of the assay was then demonstrated using a range of amphibian samples collected in 2016 and 2017.

MATERIALS AND METHODS

Gene fragments

Target amplicons from all 3 pathogens were commercially synthesized in a single gene block (gBlock®; Fig. 1) for use as positive control and quantitation standard. For *Bd* and *Bsal* detection, 146 and 161 base pair (bp) portions of the internal transcribed spacer (ITS-1) and 5.8 ribosomal genes from *Bd* and *Bsal* respectively (GenBank accession numbers AY598034, KC762295) were targeted as previously described (Boyle et al. 2004, Blooi et al. 2013, Martel et al. 2013). For FV3 detection, a 54 bp segment of portion of the major capsid protein (MCP) (Mao et al. 1997, Allender et al. 2013) was incorpo-

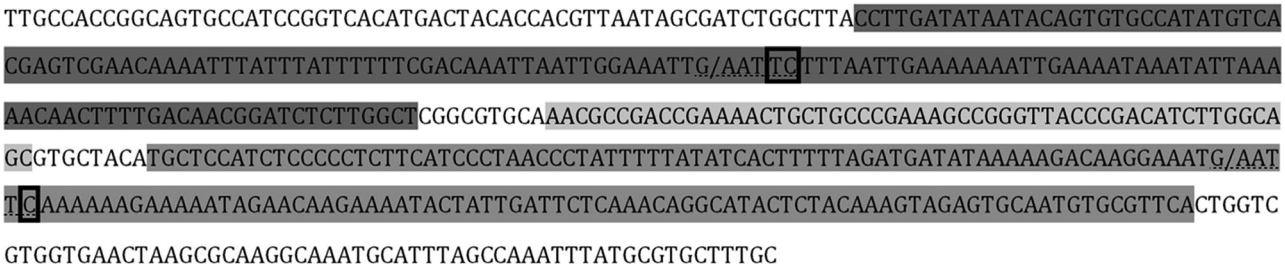

Fig. 1. gBlock® design. The 498 bp synthetic gBlock comprised 146 and 161 base pair (bp) portions (dark and mid-grey, respectively) of the internal transcribed spacer (ITS-1) and 5.8 ribosomal genes from *Batrachochytrium dendrobatidis* and *B. salamandrivorans,* respectively (GenBank accession numbers AY598034, KC762295). A 54 bp segment (light grey) of the ranavirus type species *Frog virus 3* (FV3) major capsid protein (MCP) gene was also incorporated into the gBlock (Allender et al. 2013). Additionally, 3 point transversions (identified with bold boxes) were incorporated to form 2 *Eco*RI restriction sites (G/AATTC)

rated into the gBlock®. Additionally, 3 point transversions of the native sequences of both *Bd* and *Bsal* were made to form 2 *Eco*RI restriction sites (G/AATTC) within the gBlock®. These restriction sites allowed for discrimination of native sequence and gBlock® contamination. Additional protective bases were added between each target amplicon and on each end of the gBlock®, totaling 498 bps with a molecular weight of 307536.2 g mol^{-1}. The gBlock® was initially rehydrated with 30 µl of 1× Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0; Sigma-Aldrich). The gBlock® was then quantified using a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific) and used to calculate copy concentration using the following formula:

$$\text{gBlock® copies } \mu l^{-1} = \frac{(\text{ng DNA } \mu l^{-1}) \times (6.022 \times 10^{23} \text{ copies mol}^{-1})}{(307536.2 \text{ g mol}^{-1}) \times (1 \times 10^{9} \text{ ng g}^{-1})}$$

Each 25 µl reaction contains 2.5 µl of gBlock® diluted using 100 ng ml^{-1} tRNA (Carrier RNA; Qiagen) in RNase-free H_2O (Thermo Fisher Scientific). Ten-fold serial dilutions were used to create an initial standard curve ranging from 10^8 to 1 copy $reaction^{-1}$.

qPCR

Optimization, validation, and field testing were conducted using a CFX96 Touch™ (Bio-Rad Laboratories). Amplification for both the singleplex and multiplex assays consisted of 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. The reagent concentrations were initially based on previous publications (Boyle et al. 2004, Allender et al. 2013, Blooi et al. 2013). Various master mixes were examined for the function in the multiplex, including the TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific), TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific), and the PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies). Master mixes were evaluated in triplicate reactions using the standard curve of 10-fold dilutions from 10^8 to 10 copies. The reaction efficiency, slope, and R^2 values were calculated with Bio-Rad CFX Manager v.3.0 (Bio-Rad Laboratories) using the Baseline Subtracted Curve Fit mode. The PrimeTime® Gene Expression Master Mix demonstrated the greatest efficiency, sensitivity, and R^2 values across all 3 targets simultaneously and therefore was selected for use in subsequent titration and sample analysis.

To decrease reagent competition during co-amplification within the assay, primer and probe concentrations were also assessed. Each pathogen assay was first optimized independently. The optimal concentrations for the *Bd* forward (Table 1; ITS1-3 Chytr; 5′-CCT TGA TAT AAT ACA GTG TGC CAT ATG TC-3′) and reverse (5.8S Chytr; 5′-AGC CAA GAG ATC CGT TGT CAA A-3′) primers were identified using a checkerboard titration in 0.1 µM increments from 0.9 to 0.1 µM using 0.15 µM of the FAM-labeled *Bd* probe (Chytr MGB2; 5′-FAM-CGA GTC GAA CAA AAT-MGBNFQ-3′). Ideal primer concentrations were identified as the minimum concentration needed to maintain the lowest cycle threshold (C_q) values throughout the standard curve. Probe concentrations were similarly titrated in 0.05 µM increments from 0.4 to 0.05 µM with the optimal concentration yielding the greatest relative fluorescent units (RFUs)

Table 1. Primer and probe sequences used in multiplex quantitative polymerase chain reaction (qPCR) for detection of *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*) and ranavirus (frog virus 3, FV3)

Name	Sequence (5′–3′)	Reference
Bd forward (ITS1-3 Chytr)	CTT GAT ATA ATA CAG TGT GCC ATA TGT C	Boyle et al. (2004)
Bd reverse (5.8S Chytr)	AGC CAA GAG ATC CGT TGT CAA A	Boyle et al. (2004)
Bd probe (Chytr MGB2)	FAM-CGA GTC GAA CAA AAT-MGBNFQ	Boyle et al. (2004)
Bsal forward (STerF)	TGC TCC ATC TCC CCC TCT TCA	Martel et al. (2013)
Bsal reverse (STerR)	TGA ACG CAC ATT GCA CTC TAC	Martel et al. (2013)
Bsal probe (STerC)	Cy5-ACA AGA AAA TAC TAT TGA TTC TCA AAC AGG CA-IAbRQSp	Blooi et al. (2013)
FV3 forward	AAC GCC GAC CGA AAA CTG	Allender et al. (2013)
FV3 reverse	GCT GCC AAG ATG TCG GGT AA	Allender et al. (2013)
FV3 probe	TexRed-CCG GCT TTC GGG C-IAbRSQSp	Allender et al. (2013)[a]

[a]While the probe sequence was described by Allender et al. (2013), the reporter and quencher molecules have been changed for use in this multiplex assay

without affecting the C_q values. An identical process was conducted to identify the ideal concentrations of the *Bsal* forward (STerF; 5′-TGC TCC ATC TCC CCC TCT TCA-3′), *Bsal* reverse (STerR; 5′-TGA ACG CAC ATT GCA CTC TAC-3′), and the Cy5-labeled probe (STerC; 5′-Cy5-ACA AGA AAA TAC TAT TGA TTC TCA AAC AGG CA-IAbRQSp-3′). Alterations were made to the previously described reporter and quencher molecules of the probe (Allender et al. 2013). The TexasRed fluorophore was selected for compatibility with the other pathogen targets and thermocycler detection channels; alternatively, the Tetrachlorofluorescein (TET) fluorophore was also explored and found compatible, although was not used due to incompatibility with many commercially available internal positive controls (IPCs). Concentrations of the FV3 forward (5′-AAC GCC GAC CGA AAA CTG-3′) and reverse (5′-GCT GCC AAG ATG TCG GGT AA-3′) primers were optimized as previously described at a probe (5′-TexRed-CCG GCT TTC GGG C-3IAbRSQSp-3′) concentration of 0.250 µM. The TexasRed probe was titrated from 0.7 to 0.1 µM in 0.05 µM increments. The assay described by Allender et al. (2013) likely has the ability to amplify a range of ranaviruses as the primer and probe sequence exactly match the MCP gene sequence of numerous FV3-like ranaviruses and Bohle iridovirus isolates (GenBank accession numbers: KM516716, JN615141, KJ703120, DQ335253, AB4744588,U82553, KX185156). Additionally, there are only 2 bp mismatches with isolates of epizootic haematopoietic necrosis virus (FJ433873), European catfish virus (KT989884), *Ambystoma tigrinum* virus (AY150217), pike-perch iridovirus (FJ358610), and the common midwife toad ranavirus (KJ703123). More extensive mismatches occur between isolates of the Singapore grouper iridovirus (AY521625) and Santee-Cooper ranavirus (KU507317). The highly conserved nature of the ranavirus major capsid gene (Jancovich et al. 2015) does suggest the possibility of this assay to amplify other species within the *Ranavirus* genus; however, this was not addressed within this study.

qPCR validation

A standard curve consisting of the linear range of 10-fold dilutions of gBlock® ranging from 10^8 to 10 copies reaction^{-1} was used to compare each singleplex assay to the multiplex assay. Triplicate reactions containing each gBlock® concentration were run with the multiplex and each singleplex assay individually. Multiplex C_q values were plotted against singleplex C_q values and analyzed using linear regression.

Multiplex efficiency and variability were evaluated using triplicate reactions (intra-assay variation) and compared to triplicate reactions on a subsequent plate for determining the inter-assay variation. Standard deviation and coefficients of variation of the mean C_q values at each gBlock® concentration was determined for all 4 targets: *Bd*, *Bsal*, FV3, and the TaqMan® Exogenous IPC. Additionally, the efficiency, R^2, and slope of the standard curve for all 3 targets were evaluated across 4 replicate plates containing triplicates of each standard containing 10^8 to 10 copies reaction^{-1} and plotting the C_q values versus $\log_{10}$ copies reaction^{-1}.

Assay sensitivity was assessed using a second standard curve with 10-fold serial dilutions ranging from 2.5×10^7 to 2.5 copies reaction^{-1}. This curve

was created to encompass greater sensitivity at lower target concentrations. The limit of detection (LOD), defined as the target concentration at which >95% of all 3 standards amplified, was determined by running 32 replicate reactions of 250, 25, and 2.5 copies reaction^{-1}. The limit of quantification (LOQ) was evaluated by running 12 replicates of 6 different standards 2.5×10^7, 2.5×10^5, and 10-fold dilutions from 2.5×10^3 to 2.5 copies reaction^{-1}. Standard values were assigned to 4 replicates, the remaining 8 were then used to calculate and evaluate the starting quantity (SQ) mean and SQ coefficient of variation (CV). The LOQ was determined as the lowest copy number with a SQ CV< 25% (Kralik & Ricchi 2017).

Amplicon digestion

Restriction sites were incorporated within the gBlock® to provide additional assurance that detections were not simply contamination. Conventional PCR (cPCR) was used to amplify the coding region within the gBlock®. Briefly, cPCR was conducted using Platinum® PCR SuperMix (Thermo Fisher Scientific) following the manufacturer's recommendations. To amplify the 378 bp region encoding the *Bd*, *Bsal*, and FV3 amplicons, 200 nM of both the ITS1-3 Chytr and STerR primers were added to 50 µl reactions. Individual *Bd* and *Bsal* amplicons were also amplified using the corresponding ITS1-3 Chytr — 5.8S Chytr and STerF — STerR primer sets respectively. All reactions were conducted using the following parameters: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 35 s, and terminating with 72°C for 7 min. Following amplification, restriction digests were conducted using *Eco*RI (Thermo Fisher Scientific) following the manufacturer's protocol. Digestion reactions were incubated at 37°C for 14 h followed by 20 min at 65°C for inactivation of the enzyme. The reactions were visualized using the E-Gel Electrophoresis System (Thermo Fisher Scientific) and comparing bands to the TrackIt™ 100 bp ladder (Thermo Fisher Scientific).

Amphibian sampling

Amphibian samples were collected in 2016 and 2017 for the purpose of validating and field testing the multiplex assay. Numerous species and life stages were sampled throughout the study. Sites were selected around the La Crosse, Wisconsin (USA) area to encompass a range of amphibian species and habitats (Table 2). Sampled species included green frog *Lithobates clamitans*, bullfrog *Rana catesbeiana*, leopard frog *Lithobates pipiens*, American toad *Anaxyrus americanus*, pickerel frog *Lithobates palustris*, spring peeper *Pseudacris crucifer*, boreal chorus frog *Pseudacris maculate*, central newt *Notophtalmus viridescens*, eastern redbacked salamander *Plethodon cinereus*, bluespotted salamander *Ambystoma laterale*, gray tree frog *Hyla* spp., wood frog *Lithobates sylvaticus* and commercially collected common mudpuppy *Necturus maculosus*. A total of 387 amphibian samples representing 13 species were collected from 14 sites over the 2016 and 2017 seasons (Table 2, see Fig. 2).

Sampling was conducted in accordance with the swabbing protocol described by Hyatt et al. (2007). Sterile rayon fine-tipped swabs (Puritan Medical Products) were used to first swab keratinized epidermal areas such as the tooth rows in larval anurans (Fellers et al. 2001, Kadekaru et al. 2016) followed by swabbing of the cloacal area (Gray et al. 2012). Stringent biosecurity and disinfection measures were followed between individuals and sampling areas as detailed by Gray et al. (2017). Nets were rinsed in source water and fresh gloves, sterile bags and tubes were used for each individual. All equipment (including buckets, coolers, waders, boots, nets, etc.) was disinfected between sites using a 2% solution of Virkon® Aquatic (DuPont) (Gray et al. 2017).

Swabs were stored at 4°C for immediate extraction or −20°C if extraction could not take place within 1 wk of collection. For swabs, sample DNA was extracted using 150 µl of the PrepMan™ Ultra Sample Preparation Reagent (Thermo Fisher Scientific) following the manufacturer's instructions. Beginning with the 2017 sampling season, following extraction, DNA was quantified using a Qubit™ 3 Fluorometer (Thermo Fisher Scientific) and either the dsDNA BR or HS Assay Kits (Thermo Fisher Scientific) using the manufacturer's instructions. Extracted samples were stored at 4°C until analysis.

Following the initial analysis, samples were stored at −20°C. A randomly selected subset of 80 field samples were used to further compare the performance of the multiplex and singleplex assays. For each sample, duplicate reactions of the multiplex, *Bd* singleplex, and FV3 singleplex assays were analyzed as described using the gBlock®.

Table 2. Non-lethal amphibian sampling summary, including site designation (see Fig. 1), year of sampling, habitat type, and county (in Wisconsin, USA), as well as the number of each species sampled

Site	Year	Habitat type	County	Sample	n
A	2016	Marsh/swamp	La Crosse	American toad *Anaxyrus americanus*	1
A				Green frog *Lithobates clamitans*	5
A				Leopard frog *Lithobates pipiens*	2
B	2016	River	La Crosse	Bullfrog *Rana catesbeiana*	1
B				Green frog	1
B				Leopard frog	4
B				Leopard frog tadpole	2
B				Pickerel frog *Lithobates palustris*	1
C	2016	Lake	Vernon	American toad	1
C				Green frog	2
C				Green frog tadpole	21
D	2016	Spring-fed pond	Monroe	American toad	2
D				Bullfrog	1
D				Green frog tadpole	3
E	2016	Lake	Vernon	Green frog tadpole	5
F	2017	NA	NA[a]	Mudpuppy *Necturus maculosus*	62
G	2017	Spring-fed pond	Vernon	American toad eggs	1
G				American toad tadpole	4
G				Green frog	5
G				Green frog tadpole	25
G				Tree frog tadpole *Hyla* sp.	16
H	2017	Spring-fed pond	Vernon	American toad eggs	1
H				Boreal chorus frog *Pseudacris maculate*	3
H				Boreal chorus tadpole	1
H				Green frog	7
H				Green frog juvenile	10
H				Green frog tadpole	11
H				Spring peeper *Pseudacris crucifer*	3
H				Spring peeper tadpole	1
H				Wood frog *Lithobates sylvaticus*	6
H				Wood frog juvenile	5
I	2017	Mature forest	Bayfield	Eastern red-back salamander *Plethodon cinereus*	1
C	2017	Lake	Vernon	American toad	4
C				American toad tadpole	2
C				Green frog	1
C				Green frog juvenile	3
C				Green frog tadpole	6
J	2017	Urban	Monroe	American toad juvenile	17
A	2017	Marsh/swamp	La Crosse	Green frog tadpole	4
D	2017	Spring-fed pond	Monroe	American toad eggs	6
D				American toad tadpole	1
D				Bullfrog	4
D				Green frog	7
D				Green frog juvenile	3
D				Green frog tadpole	32
D				Tree frog tadpole	1
K	2017	Spring-fed pond	La Crosse	American toad eggs	1
K				Bullfrog tadpole	4
K				Central newt *Notophtalmus viridescens*	3
K				Green frog	1
K				Green frog juvenile	2
K				Green frog tadpole	9
K				Tree frog tadpole	5
L	2017	River/marsh	La Crosse	American toad	1
L				Green frog	1
L				Green frog juvenile	11
L				Green frog tadpole	8
L				Leopard frog	17
M	2017	Spring-fed pond	Monroe	American toad	1
M				Green frog	1
M				Green frog juvenile	5
M				Green frog tadpole	3
N	2017	Mature forest	Fond du Lac	Blue-spotted salamander *Ambystoma laterale*	9
Total					387

[a]Wild common mudpuppy were collected from an unknown location(s) by a commercial harvester and sampled following sale

RESULTS

qPCR conditions

Primer and probe concentrations were optimized for a 25 µl multiplex reaction, with each reaction containing 2.5 µl of either template DNA or diluted gBlock®. Optimal reaction conditions included the addition of 12.5 µl of the 2× PrimeTime® Gene Expression Master Mix, 0.4 µM of both the *Bd* forward (ITS1-3 Chytr) and reverse primers (5.8S Chytr), 0.4 µM of *Bsal* forward (STerF) and reverse (STerR) primers, and 0.1 µM of both the *Bd* (Chytr MGB2) and *Bsal* (STerC) probes. The assay also included 0.4 µM of both the FV3 forward and reverse primers and 0.3 µM of the FV3 probe. Additionally, to rule out the occurrence of inhibition of target amplification, the TaqMan® Exogenous IPC (Thermo Fisher Scientific) was added to one of each of the duplicate reactions following the manufacturer's instructions.

Validation

Throughout validation, the multiplex performed well. The linear range was between 10^8 and 10 copies reaction^{-1}, so these standards were utilized for the subsequent testing. The multiplex performed equally well against each individual singleplex assay. Linear regression of multiplex C_q values plotted against singleplex C_q values demonstrated slopes of 1.004, 1.008, and 0.996 for *Bd*, *Bsal*, and FV3 respectively and R^2 values of 0.999 for all 3 pathogens (data not shown). The efficiency of the standard curve across all 3 targets ranged from 91.9 to 99.9% with mean efficiencies of 95.3, 93.5, and 93.3% for *Bd*, *Bsal*, and FV3 respectively. The R^2 values of the standard curve were consistent, ranging from 0.991 to 0.999, indicating high efficiency and accuracy within the assay and standard curve.

The assay was also highly consistent with the intra-assay CVs of all 3 pathogen targets and the IPC ranging from 0.05 to 3.45% across all concentrations (Table 3). While the inter-assay CVs ranged from 0.19 to 4.91%, the greatest variation occurred within the IPC C_q values. We associated inhibition of unknown samples with a CV > 5% compared to IPC C_q values of the standards.

Single digit gene copies were readily detectable, with amplification of all 3 targets in 87.5% (28 of 32 replicates containing 2.5 copies reaction^{-1} amplified). However, we selected 25 gene copies for both our LOD and LOQ. This threshold was selected because 25 copies reaction^{-1} was detectable in 100% (32 of 32) of replicates and the SQ CV values remained below 25% (Table 4).

Table 3. Mean, standard deviation (SD), intra and inter-assay variation of quantification cycle (C_q) values. Amplification of serially diluted gBlock®, ranging from 10^8 to 10 copies reaction^{-1} of *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*), and ranavirus (frog virus 3, FV3). Concentrations of the internal positive control (IPC) were constant and co-amplified in each reaction. Triplicate reactions were conducted at each template concentration to examine intra-assay mean C_q values, SD and coefficients of variation (CV) of C_q values. Triplicate reactions were conducted on a subsequent plate for determining the inter-assay variation

gBlock® copies	C_q mean				C_q SD				C_q CV (%)			
	Bd	*Bsal*	FV3	IPC	*Bd*	*Bsal*	FV3	IPC	*Bd*	*Bsal*	FV3	IPC
Intra-assay												
100000000	14.26	14.29	14.13	25.04	0.05	0.04	0.20	0.37	0.37	0.28	1.39	1.48
10000000	17.53	17.76	17.43	25.44	0.05	0.08	0.14	0.19	0.27	0.45	0.81	0.75
1000000	20.87	21.03	21.07	25.00	0.06	0.01	0.05	0.35	0.29	0.05	0.24	1.40
100000	24.36	24.42	24.41	25.28	0.03	0.06	0.08	0.10	0.11	0.24	0.35	0.41
10000	27.68	27.94	27.99	25.37	0.06	0.08	0.05	0.13	0.22	0.29	0.18	0.50
1000	31.20	31.50	31.47	25.18	0.12	0.07	0.19	0.08	0.39	0.23	0.61	0.30
100	35.07	34.56	34.85	25.53	0.11	0.16	0.15	0.08	0.33	0.46	0.43	0.29
10	38.81	39.25	38.52	25.48	0.03	0.60	1.33	0.24	0.08	1.54	3.45	0.94
Inter-assay												
100000000	14.23	14.22	14.23	25.69	0.05	0.08	0.23	0.92	0.36	0.54	1.59	3.59
10000000	17.59	17.71	17.55	25.86	0.07	0.08	0.18	0.60	0.42	0.47	1.01	2.33
1000000	20.91	21.04	21.17	25.73	0.06	0.05	0.42	0.95	0.30	0.24	1.98	3.71
100000	24.60	24.33	24.76	26.06	0.05	0.10	0.36	0.97	0.19	0.40	1.46	3.70
10000	27.72	27.89	28.44	26.00	0.06	0.09	0.45	0.81	0.23	0.31	1.59	3.12
1000	31.11	31.35	31.66	26.20	0.17	0.18	0.90	1.29	0.56	0.58	2.84	4.91
100	34.71	34.63	34.92	26.33	0.40	0.14	1.16	0.93	1.14	0.39	3.32	3.53
10	38.69	39.45	37.89	26.34	0.35	0.47	0.94	0.84	0.90	1.19	2.49	3.18

Amplicon digestion

We demonstrated that following qPCR, cPCR and restriction digestion can be used as an additional confirmatory process through the incorporation of *Eco*RI restriction sites into the gBlock®. Following cPCR, *Eco*RI restriction enzymes were used to digest any amplicons that were produced. A lack of digestion indicates pathogen presence, while successful digestion allows for the visualization of any potential gBlock® contamination (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d129p001_supp.pdf).

Field sampling

A total of 52 samples were collected and tested in 2016, and 348 samples were collected, quantified, and analyzed in 2017 (Table 2, Fig. 2). The extraction efficiency of swabs appeared to be relatively consistent. DNA yields across species ranged from 1.09 ± 0.44 to 8.86 ± 2.47 ng μl^{-1} (see Table S1 in the Supplement). To increase confidence in the results of field sampling, a conservative threshold was adopted (100 copies $reaction^{-1}$). Inhibition was observed in a few samples (<15), which were then diluted 1:10 in molecular grade water and re-run.

The prevalence of *Bd* across all sites for both years ranged from 0% in 7 sites to 71.0% for Site F (Fig. 2, see Table S2 in the Supplement), which represented a population of wild-caught, commercially collected mudpuppies. The 8 remaining sites ranged from 11.1 to 37.0%. Prevalence of FV3 varied across sites (Fig. 2, Table S2), though this is likely an artefact of sampling bias. We failed to detect FV3 in 7 sites; yet we detected FV3 from a single individual (eastern red-backed salamander) collected from Site I. Where larger sample sizes were obtained, the FV3 prevalence ranged from 2.1 to 13.7%. Individuals co-infected with both *Bd* and FV3 were rare, only found at Sites D, G, and J (Fig. 2, Table S2). At only 1 site (Site G) we noted disease signs in several green frog tadpoles exhibiting petechial hemorrhage on the ventral skin surface and lethargy in several tree frog tadpoles. Generally, throughout field sampling positive detections were not associated with observations of disease signs. *Bsal* was not detected at any of the sampling sites (data not shown).

We also looked at the detection rate among species and life stages. *Bd* was detected from nearly all species examined, except for wood frogs, pickerel frogs, leopard frogs, and the eastern red-backed salamander (Table 5). For FV3, detections were sporadic in blue-spotted salamanders, bullfrogs, eastern red-backed salamander, American toads, green frogs, and leopard frogs. Co-infection was only detected in American toads and green frogs. Also, green frog samples, regardless of life stage, showed consistent prevalence for both *Bd* (24.4 to 41.2%) and FV3 (5.9 to 7.9%) (Table 5).

As an additional step of the validation, 80 randomly selected field samples were analyzed using the multiplex and singleplex assays. Only 27 of 80 samples tested positive for *Bd*, and 16 of 80 samples positive for FV3. Linear regression demonstrated that both the FV3 and *Bd* singleplex and multiplex C_q values were highly correlated (Fig. 3), with R^2 values of 0.9878 and 0.9835 respectively. The greatest variability for both targets appeared at the lowest copy numbers (C_q > 37). However, these results clearly show that multiplexing did not affect the amplification efficiency of the individual assays.

Table 4. Mean, standard deviation (SD), intra and inter-assay variation of starting quantity (SQ) values. Amplification of 6 gBlock® standards, ranging from 2.5×10^7 to 2.5 $copies\ reaction^{-1}$ of *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*), and ranavirus (frog virus 3, FV3). Concentrations of the internal positive control (IPC) were constant and co-amplified in each reaction. A total of 12 reactions were performed for each concentration, standards values were assigned to 4 replicates, and the remaining 8 were then used to calculate and evaluate the SQ mean, SD and coefficients of variation (CV)

gBlock® copies	SQ mean			SQ SD			SQ CV (%)		
	Bd	*Bsal*	FV3	*Bd*	*Bsal*	FV3	*Bd*	*Bsal*	FV3
25000000	24450000.00	25205833.33	24691666.67	1021575.58	867548.83	1123171.35	4.18	3.44	4.55
250000	242066.67	246900.00	245166.67	11953.96	12547.44	10759.83	4.94	5.08	4.39
2500	2468.25	2475.08	2509.92	160.33	134.12	146.58	6.50	5.42	5.84
250	248.65	252.39	256.41	18.22	21.57	22.32	7.33	8.55	8.71
25	24.56	23.59	22.47	3.65	2.85	3.72	14.88	12.09	16.57
2.5	4.12	3.81	3.89	3.15	2.67	3.60	76.49	70.06	92.49

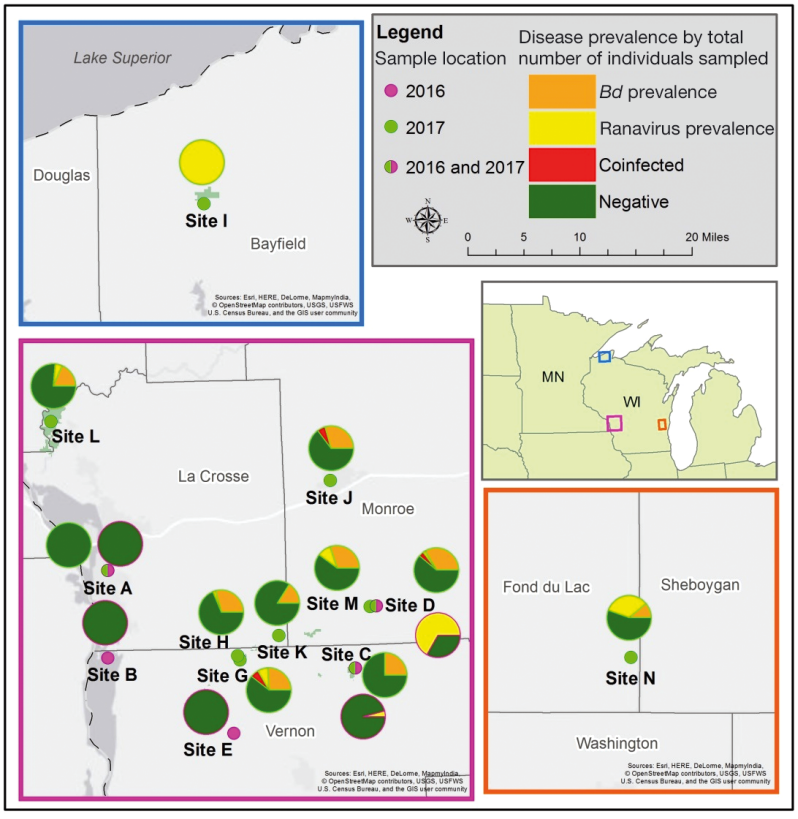

Fig. 2. *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus prevalence at Wisconsin sampling sites in 2016 and 2017. *Bd* prevalence (orange), ranavirus prevalence (yellow) co-infected individuals (red) and negative individuals (green) are shown as a portion of the total number of individuals sampled from each site. Maps throughout this paper were created using ArcGIS® software v.10.5 (ESRI)

DISCUSSION

In this study, we examined the viability of a multiplex qPCR assay for detection of *Bd*, *Bsal*, and FV3. We presented the initial validation for this multiplex method, showing that a previously published assay targeting FV3-like ranaviruses (Allender et al. 2013) could be incorporated into a duplex assay described by Blooi et al. (2013). This multiplex continues to build on numerous human and veterinary studies that have already demonstrated the value of multiplex qPCR in the detection of pathogens (Song & Hampson 2009, Janse et al. 2010, Peleg et al. 2010, Kamau et al. 2013). The ability to reliably screen for 3 pathogens in a single reaction reduces costs associated with testing as well as the time and consumables needed, making multiplex qPCR highly efficacious.

To further improve the multiplex, we explored several additional modifications. For example, we utilized a commercially available IPC, which we were able to incorporate into the assay allowing us to monitor amplification inhibition. We also employed a synthetic gBlock®, which allowed us to develop a highly accurate standard curve and quantitation method. The use of this absolute genome quantification alleviated difficulties associated with variation between *Bd* strains in the ITS-1 copy number (Longo et al. 2013). Moreover, by incorporating several point transversions into the gBlock® sequence to form *Eco*RI restriction sites we were able to easily distinguish between true positive detections or gBlock contamination.

Upon achieving a functional assay, we wanted to demonstrate its viability in the field for non-lethal sampling and surveillance efforts. For these purposes, the multiplex performed well. It should be noted that swabbing is a common non-lethal sampling method for chytrid (Hyatt et al. 2007, Burrowes et al. 2011), FV3 (Gray et al. 2012) or with samples being used to evaluate both pathogens in separate qPCR reactions (Kolby et al. 2015). However, ranavirus is primarily identified from liver tissue, toe or tail clips, as these samples allow for the detection of early stages of infection (St-Amour & Lesbarrères 2007, Gray et al. 2012) and we believe that these methods should work equally well on tissue samples if preferred. Our results demonstrate that the multiplex can reliably detect both *Bd* and FV3 in various species and locations. Both *Bd* and FV3 appear widespread; at least 1 of the 2 pathogens was detected from all but 3 locations (Sites A, B, and E), though no evidence of *Bsal* was found. These findings appear to corroborate previous studies which have shown the widespread nature of *Bd* (Rothermel et al. 2008, Bales et al. 2015). It was interesting that we were able to detect *Bd* in differing habitats; though not from sites closely associated with the upper Mississippi River (Sites A and B). It is unclear what might account for the lack of detections, though it could possibly be related to temperature or a temporal bias from sampling these sites earlier in the field season. We also note that lack of detection from the assay does not always mean a lack of presence of a patho-

Table 5. Prevalence of *Batrachochytrium dendrobatidis* (*Bd*), ranavirus (frog virus 3, FV3), and co-infected individuals by species. Prevalences are the compiled results of all species and samples examined

Sample	n	*Bd* (%)	FV3 (%)	Co-infection (%)
American toad adult	10	30.0	10.0	0.0
American toad eggs	9	0.0	0.0	0.0
American toad juvenile	17	35.3	5.9	5.9
American toad tadpole	7	14.3	28.6	0.0
Blue-spotted salamander	9	11.1	33.3	0.0
Boreal chorus frog	3	33.3	0.0	0.0
Boreal chorus frog tadpole	1	0.0	0.0	0.0
Bullfrog adult	6	33.3	16.7	0.0
Bullfrog tadpole	4	0.0	0.0	0.0
Central newt	3	66.7	0.0	0.0
Eastern red-backed salamander	1	0.0	100.0	0.0
Green frog adult	31	35.5	6.5	3.2
Green frog juvenile	34	41.2	5.9	2.9
Green frog tadpole	127	22.0	7.9	3.1
Leopard frog adult	23	0.0	4.3	0.0
Leopard frog tadpole	2	0.0	0.0	0.0
Mudpuppy	62	71.0	0.0	0.0
Pickerel frog adult	1	0.0	0.0	0.0
Spring peeper adult	3	33.3	0.0	0.0
Spring peeper tadpole	1	0.0	0.0	0.0
Tree frog tadpole	22	13.6	0.0	0.0
Wood frog adult	6	0.0	0.0	0.0
Wood frog juvenile	5	0.0	0.0	0.0
Total	387	NA	NA	NA

gen at a site. Early infection levels may be difficult to detect (Green et al. 2002, Currylow et al. 2014, Hall et al. 2016) and consideration of this must be accounted for in overall sampling efforts.

The low prevalence of ranavirus detected within larval amphibian communities also mirrors previous studies (Brunner et al. 2015, Hall et al. 2016). However, the widespread nature of FV3 does underscore the importance of biosecurity and stringent disinfection protocols when performing sampling and surveillance efforts. This is particularly true since amphibian populations are declining and we are just beginning to gain a better understanding of the range of ranavirus hosts. For example, FV3-like viruses are capable of transmission between different classes of ectothermic vertebrates (Brenes et al. 2014ab). The endangered pallid sturgeon *Scaphirhynchus albus* is especially susceptible to FV3 (Waltzek et al. 2014), and the virus has been responsible for several pallid sturgeon mortality events (Chinchar & Waltzek 2014), threatening restoration efforts for this species. The usage of this multiplex in conjunction with other sampling efforts can provide a rapid assessment of an amphibian population, whether it be focused on tissue or swab sample collection.

It is clear that the ability to detect these pathogens will further increase our understanding of the complexities associated with their disease dynamics,

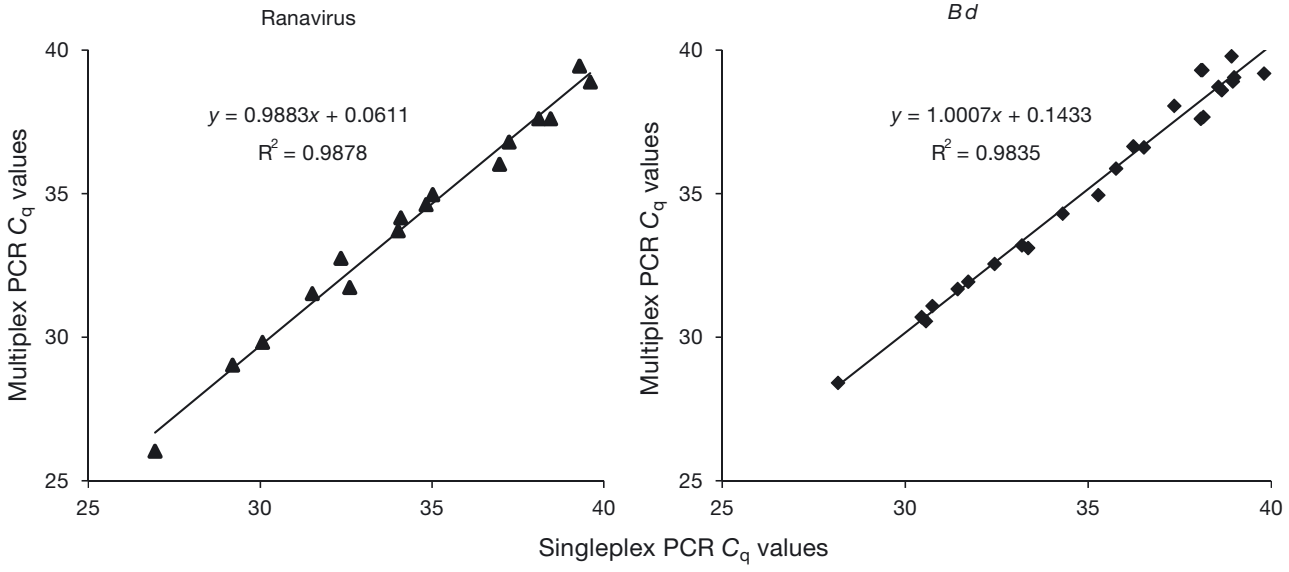

Fig. 3. Multiplex versus singleplex quantification cycle (C_q) values of field samples. Positive results of 80 randomly selected 2017 field samples run using the multiplex PCR assay and singleplex assays for *B. dendrobatidis* (*Bd*) and ranavirus (frog virus 3, FV3). The average of duplicate multiplex C_q values are plotted against the corresponding average of duplicate singleplex C_q values and analyzed using linear regression. Only positive samples ($C_q < 40$) are included

transmission, and epidemiology. However, additional sampling is necessary to determine the presence of yearly or seasonal variation in pathogen prevalence in the sampling locations, as has been documented previously in ranaviral infections (Green et al. 2002, Gray et al. 2007, Brunner et al. 2015). This work can also help inform future targeted sampling strategies for pathogen studies. For instance, *Bd* was not detected from any wood frog or leopard frog samples, but was found in samples from each green frog life stage. Moreover, *Bd* and ranavirus co-infection was detected in green frogs at each life stage. However, of all the species that were tested, the fully aquatic species, central newts and mudpuppies, had the highest *Bd* prevalence. It should be noted that the mudpuppies were housed together during transport, which could have resulted in higher chytrid prevalence, and may not be reflective of a natural infections. Continued sampling with larger sample sets including various populations would help clarify whether these trends are observed elsewhere. Additionally, this research emphasizes the importance of collecting diverse samples (e.g. from different species and sites), as the results provide a better grasp of pathogen–host interactions.

In conclusion, we found that the use of this multiplex coupled with non-lethal sampling was sufficient to detect both *Bd* and FV3, and should have utility for surveillance of *Bsal*. The assay appears to be reliable, sensitive, and minimizes the impacts of sampling on populations. Environmental and non-lethal sampling have proven to be a viable sampling strategy in the past (Gray et al. 2012, Hall et al. 2016), though we acknowledge the potential drawback associated with false negative and positive rates using this method (Gray et al. 2012). By using a highly conservative positive/negative threshold for initial field sampling, we decreased the possibility of false positives. Regardless, we suggest that environmental and non-lethal sampling methods have a place in future surveillance efforts (Hall et al. 2016), and that this assay can serve as a useful tool. Perhaps the best outcome of this study was that *Bsal* was not detected. Nevertheless, continued screening and collaboration between managers, scientists, and citizens is needed to ensure that *Bsal* remains absent in North America to maintain healthy amphibian populations.

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Editorial responsibility: Louise Rollins-Smith, Nashville, Tennessee, USA

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