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Evaluating the Sensitivity of Conventional PCR and Re-PCR for Detecting Pathogenic *Prototheca* Species

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Abstract: *Prototheca* species are achlorophyllic algae that have emerged as opportunistic pathogens in both humans and animals, causing conditions such as bovine mastitis and human protothecosis. Their identification in clinical and environmental samples remains challenging due to low organism burden and limited routine diagnostics. This study aimed to evaluate the molecular detection limit (Limit of Detection, LOD) of two pathogenic *Prototheca* strains using conventional PCR and re-amplification PCR (re-PCR) methods. Genomic DNA samples from *Prototheca ciferrii* (SAG 2063) and *Prototheca bovis* (LZ-5) were subjected to 1:2 serial dilutions and evaluated by PCR. In conventional PCR, amplification was observed up to approximately $9.1x10^{-4}$ ng/µL for *P. ciferrii* and $5.34x10^{-6}$ ng/µL for *P. bovis*. Re-PCR allowed the detection of amplification products at even lower DNA concentrations in both strains. These findings demonstrate that *Prototheca* species can be detected at very low DNA concentrations using molecular techniques and that re-PCR may enhance diagnostic sensitivity. These results are significant as they enable the detection of *Prototheca* even in sources with very low organism loads.

Patojen *Prototheca* Türlerinin Tespitinde Konvansiyonel PCR ve Re-PCR Yöntemlerinin Duyarlılığının Değerlendirilmesi

Anahtar Kelimeler
Mikroalg,
Tanısal eşik,
Nadir patojenler,
Çevresel tespit

Öz: *Prototheca* türleri hem insanlarda hem de hayvanlarda fırsatçı patojenler olarak ortaya çıkan klorofilsiz alglerdir ve sığır mastitisi ve insan prototekozu gibi enfeksiyonlara neden olabilmektedir. Klinik ve çevresel örneklerde tanımlanmaları, düşük organizma yükü ve rutin tanı yöntemlerinin yetersizliği nedeniyle halen önemli bir zorluk teşkil etmektedir**.** Bu çalışmada, iki patojen *Prototheca* suşunun konvansiyonel PCR ve yeniden PCR (re-PCR) yöntemleriyle moleküler olarak tespit edilebilirlik sınırı (Limit of Detection, LOD) değerlendirilmiştir. *P. ciferrii* (SAG 2063) ve *P. bovis* (LZ-5) suşlarına ait DNA örnekleri, 1:2 oranında seri dilüsyonlara tabi tutulmuş ve PCR yöntemiyle değerlendirilmiştir. Konvansiyonel PCR ile *P. ciferrii* suşunda yaklaşık $9.1x10^{-4}$ ng/µL, *P. bovis* suşunda ise yaklaşık $5.34x10^{-6}$ ng/µL DNA konsantrasyonuna kadar amplifikasyon gözlenmiştir. Re-PCR uygulaması sayesinde her iki suşta da daha düşük DNA düzeylerinde bant elde edilmiştir. Bu veriler, *Prototheca* türlerinin çok düşük konsantrasyonlarda bile moleküler düzeyde saptanması için yeterli hassasiyete sahip olduğunu ve re-PCR uygulamasının tanısal duyarlılığı artırabileceğini göstermektedir. Bu bulgular, *Prototheca*'nın çok düşük miktarda bulunduğu örneklerde bile tespit edilebilmesini sağladığı için büyük önem taşımaktadır.

1. Introduction

Prototheca is a genus of achlorophyllous, free-living green algae (Chlorophyta) classified within the family Chlorellaceae, order Chlorellales, and class

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Trebouxiophyceae [1, 2]. Although *Prototheca* species have lost their photosynthetic ability, they retain functional plastids involved in various metabolic processes [3, 4]. Their asexual reproduction through endospore formation, along with the presence of sporopollenin—a highly resilient biopolymer—in their cell walls, confers remarkable resistance to extreme environmental conditions such as acidity, alkalinity, enzymatic degradation, and physical stress [1, 5]. These adaptations allow *Prototheca* species to thrive in diverse moist environments rich in organic matter, including decaying vegetation, soil, slime fluxes, animal waste, and sewage systems [6, 7].

Despite their environmental ubiquity, *Prototheca* species are increasingly recognized as opportunistic pathogens, causing protothecosis infections [8]. Clinical manifestations range from cutaneous lesions and olecranon bursitis to disseminated systemic infections, affecting both humans and a wide variety of animals [9-11]. Bovine mastitis remains the most prevalent and economically significant protothecal infection worldwide, substantially impacting dairy cattle health and milk production [12]. In addition to cattle, *Prototheca* infections have been documented in cats, dogs, pigs, goats, fish, bats, flying foxes, deer, beavers, snakes, and horses [1, 14- 17].

Among the *Prototheca* species, *Prototheca bovis* and *Prototheca ciferrii* are particularly notable. *P. bovis*, a major pathogen associated with bovine mastitis, forms opaque, white, smooth colonies with a buttery consistency, and cells measuring approximately 15×13 µm (mother cells) and 9×7 µm (daughter cells) [12, 18]. *P. ciferrii* exhibits similar colony morphology but is less frequently isolated from clinical cases, despite its environmental prevalence on dairy farms [1, 18].

A significant challenge in the management of *Prototheca* infections is the difficulty of accurate and timely diagnosis. It is important to note that *Prototheca* cells are frequently misidentified as yeast. This is due to their morphological similarities. This misidentification can lead to delays and inappropriate treatments. Moreover, *Prototheca* cultures exhibit significantly slower growth rates than typical bacterial pathogens, sometimes requiring up to seven days for visible colony formation [18]. As a result, conventional culture-based diagnostics are often inadequate, especially for detecting low pathogen loads or subclinical infections.

To overcome these limitations, molecular diagnostic techniques, particularly PCR-based methods, have emerged as powerful alternatives for rapid and sensitive detection of Prototheca species [18]. A critical parameter for the validation of such assays is the Limit of Detection (LOD), which defines the minimal quantity of target DNA reliably detectable.

The LOD value is of paramount importance in scenarios where clinical samples exhibit low pathogen loads or when surveillance studies are undertaken for the purpose of identification. The detection of *Prototheca* DNA at low concentrations is a critical tool for the early diagnosis of subclinical mastitis cases and for monitoring the farm environment [19].

Previous studies have demonstrated the feasibility of sensitive molecular detection: for instance, a qPCR assay targeting the *cytb* gene achieved an LOD of 20.9 CFU/mL for *P. bovis* in milk samples [20], while a multiplex PCR approach detected *Prototheca* DNA at concentrations as low as 0.8 pg/µL [21]. These findings highlight the necessity of optimizing detection thresholds to ensure reliable diagnosis, particularly in cases with low pathogen burden.

The objective of this study was to ascertain the detection limits of PCR assays for *Prototheca* species. To this end, serial dilutions of DNA extracted from two pathogenic strains were prepared and analyzed using conventional PCR. For dilution steps where no visible bands were observed, re-amplification (re-PCR) was performed to enhance detection sensitivity. The findings of this study are expected to provide valuable insights into the diagnostic sensitivity of molecular tools used for the detection of *Prototheca*-associated infections, particularly in samples with low pathogen load.

2. Material and Method

2.1. Microalgae cultures

P. ciferri (SAG 2063) and *P. bovis* (LZ-5) were obtained from the Culture Collection of Algae at Göttingen University. The strains were sub-cultured aerobically on *Prototheca* Isolation Medium (PIM) plates, which were prepared by supplementing the medium with 20 g/L agar. The plates were incubated in the dark at 16°C for long-term storage (Table 1). This study utilized these reference strains obtained from the culture collection. For the DNA extraction the isolates transferred into 5 mL liquid PIM medium containing glass tubes and incubated at 25°C for 3 days without light.

Table 1. Contents of *Prototheca* Isolation Medium (PIM).

***Prototheca* Isolation Medium (PIM)**	**Amount (g/L)**
Potassium hydrogen phthalate	10
Sodium hydroxide	0.9
Magnesium sulfate	0.1
Dipotassium phosphate	0.2
Ammonium chloride	0.3
Glucose	10
Thiamine Hydrochloride	0.001
Agar	20

2.2. DNA extraction

Genomic DNA was extracted from *Prototheca* cultures using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Briefly, the strains were incubated in liquid medium for 3 days, after which 2 mL samples were taken from cultures with an optical density of 2.7 at 600 nm. These samples were centrifuged at 8,000 rpm for 2 minutes to remove the medium. The resulting pellets were then mechanically disrupted using 8 mm sterile glass beads. Cell lysis was performed in Buffer AP1 supplemented with RNase A, followed by incubation at 65°C. After the addition of Buffer P3 and incubation on ice, samples were centrifuged and passed through QIAshredder columns for homogenization. DNA purification was completed using spin columns with Buffer AW1 and AW2 wash steps. DNA was eluted in Buffer AE in two steps and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). All DNA samples were stored at –20°C until further analysis.

2.3. Polymerase chain reaction (PCR)

A fragment of the mitochondrial cytochrome b (cytb) gene, a commonly used molecular target for species-level identification of *Prototheca* spp., was selected due to its moderate sequence variability and high species-specific resolution within the genus [7]. Amplification was performed using specific primers (Table 2), as described by [7]. PCR reactions were carried out in a final volume of 25 µL, containing 10× DreamTaq Buffer, 10 mM dNTP mix, 10 mM of each primer, 0.5 µL DreamTaq DNA polymerase (5 U/µL), and 2 ng of template DNA.

PCR amplification was initiated with a denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 50°C for 10 seconds, and extension at 72°C for 20 seconds. A final extension was performed at 72°C for 10 minutes.

Table 2. Primer sequences used for amplification of the cytochrome b gene region.

Primer Name	Sequence (5'→3')
cytb-F1	5'-GyGTwGAACAyATTATGAGAG-3
cytb-R2	5'-wACCCATAArAArTACCATTCwGG-3'

PCR products were analyzed via electrophoresis on a 1.5% agarose gel prepared in TAE buffer. A DNA ladder was used as a molecular weight marker. Samples were mixed with loading dye and run at 5 V/cm. Gels were visualized under UV light using a gel documentation system (Axygen, USA).

2.4. DNA dilution series

To determine the detection limit of the PCR assay, genomic DNA was first extracted to obtain a stock solution for each strain. Serial 1:2 dilutions were prepared by mixing 5 µL of DNA with 5 µL of elution buffer (Buffer AE) at each step, resulting in gradually decreasing DNA concentrations. A total of 23 dilution steps were performed for *P. bovis* and 15 for *P. ciferrii*. Each dilution was used as a PCR template under the same amplification conditions. The presence or absence of PCR products was evaluated by agarose gel electrophoresis. The lowest dilution that yielded a visible band was recorded as the limit of detection (LOD) for each strain. In cases where no visible band was observed in higher dilutions, a second round of PCR (re-PCR) was conducted using the initial PCR product as the template. The same primers and amplification conditions were used. This step aimed to confirm the presence of low-copy DNA and enhance detection sensitivity near the LOD.

3. Results

3.1. Microalgae culture

Both *P. bovis* and *P. ciferrii* strains were successfully cultured on *Prototheca* Isolation Medium (PIM) and maintained under the incubation conditions described in the Materials and Methods section (Figure 1).

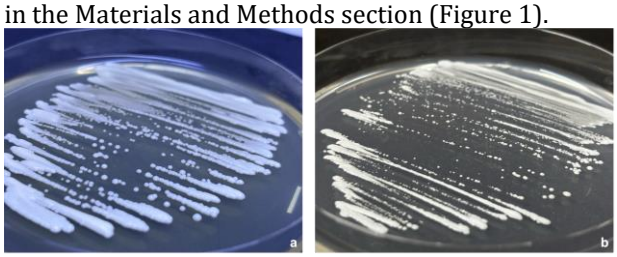

Figure 1. Cultured *Prototheca* species in plates containing Prototheca Isolation Medium (PIM): a) *P. ciferrii* (SAG 2063) and b) *P. bovis* (LZ-5) [22].

3.2. DNA extraction and serial dilutions

DNA was extracted from cultured microalgae and quantified using a NanoDrop spectrophotometer (ThermoScientific). The initial DNA concentrations were determined as 30.00 ng/µL for *P. ciferrii* and 42.9 ng/µL for *P. bovis*.

To evaluate the analytical sensitivity of the PCR assays, two-fold serial dilutions were prepared from each DNA sample. A total of 15 dilution steps were performed for *P. ciferrii*, and 23 steps for *P. bovis*. The number of dilution steps varied between species because dilutions were continued until the PCR bands were no longer visible on agarose gels. DNA concentrations at each dilution step were re-measured using NanoDrop (ThermoScientific, USA) to ensure accuracy. The initial and post-dilution values are presented in Table 3.

Table 3. Initial and final concentrations of diluted DNAs of *Prototheca ciferrii* and *Prototheca bovis*.

Strain	Initial DNA Concentration (ng/µL)	Total Dilutions Performed	PCR Detection Limit (ng/µL)	Re-PCR Range (ng/µL)
P. ciferrii	30.0	15	0.47	9.1 x10^{-4}
P. bovis	42.9	22	0.09	5.34 x10^{-6}

For *P. ciferrii*, conventional PCR yielded visible amplification bands up to the sixth dilution (2^{-6} = 0.47 ng/µL). To further enhance detection sensitivity, re-PCR was performed from the fifth (2^{-5} = 0.94 ng/µL) to the fifteenth (2^{-15} = 9.1×10^{-4} ng/µL) dilution steps (Figure 2). Amplification was successfully observed at all levels except the final dilution, where no visible band was detected.

In the case of *P. bovis*, the initial DNA concentration was higher (42.9 ng/µL), and a total of 22 dilution steps were prepared. Conventional PCR resulted in detectable amplification up to the 8th dilution (2^{-8} = 0.09 ng/µL) (Figure 3). Re-PCR was performed from the 12th (2^{-12} = 0.0105 ng/µL) to the 22nd (2^{-22} = 10.23 pg/µL) dilution steps (Figure 3). Amplification was successfully observed at all levels except the final dilution, where no visible band was detected.

These findings indicate that the re-PCR method significantly improves detection at lower DNA concentrations compared to conventional PCR alone. Furthermore, despite differences in initial DNA concentrations, *P. bovis* demonstrated a higher diagnostic sensitivity under the tested conditions.

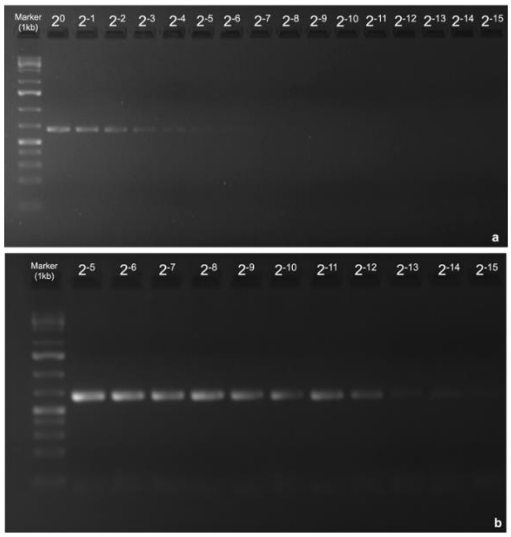

Figure 2. Agarose gel electrophoresis results of a) PCR and b) re-PCR of *P. ciferrii.* A 1 kb DNA ladder (ThermoScientific) was used as a molecular size marker. Bands at approximately 599 bp were considered positive and matched the expected amplicon size.

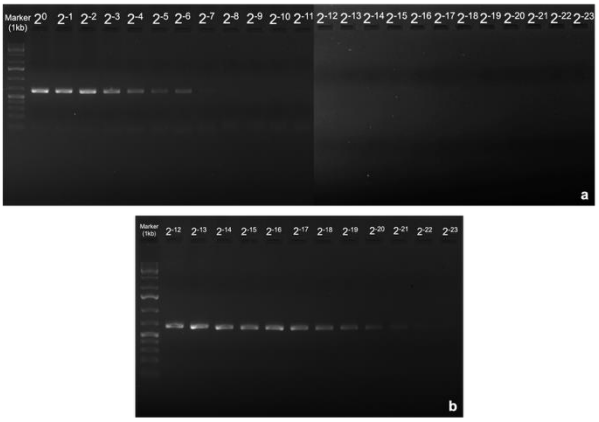

Figure 3. Agarose gel electrophoresis results of a) PCR and b) re-PCR of *P. bovis.* A 1 kb DNA ladder (ThermoScientific) was used as a molecular size marker. Bands at approximately 599 bp were considered positive and matched the expected amplicon size.

4. Discussion and Conclusion

Prototheca spp. are non-photosynthetic green algae that have been observed to cause opportunistic infections in both humans and animals. Due to their pathogenic potential, accurate species-level identification is critical for guiding effective therapeutic interventions. Historically, diagnostic approaches for pathogenic microorganisms have included histopathological examination, serological assays, and culture-based techniques [23]. In recent years, PCR-based molecular diagnostics have emerged as a routine and sensitive method for detecting pathogenic DNA [24]. However, in cases where the target DNA concentration is extremely low, PCR may yield false-negative results [25]. Therefore, establishing the minimum detection limit of the assay is essential to ensure reliable diagnosis, particularly in clinical or environmental samples with low pathogen load.

In this study, the detection limits of *P. ciferrii* and *P. bovis* were determined through serial dilutions of genomic DNA, with successful amplification observed at concentrations as low as 9.1×10^{-4} ng/µL and 5.34×10^{-6} ng/µL, respectively. These findings demonstrate that conventional PCR, particularly when coupled with re-amplification (re-PCR), can provide high analytical sensitivity for the detection of *Prototheca* spp. Comparable levels of sensitivity have been reported in previous studies. For example, [20] reported a detection limit of 20.9 CFU/mL for *P. bovis* using a qPCR assay targeting the *cytb* gene. Similarly, [21] showed that multiplex PCR assays employing mitochondrial and chloroplast-specific primers were capable of detecting *Prototheca* DNA at concentrations as low as 0.8 pg/µL.

Molecular diagnostic techniques have been shown to outperform conventional methods in various organisms, as demonstrated by studies on Mycoplasma spp. [24] and Strongylus vulgaris [27]. In

line with these findings, our study showed that while conventional PCR could detect P. ciferrii and P. bovis at low DNA concentrations, re-amplification (re-PCR) improved detection in samples where initial amplification failed, highlighting its value as a sensitive diagnostic approach, especially in low pathogen load scenarios.

The positivity rate with PCR (9.73%) was slightly higher than that of larval culture (8.85%), reinforcing PCR's reliability, particularly in low-intensity infections. These findings support the broader application of PCR in diagnostic settings, especially when culture methods are slow, labor-intensive, or insufficiently sensitive.
Similarly, previous studies by [28] and [29] have shown that PCR-based techniques are more sensitive and reliable than traditional culture methods. [28] detected fungal DNA in clinical samples where culture tests were negative, especially in patients receiving antifungal therapy. Likewise, [29] reported that universal PCR detected mollicutes in 73% of bovine milk samples, while culture methods identified only 52%. These discrepancies underscore the significance of factors such as slow microbial growth, stringent culture requirements, and prior antimicrobial exposure in hindering culture-based detection. These limitations can be surmounted by molecular methods through the identification of microbial DNA irrespective of cell viability.

Subclinical mastitis poses a particular diagnostic challenge due to the absence of overt clinical signs. In this context, PCR-based assays offer a notable advantage over culture techniques in terms of sensitivity. PCR-based methods have demonstrated higher sensitivity than conventional culture techniques in detecting subclinical mastitis pathogens, including Prototheca sp. [21]. These findings emphasize the crucial role of molecular diagnostics in pathogen detection. Given that P. bovis and P. ciferrii are also associated with subclinical mastitis, the application of PCR-based sensitive detection techniques is essential—not only for identifying well-established mastitis pathogens, but also for improving the recognition of overlooked or misdiagnosed agents such as Prototheca spp., which are often ignored in routine diagnostics. Since conventional treatments like antifungals and antibiotics are ineffective against Prototheca infections, early molecular detection is critical for guiding appropriate herd management and avoiding treatment failures.

The conventional PCR and re-PCR strategy employed in this study is not limited to pure cultures and may hold potential for application to environmental or clinical samples, provided that appropriate sample preparation protocols are developed. The low detection thresholds observed suggest promise for detecting Prototheca in samples with minimal organism burden. However, the applicability of this approach to such complex sample types requires further validation through studies involving larger sample sizes and clinically relevant datasets. While re-PCR may serve as a complementary tool in diagnostic workflows, its broader implementation necessitates thorough evaluation and methodological optimization.

In our study, the re-amplification (re-PCR) method was employed specifically to enhance diagnostic performance in samples with low pathogen load. Conventional PCR may fail to produce visible bands in certain cases due to weak amplification, which can give the false impression that no DNA is present in the sample. However, trace amounts of target DNA may still be present. In such instances, re-PCR enables the re-amplification of weak initial products, making them detectable and thereby minimizing the risk of false-negative results. Furthermore, re-PCR increases diagnostic sensitivity without the need for advanced molecular equipment, making it both cost-effective and easily applicable in laboratories equipped with basic PCR systems.

Although quantitative PCR (qPCR) is widely regarded as the gold standard for sensitive nucleic acid detection, it requires specialized equipment and reagents that may not be readily available in all laboratory settings, particularly in resource-limited environments. In contrast, the implementation of re-amplification (re-PCR) in this study demonstrated a clear diagnostic advantage at low DNA concentrations, successfully producing visible amplicons in dilution steps where conventional PCR failed. Given its cost-effectiveness and operational simplicity, re-PCR offers a practical and accessible alternative to qPCR for routine detection of Prototheca species or other low-abundance targets, especially in laboratories lacking access to real-time PCR systems.

In conclusion, this study established the minimum detection limits of two pathogenic Prototheca species (P. ciferrii and P. bovis) using conventional PCR. Our findings demonstrate that, despite the widespread use of qPCR in diagnostic workflows, conventional PCR remains a cost-effective and accessible alternative capable of achieving high analytical sensitivity when optimized appropriately. This approach holds particular value in resource-limited settings where advanced molecular platforms may not be readily available.

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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