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Comparison of methods for detection of chytrid fungus (*Batrachochytrium dendrobatidis*) in bullfrog tadpole mouthparts

Sho KADEKARU[1)] and Yumi UNE[1)]*

[1)]Laboratory of Veterinary Pathology, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

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ABSTRACT. We previously reported that the tadpole of bullfrog (*Lithobates catesbeiana*) is a useful model for the field surveillance of the *Batrachochytrium dendrobatidis* (Bd) distribution. In the present study, we compared Bd detection rates in swab-scraped and resected mouthpart samples, using nested polymerase chain reaction (PCR). The resulting detection rates for swab-scraped and resected specimens were 67 and 65%, respectively, with no significant difference. Furthermore, we performed a histopathological examination for Bd distribution in the mouthparts; we found that Bd infection occurred in the tip and basement of the jaw sheaths and tooth rows. We recommend using swab-scraped samples for Bd detection. Moreover, careful attention should be paid to scraping the tip and basement of the jaw sheaths and the entire oral cavity to reduce the rates of false-negative results on nested PCR of the mouthparts of bullfrog tadpoles.

KEY WORDS: *Batrachochytrium dendrobatidis*, bullfrog tadpole, mouthpart, nested polymerase chain reaction, swab

The World Organisation for Animal Health (OIE) has listed the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) as a serious pathogen of amphibian species and has developed a strategy for amphibian conservation [8, 9]. The establishment and implementation of conservation measures requires understanding Bd distribution; therefore, a useful investigation method needs to be developed considering the target animal (host), and its developmental stages, samples, and detection method for the fungus.

We previously confirmed that the wild bullfrog *Lithobates catesbeiana* tadpole is a suitable animal model for investigating Bd distribution in Japan [5]. Thus, in the present study, we compared the sensitivity of two detection methods for Bd: nested polymerase chain reaction (PCR) for Bd genomic DNA from (1) swab-scraped mouthpart samples (hereon referred to as "swab nested PCR") and (2) resected mouthpart samples (hereon referred to as "tissue nested PCR"). In addition, tissue nested PCR was followed by histopathological examination to determine the most effective sampling site in the mouthparts for Bd detection.

The detection rates of swab nested PCR and tissue nested PCR were investigated using samples from 120 wild bullfrog tadpoles. The tadpoles were collected from two sites: 60 from Saihaku county in Tottori prefecture on May 13, 2012, and 60 from Seihi town, Saikai city of Nagasaki prefecture on February 17, 2013; these collection times corresponded to the time at which the Bd infection rate is reportedly high [5, 10], in conformity with the Invasive Alien Species Act [7]. The collected bullfrog tadpoles were anesthetized and then euthanized, as described previously [5], for measuring body length and body weight. The bullfrog tadpoles collected from the two sites were at developmental stages 25 to 41 [3]; the average length and body weight of tadpoles collected in Tottori and Nagasaki were 9.7 cm (6.2–11.6 cm) and 14.1 g (4.1–22.6 g), and 8.8 cm (5.3–11.0 cm) and 9.0 g (3.6–14.3 g), respectively. Because the mouthparts were too small for the specimens to be used for nested PCR and histopathological analysis for the same individual, the bullfrog tadpoles were randomly divided into two groups of 60 individuals each, with no difference in Bd infection rate between groups, as revealed through macroscopic examination: there were 14 and 15 tadpoles with oral chytridiomycosis in Group 1 (nested PCR group) and Group 2 (histopathological analysis group), respectively.

All mouthparts of tadpoles in Group 1 were swabbed with sterile cotton swabs because Bd was previously reported to occur on both the jaw sheaths (the tip and the basement) and tooth rows [5], and the mouthparts were then resected. The swabs and resected mouthparts were stored frozen in a sterile microtube at −20°C for nested PCR. Bd DNA extraction from the swab samples was conducted using the NucleoSpin® Tissue kit (Macherey-Nagel, Duren, Germany) in accordance with a previous method [5].

*Correspondence to: Une, Y.: une@azabu-u.ac.jp

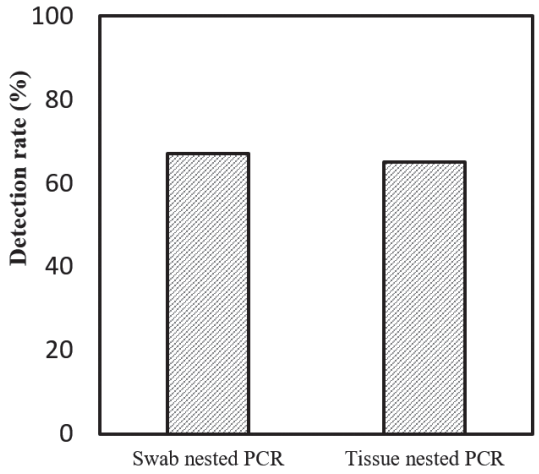

Fig. 1. Detection rates of the swab nested PCR and tissue nested PCR. n=60 per group.

Table 1. Sites of *Batrachochytrium dendrobatidis* in the mouthparts of bullfrog tadpoles detected by histopathological examination

No.	Jaw sheaths		Tooth rows	
	Upper	Lower	Upper	Lower
1	-	-	-	+
2	-	+	-	+
3	-	-	+	+
4	-	-	+	-
5	-	-	-	+
6	-	-	-	+
7	-	-	-	+
8	-	+	-	-
9	+	-	+	-
10	-	+	-	-
11	+	-	-	-
12	-	+	-	-
13	-	+	-	-
14	-	+	-	-
15	-	+	-	-
16	-	-	+	-
17	+	-	-	-
18	-	-	-	+
19	-	+	-	-
20	+	-	-	+

+ indicates the tadpole that observed Bd infection. − indicates the tadpole that not observed Bd infection.

Bd DNA was extracted from resected mouthparts using the same method after homogenization with a disposable homogenizer (BioMasher®, Nippi Inc., Tokyo, Japan). Nested PCR was performed as described by Goka *et al.* [2].

The resected mouthparts of tadpoles in Group 2 were fixed in 10% neutral-buffered formalin for over 24 hr. The fixed mouthparts were then cut along the longitudinal midline and processed routinely for tissue preparation [5]. On histopathological examination, the infected site (upper and lower jaw sheaths, and upper and lower tooth rows) was determined in the mouthparts of Bd-infected tadpoles.

The chi-square test was used to evaluate the difference in detection rates of swab nested PCR and tissue nested PCR; $P<0.05$ was considered statistically significant.

Among the 60 samples per group, Bd was detected in 40 (67%) and 39 (65%) tadpoles in the swab nested PCR and tissue nested PCR groups, respectively (Fig. 1); this difference was not significant. Histopathological examination revealed that the sites of infection of Bd were the jaw sheaths in 12 tadpoles, tooth rows in 11 tadpoles, and both sites in 3 tadpoles (Table 1). Although Bd was observed on the tips and bases of the jaw sheaths and on the tooth rows, the Bd-infected site differed among individual tadpoles.

Bd infects the whole skin surface of adult frogs, especially the ventral surface and digital skin; however, in tadpoles, Bd primarily infects the surface of the mouthparts, and is distributed on only the keratinized layer because Bd primarily metabolizes keratin. Therefore, the skin of frogs and the mouthparts of tadpoles are the main targets of diagnostic tests using swab PCR or tissue PCR and histopathological examination samples [1, 4, 6, 8]. Indeed, some previous studies have compared the sensitivities of these detection methods. Hyatt *et al.* [4] compared the detection rates on quantitative TaqMan PCR (TaqMan PCR) and histopathological examination using experimentally infected adult great barred frog (*Mixophyes fasciolatus*), and found that the sensitivity of TaqMan PCR was higher than that of histopathological examination [4]. They also reported that both the tissue and swab samples of the mouthparts from two species of *Litoria* were extremely effective for diagnosing a Bd infection with TaqMan PCR [4]. Retallick *et al.* [11] compared the sensitivities of detection with swab and resected mouthparts through TaqMan PCR, using experimentally Bd-infected *Rana subaquavocalis*, and found that the prevalence of Bd detected with resected mouthparts was consistently higher than that detected from the swab samples.

Since the aforementioned frog species are not found in Japan, it is essential to establish an effective Bd detection test that specifically targets Japanese wild amphibians, especially because the prevalence of Bd differs among amphibian species. In addition, TaqMan PCR is not suitable for diagnosing Bd infections among wild amphibians in Japan, since this method can only detect the four Bd species that are the primary causes of global pandemics; however, many more Bd haplotypes are distributed in Japan [2]. Goka *et al.* [2] developed a nested PCR method that can detect at least 26 haplotypes and has higher sensitivity than TaqMan PCR. However, the sensitivity of different samples in nested PCR for Bd infection of the tadpole mouthparts has not yet been validated. In this study, we verified that swab and resected tissue nested PCR are equally effective methods for detecting Bd

infection in the mouthparts of bullfrog tadpoles. Since there was no significant difference in the detection rates between nested PCR from swab and resected tissue samples, we recommend that swab nested PCR should be applied for the diagnosis of Bd infections in amphibian species. However, swab nested PCR has also been associated with a high rate of false-negative results when sampling is poor because the results can be influenced by the specific technique of the swab sampler [4, 11]. Moreover, our previous results contrast the present results, since the detection rate on histopathological examination was actually higher than that on swab nested PCR [5]. In our previous study, histopathological examination revealed a Bd infection on the tip and basement of the jaw sheaths, whereas Bd infection on the tooth rows was not consistently observed [5]. However, in the present study, 12 samples were positive for Bd on the jaw sheaths and 11 samples were positive for Bd on the tooth rows, and there was no significant difference in the detection rate with reference to the site in the 20 Bd-positive tadpoles. In addition, only three tadpoles (15%) had a Bd infection on the jaw sheaths and tooth rows simultaneously. Therefore, it is important that all mouthparts are collected for accurate Bd detection. The positivity rate of the lower part is also twice as high as that of the upper part for both the jaw sheath and tooth rows; therefore, care should be taken while swabbing the lower parts during sample collection.

Therefore, during swab sampling, care should be taken while scraping the tip and basement of the jaw sheaths and the entire oral cavity to reduce the rate of false-negative results.

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