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Amphibian: A Case Definition and Diagnostic Criteria for *Batrachochytrium salamandrivorans* Chytridiomycosis

A newly described chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), has caused several die-offs in salamanders in Europe (Martel et al. 2013; Cunningham et al. 2015). This pathogen is similar to the species *B. dendobatidis* (*Bd*) that has caused massive amphibian population declines globally. As the United States is home to the world's most diverse salamander assemblage, the United States Fish and Wildlife Service has sought to increase protection of native salamander populations via an interim rule under the Lacey Act (18 USC 42- USFWS 2016). This rule, effective 28 January 2016, limits international and interstate movement of 20 genera of salamanders. Concurrent with this work, a U.S. *Bsal* Task Force (Grant et al. 2016) was formed to help prevent and manage cases that might occur in North America. The diagnostics committee of this Task Force developed the following *Bsal* case definition to promote standardized communication of results and consistent national reporting of this invasive pathogen among multiple diagnostic laboratories in order to provide reliable information to wildlife managers.

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Case definitions typically include field, gross, histopathology, laboratory, and epidemiologic criteria for assigning an individual to a specific disease or condition. The diagnostic criteria including field signs, gross pathology and histopathology have been established from *Bsal* cases in free-ranging salamanders in Europe and North American species examined during experimental infection trials (Martel et al. 2013; A. P. Pessier, unpubl. data). As an emerging pathogen some aspects of *Bsal* ecology, including variation in species susceptibility and field signs for North American species, are unknown. The case definition, therefore, errs on the side of specificity over sensitivity by requiring both histopathologic and laboratory confirmation to be considered a confirmed *Bsal* case. However, we also included a category for reporting the presence of the *Bsal* fungus in the absence of corroborating evidence of disease (i.e., histopathology) since consistent reporting of this information will be useful for examining pathogen spread in new geographic locations and susceptibility in new host species.

The case definition presented here represents the current knowledge of *Bsal* infection in amphibians and is intended to provide background information on this new pathogen and the clinical and histopathogical presentation of the disease. Criteria for diagnoses are provided and should be used for determining disease presence. However, it should be noted that since this disease has not been found in free-ranging North American amphibian species, variation in its presentation for these new populations is unknown, and the definitions proposed below may evolve with geographical, environmental, and host expansion.

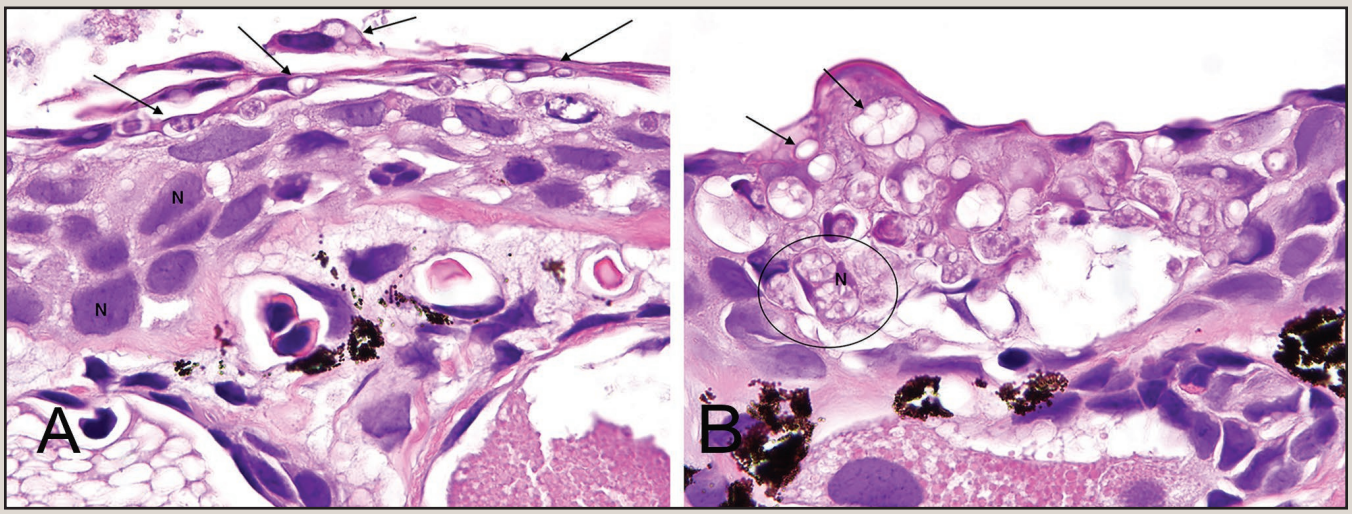

FIG. 1. Comparison of *Bd* and *Bsal*-type chytridiomycosis. *Notophthalmus viridescens*. Hematoxylin and eosin stain 1000×. *Bd*-type chytridiomycosis (A) is proliferative with epidermal hyperplasia and hyperkeratosis and numerous chytrid thalli in superficial keratinocytes (arrows). The keratinocytes are arranged in distinct layers with intact nuclei (N). In contrast, *Bsal*-type chytridiomycosis (B) has almost full-thickness necrosis of keratinocytes with myriad chytrid thalli (arrows) that frequently have internal septa (colonial thalli). Note the loss of distinct layers of keratinocytes. At the periphery of this area of necrosis, a remaining keratinocyte has two intracytoplasmic chytrid thalli that marginate the nucleus (circle).

CHYTRID FUNGUS IN THE ORDER RHIZOPHYDIALES, GENUS *BATRACHOCHYTRIUM*, SPECIES *SALAMANDRIVORANS*

I. Individual, Place, and Time

Individual.—Post-metamorphic amphibians (especially salamanders) are known to be susceptible. Although larval Fire Salamanders (*Salamandra salamandra*) are not susceptible (Van Rooij et al. 2015), larval susceptibility in other species is unknown. There is increased index of suspicion for mortality events involving multiple salamander species (and notably not involving co-occurring frog species), or North American species with laboratory confirmed susceptibility to *Bsal* chytridiomycosis, specifically *Notophthalmus viridescens* (Eastern Newt), *Taricha granulosa* (Rough-skinned Newt).

Place.—There are currently no specific restrictions for where *Bsal* can occur.

Time.—There are currently no specific restrictions for when *Bsal* can occur.

II. Diagnostic Description

Field observations.—A clinically compatible case includes amphibians with skin ulcers and lethargy, leading to a typically high mortality rate. Weak or erratic swimming may also occur.

Gross necropsy observations.—On post-mortem examination, clinically compatible cases may present with subtle to severe skin ulcers or erosions; some cases will have no lesions visible on gross post-mortem examination. In addition to *Bsal* infection, biologists, veterinarians, and diagnosticians are reminded to consider differentials for ulcerative or erosive lesions in amphibians such as viral, bacterial, parasitic or other fungal infections as well as lesions due to trauma or environmental conditions (Green 2001). Lesions due to *Bsal* infection may occur at any site on the head, body, limbs, or tail of infected individuals. Chytridiomycosis is not known to be associated with internal gross lesions (Martel et al. 2013). Although clinical compatibility may raise the index of suspicion for a positive diagnosis, its presence is not required for diagnostic categorization.

Histopathology.—Histopathological evidence most suggestive of *Bsal* infection in the epidermis of salamanders is multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular chytrid-type fungal thalli (Fig. 1) (Martel et al. 2013; A. P. Pessier, unpubl. data). Thalli have a diameter of 6.9–17.2 µm (average 12.2 ± 1.9 µm, N = 50). Lesions multifocally have areas of erosion or ulceration. In early lesions and at the periphery of older lesions, chytrid thalli may be observed within keratinocyte cytoplasm with margination of cell nuclei.

Necrotizing lesions most suspicious for *Bsal* (*Bsal*-type chytridiomycosis) can be contrasted with those typical of *Bd* infection (*Bd*-type chytridiomycosis) in which the epidermis is predominantly hyperplastic and hyperkeratotic with many superficial intracytoplasmic chytrid-type fungal thalli (Fig. 1). However, there is overlap in these infection patterns.

General histologic features of chytrid (*Batrachochytrium* spp.) fungal thalli are 5–20 µm diameter, round to slightly oval organisms, that may contain 2–3 µm basophilic spores (zoospores) or a discharge tube (giving a flask-like appearance) (Berger et al. 2005). However, developing thalli or empty thalli (those that have already discharged zoospores) often predominate. A highly characteristic feature of chytrid thalli are colonial thalli that have internal septa. Colonial thalli are most easily observed when empty or with stains that highlight fungal cell walls (e.g., Grocott's methenamine silver or periodic acid-Schiff). Features of chytrid thalli that are more consistent with *Bsal* over than *Bd* are numerous colonial thalli (compared to infrequent) with multiple internal septa (rather than single). However, these two species cannot be definitively distinguished histologically at this time.

III. Laboratory Criteria for Diagnosis

Current molecular methods for verification of *Bsal* via polymerase chain reactions (PCR) are provided by Martel et al. (2013) and via quantitative PCR by Blooi et al. (2013). Methods for culturing *Bsal* are available in Martel et al. (2013).

Case Classification

Below we provide criteria for three diagnostic categories of *Bsal* based on diagnostic criteria. PCR and culturing methods allow for confirmation of the presence of *Bsal* but do not necessarily indicate infection or the presence of disease. Histopathology can provide support for chytridiomycosis in the salamander, but as some histopathological findings such as necrosis and ulceration in the absence of chytrid fungi are non-specific and can be caused by a wide variety of pathogens and environmental conditions (Green 2001), a definitive diagnosis must include both identification of the pathogen and demonstration of disease in the salamander.

Bsal present.—Positive *Bsal* PCR or *Bsal* culture from skin tissue of an animal for which compatible gross lesions were not observed and for which histopathology was either not done or was not consistent with *Bsal* histopathology (lacking epidermal necrosis associated with chytrid zoosporangia).

OR

Positive *Bsal* PCR or *Bsal* culture from a sample (e.g., swab from individual or environmental sample) for which gross pathology was not observed or recorded and for which histopathology was not done or was not consistent with *Bsal* histopathology (lacking epidermal necrosis associated with chytrid zoosporangia).

Further verification (via additional samples or alternative methodology) of a *Bsal* positive sample in a new area of detection is highly recommended.

Suspect Bsal chytridiomycosis.—Histopathology consistent with *Bsal* infection (epidermal necrosis associated with chytrid zoosporangia) in the absence of corroborating *Bsal* PCR or *Bsal* fungal culture (either negative results or specimen unsuitable for analysis).

Further verification (via additional diagnostics such as PCR or culture) of a *Bsal* suspect sample is necessary for a definitive diagnosis.

Confirmed Bsal chytridiomycosis.—Histopathology consistent with *Bsal* infection (epidermal necrosis associated with chytrid zoosporangia) and positive *Bsal* PCR or *Bsal* culture from skin samples

OR

Histopathology consistent with chytridiomycosis AND positive *Bsal* PCR or *Bsal* culture and *Bd* PCR or culture negative.

If *Bd* is also detected, further investigation into the cause of the disease is highly recommended.

Conclusion.—As with all emerging diseases there are limited data and many knowledge gaps for *Bsal*. This case definition provides guidelines for reporting *Bsal* chytridiomycosis. Any diagnosis of *Bsal* should be reported immediately to a regional, state, or federal wildlife agency. This case definition will be updated as further information is reported and a current version will be located at www.salamanderfungus.org.

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