CHAPTER 2.1.2.

INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

1. Scope

Infection with *Batrachochytrium salamandrivorans* means infection of amphibians with the pathogenic agent *Batrachochytrium salamandrivorans*, of the Division Chytridiomycota and Order Rhizophydiales.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The type strain of the pathogenic chytrid fungal agent *Batrachochytrium salamandrivorans* (Bsal) is AMFP13/1. Three more isolates have been described (Martel *et al*., 2014) but no information is available on genetic structuring or phenotypic variation. Phylogenetic analyses show that Bsal forms a clade with its sister species *B. dendrobatidis* (Martel *et al*., 2013). The genome size of the type strain was determined at 32.6 Mb with 10,138 protein-coding genes predicted (Farrer *et al*., 2017). The contribution of these proteins to virulence is currently not clear.

2.1.2. Survival and stability inside the host tissues

Bsal is an intracellular pathogen that develops inside epidermal cells. The presence of Bsal could be demonstrated using real-time polymerase chain reaction (PCR) on dorsal skin swabs up to 7 days on average post-mortem and using histopathology of dorsal skin tissue up to 3 days on average post-mortem (Thomas *et al*., 2018). It is not clear how long Bsal can survive inside tissues of a dead host and how long a dead host remains infectious.

2.1.3. Survival and stability outside the host

Encysted spores have been shown to remain infectious in pond water for up to at least 31 days (Stegen *et al*., 2017) and are considered more resistant in the environment compared with zoospores. Experimentally inoculated forest soil was demonstrated to remain infectious to fire salamanders for 48 hours (Stegen *et al.,* 2017). However, Bsal DNA was detected in contaminated forest soil for up to 28 weeks (Stegen *et al*., 2017). Whether this reflects the presence of viable Bsal organisms is not clear. The effect of desiccation on Bsal survival has not been studied.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species [under study]

Species that fulfil the criteria for listing as susceptible to infection with Bsal according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are under study and may include:

Family	Scientific name	Common name
Plethodontidae	*Hydromantes strinatii*	French cave salamander
Salamandridae	*Cynops cyanurus*	blue-tailed fire-bellied newt
	Cynops pyrrhogaster	Japanese fire-bellied newt
	Euproctus platycephalus	sardinian brook salamander
	Ichthyosaura alpestris	Alpine newt
	Lissotriton italicus	Italian newt

Family	Scientific name	Common name
	Neurergus crocatus	yellow spotted newt
	Nothophthalmus viridescens	eastern newt
	Paramesotriton deloustali	Tam Dao salamander
	Pleurodeles waltl	Spanish ribbed newt
	Salamandrina perspicillata	northern spectacle salamander
	Salamandra salamandra	fire salamander
	Taricha granulosa	rough-skinned newt

2.2.2. Species with incomplete evidence for susceptibility [under study]

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: [under study]

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Bsal is a pathogenic agent that mainly affects urodeles. Evidence from experimental infections and disease outbreaks in the wild and in captivity show that at least most, if not all, species of the Family *Salamandridae*, as well as species of the Family *Hynobiidae* are likely to become infected when exposed to Bsal. However, differences in susceptibility to infection between species do exist: for example, for fire salamanders, the infectious dose of Bsal was determined to be a theoretical one zoospore, whereas a significantly higher dose was necessary to infect Alpine newts; Stegen *et al*., 2017) and one western Palearctic species (*Lissotriton helveticus*) may be more resistant to infection (Martel *et al*., 2014). For the largest Family of salamanders (*Plethodontidae*), little information is currently available; at least one European species (*Speleomantes strinatii*) can be infected but other, North American species (*Gyrinophilus porphyriticus*, *Plethodon glutinosus,* Ambystomatidae) seem less susceptible to infection (Martel *et al*., 2014). Susceptibility of the Family of *Cryptobranchidae* is not clear, with a single infection found in a farmed Chinese giant salamander (*Andrias davidianus*; Zhiyong *et al*., 2018). No information is available on the urodele families *Proteidae, Rhyacotritonidae* and *Amphiumidae*. Bsal infection in anurans has only been detected in two species, in captivity, the wild and in lab trials (Nguyen *et al*., 2017; Stegen *et al*., 2017).

Thus far, infections with Bsal have been demonstrated only in amphibians post-metamorphosis. In one experimental infection trial, larvae of fire salamanders were exposed to Bsal, but did not become infected (Van Rooij *et al*., 2015). The extent to which factors such as age and sex affect susceptibility to infection post-metamorphosis is unknown.

In Europe, Bsal has been detected in captive collections of urodeles (Fitzpatrick *et al*., 2018, Sabino-Pinto *et al*., 2015) and the pet trade in salamanders and newts has been hypothesised to play a central role in the distribution of this fungus (Fitzpatrick *et al*., 2018; Yap *et al*., 2015; Zhiyong *et al*., 2018). Hence, urodeles that come into contact with traded urodeles, either directly (via co-housing or contact with wild animals or released or captive animals) or indirectly (via materials, contaminated water or soil), may have a high likelihood of exposure to infection with Bsal.

For the purposes of Table 4.1, salamander larvae with gill buds may be considered early life stages, larvae with developed or developing gills and limbs are juveniles and salamander with full developed gills and limbs are adults.

2.2.4. Distribution of the pathogen in the host

Bsal only infects the skin, where it remains limited to the epidermis.

2.2.5. Aquatic animal reservoirs of infection

A large number of salamanders, mainly belonging to the families *Salamandridae* and *Hynobiidae*, may survive episodes of infection (for example Alpine newts) or be considered tolerant, resulting in persistent subclinical infections. Although persistent infection has not been demonstrated for all species, in the native Bsal range in east Asia, Bsal infection and disease dynamics appear to be consistent for all species examined and appear capable of long-term persistent infections (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018).

In its invasive range, persistent infections (e.g. in Alpine newts) have been implicated in the local extinction of a highly susceptible species (fire salamanders). It is currently not clear which of the species, mentioned in Section 2.2.1 may sustain persistent infections in the invasive Bsal range. At least some species (the best-known example is the fire salamander) are highly susceptible and invariably die shortly after exposure (Martel *et al*., 2014; Stegen *et al*., 2017), making them unlikely to sustain persistent infections.

It is not known whether other, biotic reservoirs of Bsal exist.

2.2.6. Vectors

There is evidence that birds may carry zoospores attached to their feet (Stegen *et al*., 2017) and thus may act as vectors for Bsal.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In its native range in east Asia, Bsal has been demonstrated to be present in the wild at a prevalence of between 2 and 4% on average (data from China [People's Rep. of], Japan, Thailand, and Vietnam; Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018), but in the absence of any observed morbidity or mortality under natural conditions. In some populations (*Paramesotriton hongkongensis*), prevalence may reach 50% (Zhiyong *et al*., 2018). In its invasive range in Europe, Bsal was present in a population of fire salamanders at a prevalence of between 25 and 63% (Stegen *et al*., 2017). Bsal occurrence and associated mortality has been detected in captive collections of urodeles in Europe, including Germany (1), the United Kingdom (4), Belgium (1), the Netherlands (2) and Spain (1) (number in brackets indicates number of collections). When left untreated, morbidity and mortality can reach 100%, at least in members of the genus *Salamandra*.

Morbidity, mortality and minimum infectious dose vary considerably between species (Martel *et al*., 2014; Stegen *et al*., 2017). Based on natural outbreaks in captivity and in the wild and in infection trials, the case morbidity and case mortality rate in fire salamanders can reach 100%, independent of the initial level of Bsal exposure. This has resulted in the loss of over 99.9% of the fire salamander population at the Bsal index outbreak site in the Netherlands (Spitzen-van der Sluijs *et al*., 2016). All tested western Palearctic urodeles, except for *Lissotriton helveticus* and *Salamandrella keyserlingii*, showed 100% morbidity and mortality when exposed to a single, high dose of Bsal (Martel *et al*., 2014). However, at least for Alpine newts, the case morbidity and case fatality rates depend on the Bsal dose that the animal is exposed to: a high dose resulting in the highest mortality, while a low dose does not necessarily result in morbidity or mortality.

Morbidity and mortality also depend on environmental temperature. For the Bsal type strain, temperatures above 20°C reduces the level of infection and temperatures above 25°C eventually result in killing of Bsal and elimination of infection (Blooi *et al*., 2015b). Exposure of infected animals to conditions that inhibit Bsal growth may thus result in non-clinical or sub-clinical infections in susceptible species.

Co-occurrence of highly susceptible species such as fire salamanders with less susceptible species, such as Alpine newts may facilitate density independent disease dynamics that lead to the local extinction of the highly susceptible species (Stegen *et al*., 2017).

2.3.2. Clinical signs, including behavioural changes

Chytridiomycosis caused by Bsal may be accompanied by a combination of the following signs: epidermal ulcerations (ranging from discrete to extensive), excessive skin shedding, skin haemorrhages and/or fluid loss, anorexia, apathy, abnormal body postures and convulsions (Martel *et al.,* 2013).

2.3.3 Gross pathology

Skin anomalies (haemorrhages, ulcerations, presence of sloughed skin) are the main pathological findings (Martel *et al.,* 2013).

2.3.4. Modes of transmission and life cycle

Colonial or monocentric thalli of this fungus develop inside host epidermal cells and produce motile zoospores or walled, encysted spores, both of which are infectious stages. Zoospores are released through one or several discharge tubes. While motile spores actively swim towards a suitable substrate (e.g. a host), the encysted spores float at the water–air interface and passively adhere to a passing host (Stegen *et al.,* 2017). *In vitro*, developing thalli form fine rhizoids. Mature thalli *in vitro* are between 16 and

50 µm in diameter, *in vivo* between 7 and 17 µm; zoospores are approximately 5 µm in diameter. Motile zoospores are roughly spherical, the nucleus is located outside of the ribosomal mass, with aggregated ribosomes, multiple mitochondria and numerous lipid globules. The position of the non-flagellated centriole in free swimming zoospores varies from angled to parallel to the kinetosome (Martel *et al.,* 2013).

There are no indications of vertical transmission. However, this cannot be excluded in species giving birth to metamorphosed offspring (e.g. *Salamandra atra*, *Salamandra lanzai*, *Lyciasalamandra helverseni*). Horizontal transmission occurs through direct contact or contact with contaminated soil or water (Stegen *et al*., 2017). Infectious stages include the motile zoospore and the environmentally resistant encysted spores (Stegen *et al*., 2017). Infections can be reproduced under experimental conditions by topically applying a Bsal inoculum on the dorsum of amphibians and housing the exposed animals at 15°C (Martel *et al*., 2013; 2014; Stegen *et al*., 2017). This inoculum can either contain motile zoospores or the immobile, encysted spores.

Pathways of Bsal dispersal within Europe are poorly understood but may be anthropogenic (e.g. through contaminated material). Zoospores attach to bird feet, suggesting birds may spread Bsal over larger distances (Stegen *et al*., 2017). Direct animal-to-animal contact is necessary for transmission of Bsal: salamanders only separated by 1 cm from infected conspecifics were not infected in laboratory trials, in contrast to co-housed animals (Spitzen-van der Sluijs *et al*., 2018). Overall, dispersal ability of Bsal in Europe currently seems limited: Bsal was found not to be transmitted to a neighbouring site in the Netherlands, despite being downstream of a small stream, and the current distribution of Bsal in Europe is probably not continuous (Spitzen-van der Sluijs *et al*., 2018).

Although Bsal dispersal between populations is now hypothesised to be mainly human mediated, other factors (e.g. wildlife, water) may play key roles and critical knowledge about Bsal dispersal is currently lacking.

2.3.5. Environmental factors

The Bsal type strain AMFP13/1 tolerates temperatures up to 25°C but is killed at higher temperatures (Blooi *et al*., 2015b). As Bsal infections have been demonstrated in aquatic newts at water temperatures above 25°C (Laking *et al*., 2017; Zhiyong *et al*., 2018), it is likely that thermal tolerance may be Bsal lineage dependent. A temperature of 4°C results in slower progression of infection but does not reduce morbidity or mortality (Stegen *et al*., 2017). Desiccation is likely to be poorly tolerated by Bsal, although data are currently lacking, and the encysted spore may be resistant to drying (Stegen *et al*., 2017; Van Rooij *et al*., 2015). It is not known to what extent Bsal tolerates freezing.

2.3.6. Geographical distribution

Asia is currently considered the region of origin of Bsal (Martel *et al*., 2014), where the infection appears to be endemic in amphibian communities across a wide taxonomic, geographical and environmental range, albeit at a low prevalence between 2 and 4% (Zhiyong *et al.,* 2018). In Asia, Bsal was shown to be widely present in urodele populations in China (People's Rep. of), Japan, Thailand and Vietnam. East Asia is presumed to be the native range of the fungus (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al.,* 2018).

Europe is considered the invasive range of the fungus where Bsal was first identified during a mortality event in fire salamanders in Bunderbos, the Netherlands (Martel *et al.,* 2013). In Europe, Bsal was detected by surveys of wild susceptible species in Belgium, Germany and the Netherlands (Martel *et al.*, 2014; Spitzen-van der Sluijs *et al.,* 2016), and in captive urodele populations in Belgium, Germany, the Netherlands, Spain, and the United Kingdom (Fitzpatrick *et al.,* 2018; Sabino-Pinto *et al.,* 2015).

Bsal has not been reported in Africa or the Americas.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Not available.

2.4.2. Chemotherapy including blocking agents

A combined treatment using Polymyxin E, voriconazole and a temperature regime of 20°C has been shown to be effective in eradicating Bsal from infected hosts (Blooi *et al*., 2015c). If the treatment is not performed properly and does not achieve eradication, low level carriers are created and the likelihood of Bsal detection, is reduced.

2.4.3. Immunostimulation

Not available.

2.4.4. Breeding resistant strains

No information available.

2.4.5. Inactivation methods

Bsal is sensitive to a wide variety of disinfectants (Van Rooij *et al*., 2015). Inactivation using formalin has been shown to hamper DNA detection using real-time PCR. Bsal is killed within 30 seconds in 70% ethanol (Van Rooij *et al.,* 2017). Inactivation in 70% ethanol allows for subsequent molecular tests yet is less suitable for histopathology. The Bsal type strain AMFP 13/1 is killed at temperatures exceeding 25°C; consequently, inactivation of this fungus can be achieved through heat treatment by autoclaving (Martel *et al.*, 2013).

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

In captivity, pathogen detection is difficult due to low prevalence in subclinically infected animals that often carry Bsal at low intensities (Martel *et al*., 2014; Zhiyong *et al*., 2018). These subclinically infected animals often belong to (but are not restricted to) taxa of Asian urodeles. Highly susceptible species (such as fire salamanders) may serve a sentinel function. Temperature regimes in captivity may strongly interfere with pathogen detection. Temperatures higher than 20°C (and below 25°C) severely impair pathogen proliferation in the host skin (Blooi *et al*., 2015b) and may result in infections that cannot be detected.

Heat treatment can be used to clear infection with Bsal in thermotolerant salamander species (Blooi *et al*., 2015a).

Barriers to pathogen dispersal, for example those preventing migration of infected hosts such as amphibian fences or roads, or those preventing transmission by potential Bsal vectors including humans, fomites and wildlife, may prevent transmission at small spatial scales (Spitzen-van der Sluijs *et al*., 2018).

3. Specimen selection, sample collection, transportation and handling

This Section draws on information from Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

In cases of disease or mortality in urodeles in captivity, sampling should be focused primarily on diseased or moribund animals (i.e. those showing skin lesions and abnormal behaviour). In a population with ongoing disease and mortality, live but diseased animals are preferentially sampled. The second choice is dead animals. Only freshly dead animals should be sampled as detectability of Bsal deteriorates post-mortem (Thomas *et al*., 2018). However, in the absence of diseased or freshly dead animals, apparently healthy animals can be sampled.

Similarly, in wild populations, samples should be taken preferentially from diseased, moribund or freshly dead animals; however, as these may quickly be removed (i.e. through predation, scavenging) only healthy animals may be available. Populations which have declined or where dead animals have been observed should be targeted.

3.2. Selection of organs or tissues

The only relevant tissue is skin tissue and probably only from amphibians post-metamorphosis. Both invasive (skin biopsies) and non-invasive (medical swabs) samples are appropriate, given the apical shedding of Bsal spores. In dead animals, dorsal skin is the preferred tissue, given its slower post-mortem decay (Thomas *et al*., 2018).

3.3. Samples or tissues not suitable for pathogen detection

Tissues other than skin are not suitable for the detection of Bsal in amphibians.

3.4. Non-lethal sampling

Non-lethal sampling is possible, either by collecting skin biopsies (toeclips or tailclips) or by non-invasively collecting samples using medical swabs. The latter is preferred given its minimal impact on animal welfare. As Bsal is limited to the superficial skin layers of the amphibian host, non-lethal sampling results are equivalent to lethal sampling results. Large numbers of animals can be sampled using skin swabs with minimal effects on animal welfare. Medical swabs should be rubbed firmly over the abdomen (10 times), the underside of a foot (10 times) and the ventral tail (10 times) using the tip of the swab. The use of disposable gloves for manipulating amphibians is highly recommended. Swabs should be transported immediately to the diagnostic laboratory or should be frozen until transfer.

3.5. Preservation of samples for submission

3.5.1. Samples for pathogen isolation

Bsal isolation is a laborious procedure, requiring up to two months to obtain a pure culture from a clinical sample. Isolation from animals that died due to Bsal infection is hampered by bacterial overgrowth. The best sample for Bsal isolation is a diseased, living animal, which is euthanised just prior to an isolation attempt. Before sampling, diseased animals should be kept at temperatures between 5 and 15°C to avoid clearance of infection (Blooi *et al*., 2015b).

3.5.2. Preservation ofsamples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Skin swabs should be stored dry and preferably frozen.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Skin samples for histopathology should be fixed immediately after collection. The recommended ratio of formalin (10%) to tissue is 10:1.

3.5. 4. Samples for other tests

Not applicable.

3.6. Pooling of samples

Pooling of up to four skin swab samples appears to allow reliable detection of Bsal in clinically affected animals (Sabino-Pinto *et al*., 2019a; 2019b) but estimates of the impact on diagnostic performance of the test have not been determined. Given low infection intensities in subclinically infected animals, sampling and testing of individual animals is recommended.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ =	Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
++ =	Suitable method(s) but may need further validation;
+ =	May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;
Shaded boxes =	Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts	+	+	+	1	+	+	+	1				
Histopathology[3]	+	+	+	1	++	++	++	1				
Cell culture									+	+	+	1
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Conventional PCR												
Amplicon sequencing[4]												
***In-situ* hybridisation**												
LAMP												
Lateral flow assay					+	+	+	1				
Immunohistochemistry												

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2.); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

[2]Early and juvenile life stages have been defined in Section 2.2.3.

[3]Cytopathology and histopathology can be validated if the results from different operators has been statistically compared.

[4]Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Wet mounts of skin scraping or pieces of shed skin can be examined at magnification 10× using light microscopy. The presence of motile spores of approximately 5 µm are indicative of amphibian chytrid infection.

4.2. Histopathology and cytopathology

Histopathology of skin in amphibians post-metamorphosis may provide strong indications of Bsal infection. In haematoxylin/eosin stained sections, histopathological evidence suggestive of Bsal infections of skin, multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular chytrid-type fungal thalli provides histopathological evidence of Bsal infection (Martel *et al*., 2013; White *et al*., 2016). Using immunohistochemistry, Bsal thalli can be stained, which aids in detecting low level infections (Thomas *et al*., 2018). Histopathology is highly indicative, yet does not allow definitive identification of Bsal, which needs further confirmation. In randomly collected skin samples from experimentally infected salamanders, histopathology was capable of detecting Bsal in only a minority of the samples (Thomas *et al*., 2018). In dead animals, post-mortem decay of the epidermis may mask the lesions (Thomas *et al*., 2018). Lesions can be so extensive, that the epidermis is entirely eroded and no fungal thalli can be observed. Mild infections can be missed due to the multifocal and small lesions (Thomas *et al*., 2018). In subclinically infected animals, diagnostic sensitivity should be rated low. In clinically affected animals, sensitivity and specificity of histopathology and immunohistochemistry have not been quantified.

No reports are available on the use of cytopathology.

4.3. Cell culture for isolation

Bsal can be isolated and cultured on artificial media, yet this is a laborious and difficult procedure, typically requiring between 4 weeks and 2 months. There is a significant probability of bacterial overgrowth, which hampers fungal isolation, resulting in poor sensitivity. The protocol of Fisher *et al*. (2018) can be used. Small (approximately 1 mm^2) pieces of skin from an infected, diseased animal should first be thoroughly cleaned by wiping through agar plates. The cleaned pieces of skin can then each be transferred to a well of a 96-well plate, containing tryptone-gelatin hydrolysate lactose broth (TGhL) containing penicillin/streptomycin (200 mg/litre) and incubated at 15°C. Wells showing chytrid growth without bacterial contamination can be used for subculturing (Martel *et al*., 2013). Chytrid growth can be visualised by examining the wells under an inverted microscope (10–40 × magnification).

Given the difficulties to isolate Bsal from infected animals and the high uncertainty to obtain a viable culture, this method is not appropriate as a routine diagnostic method, but in rare cases may be useful to confirm infection or to obtain isolates for research.

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

The following information is derived from Blooi *et al*. (2013), Thomas *et al*. (2018) and Sabino Pinto *et al*. (2018). DNA from skin swabs can be extracted using commercial DNA extraction kits. Extracted DNA is diluted tenfold to minimise possible PCR inhibition. Controls should be run with each assay: at least a negative extraction control and a positive control; preferably, an internal PCR control is included. Positive control consists of DNA extracts of a tenfold dilution series of Bsal zoospores from 1 to 100,000 to allow quantification.

A TaqMan PCR has been partially validated to level 3 without however, stating its intended purpose (Thomas *et al*., 2018). SYBR green real-time PCR, may be used as well but needs further validation to determine specificity and sensitivity (Martel *et al*., 2013). The TaqMan PCR can either be used as simplex PCR or in combination with primers to detect *B. dendrobatidis* in a duplex PCR (Blooi *et al*., 2013) and uses the forward primer STerF (5'-TGC-TCC-ATC-TCC-CCC-TCT-TCA-3'), reverse primer STerR (5'-TGA-ACG-CAC-ATT-GCA-CTC-TAC-3') and Cy5 labelled probe STerC (5'-ACA-AGA-AAA-TAC-TAT-TGA-TTC-TCA-AAC-AGG-CA-3') to detect the presence of the 5.8S rRNA gene of Bsal. Intra- and interassay efficiency were 95.7 and 96%, respectively (Blooi *et al*., 2013). This TaqMan duplex PCR does not decrease detectability of both Bd and Bsal, except in case of mixed infections (Thomas *et al*., 2018). The use of simplex Bsal-specific PCR is therefore recommended in case Bd has been detected in the sample. The sensitivity of this real-time PCR is between 96 and 100% and diagnostic specificity 100% (95% CI: 73–100%; Thomas *et al*., 2018) when used in clinically affected animals. Although DNA quantities as low as 0.1 genomic equivalent can be detected (Blooi *et al*., 2013), Thomas *et al*. (2018) recommend a threshold of 1 genomic equivalent per reaction to reduce the likelihood of false positive

results. Borderline results (≤ 1 GE per reaction) should be classified as suspect and need confirmation by sequencing (or isolation).

Samples are preferably run in duplicate. A sample is considered positive based on the combination of (1) the shape of the amplification curves (2) positive results in both duplications, (3) returning GE values above the detection threshold (1 GE per reaction) (4) low variability between duplicates (< 0.3 Ct value).

4.4.2. Conventional PCR

No conventional PCR protocol has been validated.

4.4.3. Other nucleic acid amplification methods

None validated.

4.5. Amplicon sequencing

No conventional PCR protocol has been validated.

4.6. *In-situ* hybridisation

No *In-situ* hybridisation validated protocols are available.

4.7. Immunohistochemistry

Immunohistochemistry is currently not Bsal specific, due to the lack of Bsal specific antibodies (Dillon *et al*., 2017; Thomas *et al*., 2018).

4.8. Bioassay

Not available.

4.9. Antibody- or antigen-based detection methods

A lateral flow assay (LFA) using an IgM monoclonal antibody (MAb) was developed to detect infection in amphibian skin samples. This MAb does not discriminate between *B. salamandrivorans, B. dendrobatidis* and *Homolaphlyctis polyrhiza* (Dillon *et al.*, 2017). The sensitivity of this test is likely to be lower than that of the real-time PCR (Dillon *et al.*, 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using real-time PCR. This would make this technique most useful in animals with high infection loads. Such techniques may be useful for point-of-care testing if specificity is increased and provided thorough validation.

4.10. Other methods

Not applicable.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The use of real-time PCR on skin swabs is recommended for surveillance.

6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or in the presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

6.1. Apparently healthy animals or animals of unknown health status[1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

Such surveys typically consist of non-invasive sampling using skin swabs that are examined for the presence of Bsal using real-time PCR. When applied to animals in the wild, confirmation by using a complementary technique, other than sequencing the PCR product, is often not feasible.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with Bsal shall be suspected if one of the following criteria is met:

i) Positive result by real-time PCR;

ii) Histopathological changes consistent with the presence of the pathogen or the disease;

iii) The presence of motile spores, compatible with chytrid zoospores, in wet mount of urodele skin.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with Bsal is confirmed if, in addition to the criteria in Section 6.1.1, one of the following criteria is met:

i) Positive result by real-time PCR;

ii) Pathogenic agent isolation from the skin in culture and identification by real-time PCR.

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with Bsal shall be suspected if at least one of the following criteria is met:

i) Clinical signs (haemorrhages, ulcerations, presence of sloughed skin, see Section 2.3.2), notably the presence of skin ulcers and/or disecdysis;

ii) Positive result by real-time PCR;

iii) Histopathological changes consistent with the presence of the pathogenic agent or the disease;

iv) Visual observation (by microscopy) of motile spores, compatible with amphibian chytrid zoospores, in a wet mount of the skin of at least one diseased urodele;

v) Positive result of antigen detection by LFA.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with Bsal is confirmed if, in addition to the criteria in Section 6.2.1, one of the following criteria is met:

i) Positive result by real-time PCR;

ii) Pathogenic agent isolation from the skin in culture and identification by real-time PCR.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with Bsal is provided in Table 6.3.1. This information can be used for the design of surveys for infection with Bsal, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic

1 For example transboundary commodities.

performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

Table 6.3.1. *Diagnostic performance of tests recommended for surveillance or diagnosis*

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR	Diagnosis	Experimentally infected salamanders (clinical and subclinical infection)	Skin swabs	*Salamandra salamandra*	96–100 (26)	100 (12)	Droplet digital PCR	Thomas *et al.* (2018)

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study.

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NB: At the time of publication (2021) there was no OIE Reference Laboratory for Infection with *Batrachochytrium salamandrivorans*
(see Table at the end of this *Aquatic Manual* or consult the OIE web site:
https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2021.