Preprint, 2009
doi: 10.3354/dao02053

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online May 6, 2009

Contribution to DAO Special 4 'Chytridiomycosis: an emerging disease'

BSA reduces inhibition in a TaqMan® assay for the detection of *Batrachochytrium dendrobatidis*

Stephen Garland[1,*], Anthony Baker[2], Andrea D. Phillott[1], Lee F. Skerratt[1]

[1]**Amphibian Disease Ecology Group, James Cook University, and the School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia**

[2]**School of Veterinary and Biomedical Science, James Cook University, Townsville, Queensland 4811, Australia**

ABSTRACT: A TaqMan® assay for the causative agent of chytridiomycosis in amphibians (*Batrachochytrium dendrobatidis*) can be inhibited by phenolic compounds, including humic and tannic acids, resulting in false negatives. Bovine serum albumin (BSA) is known to reduce inhibition of PCR when samples are contaminated with these inhibitors. We assessed the effect of BSA in reducing inhibition of the TaqMan® assay when analyzing skin swabs for *B. dendrobatidis*. We found that the addition of BSA to the TaqMan® reaction reduced inhibition to insignificant levels. BSA did not appreciably affect the efficiency or analytical sensitivity of the TaqMan® reaction in the analysis of standard DNA solutions free from environmental inhibitors. We recommend the addition of 400 ng μl^{-1} of BSA to the standard TaqMan® assay to reduce inhibition associated with sampling wild amphibians.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · TaqMan assay · Quantitative PCR · Inhibition · Bovine serum albumin · BSA

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) is the causative agent of chytridiomycosis in amphibians, and the disease is a major primary cause of declining frog populations (Skerratt et al. 2007). Boyle et al. (2004) developed a sensitive TaqMan® (Applied Biosystems) assay for the detection of *Bd*, and Hyatt et al. (2007) developed a recommended sampling and detection strategy based on this assay. The assay involves the rapid extraction of DNA using Prepman® Ultra sample preparation reagent (Applied Biosystems) from swabs run over the ventral surfaces of amphibians. Hyatt et al. (2007) stated that inhibition of the assay can be a significant problem due to contamination of the sample (e.g. swab) with soil, detritus and possibly exudates from the amphibian epidermis. These authors indicated that 25% of their PCR assays conducted on submitted swabs demonstrated complete inhibition. Phenolic compounds including humic and tannic acids inhibit PCR and are produced during the decomposition of plant material. They are present in soil, sediment, natural water environments and detritus. Kreader (1996) found that 400 ng μl^{-1} BSA reduced inhibition of PCR due to tannic and humic acids by at least 100-fold. Similar results were achieved for unknown inhibitors from herbivore feces and natural freshwater samples.

We assessed the use of BSA for the reduction of inhibition of the TaqMan® assay when analyzing swabs for the presence of *Bd*.

MATERIALS AND METHODS

Sample selection and processing. Our laboratory receives swabs from collaborating researchers for quantification of *Bd* using the chytrid TaqMan® assay (Boyle et al. 2004). Skin swabs are often taken from wild frogs during field studies and may be contaminated with soil and/or detritus. Samples that record a negative amplification are screened for inhibition

*Email: stephen.garland@jcu.edu.au

© Inter-Research 2009 · www.int-res.com

using Applied Biosystem's TaqMan® exogenous internal positive control (IPC). A submission of 155 swabs was received for analysis. The swabs had been used to sample individuals from 6 marsh frog species representing the genera *Limnodynastes*, *Litoria* and *Crinia*. Many of the skin swabs were dirty and contaminated with inorganic and/or organic matter. DNA was extracted and batched into 40 groups (3 or 4 extracts per group) for PCR analysis. Twenty-seven of the group extracts (67.5%) produced high to complete inhibition of the IPC. These 40 group extracts were selected for further experimentation to test the efficacy of BSA in reducing inhibition and improving test sensitivity.

DNA extraction and real-time PCR. DNA was extracted from swabs as described by Hyatt et al. (2007). Real-time PCR analysis, including reaction mix and cycling conditions, followed Boyle et al. (2004) with the following modifications. The reaction mix, which included the primers and probe for the detection of *Bd* as described by Boyle et al. (2004), incorporated the TaqMan® Exogenous IPC (0.6× Exo IPC Mix, 0.6× Exo IPC DNA) with and without the addition of 400 ng μl^{-1} of bovine serum albumin (BSA) (Sigma A4161). The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. A 15 µl reaction volume was produced by loading 10 µl of PCR master mix and 5 µl of the diluted group extract, the diluted negative extraction control (NEC, extraction of a new swab) or water into Gene-Disc tubes with a CAS-1200™ pipetting robot (Corbett Robotics). The group extracts and NEC were diluted 1 in 16.7 to obtain the same template/master mix ratio as indicated by Boyle et al. (2004). Individual IPC analyses were performed on 40 DNA group extracts and 5 NECs, and triplicate analyses were performed on water. Three no amplification control reactions (containing the PCR inhibitor supplied with the IPC kit at the recommended concentration) were also included to ensure that increased fluorescence was due to amplification and not to probe degradation by some other mechanism. Threshold cycle (C_T) values (PCR at the point of detection) were calculated by Rotor-Gene™ 1.7 software using a threshold of 0.01. Five group extracts were arbitrarily selected for repeat triplicate analysis for confirmation of observed trends.

Quantitative standards and PCR efficiency. We assessed the effect of BSA on the analytical sensitivity (C_T value) of the chytrid TaqMan® assay for *Bd* by analyzing standard zoospore extracts (n = 4) with and without the addition of 400 ng μl^{-1} of BSA (IPC reagents not included). A standard extract of 200 zoospore equivalents (ZSE) μl^{-1} was supplied by the Australian Animal Health Laboratory (CSIRO Australia) and prepared as described by Boyle et al. (2004). This solution was diluted to 20 ZSE μl^{-1} with molecular biology grade water (Sigma W4502) and stored at –20°C in 70 µl aliquots. A fresh serial dilution representing 20, 2, 0.2 and 0.02 ZSE μl^{-1} was prepared from a thawed aliquot just prior to the PCR analysis. The loading of 5 µl of the DNA standards into the PCR reaction produced the equivalent of 100, 10, 1 and 0.1 ZSE per reaction. C_T values and PCR amplification efficiencies were determined by Rotor-Gene™ 1.7. Efficiencies were measured as a proportion of the theoretical maximum increase in fluorescence during exponential amplification. The theoretical maximum efficiency of 100% is represented by a doubling of fluorescence per cycle.

Statistical analysis. Mean C_T values were statistically compared by *t*-test and ANOVA using SPSS 14.0 software.

RESULTS

Effect of BSA on inhibition

In the absence of BSA, 72.5% of the group extracts caused complete failure of the IPC amplification and the remainder produced C_T values ranging from 27.5 to 43.4 (mean ± SD = 31.5 ± 4.50). When BSA was included, the IPC amplified for all group extracts, which is conclusively different to that obtained without BSA. Due to the high proportion of extracts producing complete failure of the IPC in the absence of BSA, no statistical comparisons were made with the BSA treatment and negative control extracts.

When BSA was included in the TaqMan® reaction for the IPC, the mean C_T for the 40 group extracts was 29.4 ± 0.87 (Table 1). This value was not significantly different to the mean C_T produced by the NEC with BSA (28.7 ± 0.53) or without BSA (28.7 ± 0.40). How-

Table 1. Mean threshold cycle (C_T) values for the analysis of a commercial internal positive control (IPC, Applied Biosystems) in the assessment of bovine serum albumin (BSA) for the reduction of inhibition. Black horizontal lines represent non-significant differences in mean C_T values. Mean C_T values for water were significantly different ($p < 0.02$) to those of the skin swab extracts (grey lines). Sample: skin swab extraction; NEC: negative extraction control

	Sample	NEC		Water	
	with BSA	No BSA	With BSA	No BSA	With BSA
Sample size	40	5	5	3	3
Mean C_T for IPC	29.4	28.7	28.7	27.8	27.6

$p < 0.02$

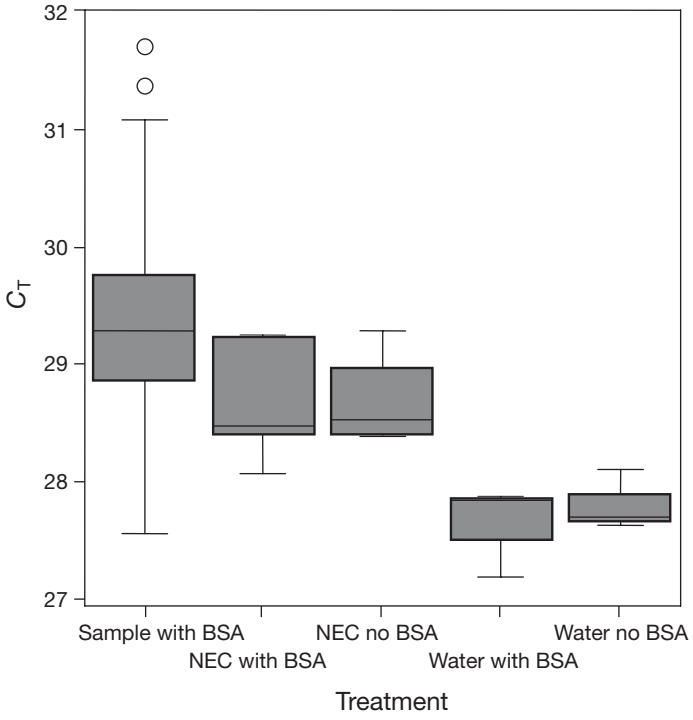

Fig. 1. Analysis of a commercial internal positive control (IPC, Applied Biosystems) in the assessment of bovine serum albumin (BSA) for the reduction of inhibition. Box plots show median values (horizontal line within box), interquartile range (box), range (whiskers) and extreme values (O) as determined by SPSS (version 14). C_T: threshold cycle; sample: skin swab extraction; NEC: negative extraction control

ever, it was significantly higher than the result for water in the absence or presence of BSA ($p < 0.02$), suggesting that the materials used in sampling and extraction may cause slight inhibition. The results are summarized in Table 1 and Fig. 1. There was a wider range of C_T values for the skin swab extracts in comparison to the negative controls (Fig. 1). Of particular interest are those C_T values above 30, which are still suggestive of slight inhibition despite the addition of BSA.

Ten group extracts were positive for the chytrid TaqMan® assay when analyzed in the multiplex reaction with the IPC. The mean C_T for the IPC of those extracts positive for *Bd* was 29.2 ± 0.63 and was not significantly different to the mean for those negative for *Bd* (29.5 ± 0.94, n = 30, $p = 0.47$). In other words, amplification of the *Bd* fragment in the multiplexed reaction did not have a detectable influence on the IPC results.

Repeat analyses and further evidence

In order to confirm the observed trend of reduced inhibition due to the addition of BSA, 5 group extracts were arbitrarily selected for repeat triplicate IPC analysis. The results of the repeat analysis were consistent with the original assessments. When no BSA was included, 3 extracts that produced complete inhibition in the original analysis also produced complete inhibition for the repeat analyses. One extract produced 2 positive reactions with C_T values above 45 for the repeat analyses, which was comparable to an original C_T value of 43.4. The fifth extract produced 3 positive reactions with a mean C_T of 34.6 ± 1.01, similar to the original C_T value of 35.4. When BSA was included in the triplicate analyses of the 5 group extracts, all reactions were positive with mean C_T values ranging from 28.4 to 29.3, confirming the original results.

Further support was obtained when the 40 group extracts were assessed in triplicate for the presence of *Bd* through the analysis of the chytrid TaqMan® assay (IPC not included). Without the addition of BSA, only 5% of the group extracts produced a clearly positive reaction for *Bd* (3 positive reactions in a triplicate analysis). However, 35% of the group extracts were positive for *Bd* when BSA was added to the reaction (data not shown).

Effect of BSA on the analytical sensitivity of the chytrid TaqMan® assay for *Bd*

To test the effect of BSA on the chytrid TaqMan® assay for *Bd* we analyzed standard solutions containing known ZSE. The results are presented in Table 2. Mean C_T values and PCR efficiencies were highly similar for each standard with or without the addition of BSA.

Table 2. Effect of the addition of 400 ng μl^{-1} of bovine serum albumin (BSA) to the chytrid TaqMan® assay in the analysis of standard extracts representing 100, 10, 1 and 0.1 zoospore equivalents (ZSE) (n = 4). Threshold cycle (C_T) values and reaction efficiencies are presented. BSA had no significant effect ($p > 0.2$) on C_T values

	100 ZSE		10 ZSE		1 ZSE		0.1 ZSE	
	With BSA	No BSA	With BSA	No BSA	With BSA	No BSA	With BSA	No BSA
Mean C_T	22.2	22	26	25.9	29.6	29.7	34.2	33.6
SD	0.09	0.24	0.21	0.11	0.36	0.17	0.54	1.08
Efficiency (%)	89	88	90	88	94	90	87	89

DISCUSSION

BSA did not appreciably affect the efficiency or analytical sensitivity of the chytrid TaqMan® reaction in the analysis of standard DNA solutions free from environmental inhibitors. The IPC analyses of skin swab extracts and results from the chytrid TaqMan® assay for *Bd* confirmed the efficacy of BSA in reducing inhibition and increasing test sensitivity. However, some skin swab extracts (Fig. 1) produced C_T values higher than those of the NECs despite the addition of BSA and probably indicated residual inhibition. This may be problematic for some quantitative comparisons between skin swabs with different levels of residual inhibition, necessitating corrections to account for the variable sensitivity. These corrections will need to be empirically determined.

The high percentage of skin swabs that demonstrated complete inhibition without the addition of BSA (72.5%) is consistent with the findings of Hyatt et al. (2007). These authors found that 25% of swabs produced complete inhibition, but the level varied greatly between submissions and ranged from 0 to 90%. Clearly, the standard assay for *Bd* is seriously compromised by differences in the level of inhibitory substances on skin swabs and the subsequent variability in sensitivity. This situation could lead to incorrect conclusions when comparisons are made. The addition of BSA to the assay may not only improve sensitivity but also help to standardize sensitivity by largely eliminating inhibition. This will enable more accurate comparison of skin swabs within and between individuals and different amphibian species collected from different environments and at different times.

CONCLUSIONS

The addition of BSA did not appreciably affect the efficiency and analytical sensitivity of the TaqMan® reaction for *Bd* but dramatically reduced inhibition to generally insignificant levels. We recommend the addition of 400 ng μl^{-1} of BSA to the standard TaqMan® assay for *Bd* to increase test sensitivity and to reduce variability in sensitivity.

Acknowledgements. Thanks to the anonymous reviewers for constructive comments. The present study was made possible through funding from the Australian Government Department of the Environment, Water, Heritage and the Arts and the Australian Research Council.

LITERATURE CITED

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Hyatt AD, Boyle DG, Olsen V, Boyle DB and others (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Org 73: 175–192

Kreader CA (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol 62:1102–1106

Skerratt LF, Berger L, Speare R, Cashins S and others (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134

Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

Submitted: August 25, 2008; Accepted: February 27, 2009
Proofs received from author(s): April 9, 2009