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Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*

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Summary

Batrachochytrium salamandrivorans (*Bsal*) poses a major threat to amphibian, and more specifically caudata, diversity. *Bsal* is currently spreading through Europe, and mitigation measures aimed at stopping its spread and preventing its introduction into naïve environments are urgently needed. Screening for presence of *Bsal* and diagnosis of *Bsal*-induced disease in amphibians are essential core components of effective mitigation plans. Therefore, the aim of this study was to present an overview of all *Bsal* diagnostic tools together with their limitations and to suggest guidelines to allow uniform interpretation. Here, we investigate the use of different diagnostic tools in post-mortem detection of *Bsal* and whether competition between *Bd* and *Bsal* occurs in the species-specific *Bd* and *Bsal* duplex real-time PCR. We also investigate the diagnostic sensitivity, diagnostic specificity and reproducibility of the *Bsal* real-time PCR and show the use of immunohistochemistry in diagnosis of *Bsal*-induced chytridiomycosis in amphibian samples stored in formaldehyde. Additionally, we have drawn up guidelines for the use and interpretation of the different diagnostic tools for *Bsal* currently available, to facilitate standardization of execution and interpretation.

KEYWORDS
amphibians, *Batrachochytrium salamandrivorans*, chytrid fungi, diagnosis, emerging infectious disease, real-time PCR

1 | INTRODUCTION

Amphibians are the most threatened class of vertebrates in the midst of the biodiversity crisis the world is currently experiencing (Stuart et al., 2004). Underlying factors such as loss of habitat, pollution and infectious disease are continuously pressuring the survival of amphibian populations and species around the world (Catenazzi, 2015). Among the major infectious diseases, negatively impacting amphibian survival in the wild is the recently described chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*), which like its closest relative *Batrachochytrium dendrobatidis* (*Bd*), is able to cause the lethal skin disease chytridiomycosis in amphibians (Longcore, Pessier, & Nichols, 1999; Martel et al., 2013). While *Bd* has been associated with chytridiomycosis in species of all amphibian orders (Anura, Caudata, Gymnophiona), *Bsal*-induced chytridiomycosis has so far only been found in amphibian species belonging to Caudata (Van Rooij, Martel, Haesebrouck, & Pasmans, 2015). Species are therefore at risk; in case, *Bsal* should be introduced (Richgels, Russell, Adams, White, & Grant, 2016; Yap, Koo, Ambrose, Wake, & Vredenburg, 2015).

While surveillance efforts have shown that *Bd* has globalized, the only wild amphibian populations in which *Bsal* has been detected so far have been in Europe, where it is spreading and causing disease outbreaks in a wavelike pattern (Spitzen-van der Sluijs et al., 2016),

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and in Asia, where to date, no negative impact of the presence of *Bsal* has been reported (Laking, Ngo, Pasmans, Martel, & Nguyen, 2017; Martel et al., 2014). However, monitoring for *Bsal's* presence has only recently commenced, and systemic active surveillance of wild, captive and traded amphibians is needed to map *Bsal*'s current distribution. *Bsal* has been shown to be present in captive amphibian collections in several European countries (Bletz et al., 2015; Cunningham et al., 2015), and with the knowledge that *Bd*'s cosmopolitan distribution is very likely facilitated by the trade in *Bd*-infected amphibians (Fisher, Garner, & Walker, 2009), it seems only a matter of time until spread of *Bsal* to naïve regions and continents is reported (Grant et al., 2016).

The risk posed by *Bsal* has led governmental and non-governmental organizations to take actions, or to advise policymakers on implementing emergency action plans for *Bsal* in order to try to protect native salamander populations and species. As currently no methods are available to mitigate the effects of *Bsal* on natural amphibian populations once it has emerged, accurately diagnosing *Bsal*-induced chytridiomycosis and preventing introduction and spread of *Bsal* are crucial elements in emergency action plans (Garner et al., 2016). Therefore, science-based screening and monitoring efforts, based on reliable and accurate diagnostic methods, are essential.

Several diagnostic methods for *Bsal* detection have been described including histology/histopathology, PCR, qPCR and lateral flow technology. However, the limitations of some of these techniques include (i) the need for experienced operators or experts to perform these assays, (ii) the ability of the tests to only detect and not quantify the pathogen in samples, (iii) the inability to detect low numbers of the pathogen and (iv) poor diagnostic sensitivity and specificity. The *Bsal* real-time PCR adequately addresses these limitations and is one of the premiere methods for *Bsal* diagnostics in use. However, despite being utilized in several countries, the diagnostic sensitivity, specificity and reproducibility of this assay have not previously been established based on the criteria of the OIE (EFSA, 2017, OIE, 2016).

The aims of this study were (i) to validate the *Bsal*-specific real-time PCR, (ii) to give an overview of the currently available diagnostic tools for *Bsal*, (iii) to give recommendations on the use of the duplex real-time PCR and immunohistochemistry, (iv) to give recommendations on most appropriate methods for post-mortem detection and time frame for post-mortem detection using the duplex real-time PCR and histopathology and (v) to elucidate the latency period (elapsed time between pathogen exposure and detectability of *Bsal* on the host). These guidelines should promote uniform implementation and interpretation of *Bsal* diagnostic tests.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

All experiments with experimental animals were performed with the approval of the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/073, 2015/26, 2015/42, EC2015/62, EC2015/83). Five fire salamanders (*Salamandra salamandra*) were experimentally infected with *Bsal* (type strain AMFP13/1. A 1 ml suspension containing 10^5 spores was dripped on each animal (Martel et al., 2013) used for the experiment examining the post-mortem detection of *Bsal*. Twenty-six fire salamanders were used to investigate the latency time of the *Bsal*-specific real-time PCR in detecting infections in fire salamanders exposed to low *Bsal* infection dosages (100 spores per ml). Six rabbits were used for production of polyclonal antibodies against *Bd* (type strain JEL423) and *Bsal* (type strain AMFP13/1).

2.2 | Post-mortem detection of *Bsal*

Fire salamanders that died due to *Bsal*-induced chytridiomycosis were used to investigate the applicability of the different diagnostic tools in post-mortem detection of *Bsal*. Directly after death of an animal, it was transferred to a passively aerated plastic container with moist soil and leaf litter to mimic conditions under which dead *Bsal*-infected salamanders were found in the field. The containers were placed in a climate-controlled room set at 15°C. Directly after death, and after 1, 2, 3, 4, 5, 7 and 10 days, samples (swabs and tissue samples) for detection of *Bsal* were taken. Each sampling occasion consisted of collecting swabs of the ventral and dorsal skin by placing a swab on the skin and rotating it in place for 20 full rotations, collecting full thickness single skin samples of the ventral and dorsal skin with use of a 3-mm biopsy punch (Stiefel, Germany) and collecting a tail clip of the animal (tissue samples were weighed to allow correction for variability in tissue size). All samples were taken from new, unsampled areas of skin to avoid interference in the detection of *Bsal*. Swab samples and tail tissues were stored at −20°C and analysed for the presence of *Bsal* with use of a *Bsal*-specific simplex real-time PCR (Blooi et al., 2013). Skin biopsy samples were stored in formaldehyde and processed for histological examination (haematoxylin and eosin [HE] stain) for detection of *Bsal*.

2.3 | Latency time of the *Bsal* real-time PCR to detect infections in fire salamanders exposed to a low dose of *Bsal*

To investigate the latency time of the *Bsal* real-time PCR in detecting *Bsal* infections in fire salamanders that were exposed to a low dose (100 spores/ml) of *Bsal* spores, 26 fire salamanders were exposed to 100 spores of *Bsal* (*Bsal* type strain AMFP13/1) and screened with the *Bsal* real-time PCR by assaying skin swabs taken at 7-day intervals. Five animals were included as unexposed negative controls. Signs of clinical chytridiomycosis (skin ulcerations, anorexia, apathy) and/or high infection loads (Stegen et al., 2017) with *Bsal* (>10,000 GE per swab) as detected with the real-time PCR were considered as humane endpoints and resulted in the animals being withdrawn from the experiment and treated with temperature (exposure to 25°C for 10 days) (Blooi et al., 2015). On the day of *Bsal* exposure (D0), no swab samples were taken to avoid inference of

the swabbing facilitating the establishment of *Bsal* infections in the animals.

2.4 | Competition between detection of *Bd* and *Bsal* in the duplex real-time PCR

In the duplex real-time PCR, two reactions are occurring in each well simultaneously. Both reactions require dNTPs, polymerase and other constituents of the master mix. When duplex real-time PCR is performed on a sample where one target largely outnumbers the other, the more abundant target outcompetes the other for the reaction reagents. This results in the less abundant target not being amplified at the same rate. Occurrence of competition between *Bd* and *Bsal* primers and probes in the *Bd*- and *Bsal*-specific duplex real-time PCR was evaluated by assaying different relative concentrations of *Bd* and *Bsal* DNA. Ten-fold serial dilutions series of both *Bd* (strain JEL423) and *Bsal* (AMFP 13/1) were prepared to achieve concentrations ranging from 1,000 to 0.1 genomic equivalents (GEs) per 5 μl (all test samples were composed of 1:1 mixtures of *Bsal* and *Bd* zoospore suspensions of 1,000 to 0.1 GE resulting in final concentrations of 500 to 0.05 GE) (Blooi et al., 2013). All possible combinations of concentrations of *Bd* and *Bsal* were run in a checkerboard set-up, with final concentrations of both *Bd* and *Bsal* ranging from 500 to 0.05 GEs per real-time PCR reaction. All concentrations of *Bd* or *Bsal* were also analysed in the absence of the other pathogen to establish baseline values for comparison with the duplex real-time PCR results. Occurrence of competition between detection of *Bd* and *Bsal* was determined based on difference in real-time PCR values (Ct-values) and abnormal real-time PCR curves. Combinations in which the difference in Ct-values was smaller than 0.3 on average when compared to the reference values were considered to have no effect. Combinations that resulted in a difference in Ct-values >0.3 on average were considered to show a high variability in Ct-values (Karlen, McNair, Perseguers, Mazza, & Mermod, 2007). Any real-time PCR curve deviating from a normal amplification curve was considered abnormal (see Figure 3 for normal and abnormal *Bd* and *Bsal* amplification curves). All concentration combinations were assayed in sextuplicate in a total of 4 real-time PCR runs.

2.5 | Validation of *Bsal* real-time PCR

Swab samples from 26 salamanders which had been experimentally infected with *Bsal*, in addition to swabs from 12 non-infected controls, were assayed. An aliquot of each DNA sample was provided to four different laboratories, which performed the *Bsal*-specific real-time PCR (laboratories 1–3) (Blooi et al., 2013) or droplet digital PCR (ddPCR, laboratory 4). The *Bsal* ddPCR fractionates each sample into 20,000 nanolitre-sized water-in-oil droplets, with subsequent amplification of the PCR target occurring within each of these droplets (Hindson et al., 2011; Pinheiro et al., 2012). The number of target copies in each individual droplet is determined, and based on Poisson statistics, the results generated by each individual droplet are combined to calculate the overall number of target copies present in the sample. This method is highly comparable in nature with the more conventional Taqman probe PCRs, sharing reagents, sequence of steps and run time, but with the defining feature of partitioning, the sample in nano-sized droplets in which the target copies are quantified. This similarity between the two methods justifies inclusion of the ddPCR as validation for the *Bsal* qPCR. Therefore, the results registered using this method were included in this reproducibility study as they originated from a technique which is principally highly comparable to the *Bsal*-specific real-time PCR (Hindson et al., 2011). The assay results from each laboratory were analysed using the exact binomial test, *t* test, analysis of proportions and Kendall's correlation in the R statistical software. Diagnostic sensitivity and diagnostic specificity of the results from each laboratory and correlation among the laboratories were calculated and analysed. In addition to analysing the correlation of genomic equivalents per sample detected in each laboratory, the genomic equivalents were converted into binary data with positive samples being designated "1" and negative samples being designated "0" and subjected to a *t* test and also a comparison of proportions of the positive test results in the four laboratories.

2.6 | Immunohistochemical detection of *Bsal* in amphibian skin

2.6.1 | Production of polyclonal anti-*Bsal* and anti-*Bd* antibodies

Polyclonal antibodies against *Bd* and *Bsal* were prepared by immunizing rabbits with *Bd*- and *Bsal*-antigen, respectively. Four bottles of sporulating *Bd* were transferred to one tube while three bottles of sporulating *Bsal* were transferred into another tube after which 3 ml of HBSS was added to each tube. The protein concentration was then determined by performing the BCA protein assay. In short, *Bd* (strain JEL423) and *Bsal* (strain AMFP 13/1) antigen suspensions with a concentration of 500 μg/ml antigen and 10 mg/ml Quil-A adjuvant were prepared. Six rabbits were randomly split up in two groups, with one group being immunized with *Bd*-antigen and the other with *Bsal*-antigen. Each rabbit received 200 μg of antigen through subcutaneous injections (400 μl of antigen suspension divided over four injection sites). Three booster immunizations, with identical antigen concentrations, were administered after the primary immunization with 14-day intervals. Blood collection of all rabbits took place prior to immunization (negative control serum) and 7 days after the last booster immunization (check for seroconversion in an indirect ELISA). Blood samples were collected by venepuncture of the ear vein using needles and syringes rinsed with heparin. After ascertaining seroconversion against *Bd*- and *Bsal*-antigen with use of an indirect ELISA assay, all rabbits were anaesthetized and euthanatized by exsanguination. Rabbit sera were separated and stored at −70°C.

In order to try to enhance the specificity of the polyclonal antibodies against *Bsal* and *Bd* in the rabbit sera, *Bsal*-antisera were pre-absorbed with *Bd*-antigen, and *Bd*-antisera were pre-absorbed with *Bsal*-antigen. Suspensions containing mixed (meaning all life stages)

cultures of *Bd* and *Bsal* at concentrations of 10^6 cells per ml were sonicated and used as pre-absorbent. *Bd*-antisera and *Bsal*-antisera were exposed to *Bsal* and *Bd* pre-absorbents respectively for an hour at 37°C.

2.7 | Immunohistochemistry

The immunohistochemical assay for both *Bsal* and *Bd* was performed as published previously for *Bd* (Hyatt et al., 2007). In addition, previously published methods to determine the optimal concentration of the anti-*Bsal* and anti-*Bd* sera were used (Berger et al., 2002), with the difference of testing the antisera on *Bd* and *Bsal* cultures that were grown on glass inserts in 48-well plates, instead of on agar. Occurrence of cross-reactivity of the antisera against other fungi belonging to the Chytridiomycota phylum (*Homolaphlyctis polyrhiza*, *Rhizophlyctis rosea*, *Gaertneriomyces semiglobifer*, *Rhizoclosmatium globosum*, *Podochytrium dentatum*, *Polychytrium aggregatum*, *Geranomyces variabilis* and *Monoblepharis polymorpha*) was tested.

Bsal- and *Bd*-positive skin tissues (as determined by positive real-time PCR results and presence of *Bd* or *Bsal* in HE-stained histology slides) preserved in neutral-buffered 10% formaldehyde were used to verify the applicability of the immunohistochemical stain and to test the specificity of the original and pre-absorbed antisera. All of the tissue samples were stored in neutral-buffered 10% formaldehyde for longer than 3 months, with the oldest samples being stored in neutral-buffered 10% formaldehyde for more than 3 years.

3 | RESULTS

3.1 | Post-mortem detection of *Bsal*

The ability of histological examination and real-time PCR to detect *Bsal* post-mortem in fire salamanders is summarized in Figure 1. All individuals showed high infection loads with the real-time PCR directly after death with swabs taken from the back (average of 4.4 log10 GE, *SD* of 0.14) and the abdomen (average of 3.8 log10 GE,

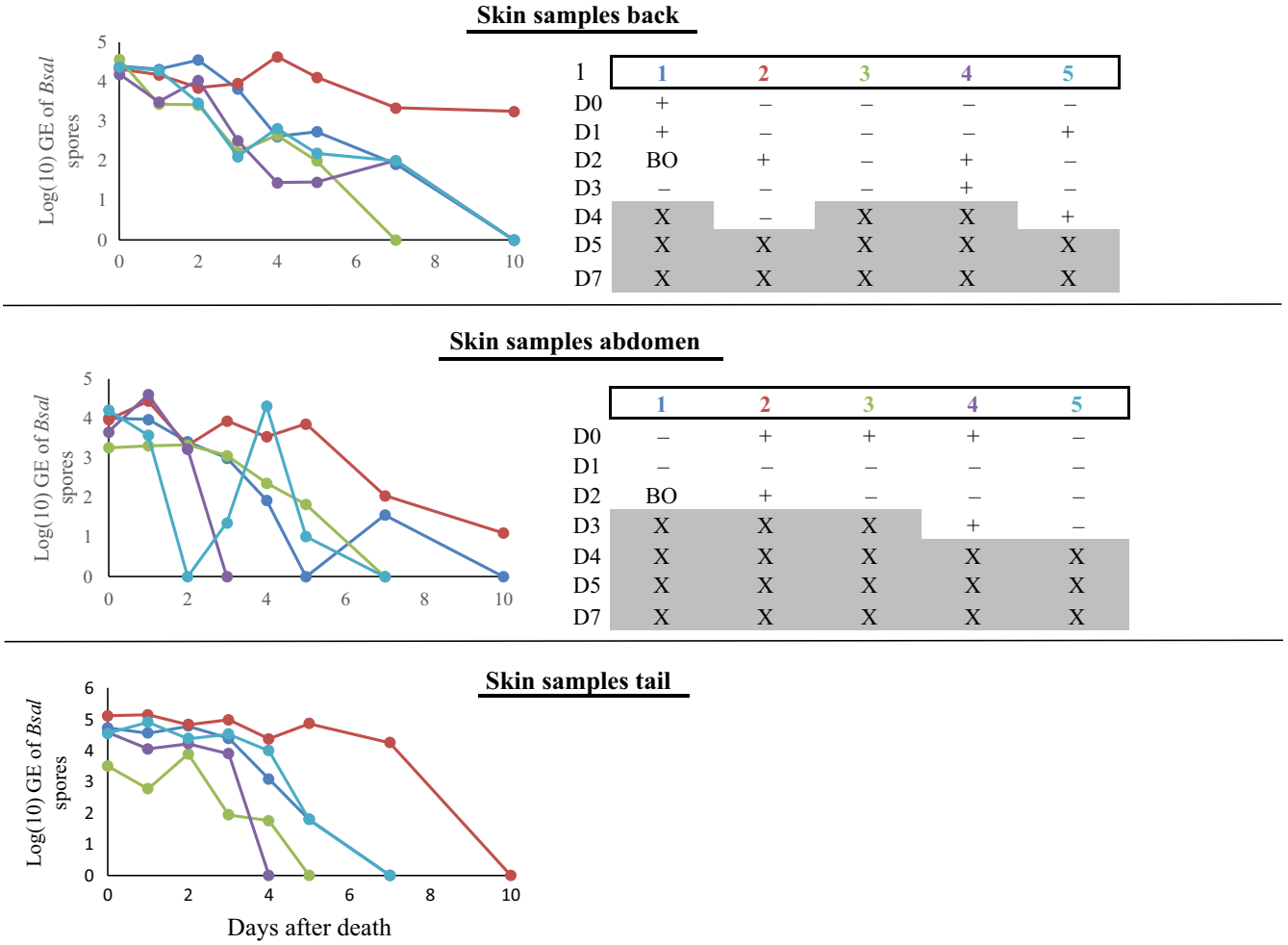

FIGURE 1 Ability of histology and real-time PCR to detect *Bsal* post-mortem. Line-graphs (real-time PCR values) and tables (histology) show the ability of the different techniques to detect *Bsal* post-mortem. Real-time PCR values are shown as genomic equivalents of *Bsal* per swab. Results of histological examination are shown as *Bsal* positive (+), *Bsal* negative (–), bacterially overgrown (BO) and no interpretation possible (X). Different colours represent different individuals

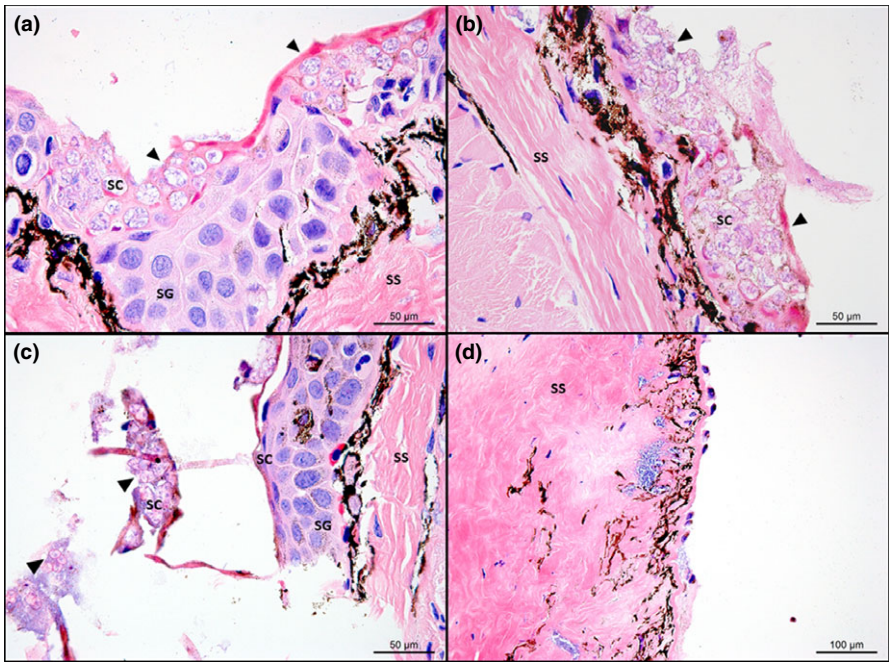

FIGURE 2 Haematoxylin and eosin (HE)-stained histological skin sections of fire salamanders infected with *Bsal*. Haematoxylin and eosin (HE)-stained 5-µm skin sections were prepared from tissue samples taken from the back and the abdomen of the fire salamanders that died due to infection with *Bsal*, in order to reveal presence of *Bsal*. (a) Multiple *Bsal* thalli present in the skin directly after death, (b and c) multiple *Bsal* thalli together with bacterial overgrowth and partial loss of the epidermis 2–3 days after death and (d) skin section lacking the complete epidermis 3–4 days after death. Arrow heads are used to indicated *Bsal* thalli. SC, stratum corneum; SG, stratum germinativum; SS, stratum spongiosum

TABLE 1 The latency time to detect *Bsal* with the *Bsal*-specific real-time PCR in fire salamanders that were exposed to a low dose of *Bsal* spores

	D0	D7	D14	D21	D28
Bsal positive	–	0/26	20/26	26/26	26/26
Bsal load (average [min–max])	–	0 (0–0)	778.7 (0–5,140)	1711.9 (8.4–6,700)	5,888 (22.6–19,990)

Twenty-six fire salamanders were exposed to 10^2 *Bsal* spores at D0 and subsequently checked for detectable presence of *Bsal* with real-time PCR on a 7-day interval. Real-time PCR values are shown as genomic equivalents of *Bsal* per swab.

SD of 0.37) and with tissue taken from the tail (average of 4.5 log10 GE, *SD* of 0.60). During the 10-day follow-up period, fluctuation in real-time PCR values was observed for all samples with most showing a decrease in GE for the last 3 days each animal was sampled. *Bsal* was detected on skin swabs taken from the back on average until 7.2 days post-mortem (*SD* of 1.8), the abdomen until 5.8 days post-mortem (*SD* of 3.0) and in tissue taken from the tail until 4.5 days post-mortem (*SD* of 0.6).

Histological examination of single skin preparations of tissue samples taken from the back did not reveal presence of *Bsal* directly after death in four of the five animals, while for single skin preparations of tissue samples taken from the abdomen two of the five animals did not show the presence of *Bsal*. Histological examination of tissue samples failed to reveal presence of *Bsal* during the follow-up phase in single skin preparations of tissue taken from the back for one individual and in single skin preparations of tissue taken from the abdomen for two individuals. Tissue samples taken from the back were suitable for histological examination for 3.4 days on average (*SD* of 0.5), and samples taken from the abdomen for 2.4 days on average (*SD* of 0.5), after which autolysis and/or (partial) absence of epidermis made histological examination impossible (see Figure 2).

3.2 | Latency time of the *Bsal* real-time PCR

Bsal infections took 2–3 weeks post-exposure to a low dose (100 spores/ml) of *Bsal* spores to manifest as detectable via PCR (see Table 1).

3.3 | Competition between detection of *Bd* and *Bsal* in the duplex real-time PCR

In most combinations, no effect on the detectability of both *Bd* and *Bsal* could be observed. However, at some combinations where one pathogen's load was 10 to 10,000 times higher than the other, the pathogen in lower concentration could not be detected (see Table 2). Also, at some of these combinations where one pathogen outnumbered the second by 10 to 100 times, high variability in Ct-values or abnormal curves was observed (see Figure 3 for normal and abnormal amplification curves). Test sensitivity was higher in the absence of the other pathogen.

3.4 | Validation of *Bsal*-specific real-time PCR (diagnostic sensitivity, diagnostic specificity and reproducibility)

Two of the laboratories demonstrated perfect diagnostic sensitivity while samples with very low GE proved challenging for the other two laboratories. In laboratories 1 and 2, the calculated diagnostic sensitivity is 100% (95% CI: 86.6%–100%), while it is 96.2% (95% CI: 80.4%–99.9%) and 92.3% (95% CI: 75%–99%) in laboratories 3 and 4 (ddPCR) respectively using the exact binomial test.

TABLE 2 Competition between detection of *Bd* and *Bsal* in the duplex real-time PCR

Combination	*Bsal*	*Bd*
Bd 500/*Bsal* 500	No effect	No effect
Bd 500/*Bsal* 50	High variability in Ct-values + Abnormal curves	No effect
Bd 500/*Bsal* 5	Failure to detect	No effect
Bd 500/*Bsal* 0.5	Failure to detect	No effect
Bd 500/*Bsal* 0.05	Failure to detect	No effect
Bd 50/*Bsal* 500	No effect	No effect
Bd 50/*Bsal* 50	No effect	No effect
Bd 50/*Bsal* 5	No effect	No effect
Bd 50/*Bsal* 0.5	Failure to detect	No effect
Bd 50/*Bsal* 0.05	Failure to detect	No effect
Bd 5/*Bsal* 500	No effect	High variability in Ct-values + Abnormal curves
Bd 5/*Bsal* 50	No effect	No effect
Bd 5/*Bsal* 5	No effect	No effect
Bd 5/*Bsal* 0.5	High variability in Ct-values + Abnormal curves	No effect
Bd 5/*Bsal* 0.05	Failure to detect	No effect
Bd 0.5/*Bsal* 500	No effect	Failure to detect
Bd 0.5/*Bsal* 50	No effect	High variability in Ct-values + Abnormal curves
Bd 0.5/*Bsal* 5	No effect	No effect
Bd 0.5/*Bsal* 0.5	No effect	No effect
Bd 0.5/*Bsal* 0.05	High variability in Ct-values + Abnormal curves	No effect
Bd 0.05/*Bsal* 500	No effect	Failure to detect
Bd 0.05/*Bsal* 50	No effect	Failure to detect
Bd 0.05/*Bsal* 5	No effect	Failure to detect
Bd 0.05/*Bsal* 0.5	No effect	High variability in Ct-values + Abnormal curves
Bd 0.05/*Bsal* 0.05	No effect	No effect

All real-time PCR values obtained for the different combinations were compared to baseline reference values composed of real-time PCR values of *Bd* and *Bsal* obtained in the absence of the other pathogen. Combinations in which the difference in Ct-values was smaller than 0.3 Ct-values on average when compared to the reference values were considered to have no effect. Combinations that resulted in a difference in Ct-values >0.3 on average were considered to show a high variability in Ct-values. Any real-time PCR curve deviating from a normal amplification curve was considered abnormal.

3.5 | All four laboratories demonstrated perfect diagnostic specificity, 100% (95% CI: 73%–100%) with the exact binomial test

In terms of reproducibility, both the statistical tests analysing the performance of the laboratories based on the positive or negative status of each sample and the tests analysing the quantification of genomic equivalents in each sample demonstrated very high levels of reproducibility among laboratories and/or among operators (see Figure 4 and Tables 3 and 4). A *t* test and analysis of proportions of positive tests revealed that there were no significant differences in proportions of samples detected as positive in all four laboratories (see Table 3), confirming the congruence of the results of the *Bsal* real-time PCR assay. In laboratories 1 and 2, all true-positive samples yielded positive results (26/26), and in laboratories 3 and 4, (25/26) positive samples and (24/26) true-positive samples tested positive, respectively. Kendall's correlation coefficient indicates very strong correlation in GE (values are very close to 1) among all the laboratories (see Table 4). The highest correlation exists between laboratories 1 and 4 (0.912), and all laboratories have ≥80% correlation which confirms a strong to very strong correlation among the results from all four laboratories.

3.6 | Immunohistochemical detection of *Bsal* in the amphibian skin

The indirect ELISA and efforts at determining the optimal concentration of both *Bsal*- and *Bd*-antisera showed high levels of reactivity, without showing specificity for *Bsal* or *Bd*. All antisera showed comparable levels of reactivity, and antisera derived from one rabbit immunized with *Bsal* and one rabbit immunized with *Bd* were chosen to further test the applicability of the antisera on amphibian skin samples. Pre-absorption of the antisera (pre-absorption of *Bsal*-antisera with *Bd*-antigen and vice versa) did not increase specificity. The concentrations at which clear immunohistochemical staining of *Bsal* and *Bd* cultures occurred, with low levels of background staining, were at a dilution of 1/5,000 and 1/2,000 for tissue sections. For the other tested Chytridiomycota, slight cross-reactivity was observed for *Homolaphlyctis polyrrhiza* for both the *Bd*- and *Bsal*-antisera. All tissue samples that were histologically positive (as determined with HE-staining) for *Bd* or *Bsal* showed clear immunohistochemical staining of *Bd* or *Bsal* (Figure 5), with no detrimental effect of long-term storage of samples in 10% formaldehyde occurring.

4 | DISCUSSION

Rapid diagnosis of disease and detection of pathogens are essential elements in controlling emerging infections, enabling responses such as specific containment measures or restrictions of movement to be implemented (Morens, Folkers, & Fauci, 2004). Therefore, shortly after *Bsal* was identified as an infectious agent capable of causing chytridiomycosis in amphibians (Martel et al., 2013, 2014), research efforts towards developing diagnostic tools that allowed rapid and reliable detection of *Bsal* were instigated (Blooi et al., 2013). The *Bsal* qPCR was subsequently developed and has since filled this diagnostic need. This assay has the advantage over the diagnostic methods histology, PCR and lateral flow assay of being able to simultaneously identify and quantify the *Bsal* pathogen in amphibian

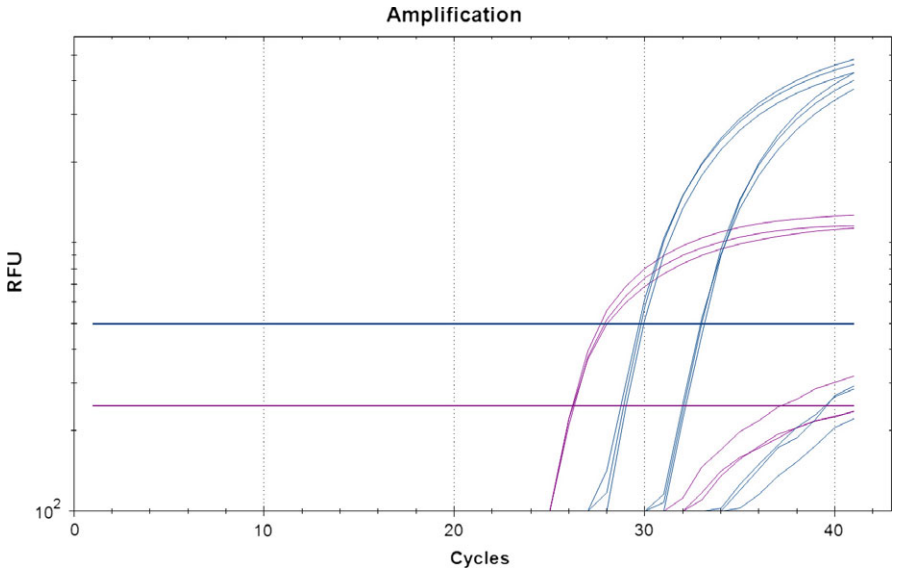

FIGURE 3 Amplification curves of the *Bd* and *Bsal* duplex real-time PCR. Amplification plot (variation of log RFU with each PCR cycle number) showing normal and abnormal amplification curves as they can be observed with the duplex real-time PCR. Blue lines depict the *Bd* component of the duplex real-time PCR, and purple lines depict the *Bsal* component of the duplex real-time PCR. Normal real-time PCR curves show an exponential increase in fluorescence as indicated by a smooth linear phase in the amplification plots. The threshold should be set halfway in the linear phase of the amplification curves

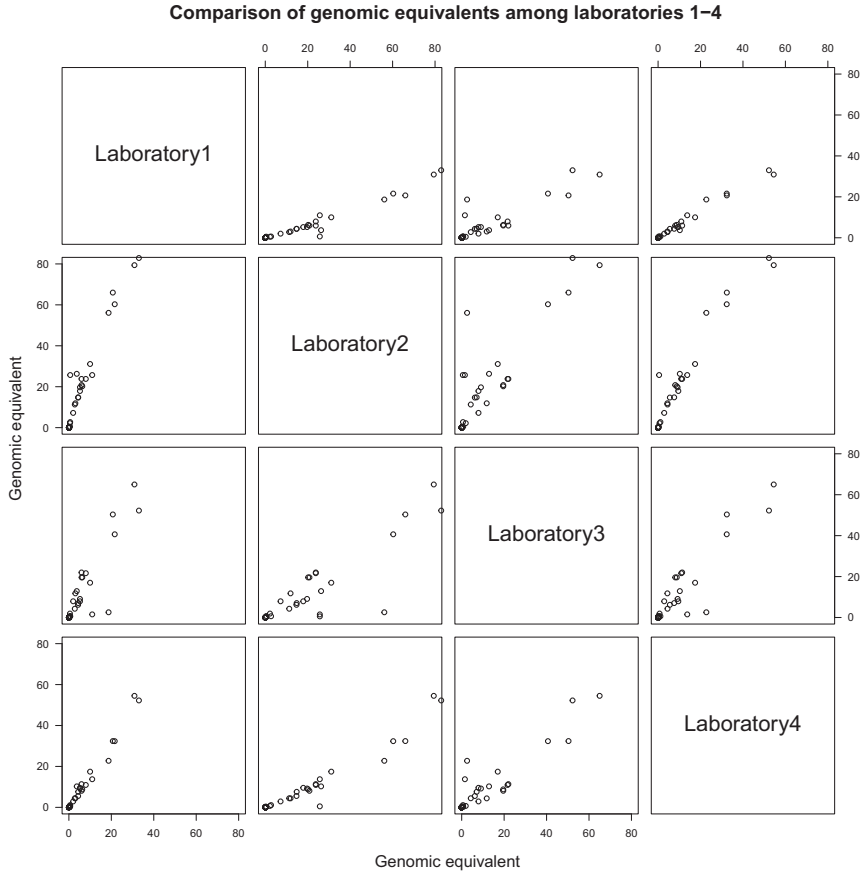

FIGURE 4 Comparison of genomic equivalents (GE) among laboratories 1–4. Scatter plot representing correlation between genomic equivalents by pairs of laboratories. All pairs demonstrate a strong to very strong positive correlation

samples (Berger, Speare, & Kent, 1999; Dillon et al., 2017; Martel et al., 2013). Based on our validation study, the *Bsal*-specific real-time PCR assay has demonstrated diagnostic sensitivity of 96.2%–100% (qPCR), 92.3% (ddPCR) and perfect specificity (100%) across all four laboratories. Like the duplex real-time PCR, the *Bsal* real-time PCR allows *Bsal* diagnosis with quantification of GE with a very high degree of sensitivity and specificity using non-invasively collected swabs and does not require specialist expertise. This study establishes that the *Bsal* real-time PCR has also demonstrated very high reproducibility both in terms of determining positive and negative samples and in the number of genomic equivalents detected across laboratories. It can thus be used to confirm negative *Bsal* test results from duplex real-time PCR assays where high GEs of *Bd* have been detected and negative histology-based tests such as immunohistochemistry and H&E tests. These characteristics indicate that this test is an extremely valuable tool in the arsenal of researchers,

TABLE 3 Comparison of laboratories (*t* test) based on proportion of positive results from total samples

Laboratories	Proportion of positive results/total samples	Proportion of positive results/total samples	*p*-value
Laboratories 1–2	Laboratory 1 0.684	Laboratory 2 0.684	1
Laboratories 1–3	Laboratory 1 0.684	Laboratory 3 0.658	.8102
Laboratories 1–4	Laboratory 1 0.684	Laboratory 4 0.632	.6341
Laboratories 2–3	Laboratory 2 0.684	Laboratory 3 0.658	.8102
Laboratories 2–4	Laboratory 2 0.684	Laboratory 4 0.632	.6341
Laboratories 3–4	Laboratory 3 0.657	Laboratory 4 0.632	.8136

Comparison of proportions of positive results from total number of samples among four laboratories. The laboratories were paired, and all participating laboratories' proportions were compared. The proportions were comparable as the detection of *Bsal* in the positive samples was 26/26 for laboratories 1&2 and 25/26 and 24/26 for laboratories 3&4, respectively.

TABLE 4 Kendall's correlation matrix of *Bsal* qPCR assay results (GE) in four laboratories

	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4
Laboratory 1	1.000	0.910	0.808	0.912
Laboratory 2	0.910	1.000	0.801	0.895
Laboratory 3	0.808	0.801	1.000	0.801
Laboratory 4	0.912	0.895	0.801	1.000

The results (genomic equivalents) of the *Bsal* qPCR assays performed in the four laboratories were analysed using a Kendall's correlation test and compared in a Kendall's correlation matrix to demonstrate the level of correlation between the number of genomic equivalents detected in each sample in each laboratory.

laboratories, clinicians and governmental agencies in identifying novel cases of *Bsal*, determining its dispersal and implementation of mitigation and/or prophylactic measures to contain its spread. The discovery that this test is able to detect *Bsal* in some hosts exposed to low fungal doses (100 spores) between 2 and 3 weeks post-exposure indicates its diagnostic efficacy and also provides a timeline where amphibians which have tested negative may be retested with even more reliable outcomes. The implications are monumental for disease prevention in naïve regions.

The developed *Bsal*- and *Bd* species-specific duplex real-time PCR allows detection of both pathogens on non-invasively collected amphibian samples. Furthermore, this test can easily be implemented by diagnostic laboratories, without the need for specialist expertise and experience required for accurate interpretation of alternative diagnostic tools for identifying the presence of chytrid fungi such as histology (Berger et al., 1999; Hyatt et al., 2007).

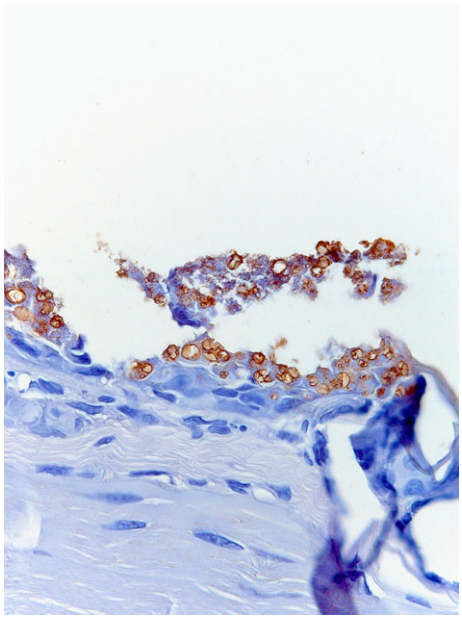

FIGURE 5 Immunohistochemical microscopy of a fire salamander with *Bsal* infection. Immunohistochemical staining of *Bsal* intracellular colonial thalli in a degraded epidermal layer

During the extensive use of the duplex real-time PCR (over 8,000 amphibian swab and tissue samples have been processed to date in our laboratory [Laking et al., 2017; Martel et al., 2014; Spitzen-van der Sluijs et al., 2016]), only rare incidents have occurred in which abnormal results have been obtained. One issue concerning the duplex real-time PCR is outlined in Table 2, namely that large differences in concentration between pathogens tended to lead to the lower concentration pathogen not being detected efficiently. We suggest that simplex *Bd* or *Bsal* assays are performed to confirm the true absence of the respective pathogen in amphibian samples, when the other pathogen is detected.

Another issue concerning the duplex real-time PCR was noted when the assay was used in post-mortem detection of *Bsal*. Repeated borderline positive results (Ct-values corresponding with GE values around or lower than 0.1 GE of *Bsal*) were obtained with the real-time PCR when it was applied to detect *Bsal* in amphibians (Alpine newts [*Ichtyosaura alpestris*]) post-mortem. *Bsal's* presence could not be confirmed with other diagnostic tools (histopathology and immunohistology), and sequencing did not confirm the presence of *Bsal*. In most cases, these results were associated with abnormal amplification curves, but normal amplification curves were occasionally observed as well (Figure 3). Therefore, when used as a post-mortem diagnostic tool, we suggest to elevate the detection limit to 1.0 GE of *Bsal* instead of 0.1 GE of *Bsal*, thus preventing an animal to be wrongly designated as *Bsal* positive. Furthermore, we stress that molecular diagnostic tools such as the duplex real-time PCR should be used in conjunction with techniques that are able to show colonization and/or pathology such as histology or histopathology, where able.

We found that the duplex real-time PCR is valuable as a post-mortem screening tool for *Bsal* (see Figure 1). Swab samples taken

from the back and abdomen and analysed with the duplex real-time PCR were able to detect *Bsal* on average for up to 7.2 days and 5.8 days after death, respectively. For tail tissue samples, post-mortem detectability was shorter with 4.5 days on average. This shows that despite advanced stages of decomposition (which occurred in all animals between 3 and 6 days post-mortem), the duplex real-time PCR is still able to detect *Bsal*, when animals have died due to *Bsal* infection (thus having a high *Bsal* infection load at time of death). Skin decomposition was found to affect diagnostic value of histological samples more rapidly, making the duplex real-time PCR a comparatively more sensitive method to show the presence of *Bsal* post-mortem.

Although histological examination failed to identify the presence of *Bsal* in most samples in this study (Figure 1), it should be noted that only single skin preparations were histologically assayed and that these preparations may not have been performed on samples from areas of the skin where there were *Bsal* lesions, whereas a higher probability of detection of chytrids can be obtained by examining multiple skin sections (Berger et al., 1999; Puschendorf & Bolaños, 2006; Skerratt et al., 2011).

Regardless of the technique used to detect *Bsal* post-mortem, on average, samples taken from the back had the longest detectability or in case of histology, the best chance of detecting *Bsal* (meaning presence of epidermis without autolysis obstructing histopathological examination). We believe that this can be attributed to more rapid degradation of DNA in the ventral area (as can be seen by the loss of dermal structure in the histological samples) which was in direct contact with soil and associated soil microorganisms. It has to be noted, however, that post-mortem detectability was assayed under experimental conditions. Therefore, environmental conditions such as temperature and/or humidity fluctuation or exposure to direct sunlight, which may also affect post-mortem detectability of *Bsal*, could not be taken into account.

For detection of *Bsal* in older and/or stored samples, histology does have an advantage over molecular methods such as real-time PCR. For instance, storage for months to years in neutral-buffered formaldehyde does not affect tissue morphology nor does it affect the ability of the immunohistochemical staining technique to detect *Bsal* (Figure 5).Whereas, detection of DNA in these stored samples utilizing genomic detection methods is challenging and often obstructed due to tissue lysis and/or DNA damage (Wandeler, Hoeck, & Keller, 2007; Zimmermann et al., 2008). Although protocols have been developed to circumvent issues with detecting chytrid DNA in archived samples, real-time PCR inhibition affecting accurate quantification still occurs to some extent and procedures are laborious (Adams et al., 2015). Instead, chytrid-specific

Application	Recommendations
Screening live animals	(1) Use the protocols for sampling, DNA extraction and PCR execution as described in Blooi *et al*. (2013). (2) Verify assay performance (precision, efficiency and linear correlation) using quantification standards of both *Bd* and *Bsal* as described in Blooi *et al*. (2013), and set the threshold halfway in the linear phase of the amplification curve. (3) Run all samples in duplicate. Interpret samples as: Positive: - Ct-values for both wells, or in three of four wells when a sample is rerun (see further down) - Normal amplification curves for all positive wells - Low variability between the duplicate (< 0.3 Ct-value) - Ct-values corresponding to GE-values ≥ 1 GE of *Bsal* Negative: - No Ct-values for both wells, or in two or less out of four wells when a sample is rerun - Ct-values corresponding to GE-values <1 GE of *Bsal* Ambiguous: - Ct-value in only one well - Abnormal amplification curves - High variability between the duplicate (> 0.3 Ct-value) - Presence of only *Bd* or only *Bsal* For all ambiguous samples, rerun the sample in duplicate and try to verify true presence/absence by sequencing and/or histology. In case the issue remains unresolved, repeat the sample collection and PCR analyses. For samples that show the presensce of only *Bd* or only *Bsal*, a simplex assay should be run to verify true absence of the other pathogen.
Post-mortem screening	(1) Collect skin swabs from the back (dorsal) side of the animal (side that has not been in contact with substrate) as soon as possible, but no longer than 5 days after death of the animal. (2) Uphold the recommendations for screening live animals, but with a cut-off for *Bsal*-positive samples of Ct-values corresponding to 1.0 GE of *Bsal* instead of 0.1 GE of *Bsal*. (3) Confirm the presence of disease lesions by histopathology. (Samples should preferably be taken from the areas of the skin with obvious lesions.)

FIGURE 6 Summary of the recommendations on the use of the duplex real-time PCR for *Bd* and *Bsal*. Recommendations on sampling, assaying and interpreting results of samples tested using the duplex real-time PCR for simultaneous detection of *Bsal and Bd*

histological staining techniques are useful tools to allow visualization of chytrids without the need for expertise in identifying chytrid stages present in the skin (Berger et al., 2002).

Although previously described, polyclonal antibodies against *Bd* and the polyclonal *Bsal* antibodies generated in this study were found not to be species-specific (even after antigen pre-absorption of antibodies). Several parameters were investigated to determine whether the specificity could be enhanced (longer incubation time, increased antigen content, increased antibody concentration and whole-cell pre-absorption); however, none of these tests were successful. Therefore, we are inclined to believe that most of the antigens to which the antibodies respond are common to *Bd* and *Bsal*. In a case like this, however, differences in pathology associated with infections of *Bd* and *Bsal* still allow for differentiation between the two pathogens in amphibian samples (White et al., 2016). A summary of our recommendations concerning the duplex real-time PCR for *Bsal* can be found in Figure 6.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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