ORIGINAL ARTICLE

A Comparative Analysis of Conventional Methods versus Oxford Nanopore Sequencing Technology in the Identification of Fungi in Ocular Specimen

Madhu Rai[1], Vaibhav Nagpal[2], Sanchi Shah[3], Anirvan Chatterjee[4], Nidhi Sehgal[5], Amrutraj Zade[6], Savitri Sharma[7]

ABSTRACT

The correct identification of fungal pathogens is critical for the efficient management of fungal diseases. Conventional approaches, such as assessing colony morphology, microscopic characteristics on lactophenol cotton blue (LPCB) staining, and biochemical tests, are still frequently employed, although they can be time-consuming and inconclusive, especially in non-sporulating fungi. With recent breakthroughs in molecular diagnostics, genome sequencing (GS) with Oxford Nanopore Technology (ONT) provides a quick and accurate alternative. This study compares traditional approaches to ONT-based sequencing for fungus identification in corneal scraping samples from keratitis patients. Ten fungal isolates from ocular specimens at a tertiary eye care institution in Mumbai were investigated. Each isolate was examined using both conventional approaches (macroscopic culture characteristics and microscopic LPCB stain morphology) and GS, which targeted the internal transcribed spacer (ITS) region. Following DNA extraction and sequencing, an automated bioinformatics analysis was performed. Both traditional techniques and ONT sequencing identified all ten fungal isolates with 90% agreement. The average turnaround time for conventional identification was 5-7 days, whereas ONT yielded results in 16-24 hours. Rapid identification by ONT facilitated early clinical decision-making, such as the beginning or optimization of antifungal medication. This study found excellent agreement between conventional and ONT-based approaches for fungal identification, underscoring ONT's usefulness as a fast, culture-independent diagnostic tool. Integrating sequencing technology into clinical microbiology can dramatically reduce diagnosis time, hence improving patient care in fungal keratitis and other mycoses.

KEY WORDS: Oxford Nanopore Sequencing, Fungal keratitis, Conventional culture methods

Introduction

Fungal keratitis is a significant cause of corneal blindness, particularly in tropical and subtropical regions[1]. In some areas, fungi are responsible for 40–50% of all microbial keratitis cases, necessitating rapid and accurate identification of the causative agent to ensure proper management[2, 3].

Conventional laboratory methods for fungal identification include gross observation of culture characteristics, microscopic examination of fungal spores using stains such as lactophenol cotton blue (LPCB), and a range of biochemical tests[4]. These methods have been in use for many years, but they can take several days to finish and may not identify fungi that don't sporulate in

Access this article online

Quick Response Code:

Website: jmsh.ac.in

Doi: 10.46347/jmsh.v12.i3.26.166

[1]Faculty Microbiology, Ocular Microbiology Service, Shantilal Shanghvi Eye Institute, Wadala East, Mumbai-400037, Maharashtra, India. [2]Junior Faculty, Cornea Service, Shantilal Shanghvi Eye Institute, Wadala East, Mumbai- 400037, Maharashtra, India. [3]Head Bioinformatics, infexn-NGS, Haystack Analytics Private Limited SINE, IIT Bombay, Powai, Mumbai – 400076, Maharashtra, India. [4]Director, Haystack Analytics Private Limited SINE, IIT Bombay, Powai, Mumbai – 400076, Maharashtra, India. [5]Technician, Ocular Microbiology Service, Shantilal Shanghvi Eye Institute, Wadala East, Mumbai-400037, Maharashtra, India. [6]Head of the Department, Research & Development, Haystack Analytics Private Limited SINE, IIT Bombay, Powai, Mumbai – 400076, Maharashtra, India. [7]||Network Director Emeritus|| Laboratory Services, LV Prasad Eye Institute, Hyderabad/Ocular Microbiology Service, Shantilal Shanghvi Eye Institute, Wadala East, Mumbai-400037, Maharashtra, India.

Address for correspondence:
Madhu Rai, Faculty Microbiology, Ocular Microbiology Service, Shantilal Shanghvi Eye Institute, Wadala East, Mumbai-400037, Maharashtra, India. E-mail: madhu.rai@ssei.ind.in

culture[5].

The advent of molecular diagnostics has revolutionized microbial identification[6]. Techniques such as polymerase chain reaction [PCR] have significantly improved diagnostic accuracy; however, PCR methods are highly targeted and are likely to have a limited number of pathogens that can be screened in an assay[7]. For over a decade, microbiological research has chosen genome sequencing [GS] due to its unparalleled comprehensiveness, sensitivity, and specificity. However, the application of GS in laboratory diagnostics has been limited due to high costs, the need for a highly skilled workforce, and non-standardized methods and kits. In a recent report, GS performed using ONT demonstrated a portable, cost-effective, and standardized method for the detection of pathogens[8]. By using automated bioinformatics, the method described demonstrated the use of GS in laboratory diagnosis for rapid and precise identification of pathogens, including bacteria, fungi, and viruses, through real-time sequencing. This capability would enable clinical decisions and could transform microbial detection, especially for fungal pathogens, and improve the chances of clinical success while minimizing complications. By avoiding delays associated with traditional methods, GS can enable better patient outcomes, reduce the misuse of broad-spectrum antimicrobials, and contribute to the global effort to combat antimicrobial resistance[9]. However, the study did not establish the use of the GS for direct detection in ocular samples.

To determine its clinical diagnostic applicability, this study compared GS with conventional methods for the identification of fungi grown from corneal scrapings of patients with fungal keratitis and other ocular specimens.

Patients And Methods

This study was conducted at Shantilal Shanghvi Eye Institute, Mumbai, India, between October 2023 and April 2024. As per the institutional protocol, a complete clinical examination, including slit-lamp microscopy and microbiological investigation of the clinical specimens, was done for all patients presenting to the clinic with suspected ocular microbial infection. Under topical anesthesia, multiple corneal scrapings were obtained in the clinic under slit lamp magnification for microbiological processing. BCL was received directly in the laboratory, while discharge from the eyelid wound was collected in the OPD. Smears for direct microscopic examination were stained with 10% KOH and 0.1% calcofluor white staining [KOH+CFW] and gram staining. The first was seen under a fluorescence microscope and the second under a light microscope. Specimens were also inoculated on 5% sheep blood agar, Sabouraud dextrose agar [SDA] with chloramphenicol, and potato dextrose agar [PDA] with chloramphenicol for fungal culture. Blood agar was incubated at 37°C, while SDA and PDA were incubated at 27°C. Fungal isolates were maintained on PDA and SDA and examined microscopically under a lactophenol cotton blue wet mount for morphological identification from both medias. A subculture was done of each fungal isolate on an SDA plate, which was sent to Haystack Analytics Pvt. Ltd. for nanopore sequencing, where the investigators were masked to patient details and morphological identification of the fungal isolates.

This was an isolate-based laboratory study conducted at a single tertiary eye care center.

Inclusion criteria: The study included only fungal isolates recovered from ocular specimens.

Exclusion criteria: Non-fungal isolates were excluded from the study.

Genome Sequencing:

Fungal isolate cultures from SDA plates were aseptically transferred to a sterile Luria-Bertani [LB] broth tube by scraping the plate surface using a sterile surgical blade and incubating at 22°C for 24-48 hours. The 1 mL broth from each tube was transferred into a sterile microfuge tube and centrifuged at 13000 g for 15 min. The pellet was then processed for DNA extraction using the DNeasy UltraClean 96 Microbial Kit [Qiagen, Cat. No. 10196-4] as per the manufacturer's protocol with a minor modification of incubation at 95°C for 1 hr post-power bead and solution SL buffer addition.

The extracted DNA was used as a template for full-length ITS amplification using the Universal ID kit [HaystackAnalytics, Cat. No. 02ID09AMR] as per the manufacturer's protocol. The kit is designed to amplify full-length 16S and ITS regions for bacterial and fungal identification, respectively. The kit also amplifies antimicrobial resistant genes [ARGs], viz., blaOXA type, blaCTX-M, blaSHV, blaTEM, blaNDM, mecA, vanA, mcr-1, and aph[9].

The amplicons were then purified and subjected to library preparation using the Native Barcoding kit protocol [ONT, Cat. No. SQL-LSK109] and sequenced on the MinION sequencing platform [ONT, Cat. No. MIN-101B]. Basecalling and demultiplexing were performed using MinKNOW. The sequence data was analyzed using an automated bioinformatics pipeline reported earlier[8]. Briefly, the fastq files were checked for adapters and primers and preprocessed, followed by annotation against a curated ITS database.

A comparison between the ONT and the standard of care method (culture) (SOC) was done at the highest taxonomic classification, which was achieved by either of the methods, to identify agreement. Concordance

between the methods was evaluated using Cohen's kappa (κ) coefficient with 95% confidence intervals (95% CI), which was calculated using the psych library in R.

Results

Ten clinical samples were collected from 10 patients. [Table. 1] displays the clinical diagnosis, the type of sample collected, demographic details, and the results of the conventional microbiological investigation. The samples included eight corneal scrapings, one discharge from an eyelid wound, and one bandage contact lens (BCL). Fungal growth was observed in all samples; however, direct microscopy revealed fungal septate filaments in corneal scrapings, which was not performed for BCL and discharge samples. All 10 fungal isolates were filamentous septate fungi belonging to different species, as shown in [Table. 1].

Table 1: Patient demographics, sample characteristics, and fungal identification based on conventional microbiological techniques and genome sequencing results

Patient no	Age/ gender	Clinical diagnosis	Specimen type	Eye	Time taken by fungal isolate to grow in days	Fungus identified by conventional method	Time for Identification by Conventional (LPCB) method after growth	Identification by ONT	Time for Identification by ONT in hours after growth
1	16/M	Microbial keratitis	Corneal scraping	OD	4	*Fusarium species*	15 minutes	*Fusarium falciforme* *Fusarium keratoplasticum*	12-18 hours
2	45/M	Fungal keratitis	Corneal scraping	OD	4	*Fusarium species*	15 minutes	*Fusarium ambrosium* *Fusarium solani*	12-18 hours
3	35/M	Fungal keratitis	Corneal scraping	OS	10	Unidentified Dematiaceous fungus	Unidentified (non-sporulating)	*Lasiodiplodia parva*	12-18 hours
4	54/M	Right facial palsy with lagophthalmos	Discharge from Eyelid wound	OD	3	*Aspergillus flavus*	15 minutes	*Aspergillus subflavus* *Aspergillus tamarii*	12-18 hours
5	68/F	Steven Johnson Syndrome with bilateral Dacrocystitis	BCL*	OD	10	*Alternaria* spp [formerly *Ulocladium spp*]	48 hours (sporulated in 48 hours after growth)	*Pseudopithomyces angolensis*	12-18 hours
6	9/F	Fungal keratitis	Corneal scraping	OS	4	*Fusarium species*	15 minutes	*Fusarium falciforme*	12-18 hours
7	38/M	Fungal keratitis	Corneal scraping	OS	4	*Fusarium species*	15 minutes	*Fusarium keratoplasticum*	12-18 hours
8	25/M	Fungal keratitis	Corneal scraping	OD	14	*Cladorrhinorum bulbillosum Podospora bulbillosa*	24 hours	*Podospora bulbillosa*	12-18 hours
9	56/F	Fungal keratitis	Corneal scraping	OD	20	*Curvularia* species	*10 days (sporulated in 10 days afer growth)*	*Curvularia soli*	12-18 hours
10	57/M	Fungal keratitis	Corneal scraping	OD	4	*Fusarium* species	15 minutes	*Fusarium fujikuroi*	12-18 hours

*Bandage contact lens; #OD- Right eye; OS- Right eye

[Table. 2] shows the clinical diagnosis and outcome of all patients included in the study. A total of 10 cases were analyzed, involving patients aged 9 to 68 years; there were 7 male patients and 3 female patients. The size of corneal infiltrates varied from 2x2.5 mm to 8x6 mm. The treatment given to the patients is summarized in [Table. 2]. Clinical outcomes were as follows. Patients 1, 3, 4, 5, 6, 8, and 9 healed with a scar; patient number 2 underwent therapeutic penetrating keratoplasty [TPK] combined with Descemet's stripping endothelial keratoplasty [DSEK] and intraocular lens [IOL] implantation. Patient number 4 had a complaint of right-side facial palsy with lagophthalmos; his eyelid had a wound with discharge, which was sent for culture. It grew methicillin-resistant *Staphylococcus aureus* [MRSA] and *Aspergillus flavus*.

Table 2: Overview of predisposing factors, clinical diagnosis, and outcomes observed in all study participants

Patient no.	Predisposing factor	VA [Log MAR] at presentation	Size of infiltrate [H x V, mm]	Hypopyon [mm]	VA at last follow up [Log MAR]	Clinical Diagnosis	Treatment	Final outcome
1	Foreign body	1.9	6x5.5	Streak hypopyon	1.6	Fungal keratitis	5% natamycin Tab Ketoconazole 200mg BD for 3 days.	Healed
2	Topical steroids	0.78	4x3.5	None	0.48	Fungal keratitis	Therapeutic keratoplasty	No recurrence of infection
3	Trauma	0.8	6.5x6.5	None	0.4	Fungal keratitis	5% natamycin	Healed
4	Trauma and Lagophthalmos	0.3	NA#	None	0.3	Surgical site Eyelid infection	Tab itraconazole 100 mg OD 2 weeks and Tab linezolid 600mg BD for 2 weeks	Healed
5	Steven Johnson Syndrome Sequelae.	3	5 x 5	None	3	Bacterial keratitis [BCL for impending corneal perforation]	0.5% Chloramphenicol eye drops	Healed with scar
6	Foreign body	0.4	3x4.3	None	0.2	Fungal keratitis	5% Natamycin	Healed with scar
7	Trauma	1.3	2x2.5	1.5mm	LTFU*	Fungal keratitis	5% Natamycin	LTFU*
8	Foreign body	0.4	5x3	None	0.2	Fungal keratitis	5% Natamycin	Healed with scar
9	Systemic Immunosuppression	0.2	3x3	None	0.2	Fungal keratitis	Tapering high dose oral steroids, 5% Natamycin	Healed with scar
10	Topical steroids	1.9	8x6	None	LTFU*	Fungal keratitis	5% Natamycin, Tab Itraconazole BD	LTFU*

* lost to follow up; # Not available

A reconstruction surgery was done, and the patient was started on antimicrobials [Table. 2], after which the wound healed. Patient number 5 was a known case of ocular sequelae of Steven Johnson Syndrome [SJS] and bilateral keratitis and had distichiasis in both eyes. Tissue adhesive application with bandage contact lens [TA + BCL] was done in both eyes, following which the right eye developed an infection. BCL was sent for culture, which grew *Staphylococcus epidermidis* and *Alternaria* spp. Two out of 10 specimens showed mixed infection with bacteria and fungus. Two patients were lost to follow-up.

Genome sequencing results:

GS of DNA extracted from cultured isolates yielded genus and species identification of all 10 isolates. ONT successfully identified all 10 fungal isolates. The species identified by ONT matched the identifications made through conventional methods except one, showing 90% concordance [Table. 1]. With the exception of Patient 7, DNA from all other samples was identified with a depth > 1000x. Unlike other molecular methods, which have been reporting multiple species due to inherent sensitivity and specificity challenges, GS of the isolates identified a single fungal species with >99% read match per isolate, which indicated high specificity. Since the GS assay consisted of 16S and ARG primers in addition to ITS primers, the absence of a bacterial signature in the bioinformatics analysis suggests the high specificity of the assay. Conventionally, isolate number 5 [Table. 1] was identified as an *Alternaria* species; however, sequencing revealed it to be *Pseudopithomyces angolensis*, underscoring the limitation of phenotypic methods in distinguishing closely related fungal species. Also, Isolate number 3 did not sporulate and hence could not be identified based on its growth on SDA and PDA; only hyphae were observed in LPCB. However, sequencing revealed it to be *Lasiodiploidia parva.* Additionally, *Curvularia* required an extra 10 days to sporulate after initial growth, resulting in a total identification time of approximately 30 days, whereas ONT identified the isolate within 12–18 hours. Highlighting the limitations of conventional morphology-based identification and emphasizing the value of molecular sequencing.

Overall agreement between ONT and culture was 90% (N=9/10). The unweighted Cohen's kappa resulted in the kappa value of 0.87 (CI 95%: 0.64 - 1.00), which is associated with perfect agreement.

Discussion

This study conclusively demonstrates the reliability and accuracy of genome sequencing using the ONT method in the identification of fungal isolates. The results were available within 12-18 hours. Given the 90% concordance with conventional methods, the potential of this method to detect fungal species in clinical samples appears high. We are currently evaluating the corneal scrapings directly for the purpose. Among the fungal species obtained in this small sample size of 10 isolates, *Fusarium solani* and *Aspergillus flavus* emerged as predominant pathogens, along with some rarer fungi like *Pseudopithomyces spp*., *Podospora bulbillosa*, and *Curvularia spp*. Studies from southern states of India have also shown a high prevalence of *Fusarium* and *Aspergillus* [10-11].

All cases of fungal keratitis were started on antifungal treatment based on the finding of fungal filaments in direct microscopic examination of the corneal scrapings. However, culture confirms the causative agent, and the determination of the species of infecting fungus further helps in estimating the prognosis. In this pilot study the cultures were tested by GS after the required fungal growth, a period that is similar to the conventional method (7-14 days). Details are in [Table. 1]. We are currently evaluating the sensitivity and specificity of this method on clinical samples.

The size of the corneal infiltrate and presence of hypopyon are well-known indicators of infection severity. A large infiltrate was noted in patient 10 [8x6 mm], who also exhibited the most severe outcome—no perception of light (No PL) and a prephthisical eye at the last follow-up. In contrast, smaller infiltrates were observed in patients 6, 7, 8, and 9 that responded well to therapy.

The rapid identification of fungal species is critical in clinical settings, particularly for fungal infections where early and targeted antifungal therapy can improve patient outcomes[12]. In this study, ONT provided accurate identification within 12-18 hours, whereas the conventional method took several days due to the need for sporulation. The faster turnaround time with ONT can facilitate earlier initiation of targeted antifungal therapy, potentially improving clinical outcomes and reducing the risk of complications[13]. ONT, which uses a portable sequencer, can potentially improve standardized diagnostics and reduce overall health costs[8].

The concordance between conventional methods and ONT observed in this study suggests that ONT has considerable potential as a complementary tool for the diagnosis of fungal keratitis. However, further studies involving larger sample sizes, direct clinical specimens, and broader fungal diversity are required to validate its routine clinical application[14].

Limitations of the Study:

A major limitation of this study is the small sample size (n = 10) from a single tertiary eye care center, which limits the generalizability of the findings. In addition, ONT was performed on cultured fungal isolates rather than directly on clinical specimens, and an independent molecular reference standard was not available for comparison. Larger, prospective studies are needed to confirm the observations and evaluate the reproducibility and clinical utility of the proposed approach.

Conclusion

ONT provides a faster alternative to conventional fungal identification methods in the diagnosis of fungal keratitis. The reduced turnaround time offered by ONT can lead to quicker clinical decision-making and improved patient outcomes. Future studies should explore the cost-effectiveness of implementing ONT in routine diagnostics, particularly in high-burden settings where fungal infections are common.

Disclosure

This study is approved by the Shantilal Shanghvi Foundation Ethics Committee No. SSF/IRB/0013/2025.

Written informed consent is obtained from all the patients.

Credit authorship contribution statement:

Madhu Rai: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft. **Vaibhav:** conceptualization, investigation, and writing—original draft. **Sanchi Shah**: Statistical Analysis. **Anirvan:** Methodology, validation, and investigation. **Nidhi:** Investigation. **Amrutraj:** Data Curation. **Dr. Savitri:** Writing—Review & Editing.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Brown L, Leck AK, Gichangi M, Burton MJ, Denning DW. The global incidence and diagnosis of fungal keratitis. The Lancet Infectious Diseases. 2021; 21(3):e49-e57. Available from: https://doi.org/10.1016/s1473-3099(20)30448-5
2. Xie L, Zhong W, Shi W, Sun S. Spectrum of Fungal Keratitis in North China. Ophthalmology. 2006; 113(11):1943-1948. Available from: https://doi.org/10.1016/j.ophtha.2006.05.035
3. Ahmadikia K, Aghaei Gharehbolagh S, Fallah B, Naeimi Eshkaleti M, Malekifar P, Rahsepar S, *et al*. Distribution, Prevalence, and Causative Agents of Fungal Keratitis: A Systematic Review and Meta-Analysis (1990 to 2020). Frontiers in Cellular and Infection Microbiology. 2021; 11. Available from: https://doi.org/10.3389/fcimb.2021.698780
4. Vengayil S, Panda A, Satpathy G, Nayak N, Ghose S, Patanaik D, *et al*. Polymerase Chain Reaction-Guided

Diagnosis of Mycotic Keratitis: A Prospective Evaluation of Its Efficacy and Limitations. Investigative Opthalmology & Visual Science. 2009; 50(1):152-156. Available from: https://doi.org/10.1167/iovs.07-1283

5. Pounder JI, Simmon KE, Barton CA, Hohmann SL, Brandt ME, Petti CA. Discovering Potential Pathogens among Fungi Identified as Nonsporulating Molds. Journal of Clinical Microbiology. 2007; 45(2):568-571. Available from: https://doi.org/10.1128/jcm.01684-06
6. Goyal PA, Bankar NJ, Mishra VH, Borkar SK, Makade JG. Revolutionizing Medical Microbiology: How Molecular and Genomic Approaches Are Changing Diagnostic Techniques. Cureus. 2023; 15(10). Available from: https://doi.org/10.7759/cureus.47106
7. Liu HY, Hopping GC, Vaidyanathan U, Ronquillo YC, Hoopes PC, Moshirfar M. Polymerase chain reaction and its application in the diagnosis of infectious keratitis. Medical hypothesis discovery and innovation in ophthalmology. 2019; 8(3):152-155. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC6778471/
8. Kuruwa S, Zade A, Shah S, Moidu R, Lad S, Chande C, *et al*. An integrated method for targeted Oxford Nanopore sequencing and automated bioinformatics for the simultaneous detection of bacteria, fungi, and ARG. Journal of Applied Microbiology. 2024; 135(2). Available from: https://doi.org/10.1093/jambio/lxae037
9. Bogaerts B, Van den Bossche A, Verhaegen B, Delbrassinne L, Mattheus W, Nouws S, *et al*. Closing the gap: Oxford Nanopore Technologies R10 sequencing allows comparable results to Illumina sequencing for SNP-based outbreak investigation of bacterial pathogens. Journal of Clinical Microbiology. 2024; 62(5). Available from: https://doi.org/10.1128/jcm.01576-23
10. Manikandan P, Abdel-Hadi A, Randhir Babu Singh Y, Revathi R, Anita R, Banawas S, *et al*. Fungal Keratitis: Epidemiology, Rapid Detection, and Antifungal Susceptibilities of*Fusarium*and*Aspergillus*Isolates from Corneal Scrapings. BioMed Research International. 2019; 2019(1):1-9. Available from: https://doi.org/10.1155/2019/6395840
11. Lalitha P, Prajna NV, Manoharan G, Srinivasan M, Mascarenhas J, Das M, *et al*. Trends in bacterial and fungal keratitis in South India, 2002–2012. British Journal of Ophthalmology. 2015; 99(2):192-194. Available from: https://doi.org/10.1136/bjophthalmol-2014-305000
12. Barnes RA. Early diagnosis of fungal infection in immunocompromised patients. Journal of Antimicrobial Chemotherapy. 2008; 61(Supplement 1):i3-i6. Available from: https://doi.org/10.1093/jac/dkm424
13. Benítez-Páez A, Sanz Y. Multi-locus and long amplicon sequencing approach to study microbial diversity at species level using the MinION™ portable nanopore sequencer. GigaScience. 2017; 6(7). Available from: https://doi.org/10.1093/gigascience/gix043
14. Nafea AM, Wang Y, Wang D, Salama AM, Aziz MA, Xu S, *et al*. Application of next-generation sequencing to identify different pathogens. Frontiers in Microbiology. 2024; 14. Available from: https://doi.org/10.3389/fmicb.2023.1329330

How to cite this article: Rai M, Nagpal V, Shah S, Chatterjee A, Sehgal N, Zade A, Sharma S. A Comparative Analysis of Conventional Methods versus Oxford Nanopore Sequencing Technology in the Identification of Fungi in Ocular Specimen. Journal of Medical Sciences and Health 2026; 12(3):320-325

Date of submission: 25.04.2026
Date of review: 13.06.2026
Date of acceptance: 17.07.2026
Date of publication: 19.08.2026