Development and Clinical Evaluation of a CRISPR/Cas12a-Based Nucleic Acid Detection Platform for the Diagnosis of Keratomycoses

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Objective: The objective of this study was to develop a rapid and accurate clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a-based molecular diagnostic assay (Rapid Identification of Mycoses using CRISPR, RID-MyC assay) to detect fungal nucleic acids and to compare it with existing conventional mycologic methods for the diagnosis of fungal keratitis (FK).

Design: This study was structured as a development and validation study focusing on the creation and assessment of the RID-MyC assay as a novel diagnostic modality for FK.

Subjects: Participants comprised 142 individuals presenting with suspected microbial keratitis at 3 tertiary care institutions in South India.

Methods: The RID-MyC assay utilized recombinase polymerase amplification targeting the 18S ribosomal RNA gene for isothermal amplification, followed by a CRISPR/Cas12a reaction. This was benchmarked against microscopy, culture, and polymerase chain reaction for the diagnosis of FK.

Main Outcome Measures: The primary outcome measures focused on the analytical sensitivity and specificity of the RID-MyC assay in detecting fungal nucleic acids. Secondary outcomes measured the assay's diagnostic sensitivity and specificity for FK, including its concordance with conventional diagnostic methods.

Results: The RID-MyC assay exhibited a detection limit ranging from 13.3 to 16.6 genomic copies across 4 common fungal species. In patients with microbial keratitis, the RID-MyC assay showed substantial agreement with microscopy (kappa = 0.714) and fair agreement with culture (kappa = 0.399). The assay demonstrated a sensitivity of 93.27% (95% confidence interval [CI], 86.62%–97.25%) and a specificity of 89.47% (95% CI, 66.86%–98.70%) for FK diagnosis, with a median diagnostic time of 50 minutes (range, 35–124 minutes).

Conclusions: The RID-MyC assay, utilizing CRISPR-Cas12a technology, offers high diagnostic accuracy for FK. Its potential for point-of-care use could expedite and enhance the precision of fungal diagnostics, presenting a promising solution to current diagnostic challenges.

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Fungal diseases are estimated to be responsible for >1.6 million deaths annually, and >1 billion people suffer from fungal infections worldwide.[1] Despite the substantial morbidity and mortality associated with fungal infections, they remain an underestimated and neglected global public health problem.[2] Moreover, the disproportionate distribution of fungal diseases in tropical developing regions, coupled with the scarcity of accessible diagnostic facilities in these areas, exacerbates the situation's complexity.[3] To effectively combat fungal infections, it is crucial to integrate technical advancements with the increased accessibility of diagnostic tools, promoting prompt and accurate diagnoses and improving disease management and control.

Ocular fungal infections, particularly fungal keratitis (FK), the most common ocular fungal infection, underscores the urgency of this need.[4,5] Expedient initiation of treatment drastically improves clinical outcomes, with time to

diagnosis being one of the most critical risk factors influencing morbidity and mortality in ocular and systemic fungal infections.[6,7] However, conventional mycological diagnostic modalities require expertise and are often time-consuming.[8] Traditional fungal culture methods can take up to 2 weeks to identify pathogens, with yeasts typically detected within 1 week and molds taking longer, potentially delaying treatment.[9] Given the fact that fungal infections disproportionately afflict the rural population in resource-limited settings (RLS), there exists an unmet clinical need for the development of newer diagnostic modalities for rapid and accurate diagnosis of fungal infections.

The rapidity, superior sensitivity, and specificity of molecular methods for fungal DNA detection, such as polymerase chain reaction (PCR), have been reported both with ocular and systemic fungal infections.[10,11] Nucleic acid detection techniques are especially advantageous in FK, where the empirical use of antimicrobial therapy decreases the sensitivity of conventional mycological diagnostic techniques. However, PCR, despite its rapidity, often requires expensive equipment and specialized personnel, and while pooled testing can reduce costs, it may extend turnaround times, thus challenging its application in RLS.[12,13]

Microbial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (CRISPR-Cas) adaptive immune systems contain programmable endonucleases with distinctive enzymatic properties that can be leveraged for the detection of microbial nucleic acids.[14] Recent studies have highlighted the potential of these CRISPR-based nucleic acid detection methods as rapid and highly sensitive diagnostic modalities to detect pathogenic bacteria and viruses.[15] However, the utility of these CRISPR-based diagnostic methods to diagnose fungal infections and their role as a potential diagnostic platform for ophthalmic infections remains to be elucidated.

Here, we describe the development of a rapid, ultrasensitive easy-to-implement CRISPR−Cas12a-based tool, Rapid Identification of Mycoses using CRISPR (RID-MyC), for the detection of fungal nucleic acids. We have also validated our method using contrived reference and clinical samples from patients with suspected microbial keratitis.

Methods

Study Design and Participants

The diagnostic accuracy of the RID-MyC assay for the detection of fungal nucleic acids was evaluated prospectively in patients with clinically suspected microbial keratitis. Informed consent was obtained from all participants in accordance with ethical guidelines and the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of Aravind Eye Hospital. The study encompassed 142 consecutive patients (Fig 1) presenting to the cornea outpatient departments of 3 tertiary eye care facilities in South India with clinical suspicion of microbial keratitis and positive findings on smear examination (indicated by the presence of fungal filaments and/or bacterial entities [such as cocci or bacilli] on potassium hydroxide mount and/or Gram stain). A total of 123 samples met the predefined quality standards and data completeness criteria and were included in the final analyses. These samples represented cases of smear-positive microbial keratitis with adequate sample volume, proper sample transport, complete patient data, and successful PCR performance. All patients underwent a standard scraping procedure for corneal debridement as previously described.[16,17] Briefly, 2 scrapings were smeared directly on separate glass slides for Gram staining and 10% potassium hydroxide wet mount, and 3 further scrapings were taken and directly inoculated onto sheep-blood agar, chocolate agar, and potato dextrose agar or Sabouraud's agar for bacterial and fungal cultures. Fungal smears were considered positive when fungal elements were seen under low-power magnification and reduced light. Fungal cultures were considered positive with growth on any 2 media or moderate to heavy growth on 1 medium. The operators of the RID-MyC assay were blinded to the results of conventional microbiological tests (including smear examination and culture) and panfungal PCR results to ensure unbiased interpretation of the assay outcomes.

PCR

Polymerase chain reaction was performed as previously described and the amplified product was visualized on an ultraviolet transilluminator using 2% agarose gel electrophoresis incorporating 0.5 mg/ml ethidium bromide. Previously described panfungal primers (Forward primer sequence, 5′- GTGAAATTGTTGAAAGGGAA-3′; and reverse primer sequence, 5′- GACTCCTTGGTCCGTGTT-3′) used in our study were specific for the 28S ribosomal RNA gene.[18]

RID-MyC Assay

Detailed protocols for the optimization of recombinase polymerase amplification (RPA) and CRISPR reactions are comprehensively described in the Supplementary Methods and Results. Briefly, for the RID-MyC assay and PCR, the affected cornea was swabbed with sterile polyester tipped applicator (Puritan Medical Products) and transferred to a 1.5 ml Eppendorf tube. DNA was isolated from both corneal scrapings using the QIAmp DNA Mini kit (Qiagen) as per the manufacturer's instructions. The RPA reaction was performed as described with 10 μl of the eluted DNA at 37°C for 30 minutes. LbCas12a trans-cleavage assays were performed as described with 13 μl of the RPA amplicon and incubated at 37°C for 30 minutes or the denoted time in figures. Both real-time and endpoint fluorescence detection was performed for all clinical samples. For the analysis of clinical samples, a RID-MyC assay result was considered positive if it was equal or greater than a cutoff threshold equal to the mean signal of the negative control samples plus 3 times its standard deviation (Table S3).

Statistical Analysis

Background-subtracted fluorescence was calculated by subtraction of the fluorescence of no-template (water only as "template" input into the RID-MyC reaction) control wells on the plate from target fluorescence values evaluated in the assay run at the same time points in the assay. The analytical sensitivity of the RID-MyC test was estimated using linear regression with a 95% confidence interval (CI). The performance indices, including sensitivity, specificity, positive predictive value, and negative predictive value, for the RID-MyC assay was calculated. Here the sensitivity was defined as the number of eyes with FK detected by the RID-MyC divided by the number of eyes with either potassium hydroxide smear- or culture-positive for fungus. Specificity was defined as the number of eyes with non-FK detected by the RID-MyC divided by the number of eyes with both smear and culture-negative for fungus. The difference between the performance indices between the test groups was performed using the McNemar test.[19] Unpaired t-
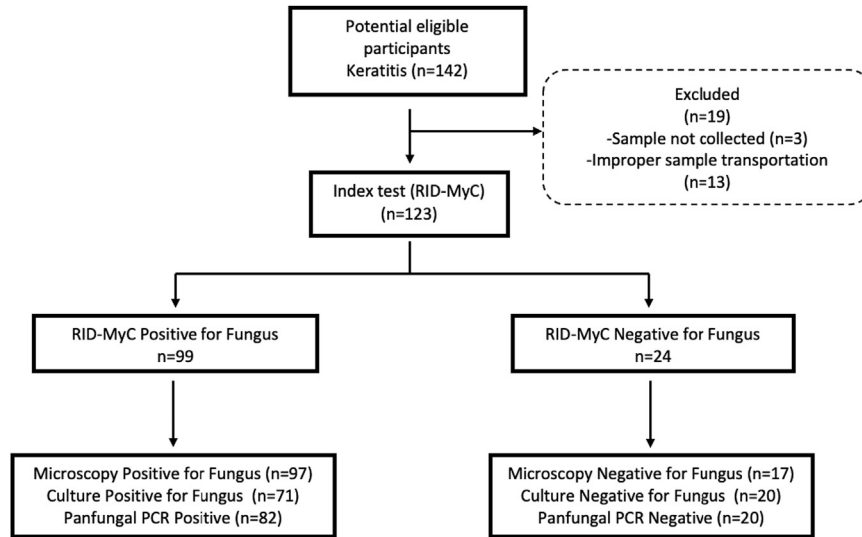
Figure 1. Participants and diagnostic outcomes in the keratitis cohorts. In the keratitis cohort, 142 patients with suspected microbial keratitis were initially assessed, of which 123 met the predefined quality standards and data completeness criteria. All underwent a standard scraping procedure for corneal debridement. The samples were then subjected to Gram staining, potassium hydroxide wet mount, and bacterial/fungal culture. Further, the samples were evaluated using polymerase chain reaction (PCR) analysis and the Rapid Identification of Mycoses using CRISPR Assay (RID-MyC).

tests were conducted to compare differences in time to diagnosis. Cohen's kappa coefficient (κ) was calculated to assess concordance and inter-rater agreement for the study. Statistical analyses were performed using Prism 8 (GraphPad Software, version 8.0.1) and R software. A *P*-value of <0.05 was considered statistically significant.

Results

Using RPA primers and CRISPRRNA sequences targeting the 18S ribosomal RNA region (Fig 2A), the RID-MyC assay demonstrated positive results for all tested control fungal DNA and negative results for bacterial and human genomic DNA (Fig 2B, Figs S6 and S7). The RID-MyC assay exhibited a linear quantification range for all tested fungal species demonstrating a limit of detection of 13.8 genomic copies for *Aspergillus flavus*, 16.6 genomic copies for *Fusarium solani*, 13.9 genomic copies for *Curvularia lunata*, and 13.3 genomic copies for *Candida albicans* (Fig 2C and Figs S8−S11).

A total of 123 samples (Fig 1 and Table S4) from patients with suspected microbial keratitis were included in the analysis. Microscopy revealed fungal elements in 104 samples (84.5%), while culture confirmed fungal growth in 75 samples (61%). Polymerase chain reaction analysis identified fungal nucleic acids in 86 samples (70%), and the RID-MyC assay (Fig 3A) demonstrated positive results in 99 samples (80%). In patients with positive fungal culture results, the most common fungal species were Fusarium (41%), Aspergillus (11%), and Curvularia species (3%), with other species contributing to the remainder. For 6% of the samples, the fungal species remained undetermined. Concordance analysis (Fig 3B) showed substantial agreement between the RID-MyC assay and microscopy (kappa = 0.714), fair agreement between the RID-MyC assay and culture (kappa = 0.399), and

moderate agreement between culture and microscopy (kappa = 0.444). The RID-MyC assay corresponded with microscopy for 97 positive and 17 negative samples, and with culture for 71 positive and 20 negative samples. Within the subset of samples yielding positive culture results but negative RID-MyC assay readings, 2 were determined to be Fusarium species, with the third sample harboring a dematiaceous fungus that was not characterized.

The median time for the RID-MyC CRISPR reaction to signal a positive result was 62.50 minutes (range 40−85 minutes) for samples negative by both microscopy and culture, significantly higher ($P < 0.05$) than the 25.5 minutes (range 8−81 minutes) for samples positive by microscopy alone and the 19 minutes (range 5−94 minutes) for samples positive by both microscopy and culture (Fig 3C). Being a nucleic acid detection modality, the diagnostic performance of the RID-MyC assay was compared with panfungal PCR. A sample was considered positive for FK if it demonstrated positive results by culture or microscopy. The RID-MyC assay displayed a sensitivity of 93.27% (95% CI, 86.62%−97.25%), specificity of 89.47% (95% CI, 66.86%−98.70%), positive predictive value of 97.98% (95% CI, 92.89%−99.75%), and negative predictive value of 70.83% (95% CI, 53.88%−83.47%). For PCR, the sensitivity was calculated at 79.81% (95% CI, 70.81%−87.04%), specificity at 84.21% (95% CI, 60.42% - 96.62%), positive predictive value at 96.51% (95% CI, 90.14%−99.27%), and negative predictive value at 43.24% (95% CI, 33.16%−53.91%) (Table 1). In a sub-analysis of 75 clinical swab samples (Fig S13), the point-of-care (POC) utility of the RID-MyC assay was evaluated via visual detection. Test outcomes were independently interpreted by 3 blinded observers, with an observed interobserver agreement of 98.6%. Additionally, excellent agreement (kappa = 1.00) was observed between real-time analysis outcomes and visually detected results.
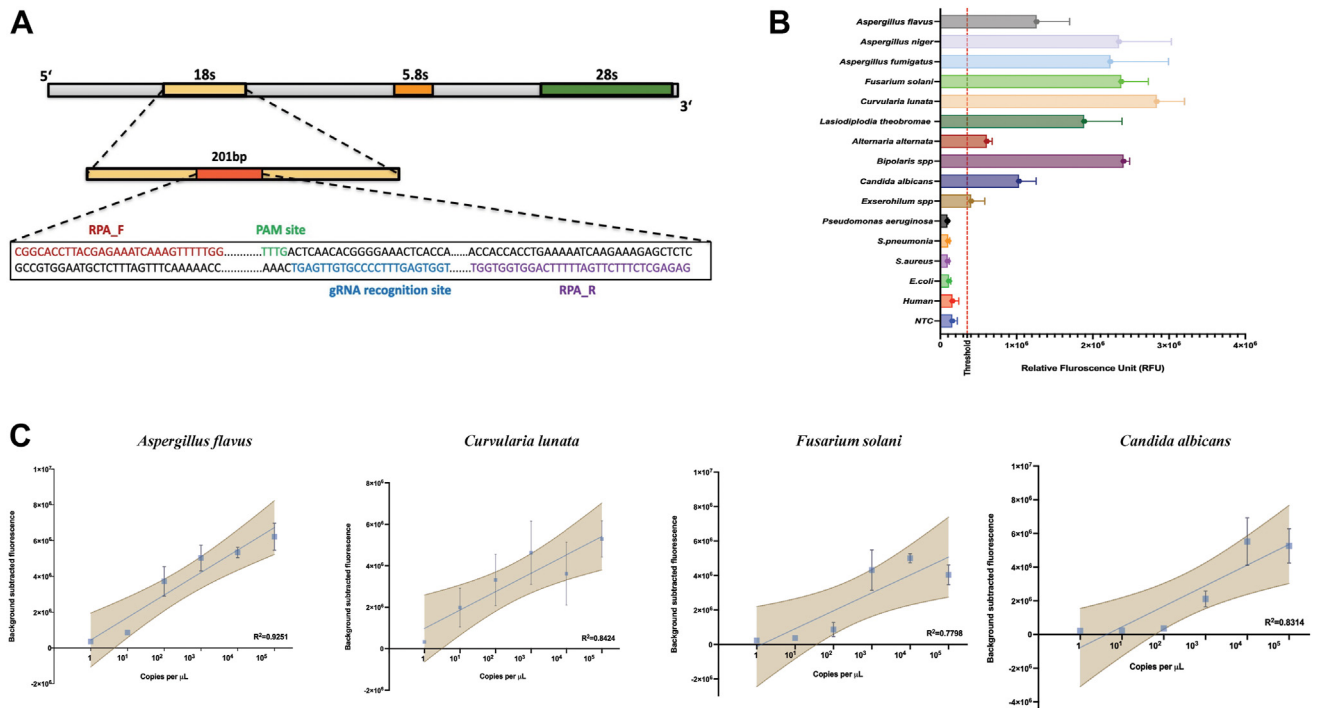
Figure 2. Analytical validation of the Rapid Identification of Mycoses using CRISPR (RID-MyC) assay. **A,** Schematic representation of the ribosomal RNA (rRNA) cluster of fungi, highlighting the location of the recombinase polymerase amplification (RPA) primers, protospacer adjacent motif (PAM), and the clustered regularly interspaced short palindromic repeats (CRISPR) guide RNA (gRNA) target sequence in the 18S rRNA region. The RPA primers (RPA_F and RPA_R) are designed to amplify the target region for subsequent detection. The PAM serves as the recognition site for the CRISPR/Cas system, allowing the activation of the gRNA-guided Cas protein for targeted cleavage. This schematic provides an overview of the molecular components and their respective positions within the rRNA cluster, highlighting the key elements involved in the RID-MyC assay. **B,** Specificity of the RID-MyC assay demonstrated through endpoint fluorescence analysis. Triplicate samples of bacterial, human, nontemplate control (NTC), and fungal specimens were evaluated. The cutoff fluorescence was determined by calculating the average NTC intensity plus 3 times the standard deviation (SD), depicted by the red horizontal dashed line. Error bars indicate the SD of the mean values obtained from triplicate measurements. **C,** RID-MyC standard curve, in which shading denotes the 95% confidence interval of the fitted line to detect the analytical sensitivity of the RID-MyC assay *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani* and *Candida albicans*.

In our cohort, 37.19% of patients had received prior antifungal therapy. The concordance between RID-MyC and culture-based methods in this subgroup was significantly lower (64.44% agreement, Cohen's k 0.20) compared with those who had not received therapy (79.49% agreement, Cohen's k 0.52), emphasizing the potential influence of prior treatment on culture test performance. Nevertheless, the concordance for RID-MyC versus PCR maintained a moderate agreement for both groups—with prior therapy (Cohen's k 0.46) and without prior therapy (Cohen's k 0.59).

To validate the RID-MyC assay's reliability in practical settings, environmental samples from the clinic, lab, and DNA extraction room were examined. Real-time fluorescence analysis, end-point fluorescence analysis, and visual RID-MyC detection (Fig S12) did not detect the presence of fungal nucleic acids in any of the examined environmental samples.

Discussion

The growing challenge of fungal infections, particularly in the context of ocular diseases like FK, underscores the critical need for timely and accurate diagnostic methods.Our study aimed to address this unmet need by developing and validating a CRISPR−Cas12a-based diagnostic platform, RID-MyC. The diagnostic effectiveness of RID-MyC is highlighted in this study through its application for the diagnosis of FK.

Over 90% of FK cases occur in developing regions, predominantly affecting young adults from rural communities.[20,21] Delayed anti-fungal therapy, largely due to long travel distances to hospitals and high access costs, has been identified as a key factor contributing to treatment failure and potential irreversible blindness in FK patients.[22,23] Corneal smears and culture are considered to be the current gold standard for the diagnosis of FK. However, culture lacks sensitivity, is time consuming, and requires ≥48−72 hours to establish a diagnosis.[24] Microscopy is a rapid, inexpensive, sensitive and specific diagnostic test but still requires clinical expertise and equipment for obtaining corneal scrapings and considerable mycological skill and knowledge for prompt identification of fungal hyphae and to rule out artefacts and contaminants. Given the above facts, the need for tertiary care facilities to diagnose FK remains the bottleneck which precludes the management of FK at the primary health care level.
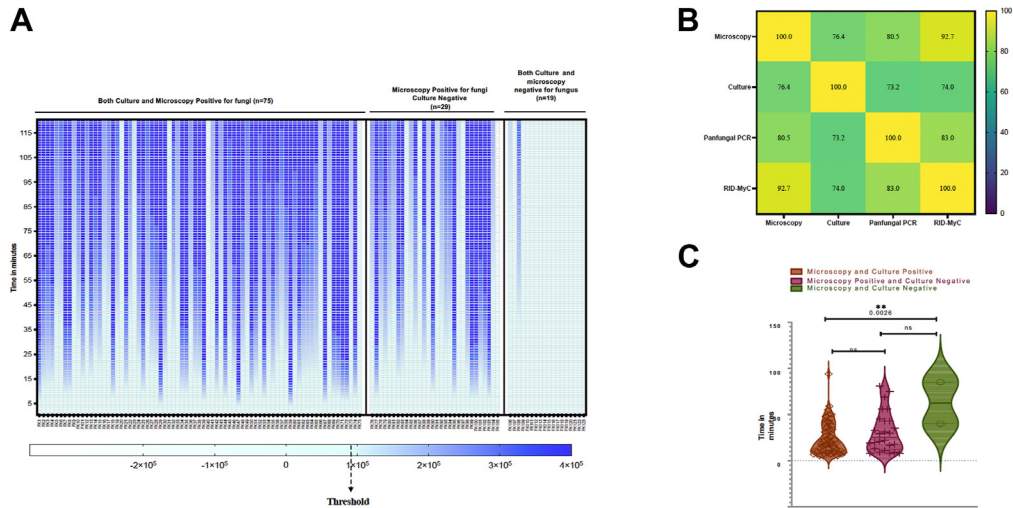
Figure 3. Performance of the Rapid Identification of Mycoses using CRISPR (RID-MyC) assay. **A,** The heat map displays the background subtracted fluorescence kinetics over time (y-axis) for different sample groups. The first group (FK1 to FK75) represents samples that were positive for fungal keratitis based on both culture and microscopy. The second group (FK76 to FK104) consists of samples that were microscopy positive for fungus but culture negative. The third group (FK105 to FK113) includes samples that were negative for fungus by both culture and microscopy. The gradient scale on the heat map provides a visual representation of the transition from negative to positive results. **B,** The heatmap displays the percentage concordance between different diagnostic methods: microscopy, culture, panfungal polymerase chain reaction (PCR), and RID-MyC. Each cell represents the percentage agreement for both positive and negative outcomes between the methods corresponding to its row and column. Colors range from light green (lower agreement) to dark green (higher agreement), with yellow indicating intermediate levels of agreement. Diagonal cells, shaded in yellow, represent 100% agreement, as they compare each method to itself. **C,** Presents a comparison of the time to positive diagnosis using the RID-MyC assay among different groups: microscopy and culture-positive, microscopy-only positive, and both negative. The statistical analysis was performed using a unpaired t-test, with nonsignificant results denoted as "ns" and statistically significant results ($P < 0.01$) indicated by "**".

Nucleic acid-based diagnostic strategies have evolved into key methods for diagnosing of invasive fungal infections.[25] The clinical utility of nucleic acid-based diagnostics relying on PCR or on sequencing have been widely reported for the diagnosis of FK.[26] Though PCR is still considered to be the gold standard technique for nucleic acid detection, the high cost and requirement of sophisticated equipment and trained personnel precludes its application in RLS where the incidence of ocular and systemic fungal infections is disproportionately high. Isothermal amplification strategies have been able to circumvent the need for thermal cyclers.[27] However, nonspecific amplification decreases their specificity, impeding their utilization in real-world settings.[28,29] The RID-MyC assay combines the cost effectiveness of isothermal amplification with the sub-attomolar sensitivity of CRISPR-Cas12a systems to create a field-applicable diagnostic for FK. Some key advantages of the RID-MyC assay over PCR include the rapid turnaround time (45−60 minutes for RID-MyC vs. 4 hours for PCR) and the integration with accessible and easy-to-use fluorescence-based reporting formats obviating the requirement for complex laboratory infrastructure. Building on these operational benefits, our preliminary assessments suggest that the cost of the RID-MyC assay is approximately $10 per test at research scale, comparable to other CRISPR-based assays and notably lower than typical PCR and culture methods. This cost advantage, coupled with the absence of a requirement for expensive, large-scale equipment, positions

the RID-MyC as a potentially more accessible and economically feasible option for various settings. Our advancements with the development of the RID-MyC assay could fill significant gaps in the diagnosis of FK by establishing a POC test which could enable the management of FK at the primary health care level.

Limitations of this assay include those intrinsic to all nucleic acid detection platforms, including the possibility of detecting nonviable fungi. Though simple visualization of test results greatly improves the ease of use of the RID-MyC assay, sample preparation and DNA extraction is still required, which increases the complexity of the procedure and potentially limiting its use as a POC test. However, other CRISPR-based diagnostic systems have optimized nucleic acid extraction-free lyophilized one-pot reactions for the diagnosis of infectious diseases, which can be utilized to improve the field deployability of the RID-MyC assay.[30] The current version of the RID-MyC assay does not provide species differentiation. While the use of Cas12 effectors does allow for future expansion to perform multiplexed and sensitive assays for fungal species differentiation, the lack of this capability remains a limitation at this stage.[31] Additionally, the RID-MyC assay targets the 18s ribosomal RNA region of the fungal genome, which has been previously reported as an effective target for species-level differentiation of medically important fungi.[10] One potential limitation to bear in mind is that the patient population in our study predominantly suffered from filamentous FK, with a relatively low proportion of bacterial infections and

Table 1. Performance of PCR and RID-MyC Assay for the Diagnosis of Fungal Keratitis

Results (n = 123)	Reference Standard*		Performance of Assay				
	Positive	Negative	% Sensitivity	% Specificity	% PPV	% NPV	P-value
PCR			79.81%	84.21%	96.51%	43.24%	0.009[†]
Positive	83	3	(70.81%−87.04%)	(60.42%−96.62%)	(90.14%−99.27%)	(33.16%−53.91%)	
Negative	21	16					
RID-MyC			93.27%	89.47%	97.98%	70.83%	
Positive	97	2	(86.62%−97.25%)	(66.86%−98.70%)	(92.89%−99.75%)	(53.88%−83.47%)	
Negative	7	17					

CRISPR = clustered regularly interspaced short palindromic repeats; NPV = negative predictive value; PCR = polymerase chain reaction; PPV = positive predictive value; RID-MyC = Rapid Identification of Mycoses using CRISPR.
*Reference standard - includes results of both microscopy and culture; considered positive if either was positive. Values within brackets in performance parameters indicate 95% confidence interval.
[†]Indicates P-value between PCR and RID-MyC calculated using the McNemar test.

Candida keratitis. This, however, reflects the disease distribution reported in other large-scale epidemiological studies from this geographical region.[32] Future studies conducted across a variety of geographic areas and disease distributions will be instrumental in confirming the global applicability of our findings.

Besides addressing the critical need for improved FK diagnosis, our study also features notable strengths. We successfully pioneered the RID-MyC assay, a CRISPR-based method, providing a reliable tool for the sensitive and precise detection of fungal nucleic acids in FK patients. Our robust testing framework, which incorporates environmental samples and healthy controls, offers a comprehensive evaluation of the assay's specificity. Collectively, these merits underscore the transformative potential of RID-MyC, positioning it as a powerful, versatile tool for FK diagnosis that could revolutionize fungal infection management and patient care.

In conclusion, the RID-MyC assay is a promising addition to the existing diagnostic armamentarium for the diagnosis of FK and will potentially enable POC testing of FK in RLS. By combining the cost-effectiveness of isothermal amplification with the high sensitivity of CRISPR-Cas12a systems, this tool overcomes key limitations of existing diagnostic methods, enabling prompt and accurate detection of fungal infections. Considering that expedient and accurate diagnosis is a huge unmet need for other localized and systemic fungal infections, we believe that the results of this study would catalyze the application and development of CRISPR-based assays for the diagnosis of other invasive fungal infections.

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Footnotes and Disclosures

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