An All-in-One Highly Multiplexed Diagnostic Assay for Rapid, Sensitive, and Comprehensive Detection of Intraocular Pathogens

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• PURPOSE: Intraocular infections are sight-threatening conditions that can lead to vision loss. Rapid identification of the etiologies plays a key role in early initiation of effective therapy to save vision. However, current diagnostic modalities are time consuming and lack sensitivity and inclusiveness. We present here a newly developed comprehensive ocular panel designed to improve diagnostic yields and provide a tool for rapid and sensitive pathogen detection.

• DESIGN: Experimental laboratory investigation.

• METHODS: A panel containing 46 pathogens and 2 resistance/virulence markers that are commonly detected in intraocular infections was developed. Genomic targets were scrutinized for stretches predicted to be specific for a particular species while being conserved across different strains. A set of primers for sample enrichment, and two 50mer NanoString compatible probes were then designed for each target. Probe−target hybrids were detected and quantified using the NanoString nCounter SPRINT Profiler. Diagnostic feasibility was assessed in a pilot clinical study testing samples from infectious retinitis (n = 15) and endophthalmitis (n = 12) patients, for which the etiologies were confirmed by polymerase chain reaction (PCR) or culture.

• RESULTS: Analytical studies demonstrated highly sensitive detection of a broad spectrum of pathogens, including bacteria, viruses, and parasites, with limits of detection being as low as 2.5 femtograms per reaction. We also found excellent target specificity, with minimal cross-reactivity detected. The custom-designed NanoString ocular panel correctly identified the causative agent from all clinical specimens positive for a variety of pathogens.

• CONCLUSION: This highly multiplexed panel for pathogen detection offers a sensitive, comprehensive, and uniform assay run directly on ocular fluids that could significantly improve diagnostics of sight-threatening intraocular infections. (Am J Ophthalmol 2023;250: 82–94. © 2023 Elsevier Inc. All rights reserved.)

Laboratory-based diagnosis of ocular infections relies largely on the use of multiple tests based on outdated methods (eg, microscopy and culture) that are time consuming and lack sensitivity of detection, especially for ocular specimens that are markedly small in size and contain minute amounts of microbes available for detection.[1,2] As a result, most patients presenting with an intraocular infection are treated for long periods with empirical trial therapies of various broad-spectrum antimicrobials. These shotgun empirical approaches can lead to increased toxicity,[3] select for resistant organisms,[4] and are becoming increasingly compromised by the global emergence of antimicrobial resistance.[5]

The description of the modern-day polymerase chain reaction (PCR) by Kary Mullis and collaborators in the 1980s[6] revolutionized the diagnostic field, providing a new and improved tool for rapid and more sensitive detection of pathogens in clinical specimens that can support early initiation of appropriate therapy.[7,8] The first clinical applications of PCR for molecular diagnosis of ocular diseases were published in the late 1980s and early 1990s, and centered on the detection of herpesviruses from corneal scrapings and intraocular specimens of patients presenting with keratitis and retinitis, respectively.[9-11] In the subsequent years, many PCR-based assays have been developed that have successfully improved pathogen detection and diagnostic yields in infectious uveitis and endophthalmitis.[12-26] Despite their usefulness in generating a clinically actionable result, PCR-based assays are constrained in the number of targets that can be simultaneously detected. This results in diagnostic workups that rely on the use of multiple sequential and separate tests that can be challenging to perform when only a minute amount of ocular sample is available for processing, and also increases the total turnaround time for diagnosis. In cases in which a universal target is used (eg, ribosomal genes) and virtually all species of bacteria (16S rRNA) or

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fungi (28S rDNA and ITS) are detected by a single PCR test, identification of genus and species is dependent on post-PCR characterization of amplicons such as Sanger sequencing and fragment restriction analysis, among others, adding a significant number of hours to the diagnostic process. Metagenomic next generation sequencing is increasingly being explored as an unbiased method for comprehensive detection of ocular pathogens[27]; however, to date, this method remains costly, slow depending on the method used, and technically challenging and complex to perform and analyze in routine clinical laboratories, and is limited by low signal-to-noise due to dominance of host DNA over the microbial fraction.[28]

Because of the wide breadth of etiologies involved in intraocular infections that result in a laborious and slow diagnostic workup requiring the use of multiple assays with limited clinical sensitivity, we sought to create a novel all-in-one targeted molecular diagnostic assay that could detect more than 90% of pathogens known to be associated with these infections with high accuracy, using only a small amount of sample, and in a timely manner. Our innovative approach to diagnose sight-threatening intraocular infections leverages the advantages of the NanoString technology, which enables simultaneous detection and quantification of hundreds of different DNA or RNA molecules while maintaining excellent sensitivity per target. The NanoString technology relies on the use of highly multiplexed pools of specific 50mer hybridization probes marked with fluorescent reporters that are mixed with crude or enriched samples. Biotinylated probe—target hybrids are then captured onto a streptavidin-coated imaging surface and counted using fluorescence microscopy.[29] A handful of studies have reported the use of custom-designed NanoString panels to improve diagnosis and surveillance of infectious diseases,[30-34] including 1 proof-of-principle study from our group demonstrating the feasibility of this method to rapidly detect keratitis-causing pathogens.[35] Here we present the development and evaluation of our novel and highly multiplexed assay for diagnosis of infectious uveitis and endophthalmitis that is able to simultaneously detect 46 pathogens and 2 resistance/virulence markers in a single reaction.

METHODS

This is an experimental laboratory investigation study approved by the Mass General Brigham Institutional Review Board (MGB IRB) (reference: 2019P000551; excess human material protocol). Protocols for collection of discarded intraocular specimens were approved by the MGB IRB for prospective sampling, and the need for informed consent was waived. The study was in adherence to the tenets of the Declaration of Helsinki and is in accordance with Health Insurance Portability and Accountability (HIPAA) regulations.

• **SELECTION OF TARGET SEQUENCES AND PANEL DESIGN:** Using the epidemiological information on the etiologies of ocular infections seen at our hospital and in combination with literature review, we identified 46 pathogens and 2 resistant/virulence markers that are commonly associated with ocular infections. This panel of organisms (Figure 1 and Table S1) accounts for >90% of pathogens isolated from intraocular infections in our service (data not shown). Genomic targets commonly used for PCR-based detection of these pathogens were scrutinized for stretches that are predicted to be specific for a particular species while being conserved across different strains from the same species. Regions of 150 to 300 base pairs in length were selected and a set of primers for sample enrichment, and two 50mer probes per target were designed to these sequences in collaboration with NanoString to incorporate their proprietary barcode sequences, and to use validated bioinformatics algorithms for probe design that minimize cross-reactivity and optimize thermodynamic profiles for hybridization.

• **MULTIPLEX TARGETED ENRICHMENT:** Because of the low pathogen biomass in ocular specimens, analytical sensitivity of an assay is critical for its clinical application. To deal with that issue, we developed an optimized multiplex targeted enrichment step previous to detecting genus- or species-specific DNA segments with barcoded probes. Primers flanking the targeted genomic regions that bind to the specific probes were designed (described above). A 7-μL polymerase chain reaction was set up using 3.5 μL of 2X TaqMan Fast Advanced master mix, 1 μL of the primer mixture at 0.5 nM per oligonucleotide, and 2.5 μL of purified DNA. PCR was performed under the following conditions: 95°C denaturation for 5 minutes, followed by 14 cycles of 15 seconds at 95°C and 4 minutes at 60°C. This number of cycles was found to improve sensitivity while maintaining a low level of background noise (Figure S3).

• **DNA DETECTION:** DNA from control organisms used for analytical validation were extracted and purified using the QIAamp UCP Pathogen mini kit (Qiagen), following the manufacturer's protocol. Extraction of genomic DNA from clinical specimens was performed on 100 μL of aqueous or vitreous mixed with 1X DNA/RNA Shield (Zymo) using the high-sensitivity ultra-pure Quick-DNA/RNA Microprep Plus Kit (Zymo). To guarantee that tough-to-lyse cells such as gram-positives, acid-fast bacteria, and fungi were adequately disrupted, sample mixes (intraocular fluid + DNA/RNA shield) were mechanically homogenized in the FastPrep-24 for 2 × 45 seconds with a 5-minute intermission, using the ZR BashingBead Lysis Tubes (0.1 and 0.5 mm) (Zymo). DNA was eluted in 30 μL of DNase/RNase-free water and, for clinical specimens, DNA

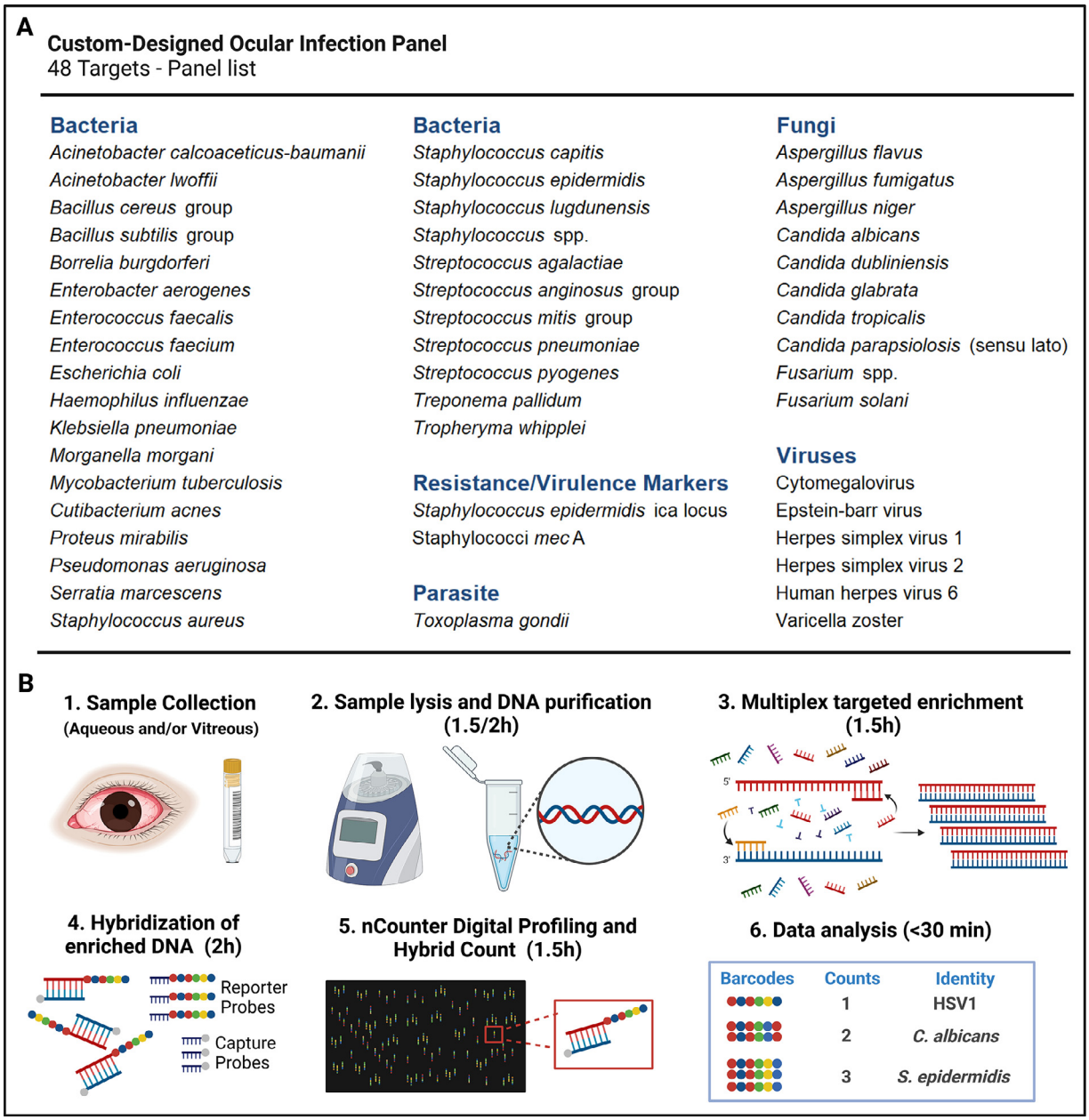

FIGURE 1. (A) Pathogens targeted in a custom-designed multiplexed panel for improved diagnosis of ocular infections. (B) Improved diagnostic workflow for patients presenting with intraocular infections. Total turnaround time was reduced from >24 hours to approximately 12 hours while maintaining excellent analytical sensitivity. Figure created using BioRender.com under a standard academic license.

quality was assessed by performing an internal control real-time PCR assay targeting the human β-globin gene as described.[14] Purified DNA was then used as input for the multiplex targeted enrichment step described above. Enriched samples were then mixed with a cocktail of 96 probes (two 50mer probes per each of the 48 targets), containing 1 capture probe that secures the hybrid of interest to a streptavidin-coated surface in the NanoString cartridge, and a second 50mer that attaches a unique color-coded barcode for each target included in the panel (Figure 1). The hybridization was performed at 67°C for varying periods of time, and the resulting products were analyzed in the NanoString nCounter SPRINT analyzer.

• **ANALYTICAL SPECIFICITY:** The analytical specificity of the panel was tested for all targeted organisms. Purified genomic DNA was isolated in our laboratory using the QIAamp UCP pathogen mini kit (Qiagen) following the man-

ufacturer's recommendations. DNA controls acquired from commercial sources came as already purified genomic DNA. Plasmids (pUC-GW-Kan) carrying the targeted region were constructed by Genewiz. A complete list of controls, strains, and their sources is shown in Table S1.

• **ANALYTICAL SENSITIVITY:** The limit of detection of our test was evaluated for selected clinically relevant pathogens targeted in the panel that have varying GC content and genome sizes. Preliminary testing was performed using purified genomic DNA from 1 gram-positive (*Staphylococcus aureus* USA300 strain) and 1 gram-negative pathogen (*Pseudomonas aeruginosa* ATCC 27853), both serially diluted (10-fold) from 2.5 pg/reaction to 2.5 fg/reaction. With an assay that was extensively optimized for timely and sensitive detection of these organisms, we then tested the analytical sensitivity of our approach using additional pathogens that are important causes of intraocular infections, including cytomegalovirus (ATCC VR-538DQ), herpes simplex virus 1 (HSV-1) (ATCC VR-539DQ), herpes simplex virus 2 (HSV-2) (Exact Diagnostics control panel), varicella-zoster virus (VZV) (Exact Diagnostics control panel), *Toxoplasma gondii* (ATCC 50174D), *Streptococcus pneumoniae* (ATCC 49619), and *Staphylococcus epidermidis* (ATCC 12228). The limit of detection (LoD) was determined as a function of the limit of blank (LoB), the latter calculated based on the background noise of the assay when blank extractions (using only the 1X DNA/RNA shield buffer as the input material), but no target DNA sequences, were used. To calculate the LoB, we performed a series of 45 blank reactions for each target tested (n = 48) and used the following standard formula for LoB calculation: mean of the blanks + 1.645 times the standard deviation of the blanks.[36]

• **PROOF-OF-PRINCIPLE CLINICAL STUDIES:** Samples were obtained either by anterior paracentesis (aqueous samples) or posterior chamber paracentesis (undiluted vitreous samples) or during pars plana vitrectomy (both undiluted vitreous and diluted vitreous washing samples). Following collection, all specimens were immediately transported to the laboratory and stored at −20°C and then transferred to a −80°C freezer for long-term storage. To assess the usefulness and accuracy of our custom-designed NanoString ocular panel to detect pathogens causing infectious uveitis and endophthalmitis, we performed a pilot clinical validation study on 27 stored samples (4 aqueous, 23 vitreous) collected from patients with clinically diagnosed herpetic retinitis (n = 9), cytomegalovirus (CMV) retinitis (n = 4), ocular toxoplasmosis (n = 2), and endophthalmitis (n = 12). Infectious uveitis samples that tested positive for herpesviruses (HSV-2, n = 5; VZV, n = 4; CMV n = 4) and *T gondii* (n = 2) using a validated PCR assay[14] were included. For endophthalmitis, only monomicrobial cases that were positive by culture were included.

RESULTS

• **IMPROVING SENSITIVITY OF PATHOGEN DETECTION WITH SAMPLE ENRICHMENT:** Because of the small sample size, ocular fluids collected from patients presenting with intraocular infections (typically 50-100 μL total) have low amounts of pathogen biomass available for detection. To overcome this problem, we designed and optimized a multiplex targeted enrichment (MTE) assay aiming to increase the amount of targeted pathogen DNA in the samples before probe detection occurs. By following NanoString recommendations for MTE (20 cycles) and hybridization time (16 hours), we were able to detect minute amounts of DNA from 2 common ocular pathogens: *S aureus* (methicillin-resistant *Staphylococcus aureus* [MRSA] strain USA300) and *P aeruginosa* (ATCC 27853). By performing serial 10-fold dilutions of DNA ranging from 2.5 pg/reaction to 2.5 fg/reaction, we found that the analytical sensitivity of this assay (or limit of detection [LoD]) for these organisms was as low as 25 femtograms (fg) of DNA per reaction (Figure S1).

• **ASSAY OPTIMIZATION PROCESS:** Although the original NanoString protocol supports robust and sensitive detection of nucleic acids (described above), it includes long hybridization steps (16-18 hours) and is considerably time consuming (>24 hours for total turnaround time). To improve the test turnaround time and tp generate clinically actionable data in a timely manner, we sought to test shorter times of hybridization using *S aureus* DNA as a control. Using the higher concentration of *S aureus* DNA tested (2.5 pg/reaction), pathogen detection was possible following 16 hours (recommended by NanoString), 8 hours, 4 hours, and 2 hours of hybridization with an MTE protocol fixed at 20x (Figure S2, A). To determine how this decrease would affect the assay's analytical sensitivity, we fixed the hybridization at 2 hours and repeated the test using DNA at the LoD (25 fg/reaction) and 1 dilution above (250 fg/reaction) (Figure S2, B). Both concentrations resulted in robust probe counts, which demonstrated that a decrease in time of hybridization could be implemented without compromising the analytical sensitivity. Although the MTE assay described above can substantially increase the sensitivity of the assay, it can also significantly increase the noise of the reaction and add unnecessary time to the diagnostic workflow. Because of that, we next sought to determine the best number of MTE cycles that would maintain robust sensitivity of pathogen detection while decreasing the noise of the reaction. To do that, we tested the ability of our assay to detect *S aureus* (Figure S3, A) and *P aeruginosa* (Figure S3, B) DNA at the LoD (25 fg/reaction) following various numbers of enrichment cycles (10, 12, 14, 16, 18, and 20), with hybridization fixed at 2 hours. We found that 14 MTE cycles optimized detection for both pathogens while preventing false-positive detection, with pathogen signal being 100-

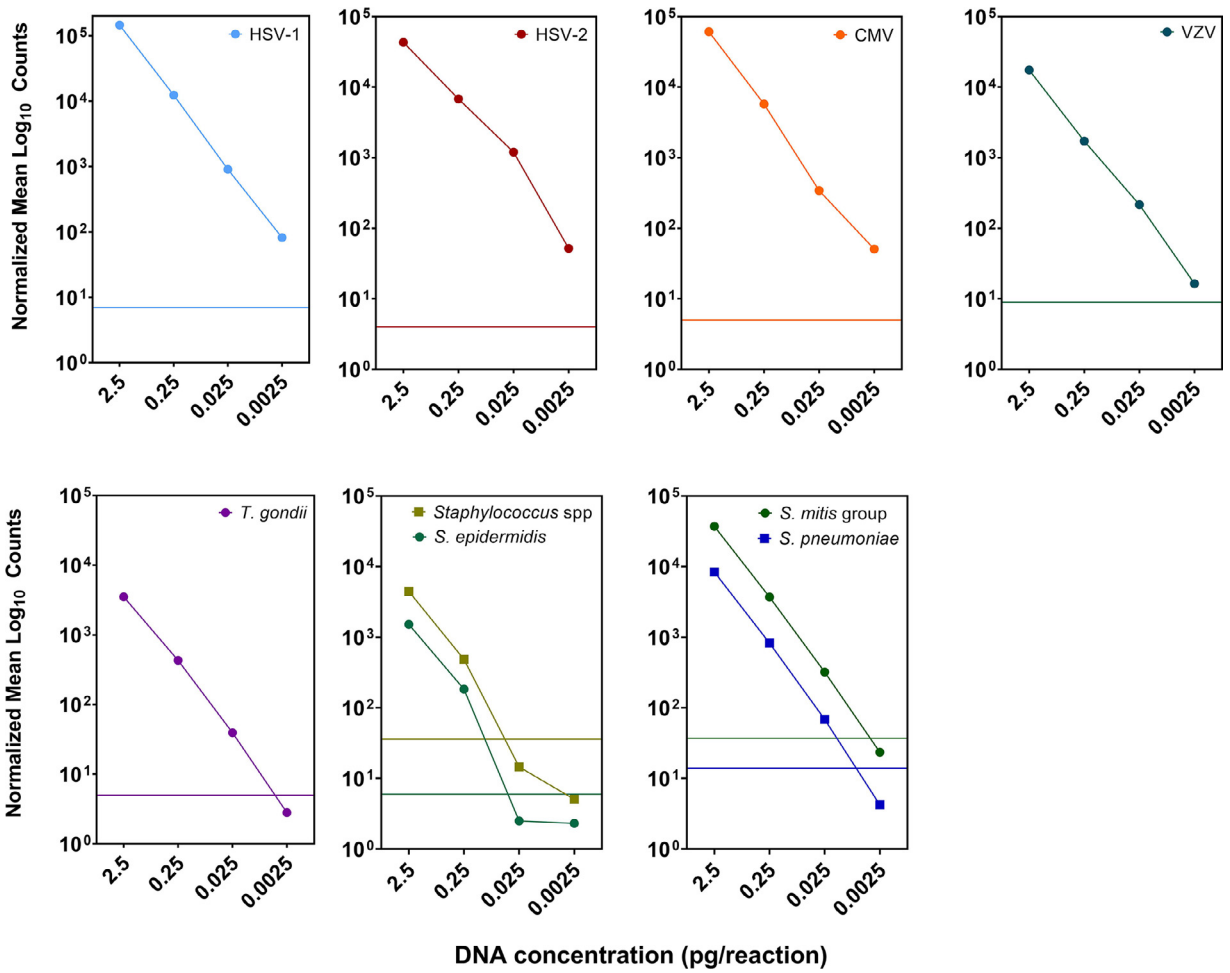

FIGURE 2. Analytical sensitivity studies using serial 10-log dilutions ranging from 2.5 picograms to 2.5 femtograms of DNA per reaction. The amount of pathogen DNA was determined by Qubit (dsDNA high-sensitivity assay). Each data point is the average log10 probe counts calculated from triplicates. Horizontal lines indicate the analytical cutoff values as determined by the limit of blank for each target in the panel, which varies according to the probe set (Table S3). Line colors match those of the target signal shown in each plot.

fold higher than that of the background noise. Following these optimization steps, an improved diagnostic workflow was developed, which reduced total turnaround time from >24 hours to around 12 hours while maintaining excellent analytical sensitivity of pathogen detection (Figure 1, B).

• **OPTIMIZED ASSAY WAS ABLE TO DETECT MINUTE AMOUNTS OF DNA:** With an assay that has been optimized for timely and sensitive detection of ocular pathogens, the analytical sensitivity studies were expanded to determine the LoD of our test using purified control DNA from organisms (other than MRSA and *P aeruginosa*) that are important causes of intraocular infections (and other ocular infections) including CMV, HSV-1, HSV-2, VZV, *T gondii*, *Streptococcus pneumoniae*, and *Staphylococcus epidermidis* (Figure 2). LoD was determined as a function of the limit of blank (LoB), the latter calculated based on the background noise of the assay when only reagents and water, but no target DNA sequences, are used. To calculate the LoB, we performed 45 blank reactions for each target tested (n = 48). Because each target can have different amplification efficiencies and hybridization dynamics that could result in varying levels of background noise, an individual LoB value was calculated for each target included in our panel (Table S3). By using their individual LoB values as analytical cutoff values, the LoD for the 4 herpesviruses tested (CMV, VZV, HSV-1 and HSV-2) was as low as 2.5 fg/reaction. The LoD of this approach to detect *T gondii* DNA was 25 fg/reaction. *S pneumoniae* is detected in our assay by a species-specific probe targeting the *lyt*A gene and also by a *Streptococcus mitis* group–specific probe that can detect any member of the mitis group. A signal for both probes was detected with as low as 25 fg of DNA per reaction. Similarly, *S epidermidis* DNA can be detected by 2 separate sets of probes, 1 species-specific probe targeting the thermonuclease gene and a genus-specific probe that targets areas of the 23S rRNA that are common to all clinically rel-

evant staphylococcal species. Both of these probes detected an *S epidermidis* signal with a LoD of 250 fg/reaction.

• **PROBES AND PRIMERS HAVE BEEN SHOWN TO BE HIGHLY SPECIFIC:** The analytical specificity of our panel was tested using a collection of control DNA from bacteria (29 group, genus- or species-specific targets, 1 antimicrobial resistance gene, and 1 biofilm-associated gene), herpesviruses (n = 6), fungi (n = 10), and parasite (n = 1) (Table S1). The 48 sets of probes and oligonucleotides tested demonstrated excellent target specificity, with minimal cross-reactivity detected for some probes targeting conserved genomic stretches of the ribosomal operon. The *Streptococcus anginosus* group_16S rRNA probe and the *S mitis* group_16S rRNA probe displayed varying levels of cross-reactivity with closely related streptococci species (Figure S4,A). These signals, however, were accompanied by strong signals for species-specific probes for the control streptococci strains tested, which would still allow direct identification of these organisms from clinical specimens to the species level. Probes designed to detect *Candida non-albicans* species (eg, *parapsilosis*, *glabrata*, *dubliniensis*, and *tropicalis*) also displayed varying levels of cross-reactivity among them. The species-specific *Candida albicans* probe set has shown excellent analytical specificity, allowing us to separate C *albicans* from all other non-*albicans* species, which are, for now, identified as a group, because many of these probes will light up together when a non-*albicans* species is present in the sample. In addition, during analytical specificity studies, we also found that probes designed to target DNA segments present in high copy number in the organism's genomes (eg, ribosomal genes) had positive signals that were ≥10 times higher when compared to species-specific probes that target single-copy genes. To avoid excessive signal and noise coming from these probes, we systematically optimized their concentrations in the reaction so that all probe sets resulted in signals of similar intensity (data not shown).

• **PROOF-OF-PRINCIPLE CLINICAL VALIDATION STUDIES ATTEST TO THE FEASIBILITY OF OUR PANEL FOR DIAGNOSING INTRAOCULAR INFECTIONS:** To determine the feasibility of our novel custom-designed NanoString ocular infection panel to detect intraocular pathogens directly from clinical specimens, 27 ocular fluid samples from 24 adult patients presenting with infectious retinitis (n = 12) and endophthalmitis (n = 12) were tested in a pilot study. The majority of the patients (75%) were male, with a mean age of 60.8 years (±20.6 years; range, 7-87 years). Among the uveitis cases, most were diagnosed with herpetic retinitis (n = 6) and CMV retinitis (n = 4). Two patients had toxoplasmosis chorioretinitis. For endophthalmitis patients, the majority presented with infections following an intraocular surgery (n = 6) or intravitreal injection (n = 4), with the remaining patients (n = 2) presenting with endophthalmitis of hematogenous origin (Table 1). To generate a validation dataset for which results from the NanoString assay could be compared to the standard of care, only patients with culture- or PCR-positive specimens were included in this initial study. In total, 27 specimens collected from 24 patients were processed by NanoString and quality-control PCR assays for detection of an internal control (human β-globin gene).

TABLE 1. Characteristics of 24 Patients With Clinical Diagnoses of Infectious Uveitis (n = 12) and Endophthalmitis (n = 12) Included in Pilot Proof-of-Principle Clinical Validation Studies.

Sex	
Male (%)	18 (75.0)
Female (%)	6 (25.0)
Age, y	
Mean (SD)	60.8 (±20.6)
Range	7-87
Uveitis Diagnoses	
Herpetic retinitis	6 (25.0)
CMV retinitis	4 (16.7)
Toxoplasmosis chorioretinitis	2 (8.3)
Endophthalmitis Diagnoses	
Post-operative endophthalmitis	6 (25.0)
Post-intravitreal injection endophthalmitis	4 (16.7)
Endogenous endophthalmitis	2 (8.3)

CMV = cytomegalovirus.

Uveitis

Most cases of uveitis were viral in etiology (10 of 12; 83.3%), with CMV (n = 4), HSV-2 (n = 3) and VZV (n = 3) being detected by PCR. Two cases were associated with toxoplasmosis (Table 2 and Table S2). The mean β-globin cycling threshold (Ct) for these cases was 29.98 (ranging from 24.42 to 36.47). All of these samples were positive for a pathogen using our NanoString assay with mean normalized probe counts ranging from 155 to 207,834 (median, 11,371) and from 2.2 to 5.3 (median, 4.1) when transformed to a $\log_{10}$ count. We found complete agreement between the results of our custom-designed NanoString panel with those of a validated in-house real-time PCR test developed to detect the most common causes of infectious uveitis (Table 2).[14] When stratified by each of the targets found in the population of specimens processed, quantification of DNA hybrids by NanoString correlated fairly well with the pathogen burden as determined by real time PCR Ct values (Figure S5). Only 1 outlier was found for a specimen positive for HSV-2 with low Ct value (21, meaning that the viral burden was probably high) and low probe counts (mean count, 301).

Endophthalmitis

Most cases of endophthalmitis were bacterial (11 of 12; 91.6%), with 1 case being caused by C *albicans* (Table 3

TABLE 2. Results of Our Custom-Designed NanoString Panel for Patients With Clinically Diagnosed and PCR-Positive Infectious uveitis.

Patient No.	Specimen (Eye)	Clinical Diagnosis	PCR Assay		NanoString Detection		Final Diagnosis
			ID	Mean Ct (SD)	Probe IDs	Mean Probe Count	
1	VW (OS)	CMV Retinitis	CMV	23.34 (0.24)	CMV	5,461	CMV retinitis
2	VW (OD)	PORN	*T gondii*	26.87 (0.34)	*T gondii*	155	Toxoplasmosis chorioretinitis
3	Vitreous (OS)	ARN	HSV-2	28.16 (0.76)	HSV-2	255	Herpetic retinitis
4	Vitreous (OD)	ARN	VZV	28.59 (0.11)	VZV	16,147	Herpetic retinitis
	VW (OD)		VZV	26.77 (0.37)	VZV	46,055	
5	Aqueous (OD)	CMV Retinitis	CMV	20.76 (0.11)	CMV	102,329	CMV retinitis
6	Vitreous (OS)	Posterior Uveitis	HSV-2	20.40 (0.07)	HSV-2	21,444	Herpetic retinitis
	VW (OS)		HSV-2	21.52 (0.02)	HSV-2	11,943	
7	VW (OS)	ARN	VZV	29.53 (1.37)	VZV	11,371	Herpetic retinitis
8	Aqueous (OD)	Viral Retinitis	HSV-2	20.87 (0.01)	HSV-2	301	Herpetic retinitis
	Aqueous (OS)		HSV-2	23.47 (0.09)	HSV-2	4,413	
9	Vitreous (OS)	CMV Retinitis	CMV	19.47 (0.14)	CMV	207,834	CMV retinitis
10	VW (OS)	ARN	VZV	25.02 (0.05)	VZV	112,551	Herpetic retinitis
11	Aqueous (OD)	CMV Retinitis	CMV	25.55 (0.55)	CMV	1,934	CMV retinitis
12	VW (OD)	Toxoplasmosis Chorioretinitis	*T gondii*	26.35 (0.59)	*T gondii*	764	Toxoplasmosis chorioretinitis

ARN = acute retinal necrosis; Ct = cycling threshold; CMV = cytomegalovirus; HSV-2 = herpes simplex virus 2; OD = right eye; OS = left eye; PCR = polymerase chain reaction; PORN = progressive acute retinal necrosis; VW = vitreous washing; VZV = varicella-zoster virus.

and Table S2). Gram-positive organisms dominated this population (10 of 12 cases; 83.3%), with most being identified as either a staphylococci (n = 6) or streptococci (n = 4) species. One *S epidermidis* isolate was resistant to methicillin. Mean β-globin Ct value for these specimens was 27.07 (ranging from 21.11 to 32.84). Mean normalized NanoString probe counts ranged from 43 to 98,978 (median, 2564) and from 1.6 to 5.0 when transformed to $\log_{10}$ counts (median, 3). There was complete agreement between culture results and NanoString-based identification at the genus level. At the species level, the correct signal was detected for the species-specific probe for all cases, with the exception of 1 patient. Patient 13 presented with an endogenous endophthalmitis with a yeast isolated by culture that was identified as a probable *C albicans* using a rapid test for presumptive identification (Remel *Candida albicans* test kit). By NanoString, we identified this as being a non-*albicans Candida* species, given that signal for multiple non-*albicans* probes was identified (Table 3 and Table S2). At the antimicrobial resistance level, one of the *S epidermidis* isolated by culture (patient 16) was phenotypically resistant to methicillin, and this was captured at the genotypic level by our panel (*mec*A gene detected with an average probe count of 4071; the average probe count for *S epidermidis* was 2564 and for the *Staphylococcus* genus probe 8147). None of the staphylococci species detected were positive for the biofilm-associated *ica* genes. The turnaround time for detection (time to growth) plus identification of these organisms by culture ranged from 2 to 5 days (median, 2.5 days). Using our custom-designed ocular panel and an optimized approach, the complete diagnostic process, from sample preparation (12 at a time) to data analysis, can be done within 12 hours.

DISCUSSION

Intraocular infections, such as infectious uveitis and endophthalmitis, are sight-threatening conditions associated with high ocular morbidity.[37-39] There are a wide range of possible infectious etiologies that cannot be easily and promptly detected using the outdated laboratory diagnostic methods currently used. As a result, patients presenting with intraocular infections can be treated for long periods using one-size-fits-all therapies that are not tailored for an individual and that do not work for everyone. With the goal of improving care of patients presenting with these infections by providing clinically actionable diagnostic results in a timely and accurate manner so that targeted and precise therapies can be initiated at early stages of the disease, we developed a novel diagnostic approach for rapid, sensitive, and comprehensive detection of pathogens directly from intraocular fluids.

Our innovative approach to diagnose sight-threatening intraocular infections leverages the advantages of the NanoString technology, which captures and counts hundreds of different DNA or RNA molecules in complex mixtures with excellent sensitivity and high multiplexing capability.[29] Additional advantages for clinical applications in-

TABLE 3. Results of Our Custom-Designed NanoString Panel for Patients Presenting With Culture-Positive Endophthalmitis.

Patient No.	Specimen (Eye)	Clinical Diagnosis	Culture Result	Time to Growth + ID	NanoString Detection	
					Probe IDs	Mean Probe Count
13	VW (OD)	Endogenous endophaltmitis	*C albicans*[a]	3 days	***C* non-*albicans***	48-255[b]
14	VW (OD)	Post-operative endophthalmitis	*S agalactiae*	2 days	***S agalactiae***	98,978
					***S anginosus* group**	89,670
					S mitis group	55,951
15	VW (OS)	Post-operative endophthalmitis	*S pneumoniae*	2 days	***S mitis* group**	16,817
					S pneumoniae	5,634
					S anginosus group	1,508
16	VW (OD)	Post-intravitreal injection endophthalmitis	MR *S epidermidis*	3 days	***Staphylococcus* genus probe**	8,147
					***mecA* gene**	4,071
					S epidermidis	2,564
17	VW (OD)	Post-intravitreal injection endophthalmitis	MS *S epidermidis*	2 days	*Staphylococcus* **genus probe**	352
					S epidermidis	109
18	VW (OD)	Post-operative endophthalmitis	*S pneumoniae*	2 days	***S mitis* group**	62,090
					S pneumoniae	17,792
					S anginosus group	10,296
19	VW (OD)	Post-operative endophthalmitis	MS *S epidermidis*	4 days	***Staphylococcus* genus probe**	180
					S epidermidis	124
20	Vitreous (OD)	Post-operative endophthalmitis	MS *S lugdunensis*	3 days	***Staphylococcus* genus probe**	2,524
					S lugdunensis	320
21	VW (OS)	Endogenous endophaltmitis	*K pneumoniae*	2 days	***K pneumoniae***	22,550
22	Vitreous (OD)	Post-operative endophthalmitis	*S mitis*	2 days	***S mitis* group**	30,397
					S anginosus group	4,636
23	VW (OD)	Post-intravitreal injection endophthalmitis	MS *S epidermidis*	5 days	***Staphylococcus* genus probe**	112
					S epidermidis	43
24	Vitreous (OD)	Post-intravitreal injection endophthalmitis	MS *S epidermidis*	3 days	*Staphylococcus* **genus probe**	4,476
					S epidermidis	1,636

MR = methicillin resistant; MS = methicillin sensitive; OD = right eye; OS = left eye; VW = vitreous washing.

Underlined and boldface probes IDs match at the genus or species level compared to culture.

[a]Identified using the Remel *Candida albicans* test kit for rapid presumptive identification of *C albicans.*

[b]*Candida* non-*albicans* species are detected by a combination of probes targeting conserved regions of the ribosomal genes of various species. Average count is the range of probe counts for the non-*albicans* probes (Figure 1 and Table S1)

clude the possibility to modify the entire experimental approach (eg, introduction of enrichment steps, modification in hybridization time, among others), meaning that assays can be developed in a way that both sensitivity and processing time can be optimized, and also include the possibility to custom design and update panels that can facilitate expansion of target lists to include new and emerging pathogens, organisms of relevance to a specific geography, and clinically relevant markers of antimicrobial resistance or virulence. This technology has been explored before in a handful of studies that used custom-designed NanoString panels to improve diagnosis and surveillance of infectious diseases,[30-34] including 1 proof-of-principle study from our group demonstrating the feasibility of this method to rapidly detect keratitis-causing pathogens.[35] In the present study, we sought to evaluate the analytical performance and to determine the usefulness of this novel molecular barcoding approach as a tool for improved diagnosis of infectious uveitis and endophthalmitis. We demonstrate that our custom-designed panel for ocular infections combined with an optimized approach for sample processing can be used for comprehensive detection of a broad range of pathogens across different domains in only 1 assay, with PCR-level sensitivity,[12,13,40-42] and in a timely fashion. In pilot vali-

dation studies on primary clinical specimens collected from patients presenting with infectious uveitis (n = 15 specimens) and endophthalmitis (n = 12 specimens) that were positive by PCR and culture, excellent identification agreement was found.

Uveitis is a sight-threatening intraocular disease caused by myriad different etiologies that can present with overlapping features, resulting in a complex and difficult diagnostic process.[18,43,44] Infectious etiologies are involved in 17% to 60% of uveitis cases.[45,46] Although herpesviruses and *T gondii* are the leading causes, bacteria, fungi, and other viruses and parasites may also be involved.[45,47-49] Infectious uveitis can have a significant impact on vision health, especially when early treatment is misguided.[43] Initial diagnosis is currently made on the basis of the patient's history and clinical examination findings, but is complicated by overlapping findings among cases caused by different infectious agents, as well as by non-infectious causes.[18,44,45,50] Laboratory diagnosis is often required to aid in treatment decisions, but because many of the etiologies are difficult to culture and identify, the diagnostic workup can often become very challenging. and requires multiple immunological or pathogen-targeted molecular tests to screen for all potential etiologies.[18,49,51,52] Multiplexed tests can help to optimize the diagnostic workflow and the use of limited ocular samples to cover a greater number of potential etiologies. Toward that end, we[14] and others[16,19,26] have developed multiplex PCR assays for rapid diagnosis of uveitis from small specimens. However, these tests are limited in their ability to test for only a handful (normally 3- 5) of leading etiologic agents associated with uveitis per reaction. A series of multiplex PCR reactions have also been developed for detection of 9 common uveitis pathogens, with only 1 to 3 different targets included per reaction, creating the need for multiple parallel reactions to be performed.[53] The highly multiplexed custom-designed NanoString ocular panel developed in the present study has the tremendous advantage of probing for dozens of clinically relevant uveitis-causing pathogens in a single reaction, while maintaining excellent PCR-level sensitivity of detection. The limit of detection for herpesviruses (HSV-1, HSV-2, and CMV) was as low as 2.5 fg of DNA per reaction (1 fg/μL) and for *T gondii* 25 fg/reaction (10 fg/μL). The high analytical sensitivity of our novel diagnostic approach is comparable to what has been reported for detection of these pathogens using PCR assays (1 to 1.7 fg for herpesviruses and 20 to 200 fg for *T gondii*) that detect only only a single or a handful of targets.[40-42]

Uveitis specimens tested in our study were PCR positive for HSV-2, VZV, CMV, and *T gondii* and included vitreous tap, vitreous washings, and aqueous humor. Correct identification of the causative agent was achieved for all of these different sample matrices using our novel diagnostic test, and results were in full agreement to those found by real-time PCR. In addition, the NanoString nCounter instrument enables quantification of DNA hybrids that can be used for determination of pathogen burden in clinical specimens, a measurement that can be useful in determining disease severity and prognosis and in monitoring of therapeutic responses.[54-57] When quantification of DNA hybrids by NanoString was stratified by each of the targets/pathogens found in the population of uveitis specimens processed, we found very good correlations with the real-time PCR Ct values, which are often used as an indirect measurement of pathogen burden. This level of correlation with the method (quantitative PCR) that is routinely used for quantitative analysis indicates that counting of DNA hybrids by NanoString could also be used in the future for indirect determination of pathogen burden in infected specimens.

Endophthalmitis is one of the most aggressive ocular infections that may lead to irreversible tissue damage and permanent vision loss within hours or a few days following symptom onset.[37] These infections are commonly associated with an intraocular surgery or injection. In these cases, bacteria that colonize the ocular surface and periocular tissues, such as staphylococci and streptococci species, are commonly involved.[37] Some cases develop following an ocular trauma that can inoculate a variety of organisms into the posterior segment including environmental bacteria (eg, *Bacillus* spp) and filamentous fungi. Endogenous endophthalmitis secondary to a distal infection seeded by the bloodstream is often caused by gram-positive and gram-negative bacteria and *Candida* spp[37] Laboratory diagnosis of endophthalmitis is based on direct microscopy visualization of the causative agent and culture of the intraocular fluids and blood for endogenous cases.[37] Unfortunately, these methods have very low sensitivity of pathogen detection in endophthalmitis specimens, with most series reporting rates of culture positivity that range from about 23% to 63%.[12,13,15,17,20-25,58] Microscopic detection can be faster than culture but also has the same sensitivity limitations and can be used for only presumptive identification (eg, gram-positive or gram-negative bacteria, yeast, or filamentous fungi structures). Rapid PCR assays for universal detection of bacteria and fungi that target the ribosomal RNA operon of these organisms have been developed and have been shown to substantially improve pathogen detection.[12,13,15,17,20-23,25] However, these methods are often dependent on post-PCR analysis (eg, Sanger sequencing) for final pathogen identification, making the diagnostic process more laborious and time consuming. In addition, although the use of universal primers targeting ribosomal regions facilitates the detection of any bacteria and fungus in a clinical specimen, contaminant organisms introduced during sample collection, processing, or laboratory reagents will also be amplified. For specimens containing very limited amounts of the actual pathogen (eg, culture-negative intraocular fluids), the low signal-to-noise ratio makes it difficult to differentiate background contamination from the pathogenic organism.

In our custom-designed NanoString ocular panel, we included many pathogens that are associated with post-operative, post-injection, post-trauma, and endogenous en-

dophthalmitis (Figure 1, A). We avoided the use of probes that are universal to all bacteria or fungi that can result in contamination issues, and all probes designed and included in our panel target regions of genes that are either specific to a species or a group of organisms. Based on the distribution of species isolated from exogenous and endogenous infections in our service, our novel diagnostic panel covers >90% of the common etiologies of both bacterial and fungal endophthalmitis. These etiologies are also the most common causes of endophthalmitis seen in other hospitals in the United States and globally.[59-66] Samples collected from 12 culture-proven endophthalmitis cases were processed using our novel diagnostic approach. We found complete agreement between culture results and molecular identification at the genus level. At the species level, the correct signal was detected for the species-specific probe for all but 1 patient. Patient 13 presented with an endogenous endophthalmitis, and cultures from the vitreous sample grew a yeast isolate identified as *C albicans* using a rapid test for presumptive identification (Remel *Candida albicans* test kit). This kit is based on the colorimetric detection of 2 enzymes, L-proline aminopeptidase and β-galactosaminidase.[67] Although the combination of these enzymes is found consistently in *C albicans*, other related species can be positive as well.[67,68] By NanoString testing, we identified this as being a non-*albicans Candida* species, given that signal for multiple non-*albicans* probes was identified (Table 3 and Table S2). Together, data for the pilot clinical validation studies demonstrate that our novel diagnostic approach is feasible in detecting the common endophthalmitis-causing pathogens with a high degree of concordance when compared to culture. Follow-up studies to determine the value of this novel method in improving diagnostic yields among culture-negative samples are now being conducted.

In addition to the low sensitivity of pathogen detection, cultures rely on our ability to grow organisms in vitro, which can be time demanding especially for fastidious organisms, fungi, and specimens containing low numbers of viable cells. For our endophthalmitis sample population, the turnaround time for pathogen growth plus culture-based identification ranged from 2 to 5 days (median, 2.5 days). Using our custom-designed ocular panel and an extensively optimized approach that substantially reduces the processing time for intraocular fluids when compared to typical NanoString protocols, a complete diagnostic process, from sample preparation to data analysis, can be completed in only 12 hours, with analytical sensitivities for bacterial detection comparable to PCR.[12,13] These advantages could result in more patients being treated with targeted antimicrobial therapies at earlier stages of the infection, and could also aid in the decision to perform early ancillary therapeutic approaches such as vitrectomy and removal of intraocular lenses, depending on the causative agents found and their associated prognoses. Surgical treatment of endophthalmitis is still largely based on the recommendations of the Endophthalmitis Vitrectomy Study (EVS), published in 1995.[69] The EVS established that early vitrectomy provided no significant benefit compared to intravitreal injection of antibiotics alone for patients presenting with visual acuity better than light perception. However, more recent studies using updated surgical techniques (eg, small-gauge microincisional vitrectomy surgery) have shown that early vitrectomy can be beneficial, especially for patients infected with highly virulent and aggressive organisms.[70,71] Endophthalmitis caused by gram-negative organisms and streptococcal species are particularly associated with poor visual outcomes.[70] These organisms commonly cause infections following intravitreal injection of anti−vascular endothelial growth factor agents (streptococci),[72] filtering blebs resulting from glaucoma surgery (gram-negative bacteria and streptococci),[73] and are also associated with outbreaks secondary to contaminated solutions (gram-negative bacteria),[74] all clinical scenarios that were not addressed in the EVS study. In a retrospective review of visual outcomes and predictive factors in the treatment of endophthalmitis caused by various *Streptococcus* species, early vitrectomy was found to be the only variable to predict improved outcomes.[71] In recently published updated recommendations for early vitrectomy, one of the indications is patients at high risk for having an infection caused by more virulent organisms such as post−intravitreal injection, bleb-related and post-trauma endophthalmitis.[70] This indication is solely based on the knowledge of the spectrum of organisms causing these infections and the more commonly expected etiologies according to the category of infection. Having a test for rapid and accurate detection of endophthalmitis pathogens such as the novel diagnostic assay presented here could be an important allied tool to support early recommendations for vitrectomy and other surgical treatments.

At the antimicrobial resistance level, one of the *S epidermidis* isolated by culture (patient 16) was phenotypically resistant to methicillin, and this was captured at the genotypic level in our panel (*mec*A gene detected with an average probe count of 4071). The rationale to include the *mec*A gene in the panel stemmed from the fact that methicillin resistance is a key phenotype in staphylococci and is associated with higher rates of co-resistance to clinically relevant antimicrobials often used in ophthalmology.[75] In addition to being resistant to all β-lactam agents, this particular isolate of methicillin-resistant *S epidermidis* was also resistant to macrolides, lincosamides, and fluoroquinolones (data not shown). Determining whether or not a given staphylococci isolate is susceptible or resistant to methicillin could help one to infer additional resistances to antimicrobials that should be avoided clinically. Another bacterial factor that impacts treatment of endophthalmitis is biofilm formation on intraocular lenses (IOLs) and surrounding tissues.[76] Biofilms are aggregations of bacterial cells in different physiological states (ie, some are metabolically active whereas others are dormant) held together by

an extracellular matrix, which are more resistant to the action of most antimicrobials and the host immune system than are planktonic cells.[76] The involvement of biofilms in the pathogenesis of post–cataract surgery is significant, especially for infections caused by *S epidermidis*.[76] Removal of IOLs containing biofilms of gram-positive bacteria have been demonstrated to help control the inflammation.[77] Detection of the *ica* locus, involved in biofilm formation in *S epidermidis* and other related species, is supported by our panel, and could serve as an additional indicator for surgical interventions in recalcitrant cases of endophthalmitis caused by biofilm-producing organisms.

In summary, we have presented here the development, analytical validation, and initial clinical evaluation of an innovative all-in-one diagnostic approach using NanoString technology for rapid and sensitive detection of >90% of pathogens that cause sight-threatening intraocular infections. We have demonstrated that this approach can be used for comprehensive detection of a broad range of pathogens across different domains using only 1 assay, with PCR-level sensitivity and in a timely fashion. These advantages may translate into an improved diagnostic test for routine clinical application that can quickly provide critical and clinically actionable results for early management of infectious uveitis and endophthalmitis, and can help save vision by reducing delays in appropriate treatment. The encouraging results presented here form the basis for additional clinical validation studies needed for implementation of this novel diagnostic test in clinical laboratory routines in the near future.

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REFERENCES

1. Taravati P, Lam D, Van Gelder RN. Role of molecular diagnostics in ocular microbiology. *Curr Ophthalmol Rep*. 2013;1(4). doi:10.1007/s40135-013-0025-1.
2. Van Gelder RN. Molecular diagnostics for ocular infectious diseases: LXXVIII Edward Jackson Memorial Lecture. *Am J Ophthalmol*. 2022;235:300–312. doi:10.1016/j.ajo.2021.12.002.
3. Sallam AB, Kirkland KA, Barry R, Soliman MK, Ali TK, Lightman S. A review of antimicrobial therapy for infectious uveitis of the posterior segment. *Med Hypothesis Discov Innov Ophthalmol*. 2018;7(4):140–155.
4. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res*. 2005;36(6):697–705. doi:10.1016/j.arcmed.2005.06.009.
5. World Healh Organiztion (WHO). *Antimicrobial resistance: global report on surveillance*. World Heath Organization; 2014.
6. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 1988;239(4839):487–491. doi:10.1126/science.2448875.
7. Buchan BW, Ledeboer NA. Emerging technologies for the clinical microbiology laboratory. *Clin Microbiol Rev*. 2014;27(4):783–822. doi:10.1128/CMR.00003-14.
8. Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D. Modern clinical microbiology: new challenges and solutions. *Nat Rev Microbiol*. 2013;11(8):574–585. doi:10.1038/nrmicro3068.
9. Crouse CA, Pflugfelder SC, Pereira I, Cleary T, Rabinowitz S, Atherton SS. Detection of herpes viral genomes in normal and diseased corneal epithelium. *Curr Eye Res*. 1990;9(6):569–581. doi:10.3109/02713689008999597.
10. Fox GM, Crouse CA, Chuang EL, et al. Detection of herpesvirus DNA in vitreous and aqueous specimens by the polymerase chain reaction. *Arch Ophthalmol*. 1991;109(2):266–271.
11. Qavi HB, Green MT, SeGall GK, Font RL. Demonstration of HIV-1 and HHV-6 in AIDS-associated retinitis. *Curr Eye Res*. 1989;8(4):379–387. doi:10.3109/02713688908996385.
12. Anand AR, Madhavan HN, Therese KL. Use of polymerase chain reaction (PCR) and DNA probe hybridization to determine the Gram reaction of the infecting bacterium in the intraocular fluids of patients with endophthalmitis. *J Infect*. 2000;41(3):221–226. doi:10.1053/jinf.2000.0731.
13. Bispo PJ, de Melo GB, Hofling-Lima AL, Pignatari AC. Detection and gram discrimination of bacterial pathogens from aqueous and vitreous humor using real-time PCR assays. *Invest Ophthalmol Vis Sci*. 2011;52(2):873–881. doi:10.1167/iovs.10-5712.
14. Bispo PJM, Davoudi S, Sahm ML, et al. Rapid detection and identification of uveitis pathogens by qualitative multiplex real-time PCR. *Invest Ophthalmol Vis Sci*. 2018;59(1):582–589. doi:10.1167/iovs.17-22597.
15. Chiquet C, Cornut PL, Benito Y, et al. Eubacterial PCR for bacterial detection and identification in 100 acute post-cataract surgery endophthalmitis. *Invest Ophthalmol Vis Sci*. 2008;49(5):1971–1978. doi:10.1167/iovs.07-1377.
16. Dworkin LL, Gibler TM, Van Gelder RN. Real-time quantitative polymerase chain reaction diagnosis of infectious posterior uveitis. *Arch Ophthalmol*. 2002;120(11):1534–1539.
17. Goldschmidt P, Degorge S, Benallaoua D, et al. New test for the diagnosis of bacterial endophthalmitis. *Br J Ophthalmol*. 2009;93(8):1089–1095. doi:10.1136/bjo.2008.152181.
18. Knox CM, Chandler D, Short GA, Margolis TP. Polymerase chain reaction-based assays of vitreous samples for the diagno-

sis of viral retinitis. Use in diagnostic dilemmas. *Ophthalmology*. 1998;105(1):37–44 discussion 44-45.
19. Kumar A, Singh MP, Bansal R, Gupta A, Ram J, Ratho RK. Development and evaluation of multiplex real-time PCR for diagnosis of HSV-1, VZV, CMV, and Toxoplasma gondii in patients with infectious uveitis. *Diagn Microbiol Infect Dis*. 2017;89(3):191–196. doi:10.1016/j.diagmicrobio.2017.08.002.
20. Lohmann CP, Linde HJ, Reischl U. Improved detection of microorganisms by polymerase chain reaction in delayed endophthalmitis after cataract surgery. *Ophthalmology*. 2000;107(6):1047–1051 discussion 1051-2. doi:10.1016/s0161-6420(00)00083-x.
21. Mazoteras P, Bispo PJ, Hofling-Lima AL, Casaroli-Marano RP. DNA extraction methods for panbacterial and panfungal PCR detection in intraocular fluids. *Curr Eye Res*. 2015;40(7):697–706. doi:10.3109/02713683.2014.957327.
22. Mishra D, Satpathy G, Chawla R, Venkatesh P, Ahmed NH, Panda SK. Utility of broad-range 16S rRNA PCR assay versus conventional methods for laboratory diagnosis of bacterial endophthalmitis in a tertiary care hospital. *Br J Ophthalmol*. 2019;103(1):152–156. doi:10.1136/bjophthalmol-2018-312877.
23. Seal D, Reischl U, Behr A, et al. Laboratory diagnosis of endophthalmitis: comparison of microbiology and molecular methods in the European Society of Cataract & Refractive Surgeons multicenter study and susceptibility testing. *J Cataract Refract Surg*. 2008;34(9):1439–1450. doi:10.1016/j.jcrs.2008.05.043.
24. Selva Pandiyan A, Siva Ganesa Karthikeyan R, Rameshkumar G, Sen S, Lalitha P. Identification of bacterial and fungal pathogens by rDNA gene barcoding in vitreous fluids of endophthalmitis patients. *Semin Ophthalmol*. 2020;35(7-8):358–364. doi:10.1080/08820538.2020.1864416.
25. Sugita S, Shimizu N, Watanabe K, et al. Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR. *Br J Ophthalmol*. 2011;95(3):345–349. doi:10.1136/bjo.2009.171504.
26. Sugita S, Shimizu N, Watanabe K, et al. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. *Br J Ophthalmol*. 2008;92(7):928–932. doi:10.1136/bjo.2007.133967.
27. Valdes L, Bispo P, Sobrin L. Application of metagenomic sequencing in the diagnosis of infectious uveitis. *Semin Ophthalmol*. 2020;35(5-6):276–279. doi:10.1080/08820538.2020.1818795.
28. Greninger AL. The challenge of diagnostic metagenomics. *Expert Rev Mol Diagn*. 2018;18(7):605–615. doi:10.1080/14737159.2018.1487292.
29. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008;26(3):317–325. doi:10.1038/nbt1385.
30. Barczak AK, Gomez JE, Kaufmann BB, et al. RNA signatures allow rapid identification of pathogens and antibiotic susceptibilities. *Proc Natl Acad Sci U S A*. 2012;109(16):6217–6222. doi:10.1073/pnas.1119540109.
31. Bhattacharyya RP, Bandyopadhyay N, Ma P, et al. Simultaneous detection of genotype and phenotype enables rapid and accurate antibiotic susceptibility determination. *Nat Med*. 2019;25(12):1858–1864. doi:10.1038/s41591-019-0650-9.
32. Bhattacharyya RP, Walker M, Boykin R, et al. Rapid identification and phylogenetic classification of diverse bacterial pathogens in a multiplexed hybridization assay targeting ribosomal RNA. *Sci Rep*. 2019;9(1):4516. doi:10.1038/s41598-019-40792-3.
33. Huang B, West NP, Vider J, et al. Diagnosis and typing of influenza using fluorescent barcoded probes. *Sci Rep*. 2017;7(1):18092. doi:10.1038/s41598-017-18333-7.
34. Koehler JW, Douglas CE, Minogue TD. A highly multiplexed broad pathogen detection assay for infectious disease diagnostics. *PLoS Negl Trop Dis*. 2018;12(11):e0006889. doi:10.1371/journal.pntd.0006889.
35. Ung L, Belanger BA, Chodosh J, Gilmore MS, Bispo PJ. Novel molecular barcoding for rapid pathogen detection in infectious keratitis. *Ophthalmol Sci*. 2021;1(4):100066. doi:10.1016/j.xops.2021.100066.
36. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. *Clin Microbiol Rev*. 2010;23(3):550–576. doi:10.1128/CMR.00074-09.
37. Durand ML. Bacterial and fungal endophthalmitis. *Clin Microbiol Rev*. 2017;30(3):597–613. doi:10.1128/CMR.00113-16.
38. Durrani OM, Meads CA, Murray PI. Uveitis: a potentially blinding disease. *Ophthalmologica*. 2004;218(4):223–236. doi:10.1159/000078612.
39. Suttorp-Schulten MS, Rothova A. The possible impact of uveitis in blindness: a literature survey. *Br J Ophthalmol*. 1996;80(9):844–848.
40. Davis JL. Diagnostic dilemmas in retinitis and endophthalmitis. *Eye (Lond)*. 2012;26(2):194–201. doi:10.1038/eye.2011.299.
41. Moshfeghi DM, Dodds EM, Couto CA, et al. Diagnostic approaches to severe, atypical toxoplasmosis mimicking acute retinal necrosis. *Ophthalmology*. 2004;111(4):716–725. doi:10.1016/j.ophtha.2003.07.004.
42. Rodriguez A, Calonge M, Pedroza-Seres M, et al. Referral patterns of uveitis in a tertiary eye care center. *Arch Ophthalmol*. 1996;114(5):593–599.
43. Tsirouki T, Dastiridou A, Symeonidis C, et al. A focus on the epidemiology of uveitis. *Ocul Immunol Inflamm*. 2018;26(1):2–16. doi:10.1080/09273948.2016.1196713.
44. Lau CH, Missotten T, Salzmann J, Lightman SL. Acute retinal necrosis features, management, and outcomes. *Ophthalmology*. 2007;114(4):756–762. doi:10.1016/j.ophtha.2006.08.037.
45. Thorne JE, Jabs DA, Kempen JH, et al. Incidence of and risk factors for visual acuity loss among patients with AIDS and cytomegalovirus retinitis in the era of highly active antiretroviral therapy. *Ophthalmology*. 2006;113(8):1432–1440. doi:10.1016/j.ophtha.2006.03.021.
46. Tran TH, Rozenberg F, Cassoux N, Rao NA, LeHoang P, Bodaghi B. Polymerase chain reaction analysis of aqueous humour samples in necrotising retinitis. *Br J Ophthalmol*. 2003;87(1):79–83.
47. McCannel CA, Holland GN, Helm CJ, Cornell PJ, Winston JV, Rimmer TG. Causes of uveitis in the general practice of ophthalmology. UCLA Community-Based Uveitis Study Group. *Am J Ophthalmol*. 1996;121(1):35–46.
48. Harper TW, Miller D, Schiffman JC, Davis JL. Polymerase chain reaction analysis of aqueous and vitreous specimens in the diagnosis of posterior segment infectious uveitis. *Am J Ophthalmol*. 2009;147(1):140–147. doi:10.1016/j.ajo.2008.07.043.

49. Rothova A, de Boer JH, Ten Dam-van Loon NH, et al. Usefulness of aqueous humor analysis for the diagnosis of posterior uveitis. *Ophthalmology*. 2008;115(2):306–311. doi:10.1016/j.ophtha.2007.05.014.
50. Nakano S, Tomaru Y, Kubota T, et al. Evaluation of a multiplex strip PCR test for infectious uveitis: a prospective multicenter study. *Am J Ophthalmol*. 2020;213:252–259. doi:10.1016/j.ajo.2019.10.031.
51. McCann JD, Margolis TP, Wong MG, et al. A sensitive and specific polymerase chain reaction-based assay for the diagnosis of cytomegalovirus retinitis. *Am J Ophthalmol*. 1995;120(2):219–226.
52. Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM. Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis*. 2003;3:7. doi:10.1186/1471-2334-3-7.
53. Short GA, Margolis TP, Kuppermann BD, Irvine AR, Martin DF, Chandler D. A polymerase chain reaction-based assay for diagnosing varicella-zoster virus retinitis in patients with acquired immunodeficiency syndrome. *Am J Ophthalmol*. 1997;123(2):157–164.
54. Asano S, Yoshikawa T, Kimura H, et al. Monitoring herpesvirus DNA in three cases of acute retinal necrosis by real-time PCR. *J Clin Virol*. 2004;29(3):206–209. doi:10.1016/s1386-6532(03)00162-8.
55. Cottet L, Kaiser L, Hirsch HH, Baglivo E. HSV2 acute retinal necrosis: diagnosis and monitoring with quantitative polymerase chain reaction. *Int Ophthalmol*. 2009;29(3):199–201. doi:10.1007/s10792-008-9198-2.
56. Kido S, Sugita S, Horie S, et al. Association of varicella zoster virus load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. *Br J Ophthalmol*. 2008;92(4):505–508. doi:10.1136/bjo.2007.125773.
57. Miyanaga M, Sugita S, Shimizu N, et al. A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis. *Br J Ophthalmol*. 2010;94(3):336–340. doi:10.1136/bjo.2008.156422.
58. Kosacki J, Boisset S, Maurin M, et al. Specific PCR and quantitative real-time PCR in ocular samples from acute and delayed-onset postoperative endophthalmitis. *Am J Ophthalmol*. 2020;212:34–42. doi:10.1016/j.ajo.2019.11.026.
59. Grandi G, Bianco G, Boattini M, et al. Bacterial etiology and antimicrobial resistance trends in ocular infections: a 30-year study, Turin area, Italy. *Eur J Ophthalmol*. 2021;31(2):405–414. doi:10.1177/1120672119896419.
60. Joseph J, Sontam B, Guda SJM, et al. Trends in microbiological spectrum of endophthalmitis at a single tertiary care ophthalmic hospital in India: a review of 25 years. *Eye (Lond)*. 2019;33(7):1090–1095. doi:10.1038/s41433-019-0380-8.
61. Liu Q, Wan L, Zhou J, Huang Y. Ten-year analysis of pathogenic factors and etiological characteristics of endophthalmitis from a tertiary eye center in North China. *Infect Drug Resist*. 2022;15:3005–3012. doi:10.2147/IDR.S367222.
62. Lu LJ, Chen X, Adelman RA. Clinical etiologies, microbial spectrum, antibiotic susceptibilities, and visual acuity outcomes of acute endophthalmitis. *J Ocul Pharmacol Ther*. 2020;36(7):534–539. doi:10.1089/jop.2018.0074.
63. Malmin A, Syre H, Ushakova A, Utheim TP, Forsaa VA. Twenty years of endophthalmitis: incidence, aetiology and clinical outcome. *Acta Ophthalmol*. 2021;99(1):e62–e69. doi:10.1111/aos.14511.
64. Melo GB, Bispo PJ, Yu MC, Pignatari AC, Hofling-Lima AL. Microbial profile and antibiotic susceptibility of culture-positive bacterial endophthalmitis. *Eye (Lond)*. 2011;25(3):382–387 quiz 388. doi:10.1038/eye.2010.236.
65. Sanchez JM, Elinav H, Tiosano L, Amer R. Endophthalmitis panorama in the Jerusalem area. *Int Ophthalmol*. 2022;42(5):1523–1535. doi:10.1007/s10792-021-02142-z.
66. Schimel AM, Miller D, Flynn Jr HW. Endophthalmitis isolates and antibiotic susceptibilities: a 10-year review of culture-proven cases. *Am J Ophthalmol*. 2013;156(1):50–52. doi:10.1016/j.ajo.2013.01.027.
67. Dealler SF. Candida albicans colony identification in 5 minutes in a general microbiology laboratory. *J Clin Microbiol*. 1991;29(5):1081–1082. doi:10.1128/jcm.29.5.1081-1082.1991.
68. Carrillo-Munoz AJ, Quindos G, Cardenes CD, et al. Performance of Bacticard Candida compared with the germ tube test for the presumptive identification of Candida albicans. *Mycoses*. 2003;46(11-12):467–470. doi:10.1046/j.0933-7407.2003.00921.x.
69. Endophthalmitis Vitrectomy Study GroupResults of the Endophthalmitis Vitrectomy Study. A randomized trial of immediate vitrectomy and of intravenous antibiotics for the treatment of postoperative bacterial endophthalmitis. *Arch Ophthalmol*. 1995;113(12):1479–1496.
70. Shao EH, Yates WB, Ho IV, Chang AA, Simunovic MP. Endophthalmitis: changes in presentation, management and the role of early vitrectomy. *Ophthalmol Ther*. 2021;10(4):877–890. doi:10.1007/s40123-021-00406-6.
71. Yospaiboon Y, Meethongkam K, Sinawat S, et al. Predictive factors in the treatment of streptococcal endophthalmitis. *Clin Ophthalmol*. 2018;12:859–864. doi:10.2147/OPTH.S161217.
72. McCannel CA. Meta-analysis of endophthalmitis after intravitreal injection of anti-vascular endothelial growth factor agents: causative organisms and possible prevention strategies. *Retina*. 2011;31(4):654–661. doi:10.1097/IAE.0b013e31820a67e4.
73. Al-Turki TA, Al-Shahwan S, Al-Mezaine HS, Kangave D, Abu El-Asrar AM. Microbiology and visual outcome of bleb-associated endophthalmitis. *Ocul Immunol Inflamm*. 2010;18(2):121–126. doi:10.3109/09273940903370730.
74. Pathengay A, Flynn Jr HW, Isom RF, Miller D. Endophthalmitis outbreaks following cataract surgery: causative organisms, etiologies, and visual acuity outcomes. *J Cataract Refract Surg*. 2012;38(7):1278–1282. doi:10.1016/j.jcrs.2012.04.021.
75. Bispo PJM, Sahm DF, Asbell PA. A systematic review of multi-decade antibiotic resistance data for ocular bacterial pathogens in the United States. *Ophthalmol Ther*. 2022;11(2):503–520. doi:10.1007/s40123-021-00449-9.
76. Bispo PJ, Haas W, Gilmore MS. Biofilms in infections of the eye. *Pathogens*. 2015;4(1):111–136. doi:10.3390/pathogens4010111.
77. Suzuki T, Uno T, Kawamura Y, Joko T, Ohashi Y. Postoperative low-grade endophthalmitis caused by biofilm-producing coccus bacteria attached to posterior surface of intraocular lens. *J Cataract Refract Surg*. 2005;31(10):2019–2020. doi:10.1016/j.jcrs.2005.02.032.