Novel Molecular Barcoding for Rapid Pathogen Detection in Infectious Keratitis

This proof-of-concept study describes the application of a novel molecular barcoding approach for rapid and comprehensive pathogen detection in infectious keratitis.

Infectious corneal ulcers are a major cause of global blindness. Standard management approaches typically involve the collection of corneal cultures and initiation of broad-spectrum antimicrobials. However, conventional microbiologic techniques—based on our ability to either directly visualize or grow pathogens in culture—are limited by poor sensitivity (<50%) and the time required to produce actionable results. Any delay in the diagnosis and treatment of infectious corneal ulcers represents a departure from the clinical maxim, “time equals vision,” limiting our ability to tailor treatments and to apply adjunct therapies, including corticosteroids. This proof-of-concept uses novel molecular barcoding on the NanoString nCounter platform for highly multiplexed nucleic acid detection, adapted to provide identification of corneal pathogens within 12 hours of specimen collection.

This study was approved by the Mass General Brigham Institutional Review Board, conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all study participants. We recruited adult patients presenting to Massachusetts Eye and Ear with infectious keratitis and who were determined, according to our emergency department treatment algorithm—the Assess, Culture, and Treat (1-2-3-ACT) Rule—to have an immediately sight-threatening lesion requiring corneal cultures. The 1-2-3-ACT Rule requires the collection of corneal cultures for any lesion meeting 1 or more of the following criteria: (1) ≥1 anterior chamber cells; (2) an infiltrate ≥2 mm in size, with or without 2 or more satellite lesions; and (3) if the edge of the infiltrate lies within 3 mm of the corneal center, that is, if the lesion involves the visual axis. After routine swab collection for microscropy and culture, an additional sample of the infected lesion was taken using a flocked nylon swab (COPAN FLOQSwab), placed into 500 µl of 1X DNA/RNA shield (Zymo), and frozen at −80°C. Some 200 µl of each sample was chemically homogenized using ZR BashingBead Lysis tubes (Zymo) in the FastPrep-24 instrument, using 2 cycles of 45 seconds at 6.5 m/s. Nucleic acids were purified using the Quick-DNA/RNA MicroPrep Plus (Zymo), and DNA quantity and purity were determined using the NanoDrop (ThermoFisher). DNA quality was assessed by performing dual internal control real-time polymerase chain reaction (PCR) assays targeting human β-globin and variable regions 3 and 4 of bacterial 16S ribosomal RNA.

Although NanoString assays can accommodate up to 800 target probes, this pilot test used an abridged panel for ocular pathogens, covering 48 targets 150 to 300 base pairs in length (Table 1). For each target, two 50 mer oligonucleotide probes were designed and synthesized (IDT Inc.), fusing proprietary NanoString barcode sequences to pathogen DNA sequences with optimal thermodynamic properties for hybridization and minimal cross-reactivity. Each 50 mer pair consists of a biotin-bound capture probe and a reporter probe bound to a fluorescent barcode unique to each pathogen sequence.

Because of the paucity of nucleic acid extracted from corneal swabs, multiplex targeted enrichment was performed in triplicate, with the protocol equilibrated to amplify DNA sufficient for detection via hybridization, while minimizing background noise (data not shown). Each 10 µl assay consisted of 5 µl of TaqMan Fast Advanced Master Mix, 2.5 µl of purified patient-microbial DNA, 1 µl of primer mixture at 0.5 nM per oligonucleotide, and 1.5 µl of nuclease-free water. Polymerase chain reaction amplification conditions recommended by NanoString were followed.

After denaturing enriched samples at 95°C for 5 minutes, 30 µl hybridization assays were performed, consisting of 10 µl enriched DNA sample, 10 µl hybridization buffer (NanoString), 5 µl TagSet (Nanostring), 100 pM of capture probe, 20 pM of reporter probe, and nuclease-free water to complete the final volume. Hybridization was conducted at 67°C for 2 hours, allowing each 50 mer to bind to target sequences within each sample. Enriched hybridized samples were loaded onto a NanoString nCounter SPRINT Profiler cartridge in triplicate, including negative controls, and run for 6 hours. Samples undergo purification to remove excess probe, followed by immobilization of probe-sequence complexes onto the cartridge via the biotin moiety on capture probes. Barcoded complexes are digitally enumerated to reveal a relative hybrid count for each target, normalized to internal controls. We set a threshold of ≥100 mean sequence-specific probes to define positive results, only reporting results for the highest taxonomic level of identification. The entire workflow, from specimen collection to data analysis, is presented in Figure 1.

Overall, 17 culture-positive specimens that had sufficient biomass, as indicated by results on β-globin and 16S ribosomal DNA (rDNA) real-time PCR, were included. All cases were bacterial in etiology, with 6 Gram-positive, 9 Gram-negative, and 2 polymicrobial cases (Table 2). Most specimens were obtained from patients with severe infections, with 14 of 17 (82.4%) having lesions satisfying ≥2 criteria when assessed using 1-2-3-ACT and 14 of 17 (82.4%) presenting with a best-corrected visual acuity of ≤20/200. Mean β-globin and 16S PCR cycling thresholds were 28.1 and 23.6, respectively, indicating sufficient biomass for each sample and adequate quality of DNA, and absence of PCR inhibitors. Mean NanoString probe counts normalized for hybridization efficiency ranged from 129.54 (standard deviation ±30.40) to 91 297.27 (±8598.04), transformed to a log10 count of 2.11 to 4.96 (Table 2). Captured sequences included genus-level targets, such as staphylococci (28S rDNA) and streptococci (16S rDNA), and species-specific targets including Staphylococcus aureus (spa), Streptococcus agalactiae (cfb), Streptococcus pneumoniae (lytA), Pseudomonas aeruginosa (proA), Serratia marcescens (gyrB), and Haemophilus influenzae (psta). There was complete agreement between culture and our multiplex panel for monomicrobial cases and partial agreement for 2 polymicrobial infections included. Compared with a median time to growth of 3 days (range, 1–5 days), all samples underwent NanoString analysis within 12 hours.
Comprehensive targeted panels strike a fine balance between other molecular diagnostic methods, ranging from singleplex PCR to metagenomic sequencing, in terms of scalability, cost, computational demand, and time to yield actionable results (Table 3). However, the application of molecular diagnostic techniques for corneal infections remains beset primarily by insufficient patient sample. Molecular approaches have found greater success in identifying pathogens that cause uveitis and endophthalmitis, for which intraocular fluids typically provide greater volumes of template nucleic acid. Although our pilot results suggest that the NanoString platform holds promise for infectious ocular diseases, including corneal infections where specimen recovery is expected to be ultra-low, extensive validation studies will be required to determine its performance characteristics within clinical settings to reconcile culture-positive samples that may not be detected due to off-target enrichment primers or hybridization probes, and inadequate swab yield. Provided these challenges can be met, novel molecular barcoding may add to our

Table 1. Species Covered on a Custom-Designed and Readily Modifiable Panel for Diagnosis of Ocular Infections

| Bacteria                        | Fungi                          | Viruses                                      |
|---------------------------------|--------------------------------|----------------------------------------------|
| Acinetobacter calcoaceticus-haemani (16S-23S intergenic spacer region) | Pseudomonas aeruginosa (proA) | Aspergillus flavus (ß-tubulin)               |
| Acinetobacter baumannii (blaOXA-134) | Serratia marcescens (gyrB) | Aspergillus fumigatus (calmodulin)          |
| Bacillus cereus group (rpoB)    | Staphylococcus aureus (spa)    | Aspergillus niger (calmodulin)              |
| Bacillus subtilis group (28S rDNA) | Staphylococcus capitis (rnc) | Candida albicans (28S rDNA)                 |
| Borrelia burgdorferi ( flaB)   | Staphylococcus epidermidis (rnc) | Candida dubliniensis (ITS1-5.8S-ITS2)     |
| Enterobacter aerogenes (gyrB)  | Staphylococcus lugdunensis (rnc) | Candida glabrata (28S rDNA)               |
| Enterococcus faecalis ( ddl)   | Staphylococcus spp. (28S rDNA) | Candida parapsilosis (28S rDNA)            |
| Enterococcus faecium ( ddl)    | Streptococcus agalactiae (cβf) | Candida tropicalis (28S rDNA)              |
| Escherichia coli ( murC)       | Streptococcus anginosus group (16S rDNA) | Fusarium spp. (28S rDNA)                |
| Haemophilus influenzae (pstA)  | Streptococcus mitis group (16S rDNA) | Virulence Markers                         |
| Klebsiella pneumoniae (clpS)   | Streptococcus pneumoniae (lyrA) | Staphylococcus epidermidis (icaAD)         |
| Morganella morganii (gyrB)     | Streptococcus pyogenes (rstC)  | Staphylococcus spp. (mecA)                 |
| Mycobacterium tuberculosis (MPB64) | Troponema pallidum (typ47) | Tropheryma whippelii (repeat sequence) |
| Propionibacterium acnes (lipase) | Protein A mirabilis (ackA) |                                          |

Virulence Markers

| Bacteria                        | Virulence Markers |
|---------------------------------|--------------------|
| Haemophilus influenzae (pstA)  | Troponema pallidum (typ47) |
| Klebsiella pneumoniae (clpS)   | Tropheryma whippelii (repeat sequence) |
| Morganella morganii (gyrB)     | Tropheryma whippelii (repeat sequence) |
| Mycobacterium tuberculosis (MPB64) | Tropheryma whippelii (repeat sequence) |

Overall, these findings suggest that comprehensive targeted panels offer a promising approach for the diagnosis of infectious ocular diseases, particularly where culture methods are insufficient. Further research is needed to validate these findings in larger, clinical settings.

Figure 1. Diagnostic NanoString workflow for patients presenting with infectious corneal ulceration. Figure created using BioRender.com under a standard academic license.
| Participant and Eye Affected | Presenting BCVA | ≥1+ AC Cells | ≥2 mm Infiltrate | Edge ≤ 3 mm of Corneal Center | Vision-threatening Event | Gram Stain | Solid Agar Growth | Days to Growth | Mean β-Globin CT (SD) | Mean 16S PCR CT (SD) | NanoString Target | Mean Probe Count (SD) | Mean Log10 Count |
|-----------------------------|----------------|--------------|-----------------|-----------------------------|------------------------|------------|------------------|--------------|---------------------|---------------------|------------------|---------------------|-------------------|
| **Gram-Positive**           |                |              |                 |                             |                        |            |                  |              |                     |                     |                 |                     |                   |
| 1 (OS)                      | 20/60          | No           | No              | Yes                         | No                     | Positive    | Streptococcus pneumoniae | 1            | 27.60 (1.18)       | 21.55 (0.25)       | S. pneumoniae lytA | 29075.14 (132.01) | 4.46             |
| 2 (OS)                      | LP             | Yes          | No              | No                          | No                     | Positive    | S. pneumoniae           | 1            | 28.60 (0.08)       | 23.82 (0.93)       | S. pneumoniae lytA | 11282.16 (577.46) | 4.05             |
| 3 (OS)                      | CF             | Yes          | Yes             | Yes                         | Yes                    | Negative    | S. pneumoniae           | 2            | 30.98 (0.36)       | 26.15 (0.15)       | S. pneumoniae lytA | 6729.42 (174.05)  | 3.83             |
| 4 (OD)                      | 20/200         | Yes          | No              | No                          | No                     | Negative    | MSSA                 | 2            | 26.49 (1.43)       | 26.57 (0.26)       | S. aureus spp     | 913.32 (157.52)   | 2.96             |
| 5 (OS)                      | HM             | Yes          | Yes             | Yes                         | LTFU                   | Negative    | CoNS                 | 3            | 30.35 (0.80)       | 24.81 (0.41)       | Staphylococcus spp. | 141.71 (16.58)    | 2.15             |
| 6 (OS)                      | CF             | No           | Yes             | Yes                         | LTFU                   | Positive    | S. agalactiae         | 3            | 30.93 (4.61)       | 26.49 (0.99)       | S. agalactiae     | 129.54 (30.40)    | 2.11             |
| **Gram-Negative**           |                |              |                 |                             |                        |            |                  |              |                     |                     |                 |                     |                   |
| 7 (OS)                      | HM             | Yes          | Yes             | Yes                         | No                     | Negative    | S. marcescens         | 2            | 26.66 (0.13)       | 21.15 (0.21)       | S. marcescens     | 91297.27 (8598.04) | 4.96             |
| 8 (OS)                      | LP             | No view      | Yes             | Yes                         | Yes                    | Negative    | Pseudomonas aeruginosa | 3            | 19.95 (0.15)       | 18.26 (0.09)       | P. aeruginosa proA | 4227.05 (493.19)  | 3.63             |
| 9 (OS)                      | LP             | Yes          | Yes             | Yes                         | No                     | Negative    | P. aeruginosa         | 4            | 22.62 (0.21)       | 21.18 (1.2)        | P. aeruginosa proA | 4220.19 (35.82)   | 3.63             |
| 10 (OD)                     | CF             | No view      | Yes             | Yes                         | No                     | Positive    | P. aeruginosa         | 3            | 31.03 (1.81)       | 24.66 (0.15)       | P. aeruginosa proA | 1585.69 (232.78)  | 3.20             |
| 11 (OD)                     | LP             | No           | Yes             | No                          | Yes                    | Negative    | Haemophilus influenzae  | 5            | 20.29 (0.49)       | 21.43 (0.37)       | H. influenzae proA | 675.41 (182.48)   | 2.83             |
| 12 (OD)                     | 20/50          | Yes          | Yes             | Yes                         | LTFU                   | Negative    | P. aeruginosa         | 2            | 27.13 (0.99)       | 26.40 (0.92)       | P. aeruginosa proA | 587.80 (69.73)    | 2.77             |
| 13 (OD)                     | HM             | Yes          | Yes             | Yes                         | No                     | Negative    | P. aeruginosa         | 3            | 27.48 (0.32)       | 23.72 (0.15)       | P. aeruginosa proA | 509.30 (32.85)    | 2.71             |
| 14 (OS)                     | HM             | Yes          | Yes             | Yes                         | No                     | Negative    | P. aeruginosa         | 2            | 26.22 (0.51)       | 27.2 (1.02)        | P. aeruginosa proA | 282.79 (56.03)    | 2.45             |
| 15 (OD)                     | 20/60          | Yes          | Yes             | No                          | No                     | Positive    | P. aeruginosa         | 3            | 31.09 (0.29)       | 23.8 (0.10)        | P. aeruginosa proA | 206.71 (42.67)    | 2.32             |
| **Polymicrobial**           |                |              |                 |                             |                        |            |                  |              |                     |                     |                 |                     |                   |
| 16 (OD)                     | HM             | Yes          | Yes             | Yes                         | No                     | Positive    | H. influenzae and MSSA | 3            | 38.82 (1.99)       | 20.56 (0.05)       | H. influenzae proA | 18019.23 (858.48) | 4.26             |
| 17 (OS)                     | LP             | No view      | Yes             | Yes                         | Positive               | S. pneumoniae and Serratia marcescens | 3 | 30.84 (0.56) | 23.0 (1.02) | S. mitis group | 8260.12 (810.23) | 3.92 |

AC = anterior chamber; BCVA = best-corrected visual acuity; CF = counting fingers; CoNS = coagulase negative staphylococci; CT = cycling threshold; HM = hand motion; LP = light perception; LTFU = lost to follow-up; MSSA = methicillin-sensitive Staphylococcus aureus; OD = right eye; OS = left eye; rDNA = ribosomal DNA; SD = standard deviation.
growing diagnostic arsenal to provide cultureless identification of pathogens responsible for highly morbid corneal infections.

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Disclosure(s):
All authors have completed and submitted the ICMJE disclosures form.
The author(s) have made the following disclosure(s): J.C.: Consultant — Food and Drug Administration; Funding — unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, National Institutes of Health (grant nos. R01EY013124 and R01EY021558).
L.U.: Employment as a postdoctoral research fellow at Mass Eye and Ear (Chodosh lab); Supported in part by the Dozoretz Family Private Foundation
M.S.G.: Funding — National Institutes of Health (grant no. R01EY031600). P.J.M.B.: Funding — National Institutes of Health (grant no. R21EY032231).
This study was funded by the Tej Kohli Foundation through the Tej Kohli Cornea Program at Massachusetts Eye and Ear. Funding agencies had no role in study design, data analysis, decision to publish, or preparation of the manuscript. The technologies described in this brief report are contained within an active patent application (by P.J.M.B. and M.S.G.), 2019, Comprehensive Microbial Panel for Molecular Diagnosis of Eye Infections (PCT/US2018/066836).

HUMAN SUBJECTS: Human subjects were included in this study. This study was approved by the Mass General Brigham Institutional Review Board. All research adhered to the tenets of the Declaration of Helsinki. All participants provided informed consent.

No animal subjects were used in this study.

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Obtained funding: N/A
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