AMERICAN ACADEMY OF OPHTHALMOLOGY®

Report

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.[1] 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.[2] This proof-of-concept uses novel molecular barcoding on the NanoString nCounter platform for highly multiplexed nucleic acid detection,[3] 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.[4] 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 microscopy 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 mechanically 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[5] and variable regions 3 and 4 of bacterial 16S ribosomal RNA.[6]

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 $\log_{10}$ 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* (*lyt*A), *Pseudomonas aeruginosa* (*pro*A), *Serratia marcescens* (*gyr*B), and *Haemophilus influenzae* (*pst*A). 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.

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

Bacteria		Fungi	Viruses
Acinetobacter calcoaceticus-baumanii (16S-23S intergenic spacer region)	*Pseudomonas aeruginosa* (proA)	*Aspergillus flavus* (β-tubulin)	Cytomegalovirus (major immediate-early gene)
Acinetobacter lwoffii (blaOXA-134)	*Serratia marcescens* (gyrB)	*Aspergillus fumigatus* (calmodulin)	Epstein-Barr virus (DNA polymerase, BALF5)
Bacillus cereus group (rpoB)	*Staphylococcus aureus* (spa)	*Aspergillus niger* (calmodulin)	Herpes simplex 1 (DNA polymerase catalytic subunit)
Bacillus subtilis group (28S rDNA)	*Staphylococcus capitis* (nuc)	*Candida albicans* (28S rDNA)	Herpes simplex 2 (DNA polymerase catalytic subunit)
Borrelia burgdorferi (flaB)	*Staphylococcus epidermidis* (nuc)	*Candida dubliniensis* (ITS1-5.8S-ITS2)	Human herpesvirus 6 (U22)
Enterobacter aerogenes (gyrB)	*Staphylococcus lugdunensis* (nuc)	*Candida glabrata* (28S rDNA)	Varicella zoster (ORF63)
Enterococcus faecalis (ddl)	*Staphylococcus* spp. (28S rDNA)	*Candida parapsilosis* (28S rDNA)	
Enterococcus faecium (ddl)	*Streptococcus agalactiae* (cfb)	*Candida tropicalis* (28S rDNA)	**Parasites**
Escherichia coli (murC)	*Streptococcus anginosis* group (16S rDNA)	*Fusarium* spp. (28S rDNA)	Toxoplasma gondii (GPDH)
Haemophilus influenzae (pstA)	*Streptococcus mitis* group (16S rDNA)		
Klebsiella pneumoniae (clpS)	*Streptococcus pneumoniae* (lytA)	**Virulence Markers**	
Morganella morganii (gyrB)	*Streptococcus pyogenes* (ntpC)	*Staphylococcus epidermidis* (icaAD)	
Mycobacterium tuberculosis (MPB64)	*Troponema pallidum* (tpp47)	*Staphylococcus* spp. (mecA)	
Propionibacterium acenes (lipase)	*Tropheryma whipplei* (repeat sequence)		
Proteus mirabilus (ackA)			

Comprehensive targeted panels strike a fine balance between other molecular diagnostic methods, ranging from singleplex PCR to metagenomic sequencing,[7] 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

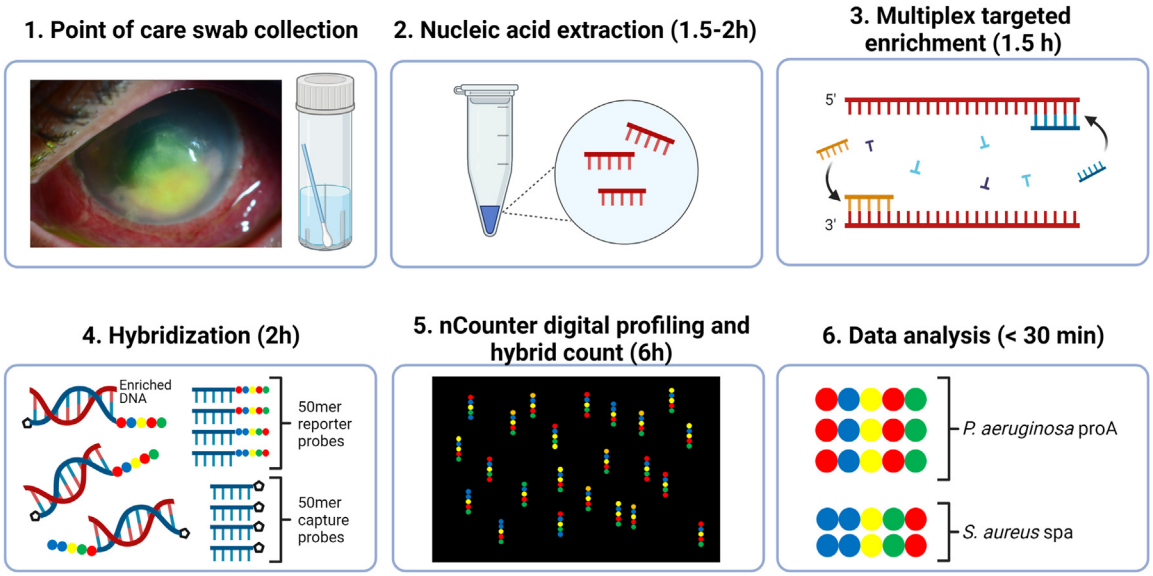

Figure 1. Diagnostic NanoString workflow for patients presenting with infectious corneal ulceration. Figure created using BioRender.com under a standard academic license.

Table 2. Overview of Culture and NanoString-Positive Cases, by Clinical Presentation, Microbiology, and Molecular Diagnostic Results

	Clinical Presentation					Culture Microbiology			Molecular Diagnostics				
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 Log_{10} 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	Yes	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	Positive	MSSA	2	26.49 (1.43)	26.57 (0.26)	*S. aureus* spa	913.32 (157.52)	2.96
5 (OS)	HM	Yes	Yes	Yes	LTFU	Negative	CoNS	3	30.35 (0.80)	24.83 (0.41)	Staphylococcus spp. 28S rDNA	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* cfb	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* gyrB	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* pstA	675.41 (182.48)	2.83
12 (OD)	20/50	Yes	Yes	Yes	LTFU	Negative	*P. aeruginosa*	3	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 (1.32)	*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* pstA	18019.23 (858.48)	4.26
17 (OS)	LP	No view	Yes	Yes	Yes	Positive	*Streptococcus mitis* and *Serratia marcescens*	3	30.84 (0.56)	23.0 (1.02)	*S. mitis* group 16S rDNA	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.

Table 3. At-a-Glance Comparison of Single-Plex Polymerase Chain Reaction, Multiplexed or Targeted Panels (e.g., the NanoString), and Clinical Metagenomics for Nonculture-based Molecular Diagnosis of Infectious Disease

Feature	Singleplex PCR	Multiplex Targeted Panels	Clinical Metagenomics
Bias	Biased	Contingent on panel design; some may be semi-unbiased if many organisms are included (e.g., NanoString)	Semi-unbiased (amplicon sequencing) Unbiased (shotgun sequencing)
Computational and Bioinformatic Expense	Low	Low	High to very high
Level of Background Noise	Low-medium	Low-medium	High
Potential for Novel Pathogen Discovery	No	No	Yes
Time to result (hrs)	≤12	≤12	>24 (often longer due to computational load)

PCR = polymerase chain reaction.

growing diagnostic arsenal to provide cultureless identification of pathogens responsible for highly morbid corneal infections.

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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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