Accompanying a semi-nested PCR assay to support histopathology findings of fungal keratitis in formalin-fixed paraffin-embedded corneal samples

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Funding information
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Abstract
Background: Fungal species are responsible for 40%-50% of all microbial keratitis cases. Due to the low amount of extracted DNA in ocular Formalin-fixed Paraffin-embedded (FFPE) samples, selecting a reliable molecular method is a substantial issue in this field.

Methods: Sixty-six samples were collected via the penetrating keratoplasty (PK) technique. Histopathology assays were performed using hematoxylin–eosin (H&E) and periodic acid Schiff (PAS) staining methods. The ITS1/ITS4 and ITS1/ITS2 primer pairs were used in a semi-nested polymerase chain reaction (PCR) to target the universal internal transcribed spacer (ITS) region. Some PCR results were validated through sequencing.

Results: Fungal DNA was detected in 44 of 66 samples (66.7%), and histopathology was positive for 41 of 66 samples (62.1%). Of 41 histopathologically proven fungal-positive cases, 39 were PCR-positive (95%). Moreover, of 44 PCR-positive samples, 39 (88.6%) were histopathology-positive, and 5 (11.3%) were histopathology-negative. Totally in 39 cases (59%), both histopathology and PCR yielded positive results. The Kappa agreement rate between the two diagnostic methods, including histopathology and PCR, was 0.77. Sensitivity, specificity, positive predictive value, and false predictive value were reported as 88.64%, 90.9%, 95.12%, and 80%, respectively.

Conclusion: As we reached the acceptable Kappa agreement rate, we concluded that applying the semi-nested PCR assay is a promising method for supporting the evidence by histopathology. Finally, we suggest targeting more specific gene regions using primer pairs that amplify smaller amplicon sizes and surveying novel molecular methods such as NGS to achieve higher sensitivity and Kappa agreement rates.

Keywords
formalin-fixed paraffin-embedded, fungal keratitis, histopathology, ocular samples, semi-nested PCR
1  |  INTRODUCTION

Fungal keratitis (FK), initially described in 1879, is a serious eye infection that accounts for 40% to 50% of all microbial keratitis cases globally. It is estimated that the incidence rate is 17%–36%, with the highest in Asia and Africa. FK is a major risk factor for corneal injury and endophthalmitis, resulting in vision loss if not treated promptly. Keratitis induced by fungus is sometimes difficult to identify, resulting in a delay in adequate treatment. As a result, it is critical to correctly identify the causative agents of keratitis to provide an appropriate treatment option.

FK can now be caused by more than 100 species (both yeast and filamentous). The most important etiologic agents are Fusarium, Aspergillus, Candida, and Alternaria. Generally, several factors determine the prevalence of specific fungi causing FK, including personal risk factors, regional temperature, climate conditions, geographic conditions, and urbanization. For example, Fusarium, Aspergillus, and other molds are more typically associated with trauma and contact lens use, whereas Candida spp. is more commonly associated with ocular surface illness and topical steroid treatment.

Corneal tissue scraping is the appropriate specimen for the laboratory diagnosis (i.e., histopathology and direct examination, culture, and molecular analysis) of FK. Because fungus tends to infiltrate deep into the cornea, a thorough corneal scraping is usually necessary for an accurate diagnosis. Therefore, a diagnostic procedure that can find even a few microorganisms in a clinical specimen is unquestionably beneficial.

Molecular techniques are becoming a hot issue in FK diagnosis. Because of their high sensitivity, molecular approaches reduce the time required for accurate diagnosis and compensate for the little amount of specimen. Among molecular methods, PCR yields the most sensitive results. An upcoming concern with this approach is that it may amplify DNA from non-pathogenic organisms, which might lead to false-positive findings. The accuracy and sensitivity of PCR assays are intimately connected to the gene areas they are designed to target. In this regard, the highly conserved rRNA gene complex, including four ribosomal rRNA genes (the large subunit [LSU] 26S-28S, the small subunit 18S, 5.8S, and 5S genes), are the most targeted regions. The entire length of ITS1-5.8S-ITS2 (pan-fungal ITS) is usually sequenced to be used in fungal classification.

Due to the inadequate amount of ocular tissue samples collected, it is impossible to allocate the sample for all of these experiments, including simultaneous microscopic and molecular tests. As a result, the samples are ideally suited for histopathology testing, considered routine but not the gold standard diagnostic approach.

A semi-nested PCR method was used in this study to detect fungal DNA in FFPE samples, even in small amounts. Using previously histopathologically proven cases, we aimed to determine the diagnostic accuracy parameters, such as sensitivity and specificity, of the semi-nested PCR method in detecting fungal pathogens. Moreover, the diagnostic accuracy of the semi-nested PCR will be compared with that of histopathology as the reference method.

2  |  MATERIAL AND METHODS

2.1  |  Samples & Patients

Sixty-six FFPE corneal blocks were collected by PK and stored for 1–7 years. Demographic data of the patients, including age, sex, type of operation, site of infection, background diseases, and final pathology report, were documented. For preparation, samples were fixed with 10% formalin, embedded in paraffin, and divided into 10 slices with a 5–7 μm thickness. Then, they were put into microtubes for further histopathological and molecular investigations. Deparaffinization of samples was carried out by the xylene method, as previously discussed. Benzene and 2 M HCl (rinsed with sterile water) were used to avoid contamination, as previously recommended.

2.2  |  Histopathological assay

The histopathological assay was performed using hematoxylin–eosin (H&E) and periodic acid schiff (PAS) staining methods, according to the protocols for FFPE sample staining. The results of the histopathology assay were documented.

2.3  |  DNA extraction

DNA extraction was performed via QIAamp DNA extraction from tissue mini kit (Qiagen, Hilden, Germany), as previously described. Briefly, 100 μl of lysis buffer, 180 μl of ATL buffer, and 20 μl of proteinase K were added to the tube samples. The tubes were washed with normal saline after the overnight incubation at 56°C. The tubes were heated in boiling water for 5 min to complete the lysis process. The tubes were incubated in boiling water and liquid nitrogen for 1 and 2 min, respectively. This step was repeated several times. Finally, they reached room temperature. This process is based on the binding of the DNA to silica columns.

2.4  |  Conventional PCR assay

The extracted DNA was tested for quality by amplifying the human globin gene using the PCR method and G1/G2 primer pairs (Table 1), yielding a 205 bp product. The total volume of the PCR reaction was 25 μl. The master mix contained 1 mM MgCl₂, 200 μM.

| TABLE 1 The sequences of PCR primers |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **ITS1**      | 5'-TCCGTAGTGGAACCTGCGG-3' | **Reverse** |
| **ITS4**      | 5'-TCCTCCGTTATGGTATGC-3' | **Forward** |
| **ITS2**      | 5'-GCTCGGTCTCCTCATCGATG-3' | **Reverse** |
| **G1**        | 5'-CAACTTCATCCACGTTCACC-3' | **Forward** |
| **G2**        | 5'-GGTTGGCCAAATCTACTCCAGG-3' | **Reverse** |
ITS1/ITS2 primer pairs targeting a 280 bp amplicon. The primer pairs and extension at 72°C for 45 s. The final extension of both reactions was carried out for 5 min at 72°C. PCR reactions contained the final concentration of 1.5 mM MgCl₂, 200 µM each dNTP, 25 µM each primer, and 1 U Taq DNA polymerase (Cinna Gene, Iran) in a final volume of 50 µl. Five microliters of the DNA extracted from corneal scraping were used as a template in the first PCR reaction, and 1 µl of the first PCR product was used in the second semi-nested PCR reaction. The PCR conditions for the first semi-nested PCR step were initial denaturation of the template DNA for 5 min at 95°C, 38 amplification cycles of denaturation for 45 s at 95°C, annealing for 45 s at 58°C, and extension for 60 s at 72°C. The conditions for the final semi-nested reaction were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. The final extension of both reactions was carried out for 5 min at 72°C.

2.5 | Semi-nested PCR Assay

The semi-nested PCR targeted the universal ITS gene region (ITS1-5.8S-ITS2) in two steps. The first reaction of the semi-nested PCR assay was carried out using ITS1/ITS4 primer pairs targeting a 510 to 879 bp amplicon. The products of the first step were amplified by ITS1/ITS2 primer pairs targeting a 280 bp amplicon. The primer pairs are listed in Table 1. PCR reactions contained the final concentration of 1.5 mM MgCl₂, 200 µM each dNTP, 25 µM each primer, and 1 U Taq DNA polymerase (Qiagen GmbH) in a final volume of 50 µl. Taq DNA polymerase (Cinna Gene, Iran), and 1 µl of (10 picomoles) of forward and reverse primers. The conventional PCR assay was carried out as follows: denaturation phase 1 cycle at 94°C for 10 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. Finally, the accuracy of β-globin gene PCR was evaluated using agarose gel by the Gel Doc XR system (Biorad, USA) and smart ladder (Eurogentec, Seraing-Belgium) (Figure 1). The positive controls were 10ng/µl of DNA of Aspergillus fumigatus and Candida albicans species that were previously identified. The negative control was sterile distilled water.

2.6 | Sequencing

Some PCR products were sequenced for species identification. The GenBank database’s BLAST tool and online software were used to analyze the sequencing results (National Institutes of Health; http://www.ncbi.nlm.nih.gov).

2.7 | Data analysis

The data were analyzed using SPSS software version 24. Briefly, descriptive data were presented as mean, standard deviation, percentages, and charts. The Chi-square and Fisher’s exact tests were used to compare the qualitative variables between the two groups. The Student’s t-test was used to compare the quantitative variables between the two groups. In addition, the agreement between the two diagnostic methods was calculated by the Kappa test, based on Viera et al. The p-value below 0.05 (p<0.05) was considered statistically significant.

3 | RESULTS

3.1 | Patients and samples

In this study, 66 corneal FFPE samples from 41 histopathologically proven FK and 25 histopathologically negative cases were examined. The patient’s age ranged from 1 to 84 years (mean±SD: 53±22 years), with 37 (56.1%) males and 29 (43.9%) females. The mean age of fungal-positive patients was 59.35±25 years. No significant association was found between PCR results, sex, or age. FK was more common in women; however, the difference was not statistically significant (75.9% vs. 64.9%, p-value: 0.422). Also, FK was more common in patients above 50 years of age (65.9%) with a p-value of 0.405, which was not statistically significant.

3.2 | Histopathological and Molecular Examinations

Sixty-six FFPE specimens were examined by histopathology and semi-nested PCR methods. Fungal DNA was detected in 44 of 66 samples (66.7%), and histopathology was positive for 41 of 66 samples (62.1%). Of 41 histopathology fungal-positive cases, 39 were PCR-positive (95%). Moreover, from 44 PCR-positive samples, 39 (88.6%) were histopathology-positive, and 5 were histopathology-negative (11.3%). Totally in 39 cases (59%), both histopathology and PCR yielded positive results (Table 2). The histopathology reports using H&E and PAS stains indicated that ulceration, necrosis, severe acute inflammation, and stromal thinning were the main histopathological manifestations. Both septate hyphae and yeast forms were observed (Figures 2 and 3). The Kappa agreement rate between the two diagnostic methods, including histopathology and PCR, was

![FIGURE 1 Electrophoretic bonds of PCR products](https://example.com/figure1.jpg)
ASHRAF et al.

0.77, which means these methods were compatible. Given that the histopathology method is known as the gold standard for the diagnosis of FK, the sensitivity, specificity, positive predictive value, and false predictive value of the semi-nested PCR assay were reported as 88.64%, 90.9%, 95.12%, and 80%, respectively.

The bold value (Cohen’s kappa) indicates substantial agreement between PCR and histopathology results.

### Table 2: Demographic data according to histopathology and PCR assays

| Diagnostic tests | Histopathology | PCR |
|------------------|----------------|-----|
|                  | Positive  | Negative | Total |
| Positive         | 39       | 5        | 44    |
| Negative         | 2        | 20       | 22    |
| Total            | 41       | 25       | 66    |

Kappa agreement: 0.77

p value: <0.001

Our results are promising for the semi-nested PCR method covering histopathology evidence in detecting fungal agents on ocular FFPE samples. Since positive results for histopathology were fewer than those of the semi-nested PCR assay (62.1% vs. 66.7%), this method is seemingly superior to the histopathology method. Although histopathological analysis is the reference method for diagnosing and approving FK in the ocular specimen, there is an urgent need for an accessible and rapid tool for diagnosing and supporting histopathology findings. Histopathology may yield false-negative results due to the small sample size, necrotic tissue with degenerated fungal hyphae, and severe inflammation. Also, PCR assays may yield false-positive results, mainly due to environmental contamination.

In time diagnosis of FK is imperative for the management of the infection. This study evaluated two diagnostic methods for detecting fungi, histopathology and semi-nested PCR, on 66 corneal FFPE tissue samples. We reported 41 (62.1%) and 44 (66.7%) positive results for histopathology and semi-nested PCR assays, respectively. In Total, 39 cases (59%) yielded positive results in both histopathology and PCR. The sensitivity, specificity, positive predictive value, and false predictive value of the semi-nested PCR assay were calculated as 88.64%, 90.9%, 95.12%, and 80%, respectively. The Kappa agreement rate between the two diagnostic methods, including histopathology and PCR, was 0.77.

Table 3.

### Sequences accessibility and results

Six PCR products were successfully identified from the 44 samples sequenced. The resulting sequences were deposited in the GenBank database (accession numbers from ON885271 to ON885276 with persistent accessible links of ON885271, ON885272, ON885273, ON885274, ON885275, ON885276). The sequencing for six PCR positive indicated three cases of Cryptococcus albidus, two cases of Candida albicans, and a black saprophyte Conocephalosporium (Alternaria) spp.

### 4 | DISCUSSION

FK is an alarming ocular infection, causing critical health problems worldwide, with 40% to 50% of all microbial keratitis cases and an incidence rate of 17%–36%. In-time diagnosis of FK is imperative for the management of the infection. This study evaluated two diagnostic methods for detecting fungi, histopathology and semi-nested PCR, on 66 corneal FFPE tissue samples. We reported 41 (62.1%) and 44 (66.7%) positive results for histopathology and semi-nested PCR assays, respectively. In Total, 39 cases (59%) yielded positive results in both histopathology and PCR. The sensitivity, specificity, positive predictive value, and false predictive value of the semi-nested PCR assay were calculated as 88.64%, 90.9%, 95.12%, and 80%, respectively. The Kappa agreement rate between the two diagnostic methods, including histopathology and PCR, was 0.77.

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Here, two cases showed negative PCR but positive histopathology results, partly because of the destruction of genomic DNA during the paraffinization process for FFPE samples and DNA extraction. False-negative results for PCR assay may be due to the effect of tissue endonuclease (RNase or DNase), degradation of nucleic acids and primers, and an inadequate amount of tissue sample. Also, DNA polymerase activity may be impeded by PCR inhibitors (heme, heparin, phenol, polyamines, plant polysaccharides, and calcium alginate), which are present in the samples or environment.

Several factors, including DNA extraction method, quality control of extracted DNA, primer pairs, targeted amplicon length, tissue cut thickness, and the time of FFPE sample banking, affect the results of PCR assays on FFPE samples to detect fungal DNA. We
| No  | Sequence                                                                 | Max score | Total score | Query cover | E value  | Ident | Accession  | Results               |
|-----|--------------------------------------------------------------------------|-----------|-------------|-------------|-----------|-------|------------|-----------------------|
| F515| GCGTCTGCTGACTGCTACATCATCATCCATAACACTGTGACCTGCTGAGCTTGCTCACAGGCACATCATGCT | 352       | 352         | 91%         | 1e-93     | 100%  | ON885271   | Cryptococcus albidus  |
| F517| TTTGTTTCTGTCAGCTTGCCCTGGCCACATCATTCATCATCAACACTGTG                         | 318       | 318         | 89%         | 2e-83     | 97%   | ON885272   | Cryptococcus albidus  |
| F535| TGCCATCTGTCACTGCGGCGGCGGCGGCAATCCATACACTCAACACTGTGACCTGCTG                | 297       | 297         | 84%         | 7e-77     | 98%   | ON885273   | Cryptococcus albidus  |
| F536| GGGTCCGAGGGTGTGCTTGCCCTCTGTAAGCGGCGGCTCTGCTTACCTATGACGTGATGGCTTGCT       | 255       | 255         | 69%         | 2e-64     | 95%   | ON885274   | Comoclathris spp.    |
| F539| CCCCTGCTTTTTCTTTGAGCAACACTGTGTTGGCCGGCGTTGGCCAGCT                          | 309       | 309         | 80%         | 1e-80     | 100%  | ON885275   | C. albicans          |
| S6  | GCCCTGTTTCTTTTGAAAACACACTGTGCTTGGCGGTTGG                                | 329       | 329         | 98%         | 1e-86     | 98%   | ON885276   | C. albicans          |
used QIAamp DNA extraction due to its accuracy, as previously confirmed.\textsuperscript{18,22-24,26} The extracted DNA was tested for quality by amplifying the human-globin gene with G1/G2 primer pairs, yielding a 205bp product, as previously done.\textsuperscript{25,26} Moreover, two sets of primers targeted the universal ITS region (ITS1-5.8S-ITS2). Here, the amplification of a 510 to 879bp region was followed by targeting a 280bp region during the second step of semi-nested PCR. This indicated that short amplicon length, especially in the second semi-nested step of PCR assay, would give more sensitive and acceptable results.\textsuperscript{34} Also, samples with 5–7 μm thickness, having been stored for 7 years, were evaluated for the presence of fungal DNA. It indicated that FFPE samples with high thickness, short storage time, and standard procedure for paraffinization and deparaffinization yielded more sensitive and acceptable results in histopathology and PCR assays.\textsuperscript{34}

Several studies have set up the semi-nested PCR assay to detect fungal DNA in FFPE samples collected from other body sites. These studies were carried out by Bialek et al., Willinger et al., and Rickerts et al. and resulted in a sensitivity rate of 60%–68%.\textsuperscript{18,27,28} Here, we reported a sensitivity of 88.64% for semi-nested PCR, which is lower than the sensitivity of molecular diagnosis in some previous studies.\textsuperscript{15,36} This difference in sensitivity between our research and the mentioned studies is due to the fact that they used direct specimens for diagnosis while we used FFPE, which gives a lower amount of DNA and lower quality. Therefore, the sensitivity and positive PCR rates of our survey (88.64% and 66.7%) seem to be logical. Moreover, we reached a Kappa factor of 0.77 between semi-nested PCR and histopathology assays, indicating substantial agreement between these two tests in the previous study.\textsuperscript{37}

In this study, 10 appropriate PCR products were selected for sequencing to verify the PCR products. It should be noted that the sequencing of PCR products of FFPE tissue samples is complicated due to the low quality of the initial extracted DNA. Here, we found that the dominant isolates were three cases of Cryptococcus albidus, two cases of Candida albicans, and a case of black saprophyte Alternaria spp. However, Fusarium spp. are the main causative agents of FK.\textsuperscript{38}

In the present study, females were affected more than males (75.9% vs. 64.9% p-value: 0.422), similar to Thomas et al.’s study.\textsuperscript{16} However, some studies reported 58–70% rates for males.\textsuperscript{39,40} It may be a consequence of the frequent usage of contact lenses by women. In this study, FR was more prevalent in patients above 50 years old, the same as in most previous studies.\textsuperscript{39,40}

5 | CONCLUSION

Due to low accuracy, diagnostic errors, the need for an expert technician in histopathology diagnosis, and the low amount of extracted DNA in ocular FFPE samples, it is imperative to confirm the results by a more sensitive molecular method. Sequencing is the reference and gold standard diagnostic method for approving FK but is an expensive and time-consuming tool. Selecting a reliable molecular method is an emerging and substantial issue in this field. This study found an acceptable agreement between histopathology and semi-nested PCR methods for detecting fungi in ocular FFPE samples. Therefore, applying the semi-nested PCR assay is a promising method for supporting histopathology evidence. Finally, we suggest targeting more specific gene regions using primer pairs that amplify smaller amplicon sizes with novel molecular techniques such as NGS to achieve high sensitivity and Kappa agreement rates.

AUTHOR CONTRIBUTIONS

Mohammad Javad Ashraf: Validation, Conceptualization, Methodology Foroogh Shamsizadeh, Saeora Hejazinia: Investigation, Formal analysis, Writing – Review & Editing Hamid Morovati: Writing – Original Draft, Writing – Review & Editing Saham Ansari, Mohammad Kord: Formal analysis, Methodology Golsa Shekarkhar: Writing – Review & Editing Keyvan Pakshir: Validation, Conceptualization, Methodology Kamiar Zomorodian: Validation, Supervision, Project administration, Finding acquisition, Writing – Review & Editing.

ACKNOWLEDGMENTS

The authors are grateful to Khalili Educational and Research Hospital, Shiraz-Iran, and the Vice President of Research, Shiraz University of Medical Sciences, for their great support of this project.

FUNDING INFORMATION

This project received financial support from the Vice President of Research, Shiraz University of Medical Sciences (Grant No. 93–7274).

CONFLICT OF INTEREST

The authors declare they have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The six sequences generated in this study were deposited in the GenBank database (accession numbers from ON885271 to ON885276 with persistent accessible links of ON885271, ON885272, ON885273, ON885274, ON885275, ON885276). All data used to support the findings of this study are available from the corresponding author upon request.

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How to cite this article: Ashraf MJ, Shamsizadeh F, Morovati H, et al. Accompanying a semi-nested PCR assay to support histopathology findings of fungal keratitis in formalin-fixed paraffin-embedded corneal samples. J Clin Lab Anal. 2022;36:e24764. doi:10.1002/jcla.24764.