BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2021, 1–7

doi: 10.1093/biomethods/bpab018
Advance Access Publication Date: 17 September 2021
Methods Article

METHODS ARTICLE

Wildlife pathogen detection: evaluation of alternative DNA extraction protocols

Vasiliki Mantzana-Oikonomaki, Martine Maan and Joana Sabino-Pinto*

Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, Netherlands

*Correspondence address. Groningen Institute for Evolutionary Life Sciences, University of Groningen, 9712 CP Groningen, Netherlands.
E-mail: joanasabinopinto@gmail.com

Abstract

Accurate detection of wildlife pathogens is critical in wildlife disease research. False negatives or positives can have catastrophic consequences for conservation and disease-mitigation decisions. Quantitative polymerase chain reaction is commonly used for molecular detection of wildlife pathogens. The reliability of this method depends on the effective extraction of the pathogen's DNA from host samples. A wildlife disease that has been in the centre of conservationist's attention is the amphibian disease Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Here, we compare the efficiency of a spin column extraction kit (QIAGEN), commonly used in *Bd* DNA extraction, to an alternative spin column kit (BIOKÈ) used in extractions from other types of samples, which is considerably cheaper but not typically used for *Bd* DNA extraction. Additionally, we explore the effect of an enzymatic pre-treatment on detection efficiency. Both methods showed similar efficiency when extracting *Bd* DNA from zoospores from laboratory-created cell-cultures, as well as higher efficiency when combined with the enzymatic pre-treatment. Our results indicate that selecting the optimal method for DNA extraction is essential to ensure minimal false negatives and reduce project costs.

Keywords: spin column extraction; QIAGEN; BIOKÈ; Chytridiomycosis; *Batrachochytrium dendrobatidis*; Chytrid

Introduction

Wildlife diseases are natural components of ecosystems and an intrinsic part of biological diversity and ecological complexity. However, they can also be anthropogenically driven and, currently, novel or introduced diseases appear more and more frequently in natural populations, with severe consequences for wildlife [1]. Over the last decades, several epidemics have caused large-scale declines in wildlife, for example the disease chytridiomycosis in amphibians, chronic wasting disease in deer, white-nose syndrome in bats, and devil facial tumour disease in Tasmanian devils [2]. Wildlife pathogens can easily transfer between wildlife species and to domestic animals or humans, as highlighted by old and new epidemics such as the current SARS-CoV-2 pandemic in humans [3]. Anthropogenic changes such as urbanization, biological invasions and the pet trade may facilitate disease emergence, making surveillance and monitoring of wildlife diseases paramount [3, 4].

The monitoring of wildlife pathogens faces several challenges related to field conditions, the necessity for non-invasive sampling and the optimization of disease detection from sampled animals [1]. Accurate monitoring of wildlife pathogens in wild populations requires the collection of high numbers of samples and subsequent analysis generally involving molecular genetics [5]. The most widely used diagnostic tests for wildlife pathogens rely on polymerase chain reaction (PCR) as it can often yield fine sensitivity and accuracy in less time than many traditional assays, like immunohistochemistry [6]. More specifically, quantitative PCR (qPCR) combines high sensitivity with high-throughput sample processing [7]. qPCR is a widely used technique that targets specific regions of the genome and

Received: 30 July 2021; **Revised:** 13 September 2021; **Editorial Decision:** 14 September 2021; **Accepted:** 15 September 2021

allows semi-quantitative estimates of the abundance of a target organism [8]. This technique is preferred as it can be more sensitive at detecting small quantities of DNA compared with traditional PCR and gel visualization [9]. Standard protocols of qPCR-based pathogen monitoring include extraction of DNA from individual samples and its amplification, along with standardized samples of known pathogen concentrations [6, 10]. Advances in qPCR protocols and their application in detection and quantification of pathogens have contributed significantly to our understanding of disease dynamics in natural host populations.

Accurate pathogen detection is critical in wildlife disease research and conservation. Sampling biases, due to variation in the probability of encountering, capturing or detecting infected individuals, and imperfect disease detection during diagnostic tests in the laboratory, may lead to false negatives and to the misidentification of infected individuals or areas [11, 12] leading to suboptimal conservation measures. To reduce the incidence of false negatives produced during laboratory diagnostic tests, it has been suggested that pathogen detection efficiency can be increased by improving the DNA extraction methods used [12].

In the present study, we focus on DNA extraction methods for the pathogen *Batrachochytrium dendrobatidis* (*Bd*, [13]), the causative agent of the potentially lethal disease chytridiomycosis in amphibians. Chytridiomycosis has been linked to the decline of over 500 amphibian species and to the extinction of an estimated 90 species of amphibians globally [14, 15]. *Bd* is a multi-host pathogen that infects a broad range of species, including 520 anuran amphibians (frogs and toads), urodeles (salamanders and newts) and caecilians [16, 17]. Most anurans are susceptible to *Bd* infection, during all life stages (excluding eggs), although morbidity and mortality vary between species and life stages [16]. The chytrid fungus *Bd* belongs to the Chytridiomycota, a phylum of fungi that have a non-mycelial morphology and can produce flagellated spores or zoospores that facilitate dispersal and site-selection [18]. *Bd* zoospores are motile flagellate spores that are specialized for dispersal and host infection [19]. The presence of these zoospores in the skin of possible hosts can be determined with DNA-based methods [19].

As for many other pathogens and for the reasons mentioned above, qPCR is the most commonly used technique to detect the presence of *Bd* [20, 21]. To monitor the occurrence of this fungal pathogen in natural populations, collection of amphibian skin swab samples is needed [22]; this is a non-invasive sampling technique that relies on skin sloughing with rayon swabs [20, 23]. DNA from these swab samples is extracted and a qPCR is performed to detect the presence and amount of *Bd* DNA, with amplification of the 5.8S-ITS1 region [6, 24]. The internal transcribed spacer (ITS1) region is a rapidly evolving nuclear ribosomal repeat unit used for fungal identification [6, 22]. In fungal genomes, it occurs in multiple copies and especially in *Bd* it can be repeated up to 169 times [5]. Since *Bd* zoospores have multiple ITS copies in the genome [25], especially when isolated from laboratory-controlled cell cultures [6], *Bd* concentrations as low as 0.1 zoospores per reaction well can be detected (for instance if the standards had 20 ITS copies per zoospore and the wild *Bd* only had 10). This leads to a scenario in which infection intensity of *Bd* in live animals can be very low and qPCR techniques can yield inconsistent and false negative results [26, 27]. In addition, the sensitivity of qPCR techniques is linked to the capacity of the extraction method to purify samples and remove enzyme inhibitors [26]. Various extraction methods have been used in combination with qPCR to detect *Bd* and several of them have been showed to perform at a suboptimal level and might result in false negatives [26].

The standardized method for extracting *Bd* DNA includes the use of a spin column extraction kit, the QIAGEN DNeasy blood and Tissue spin column extraction kit (QIAGEN no. 69504, [26, 27]). While it is indeed excellent in producing purified DNA, it is also relatively expensive, which is problematic especially when extracting large number of samples, as is often required in wildlife pathogen monitoring. In recent studies, standardized comparisons have been performed between QIAGEN and non-spin column kits; however, the standard method seems to outperform most alternatives [26–28]. Comparisons between alternative spin column extraction kits have not yet been performed to our knowledge.

In this study, we compared two different DNA extraction spin column kits to determine which one is more efficient at detecting and quantifying *Bd* DNA, when used in combination with qPCR. Our aim is to validate the alternative extraction method (BIOKÈ NucleoSpin tissue kit), a more cost-efficient kit that is commonly used in other DNA extractions [29], to reduce costs in future projects. To do so, we compared the detection efficiency of the two kits by evaluating the detection (presence/absence) and intensity (estimated zoospore equivalents) of laboratory-created *Bd* standards, using qPCR. Additionally, two different protocols (with and without an enzymatic pretreatment) were employed, for both extraction methods, to further evaluate and optimize the extractions. Our study shows that a cost–benefit analysis of the optimal method for DNA extraction can minimize false negatives while reducing project costs.

Materials and methods

Two extraction methods were evaluated for the detection of *Bd* DNA from rayon bud swabs infused with *Bd* zoospore solution. Evaluation was performed by comparing the two methods in subsequent analysis of qPCR with a *Bd* specific probe. QIAGEN blood and tissue kit (QIAGEN, Hilden, Germany, Cat. No./ID: 69506) for DNA extraction of *Bd* pathogen has been used effectively in the past and is preferred for its highly effective extraction of *Bd* DNA from rayon swabs [26]. BIOKÈ NucleoSpin tissue kit (BIOKÈ, Leiden, the Netherlands, Cat. No./ID: 740952.250) has a lower cost of almost 50% of the QIAGEN blood and tissue kit (∼500€ vs. ∼900€, per 250 samples), and to our knowledge has not been used in the context of *Bd* extraction, but it is commonly used to extract DNA from other sorts of samples [29]. Testing BIOKÈ NucleoSpin tissue kit effectiveness can be instrumental in lowering the costs of DNA extraction, especially when there are high numbers of samples to be analysed.

Sample preparation and DNA extraction

Serial dilutions of four different zoospore quantities were prepared from a standard *Bd* stock solution from laboratory-controlled cell cultures, containing 0, 1, 10, 100 and 1,000 zoospores. Rayon swabs (DELTALAB S.L, Barcelona, Spain) were inoculated with 25 μl of a solution containing the mentioned zoospore quantities and kept dry in microcentrifuge tubes at −20°C until extraction. Ten swab replicates were prepared for each zoospore load. The zero-zoospore solution consisted only of deionized water and was used to prepare the control swabs (0 zoospores). The swabs for each load were prepared four times, one set for each tested protocol (total: (Load) 5 × (swab replicates) 10 × (protocols) 4; Fig. 1).

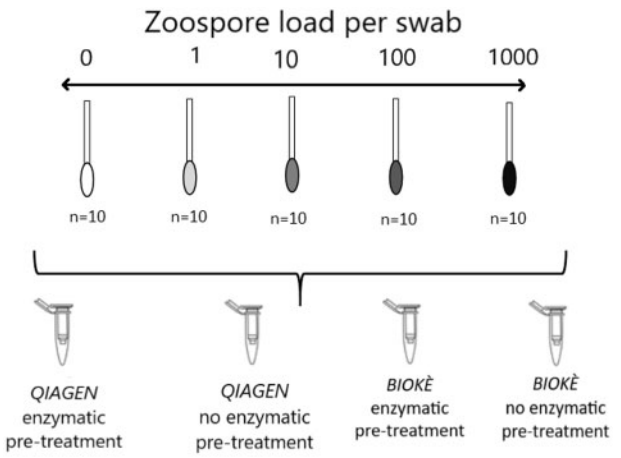

Figure 1: Rayon swabs were inoculated with a set of five different zoospore solutions made from a *Bd* stock solution. The different loads were 0, 1, 10, 100 and 1,000 zoospore equivalents. For each load, 10 replicates were created for each extraction method. The first method was the QIAGEN blood and tissue kit with enzymatic pre-treatment. The second was using again the QIAGEN blood and tissue kit but without enzymatic pre-treatment. The third method included the use of the BIOKÈ tissue kit with enzymatic pre-treatment while the fourth method included the BIOKÈ tissue kit without enzymatic pre-treatment.

DNA extraction protocol using QIAGEN blood and tissue kit

Ten replicates for each zoospore load were individually extracted with QIAGEN DNeasy blood and tissue kit. The protocol was adapted from the manufacturer's Animal Tissue protocol (Cat. No./ID: 69506, DNeasy Blood & Tissue Handbook, July 2020, HB-2061-003_HB_DNY_Blood_Tissue_0720_WW.pdf) with the following minor modifications. The first incubation time was fixed at 1 h. After adding the wash buffer AW2, centrifugation was repeated two times (the first for 1 min per protocol, the second for 3 min) to remove any residual buffer. The final elution volume was set to 50 µl and samples were incubated at room temperature for 5 min. Eluted DNA was stored at −20°C until qPCR runs. During each round of extractions, a blank sample was extracted (no swab) as an extraction negative control (extraction negative controls were all negative).

To assess the effect of enzymatic lysis, 10 replicates for each zoospore load were individually extracted with the QIAGEN DNeasy blood and tissue kit with an additional step of enzymatic lysis, to improve the lysis of the fungal cell [30]. Enzymatic lysis buffer to pre-treat gram positive bacteria was prepared with stock solutions of 1 M Tris–Cl, 0.5 M EDTA and Triton® X-100 detergent. Before each extraction trial, lysozyme was added and mixed into the lysis buffer at a 20:1 concentration. The first step of the protocol included the addition of 180 µl of enzymatic lysis buffer to the samples. Subsequently, the samples were incubated for 1 h at 37°C. Proteinase K (20 µl, 600 mAU/ml, included in kit) and 200 µl of the manufacturer's lysis buffer AL were then added, and samples were incubated at 70°C for 30 min. After that, the same procedure as described above was followed. Eluted DNA was stored at −20°C until qPCR runs. During each round of extractions, a blank sample was extracted (no swab) as an extraction negative control (extraction negative controls were all negative, data not shown).

DNA extraction protocol using BIOKÈ tissue kit

Ten replicates for each zoospore load were individually extracted with BIOKÈ NucleoSpin tissue kit. The protocol was adapted from the manufacturer's genomic DNA purification from standard protocol for human or animal tissue and cultured cells with minor alterations (Cat. No./ID: 740952.250, User Manual, NucleoSpin® Tissue, January 2017/Revision 17, UM_gDNATissue_2017.pdf), as described below. The first incubation time was fixed at 1 h and the second at 15 min. Eluted DNA was stored at −20°C until qPCR runs. During each round of extractions, a blank sample was extracted (no swab) as an extraction negative control (extraction negative controls were all negative).

To assess the effect of enzymatic lysis, 10 replicates for each zoospore load were individually extracted with the BIOKÈ NucleoSpin tissue kit with an additional step of enzymatic lysis, to improve the lysis of the fungal cell [30]. Enzymatic lysis buffer was prepared as described before in the protocol followed for DNA extraction with enzymatic lysis using QIAGEN blood and tissue kit. The first step of the protocol included the addition of 180 µl of enzymatic lysis buffer to the samples. Subsequently, 25 µl of the included proteinase K (28.84 mg/ml) and 200 µl of the included buffer B3 were added, and samples were again incubated at 70°C for 30 min. After that, the same procedure as described above was followed, with the exception that final incubation took 5 min. Eluted DNA was stored at −20°C until qPCR runs. During each round of extractions, a blank sample was extracted (no swab) as an extraction negative control (extraction negative controls were all negative).

Analysis of the samples using qPCR

After DNA extractions, qPCR was performed using established protocols and *Bd*-specific primers (*Bd* (ITS) 5.8S region, [6, 22]. qPCRs were performed on a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA). A 15 µl reaction volume, with 5 µl of template DNA, 7.5 µl IQ Taqman Universal MasterMix (1xiQ Supermix; Bio-Rad Laboratories), 1.35 µl of ITS1–3, 1.35 µl of 5.8S Chytr and 0.225 µl Chytr MGB2 FAM-labelled probe. In each qPCR run, samples were run in duplicate. When replicates of a sample presented different results, a third replicate was run (5% of samples were rerun because of such contradictory results). Each qPCR plate included a series of five plasmid-based *Bd* standards (10, 100, 1,000, 10,000 and 100,000 ITS copies) and a negative control containing deionized water. All standards consisted of a gBlock™ Gene Fragment designed by Standish et al. [31] and were run in duplicate.

Samples were considered *Bd* positive when both (or two out of the three) qPCR replicates provided an amplification signal lying in-between the amplification signals estimated for the lowest and highest standard present in the qPCR template (10–100,000 ITS copies). In addition, for a sample to be considered positive, the amplification curve had to be logarithmic and the standard error had to be smaller than the mean of the two replicates. The quantification of zoospores (i.e. zoospore equivalents, 1 zoospore equivalent = 10 ITS copies) was calculated as the average of the replicates for that sample, excluding the negative replicate in the cases where a third replicate was run.

Statistical analysis

To determine differences in load counts between the different extraction methods, linear models were fitted with load, extraction method and their interaction as fixed factors. The zero load (negative controls) were removed from the analysis as they were all negative. All extractions were compared with the standard method from the literature (QIAGEN blood and tissue kit with enzymatic pre-treatment). The normal distribution of the residuals for the LM's was controlled using Shapiro–Wilk tests.

The detected zoospore number was $\log_{10}(x)$ transformed to fulfil the criteria of normal residual distribution. All analyses were performed in R 4.0.1, using base R commands [32].

In total, four different protocols were designed and evaluated: QIAGEN blood and tissue kit (1) with enzymatic pre-treatment and (2) without, and BIOKÈ NucleoSpin tissue kit (3) with enzymatic pre-treatment and (4) without. To evaluate the different protocols, the average number of *Bd* zoospore equivalents and the total number of positive samples detected with each protocol were correlated with the standard protocol to evaluate the most efficient one.

Results

Samples extracted following the validated method of QIAGEN blood and tissue kit with the enzymatic pre-treatment led to qPCR detection of *Bd* DNA for almost all zoospore loads (Table 1). More specifically, *Bd* DNA was detected in 10/10 of the samples with the highest concentrations (10, 100 and 1,000), and in 4/10 samples with a concentration of 1 zoospore. Samples extracted with QIAGEN blood and tissue kit without the additional step of enzymatic pre-treatment led to qPCR detection of *Bd* DNA for almost all zoospore loads as well, except at the 1 zoospore per swab concentration where *Bd* DNA was detected in only 1/10 samples (Table 1). In samples extracted with the BIOKÈ NucleoSpin tissue kit with enzymatic pre-treatment, *Bd* was detected in 10/10 samples in all concentrations except the lowest concentration, where it was only detected in 6/10 samples (Table 1). Samples extracted with the BIOKÈ NucleoSpin tissue kit, without enzymatic pre-treatment, led to *Bd* DNA detection for all concentrations, but not in all samples of each concentration. *Bd* was detected in 10/10 of the samples with 1,000 and 100 zoospores, but only in 6/10 samples with 10 zoospores and in 1/10 samples with 1 zoospore (Table 1).

Overall, samples extracted with QIAGEN blood and tissue kit with enzymatic pre-treatment detected *Bd* zoospore equivalents comparable to the inoculated zoospore load (Table 1 and Fig. 2C). One exception was present at the highest concentration where the average number of *Bd* zoospore equivalents detected was 3,954.23 (±1,061.10). Samples extracted with QIAGEN blood and tissue kit without enzymatic pre-treatment detected *Bd* zoospore equivalents comparable to the load (Table 1 and Fig. 2A). The same was observed in samples extracted with BIOKÈ NucleoSpin tissue kit with enzymatic pre-treatment (Table 1 and Fig. 2D). However, samples extracted with BIOKÈ NucleoSpin tissue kit without enzymatic pre-treatment detected a different number of *Bd* zoospore equivalents than the inoculated ones (Table 1 and Fig. 2B). In samples inoculated with 1,000 zoospores, the mean *Bd* zoospore equivalents detected was 580.61 (±148.37); while for samples inoculated with 100 zoospores, the mean *Bd* zoospore equivalents detected was 317.60 (±46.50); and for samples inoculated with 1 zoospore, the mean *Bd* zoospore equivalents detected was 40.60.

There was a correlation between the number of zoospores added to the samples and the number of *Bd* zoospore equivalents detected by qPCR; however, this correlation differed between the different protocols (Fig. 2E). The standard method was considered the expected correlation and was used to select the most efficient of the other three methods and protocols. The strongest correlation, and most comparable to the standard method, was for samples extracted with the BIOKÈ NucleoSpin tissue kit with enzymatic pre-treatment (Fig. 2E). The statistical probability for detecting the presence of *Bd* did not differ significantly between the standard method (QIAGEN blood and tissue kit with enzymatic pre-treatment), and the use of BIOKÈ tissue kit with enzymatic pre-treatment (LM.: detected copies—extraction method × load, Extraction: $F_{(7:116)} = 82.15$, $P_{value} = 0.413$, Load: $P_{value} = 0.120$, Table 2). Statistical probability to detect *Bd* when samples were extracted with QIAGEN or BIOKÈ, without enzymatic pre-treatment, differed significantly from the standard method (Extraction: $P_{value(BIOKÈ)} < 0.001$, $P_{value(QIAGEN)} = 0.022$, Load: $P_{value(BIOKÈ)} < 0.001$, $P_{value(QIAGEN)} = 0.001$, Table 2).

Discussion

Reducing time and costs without minimizing detection efficiency is crucial in wildlife disease detection, especially now that infectious diseases are on the rise all over the world [33]. *Bd* is a wildlife pathogen that is in the centre of attention of amphibian conservation efforts, and current research is focused on optimizing detection of the pathogen in live animal samples [26, 33, 34]. The main objective of our study was to compare different extraction methods and evaluate detection efficiency for the *Bd* pathogen from swabs. Three different protocols were compared with a standard method, commonly used for *Bd* detection. We showed that BIOKÈ NucleoSpin tissue kit combined with enzymatic pre-treatment can detect *Bd* DNA from rayon swabs with comparable efficiency to the standard method (QIAGEN blood and tissue kit). In addition, we showed that sample enzymatic pre-treatment is an important step for maximizing detection efficiency, especially at low concentrations.

Other studies that explored the efficiency of alternative extraction methods have focused on non-spin column extraction methods [26, 34]. In our study, we included an alternative spin column kit and compared its efficiency to the standard method. The two DNA extraction kits did not differ significantly in their efficiency to detect Bd DNA via qPCR (Table 2). According to our results, differences in detection efficiency can only be observed when looking at whether the enzymatic

Table 1: detectability and zoospore amounts of the detected loads for *Bd* according to the extraction method (QIAGEN blood and tissue kit and BIOKÈ NucleoSpin tissue kit with and without pre-enzymatic treatment), and load of the inoculated swab

Load	1,000			100			10			1		
Method	O/E	GE	SE	O/E	GE	SE	O/E	GE	SE	O/E	GE	SE
QIAGEN and lysozyme	10/10	3,954.23	1,1,61.1	10/10	156	52.92	10/10	12.8	1.35	*4/10*	*7.5*	*3.17*
QIAGEN	10/10	784.43	154.26	10/10	129.4	33.6	10/10	24.18	3.07	*1/10*	*12.3*	*NA*
BIOKÈ and lysozyme	10/10	1,185.27	230.82	10/10	165.1	39.95	10/10	15.38	1.24	*6/10*	*3.82*	*0.81*
BIOKÈ	10/10	580.61	148.37	10/10	317.6	46.5	*6/10*	*33.4*	*7.37*	*1/10*	*40.6*	*NA*

O/E, number of swabs with positive signal for the chytrid fungi and the total number of swabs processed; GE, genomic equivalents, approximate number of zoospores per swab; and SE, standard error.
Italic values represent groups in which zoospores were not detected in all samples.

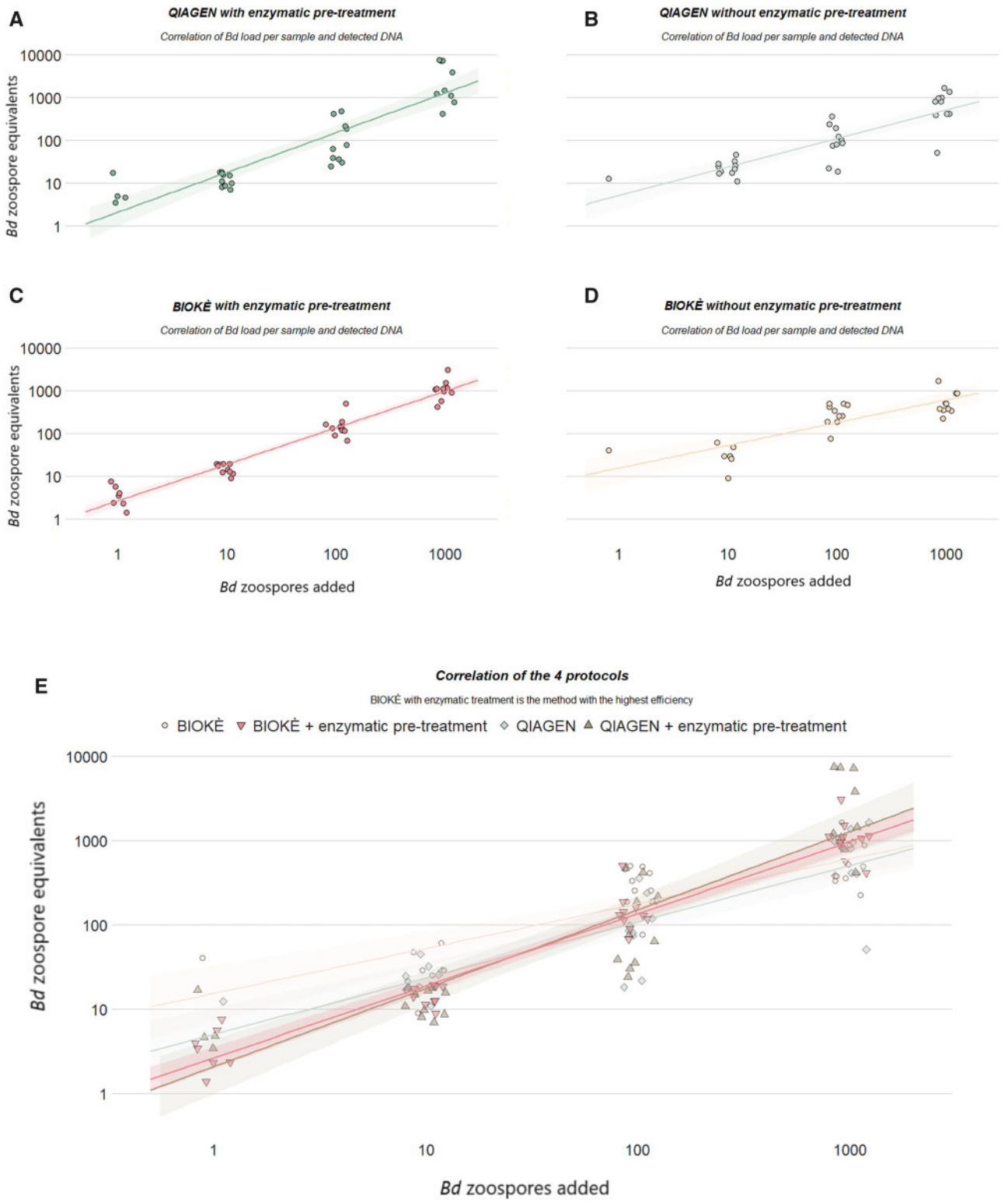

Figure 2: correlation of *Bd* zoospores added for each sample with the detected *Bd* zoospore equivalents in qPCR, for each sample and for each protocol. (**A**) Linear regression of the standard method, QIAGEN, with enzymatic pre-treatment. (**B**) Linear regression of QIAGEN DNeasy blood and tissue kit, without enzymatic pre-treatment. (**C**) Linear regression of BIOKÈ NucleoSpin tissue kit with enzymatic pre-treatment. (**D**) Linear regression of BIOKÈ NucleoSpin tissue kit, without enzymatic pre-treatment. (**E**) Correlation of all four protocols employed.

pre-treatment was included or not; protocols with enzymatic lysis resulted in higher detection of *Bd*, regardless the extraction kit used. Sample enzymatic pre-treatment can lead to higher detection efficiency, specially at lower concentrations, highlighting the importance of this step. Enzymatic lysis was performed with lysozyme that degrades chitin, facilitating the breakdown of zoospore walls, and thus resulting in better DNA isolation [35].

Table 2: statistical analysis of the interaction between load, method and detection (simple linear model formula: *Bd* detected in qPCR ~ extraction protocol * zoospore load per sample)

Factors	Protocol	Load
QIAGEN	0.02	0.001
BIOKÈ and lysozyme	**0.27**	**0.073**
BIOKÈ	6.17E−05	3.00E−05
F-value	82.15 (7,116)	
R^2	0.822	

Old highlights the non significant kit.

The probability of detecting *Bd* DNA, regardless the sample load, was the highest for BIOKÈ extracted samples with enzymatic pre-treatment (95%). The correlation between the detected *Bd* DNA copies and the zoospore load per sample showed a linear correlation comparable to the standard method, showing that this method is at least as efficient as, if not better than, the standard method (Table 1). The higher probability of detecting *Bd* with BIOKÈ and enzymatic pre-treatment shows that a higher efficiency is possible, however further investigation is needed (i.e. visual confirmation of low concentration inoculation). Detection efficiency was lowest for the protocols without enzymatic pre-treatment, with either extraction kit (67.5% for the BIOKÈ NucleoSpin tissue kit and 77.5% for the QIAGEN blood and tissue kit). It is important to keep in consideration that the zoospores-per-swab concentrations used in this study are theoretical, as they were achieved by step dilution of a high concentration zoospore stock solution and not by counting. This is particularly relevant for the 1-zoospore samples as, without visual confirmation, it is not possible to ensure that all the samples had a zoospore in them. Additionally, as mentioned above, the number of ITS1 copies varies between zoospores and between chytrid cultures due to duplications [25] and while we used the estimation of one zoospore having 10 ITS copies, it is possible that the culture used had a different copy number. Lower detection was found in similar studies; however, it is unclear if such low detectability is due to a lack of detection or to an absence of zoospores in swabs [27].

In large-scale screenings, pathogen loads can be quite low, samples might contain zoosporangia (sporangium that produces zoospores) and a high number of samples is expected to be collected [36, 37]. Our study shows that BIOKÈ NucleoSpin tissue kit shows the same efficiency as, if not higher than, the standard method, minimizing false negatives even at low concentrations. Our study also shows that the use of lysozyme is required for maximizing detection efficiency potentially through better digestion of cell walls; therefore, the breaking of the zoosporangia walls would also require the use of lysozyme. Finally, our study uses a kit that is almost 50% cheaper than the QIAGEN DNeasy blood and tissue kit, allowing to reduce the costs of large screenings. It is important to critically select the protocol used in *Bd* screening studies according to factors related to study size, known or unknown prevalence of *Bd* in the study site and expenses.

While it is convenient to use previously established methods, it is important to stay aware of alternatives and/or new opportunities. This study describes a situation in which the new method does not significantly increase the efficiency of the standard method, but drastically reduces the costs associated. This reduction in processing cost per sample allows larger sample sizes without increasing the total budget. This is particularly relevant in field screenings for invasive pathogens in regions where the presence of the pathogen is unknown. In such situations in which mortality is not observed, if present, pathogens are expected to exist at lower loads and prevalence and only by sampling a large part of the population we can be sure about the presence or absence of the pathogen.

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Funding

This project was funded by a grant from the Deutsche Forschungsgemeinschaft (DFG) to J.S.-P. (SA 3786/1-1). The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Acknowledgements

We would like to thank Prof. Dr An Martel and Prof. Dr Frank Pasmans for providing the zoospore cultures used in this project. We are grateful to Ellis Mulder and Anne Desreveaux for laboratorial assistance. Finally, we would like to thank the reviewers for their insightful comments that helped improve this manuscript. Data available in supplementary materials.

Conflict of interest statement. None declared.

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