Deep metagenomic sequencing for endophthalmitis pathogen detection using a nanopore platform

the West Midlands Collaborative Ophthalmology Network for Clinical Effectiveness & Research by Trainees (WM CONCERT); Low, Liying; Nakamichi, Kenji; Akileswaran, Lakshmi; Lee, Cecilia S.; Lee, Aaron Y.; Moussa, George; Murray, Phil; Wallace, Graham; Van Gelder, Russell N.; Rauz, Saaeha

DOI:
10.1016/j.ajo.2022.05.022

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
the West Midlands Collaborative Ophthalmology Network for Clinical Effectiveness & Research by Trainees (WM CONCERT), Low, L, Nakamichi, K, Akileswaran, L, Lee, CS, Lee, AY, Moussa, G, Murray, P, Wallace, G, Van Gelder, RN & Rauz, S 2022, 'Deep metagenomic sequencing for endophthalmitis pathogen detection using a nanopore platform', *American Journal of Ophthalmology*, vol. 242, pp. 243-251. https://doi.org/10.1016/j.ajo.2022.05.022

Deep Metagenomic Sequencing for Endophthalmitis Pathogen Detection Using a Nanopore Platform

LIYING LOW, KENJI NAKAMICHI, LAKSHMI AKILESWARAN, CECILIA S. LEE, AARON Y. LEE, GEORGE MOUSSA, PHILIP I. MURRAY, GRAHAM R. WALLACE, RUSSELL N. VAN GELDER[1], AND SAAEHA RAUZ[1], THE WEST MIDLANDS COLLABORATIVE OPHTHALMOLOGY NETWORK FOR CLINICAL EFFECTIVENESS & RESEARCH BY TRAINEES (WM CONCERT)

• PURPOSE: To evaluate the utility of nanopore sequencing for identifying potential causative pathogens in endophthalmitis, comparing culture results against full-length 16S rRNA nanopore sequencing (16S Nanopore), whole genome nanopore sequencing (Nanopore WGS), and Illumina (Illumina WGS).

• DESIGN: Cross-sectional diagnostic comparison.

• METHODS: Patients with clinically suspected endophthalmitis underwent intraocular vitreous biopsy as per standard care. Clinical samples were cultured by conventional methods, together with full-length 16S rRNA and WGS using nanopore and Illumina sequencing platforms.

• RESULTS: Of 23 patients (median age 68.5 years [range 47-88]; 14 males [61%]), 18 cases were culture-positive. Nanopore sequencing identified the same cultured organism in all of the culture-positive cases and identified potential pathogens in two culture-negative cases (40%). Nanopore WGS was able to additionally detect the presence of bacteriophages in three samples. The agreements at genus level between culture and 16S Nanopore, Nanopore WGS, and Illumina WGS were 75%, 100%, and 78%, respectively.

• CONCLUSIONS: Whole genome sequencing has higher sensitivity and provides a viable alternative to culture and 16S sequencing for detecting potential pathogens in endophthalmitis. Moreover, WGS has the ability to detect other potential pathogens in culture-negative cases. Whilst Nanopore and Illumina WGS provide comparable data, nanopore sequencing provides potential for cost-effective point-of-care diagnostics. (Am J Ophthalmol 2022;242: 243–251.

INTRODUCTION

Endophthalmitis is one of the most feared ocular infections, particularly following intraocular procedures.[1] It occurs secondary to pathogens entering the eye, either exogenously through a breach in the globe due to surgery or trauma, or endogenously via hematogenous spread from an infective focus elsewhere in the body, largely in immunocompromised patients.[1,2] Incidence rates following intraocular procedures range from 2.5 to 50 per 10,000 cases post-cataract surgery and 0.7 to 13 per 10,000 cases following intravitreal injections worldwide.[1,3-8] Despite aggressive medical and surgical interventions, endophthalmitis often results in permanent partial or total vision loss.

Conventional microbiology techniques in endophthalmitis rely on culture-based assays, but have low sensitivity, ranging between 40% to 70%.[3,4,9,10] Some causative pathogens, such as *Cutibacterium acnes* (*C. acnes*) are not easily cultured. PCR-based molecular tests are more sensitive but require prior hypothesis and only target known common pathogens.[11,12] DNA sequencing can be broadly classified into two techniques: targeted amplicon sequencing and untargeted whole genome sequencing (WGS). An example of targeted amplicon sequencing is the amplification of the universal 16S bacterial ribosomal RNA (rRNA) gene, which spans 1550 base pairs and comprises a highly conserved region interspersed with nine variable regions (V1-9), has been commonly used for assessing bacterial profiles.[13] However, due to the limitations in the Illumina sequencing platform whereby only short reads of < 500 base pairs are generated, only part of the 16S gene is able to be sequenced (eg, single variable region V4 or three vari-

AJO.com Supplemental Material available at AJO.com.

Accepted for publication May 25, 2022.

From the Academic Unit of Ophthalmology, Institute of Inflammation and Ageing, University of Birmingham, United Kingdom (L.L, P.I.M, G.R.W, S.R); Birmingham and Midland Eye Centre, Sandwell and West Birmingham Hospitals NHS Trust, Birmingham, United Kingdom (L.L, G.M, P.I.M, S.R); Department of Ophthalmology, University of Washington School of Medicine, Seattle, Washington, USA (K.N, L.A, C.S.L, A.Y.L, R.N.VG, S.R); Roger and Angie Karalis Retina Center at University of Washington

Inquiries to Saaeha Rauz, Academic Unit of Ophthalmology, Birmingham and Midland Eye Centre, Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Dudley Road, Birmingham B18 7QU, United Kingdom.; e-mail: s.rauz@bham.ac.uk

[1] Russell N. Van Gelder and Saaeha Rauz are joint senior authors

0002-9394/$36.00
https://doi.org/10.1016/j.ajo.2022.05.022

able regions V1-3), thus limiting the taxonomic resolution to genus-level classification.[14] Full-length 16S rRNA sequencing reads may provide better taxonomic resolution compared with reads that only target a certain region of the 16S gene.[15,16] Deep metagenomic WGS techniques have enabled the discovery of novel pathogens, provide better taxonomic resolution to species level, and may provide vital prognostic information for clinical outcomes.[10,17,18] Nevertheless, most clinical samples have high host DNA content and relatively lower abundance of pathogen DNA, and therefore require greater sequencing depth, leading to higher costs.[17,19] The prohibitively high running costs of Illumina sequencing platforms mean that these facilities are only available in select centers.[20]

The rapid diagnostics arena has recently seen the introduction of a portable, pocket-sized, relatively low-cost nanopore sequencer (Oxford Nanopore Technologies' MinION sequencer).[20] This technology measures the translocation of ionic currents as nucleotides pass through nanoscopic pores, which generates real-time sequencing data for analysis.[21] In comparison with Illumina short-read (~500 base pairs) sequencing platforms, nanopore sequencing is capable of generating very long reads (~1500 to 882k bases), allowing for better coverage of genomic elements.[22,23] Nanopore sequencing has been utilized for in-the-field point-of-care, real-time genomic surveillance of the Ebola, Zika, and SARS-CoV-2 viruses.[24–26] In small-scale pilot studies, nanopore sequencing has proven useful in profiling bacterial pathogens and their resistance genes directly from clinical samples of patients with respiratory tract infections, urinary tract infections, joint infections, and sepsis.[27-30] Additionally, the utility of full-length 16S rRNA nanopore sequencing has been evaluated for microbial keratitis.[31]

This study aimed to evaluate the utility of nanopore sequencing for identifying potential causative pathogens in endophthalmitis, comparing culture results against full-length 16S rRNA nanopore sequencing (16S Nanopore), whole genome nanopore sequencing (Nanopore WGS), and Illumina WGS to provide rapid point-of-care diagnostics.

METHODS

The study was approved by the United Kingdom Health Research Authority Ethics Committee [Commensal Organisms and Rapid Diagnosis of Ocular Infections (CORADAR); Reference: 11/EM/0274] and the Institutional Review Board from the University of Washington, Seattle, Washington, USA, and conducted in accordance with the Declaration of Helsinki.

• **PATIENT RECRUITMENT AND SAMPLE COLLECTION:** Patients (n = 11) presenting to the Birmingham and Midland Eye Centre, Birmingham, UK, with suspected post-procedural endophthalmitis were invited to participate in the study and written informed consent was obtained. DNA samples from intraocular fluid biopsy (aqueous humor or vitreous "tap") of patients with suspected endophthalmitis from a previously published USA cohort[10] (n = 12) were also used in this study. Patients underwent either intraocular fluid biopsy or pars plana vitrectomy according to the standard-of-care protocol for endophthalmitis at their respective clinical institutions. The vitreous tap was attempted and if unsuccessful, an aqueous tap was performed. The samples were sent for routine culture at the clinical microbiology laboratory at the respective recruiting centers in the USA and UK, and remaining samples for research were snap frozen, stored at –80 °C, and transported on dry ice to the University of Washington.

• **CLINICAL MICROBIOLOGY CULTURE:** One or two drops of intraocular fluid samples were inoculated onto agar plates and streaked out with a sterile loop for isolation of individual colonies.[32] Chocolate and blood agar were incubated at 35 to 37 °C at 5% to 10% CO_2 for 40 to 48 hours, fastidious anaerobe agar was incubated at 35 to 37 °C at anaerobic conditions for up to 10 days, and Sabouraud agar at 28 to 30 °C at atmospheric air for up to 5 days.[32]

• **HOST DNA ENRICHMENT BY SAPONIFICATION, DNA EXTRACTION, SEQUENCING, AND BIOINFORMATICS:** The intraocular fluid biopsy samples were processed for DNA extraction, nanopore full-length 16S rRNA, nanopore WGS, and Illumina WGS, as outlined in the Appendix.

• **NEGATIVE CONTROLS:** Negative control DNA extraction and sequencing were performed on reagents without a DNA template and processed in the same manner as the clinical intraocular fluid biopsy samples to account for any potential contamination.

• **DEFINITIONS:** The microorganism was considered to be a potential pathogen or credible "hit" on sequencing, taking into account the background contamination (ie, sequences present in the negative control samples) if:

1. The organism was known to be potentially pathogenic given the clinical context of the particular patient.[17]
2. The organism represented the highest bacterial load (for WGS) or most abundant reads (for 16S) in the sample.[17,33]

Bacterial load was defined as the median number of the presumed pathogen per recovered human genome by WGS,[10] calculated as:

$$\text{Bacterial load} = \frac{\text{Total number of pathogen reads}}{\text{Total number of human reads}} \times \frac{\text{Size of human genome}}{\text{Size of pathogen genome}}$$

Alignment breadth was calculated as:[10]

$$\text{Alignment breadth} = \frac{\text{Number of bases with } \geq \text{ 1 aligned base}}{\text{Size of reference pathogen genome}}$$

TABLE 1. Demographics and Clinical Details of 23 Patients

Sample ID	Gender	Age	Laterality	Clinical history	VA at presentation
1	M	84	Right	2 days post-phacoemulsification and IOL	HM
2	F	88	Left	10 days post left IOL exchange and anterior vitrectomy for subluxed IOL	PL
3	F	54	Right	2 days post IVT injection	HM
4	M	83	Left	3 days post IVT injection	HM
5	F	47	Left	Endogenous endophthalmitis secondary to *Escherichia coli* septicemia treated with antibiotics. PMH of renal transplant on tacrolimus	20/60
6	M	59	Left	14 days post corneal graft surgery	HM
7	F	75	Right	5 days post phacoemulsification and IOL	HM
8	F	87	Left	11 days post IVT injection	NPL
9	M	56	Left	3 days post-traumatic penetrating eye injury	HM
10	M	66	Left	3-day history of microbial keratitis and hypopyon treated with topical antibiotics. PMH rheumatoid arthritis on methotrexate and hydroxychloroquine	PL
11	M	62	Left	7 days post IVT injection	20/200
12	F	85	Right	5 days post phacoemulsification and IOL	LP
13	F	61	Left	2 days post phacoemulsification and IOL	HM
14	M	54	Left	1 day post IVT injection	CF
15	M	76	Left	3 days post IVT injection	HM
16	M	73	Left	5 days post phacoemulsification and IOL	HM
17	F	51	Right	5 days post IVT injection	HM
18	M	67	Left	6 days post phacoemulsification and IOL	CF
19	M	73	Left	8 days post phacoemulsification and IOL	HM
20	F	65	Right	18 days post phacoemulsification and IOL	HM
21	F	76	Right	4 days post phacoemulsification and IOL	CF
22	M	70	Right	9 days post IVT injection	20/40
23	M	61	Right	4 days post IVT injection	20/25

Abbreviations: CF = counting fingers, F = female, HM = hand movement, IOL = intraocular lens, IVT = intravitreal therapy, M = male, NPL = no perception of light, PL = perception of light, PMH = past medical history, VA = visual acuity

Alignment depth was calculated as:[10]

$$\text{Alignment depth} = \frac{\text{Sum of all aligned bases of the query sequence}}{\text{Length of regions with } \geq \text{ 1 aligned base of the reference sequence}}$$

Coverage was calculated as:[10]

$$\text{Coverage} = \text{Alignment depth} \times \text{Alignment breadth}$$

RESULTS

• **CLINICAL SAMPLES:** Twenty-three samples from 23 patients were used in the study, 22 of which were vitreous humor and one was aqueous humor. The patients had a median age of 68.5 (range 47-88) years and 14 were male (61%). The most common preceding clinical history was post-intravitreal injection in 10 patients (43%) followed by post-cataract surgery in nine patients (39%). The majority of the patients presented with visual acuity worse than 20/200 (87%) (Table 1).

• **AGREEMENT BETWEEN MICROBIAL CULTURE AND FULL-LENGTH 16S RRNA NANOPORE SEQUENCING:** Eighteen samples were culture-positive and five were culture-negative. The most commonly cultured organism was *Staphylococcus epidermidis* (*S. epidermidis*) in seven patients (39%), followed by other *Staphylococcus* spp. in three (17%), *Streptococcus* spp. in two (11%), and *Pseudomonas* spp. in two (11%) (Table 2).

Twenty samples had sufficient volume for full-length 16S rRNA nanopore sequencing. The predominant organism detected by full-length 16S rRNA nanopore sequencing agreed with culture results in 15 of 20 cases (75%) at genus level (Table 3).

Two culture-positive cases did not yield any significant organism on 16S rRNA nanopore sequencing: sample 1 grew *Pseudomonas aeruginosa*, whilst sample 6 grew *C. acnes*. In two culture-negative cases, 16S rRNA nanopore sequencing detected potential pathogens including *S. epidermidis* in sample 19 and polymicrobial *Massillia oculi* and *C. acnes* in sample 22. These two culture-negative samples were tested by Illumina WGS and the results agreed with 16S rRNA nanopore sequencing results. *S. epidermidis* was detected by Illumina WGS in sample 19. In sample 22, *C. acnes* was detected by Illumina WGS but *Massillia oculi* was not detected. One sample (sample 5) grew *Escherichia coli*

TABLE 2. Agreement Between Organism Cultured and Detected by 16S rRNA Nanopore Sequencing, Whole Genome Nanopore Sequencing, and Illumina Miseq

Sample ID	Organism cultured by conventional microbiology	Organism detected from 16S rRNA nanopore sequencing	Organism detected from whole genome nanopore sequencing	Organism detected by Illumina Miseq whole genome sequencing
1	*Pseudomonas aeruginosa*	No significant taxon	*Pseudomonas sp. LPH1*	*Pseudomonas flourescences*
2	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Pseudomonas pseudoalcaligenes*	*Pseudomonas aeruginosa*
3	No growth	No significant taxon	No significant taxon	No significant taxon
4	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
5	*Escherichia coli*	*Streptococcus sanguinis*	*Escherichia coli* *Streptococcus mitis*	No significant taxon
6	*Cutibacterium acnes*	No significant taxon	*Cutibacterium acnes*	No significant taxon
7	*Staphylococcus epidermidis*	*Staphylococcus saccharolyticus* *Staphylococcus epidermidis*	*Staphylococcus aureus*	No significant taxon
8	*Staphylococcus aureus*	*Staphylococcus aureus*	*Staphylococcus aureus* Phietavirus (*Staphylococcus bacteriophage*)	*Staphylococcus aureus*
9	No growth	No significant taxon	No significant taxon	No significant taxon
10	No growth	No significant taxon	No significant taxon	No significant taxon
11	*Staphylococcus aureus*	*Staphylococcus aureus*	*Staphylococcus aureus*	*Staphylococcus aureus*
12	Alpha-hemolytic streptococcus	*Streptococcus sanguinis*	*Streptococcus sanguinis*	*Streptococcus sanguinis*
13	*Streptococcus mitis*	*Streptococcus sanguinis*	*Streptococcus sanguinis* *Streptococcus virus 9874* (*Streptococcus bacteriophage*)	*Streptococcus sanguinis*
14	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*
15	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
16	*Staphylococcus epidermidis*	N/A	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
17	*Staphylococcus epidermidis*	N/A	*Staphylococcus epidermidis* Phietavirus (*Staphylococcus bacteriophage*)	*Staphylococcus epidermidis*
18	Coagulase-negative Staphylococcus	N/A	*Cutibacterium acnes* *Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*
19	No growth	*Staphylococcus epidermidis*	N/A	*Staphylococcus epidermidis*
20	*Staphylococcus lugdunesis*	*Staphylococcus lugdunesis*	N/A	*Staphylococcus lugdunesis*
21	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	N/A	*Staphylococcus epidermidis*
22	No growth	*Massillia oculi Cutibacterium acnes*	N/A	*Cutibacterium acnes*
23	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	N/A	*Staphylococcus epidermidis*

*N/A – denotes that there was insufficient sample to be processed for sequencing

TABLE 3. Comparison of Microbial Culture, Full-Length 16S Nanopore Sequencing, Nanopore and Illumina Whole Genome Sequencing (WGS) Results for Endophthalmitis

	16S Nanopore			Nanopore WGS			Illumina WGS		
	Positive	*Negative*	*Total*	*Positive*	*Negative*	*Total*	*Positive*	*Negative*	*Total*
Culture-positive	13	2	15	15	0	15	15	3	18
Culture-negative	2	3	5	0	3	3	2	3	5
Total	15	5	20	15	3	18	17	6	23

(*E. coli*) in culture, while the predominant organism detected by 16S nanopore sequencing was *Staphylococcus sanguinis*. Nanopore WGS for sample 5 detected polymicrobial *E. coli* and *Streptococcus mitis*; however, no significant taxon was detected by Illumina WGS.

• **AGREEMENT BETWEEN MICROBIAL CULTURE AND NANOPORE WGS:** Eighteen samples had sufficient volume for Nanopore WGS. The predominant genus detected by Nanopore WGS agreed with culture results in all cases (18 of 18) (Table 3). Speciation by Nanopore WGS agreed with culture results in 16 of 18 cases. The bacteriophage Staphylococcus phietavirus was detected in two cases of *Staphylococcus* endophthalmitis and *Streptococcus* virus 9874 (bacteriophage) was detected in one case of *Streptococcus* endophthalmitis through Nanopore WGS.

Nanopore sequencing reads of culture-positive cases were aligned to the corresponding reference genome of the predominant organism detected by Nanopore WGS (Supplementary **Table 1**). Low levels of alignment breadth were observed in samples from the UK (samples 1-11), ranging from 0.17% to 12.89%, and could either be due to application of DNase in the saponification process or bead-beating step in the DNA extraction process. These UK samples had relatively high bacterial load (by sequencing reads), with a median of 608.4 (range 5.62-418445.89). The samples from the USA cohort (samples 12-23) had higher levels of alignment breadth, ranging from 6.96% to 88.77%, compared with the UK samples.

• **AGREEMENT BETWEEN CULTURE AND ILLUMINA WGS:** All 23 samples were processed for Illumina WGS. The predominant organism detected by Illumina WGS agreed with culture results in 18 cases (78%) (Table 3). Illumina WGS detected the cultured pathogen as the predominant genus in 15 of 18 culture-positive samples (83%). The remaining three culture-positive samples grew *E. coli* (sample 5), *C. acnes* (sample 6), and *S. epidermidis* (sample 7), respectively, and were detected but did not constitute the most abundant read by Illumina WGS, as the predominant sequences were similar to the sequences in the negative controls. Fifteen samples were sufficient for culture, 16S Nanopore, Nanopore WGS, and Illumina WGS sequencing, with 11 of 15 (73%) in agreement between all four methods (Figure 1).

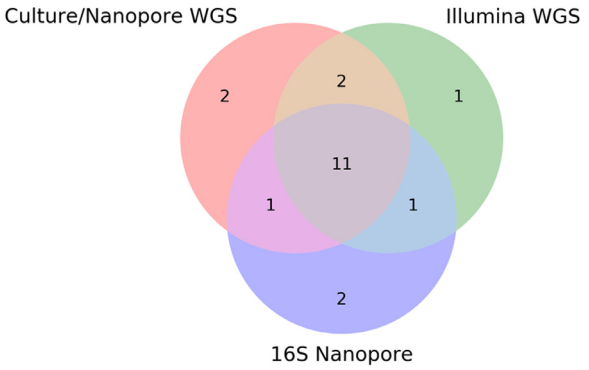

FIGURE 1. Venn diagram for agreement between microbial culture, full-length 16S nanopore sequencing (16S Nanopore), nanopore and Illumina whole genome sequencing (WGS) at genus level. Fifteen samples were sufficient for culture, 16S Nanopore, Nanopore WGS, and Illumina WGS sequencing, with 73% (11 of 15) agreement between all four methods. Culture and Nanopore WGS were in 100% agreement and are superimposed.

DISCUSSION

Of the 23 endophthalmitis cases in the present study, it was found that Illumina deep sequencing detected potential organisms in 17 cases (74%), 16S nanopore sequencing yielded potential organisms in 15 of 20 cases (75%), and nanopore WGS detected 15 of 18 cases (83%). Nanopore sequencing (16S and WGS together) identified the cultured organism in all of the culture-positive cases (18 of 18) and identified potential pathogens in two of five of culture-negative cases (40%). Nanopore WGS was able to additionally detect the presence of bacteriophages in three samples. The agreements at genus level between culture and 16S nanopore, Nanopore WGS, and Illumina WGS were 75%, 100%, and 78%, respectively. Taken together, these results suggest that nanopore sequencing may provide useful information on the pathogens associated with endophthalmitis.

In the present study, the concordance of whole genome nanopore and Illumina sequencing results with cultured organism was better than 16S amplicon sequencing. Previous

pilot studies on nanopore sequencing for endophthalmitis were based on amplicon sequencing, which utilizes PCR primers to amplify specific target regions of interest, such as 16S rRNA for bacteria or ITS for fungi. Jun and associates were able to identify pathogens in five cases of bacterial endophthalmitis and three cases of fungal endophthalmitis using 16S and ITS nanopore amplicon sequencing.[34] Similarly, Huang and associates identified pathogens in 17 of 18 cases of endophthalmitis using 16S, ITS, and rpoB gene nanopore amplicon sequencing.[35] Identification of organisms using targeted amplicon sequencing such as 16S is restricted by the primer sequence used, and quantification can be challenging given the highly variable per-genome copy number of the 16S rRNA gene.[15,36] Amplicon sequencing is also prone to bias and false-positive errors compounded by DNA amplification of contaminants and sequencing errors.[37,38] As modelled by Doan and associates, with an estimated polymerase error rate of 1×10^{-7}, an additional 27 new genera would be detected after 25 cycles of amplification.[38] Chimeric sequences can also produce spurious operational taxonomic units and be falsely classified as a novel organism.[37] Whole genome sequencing provides better bacterial taxonomic resolution to the level of species or strain compared with 16S sequencing, which is usually limited to genus level classification.[39] Furthermore, by sequencing the entire community of genomes, the sequences of all organisms including viruses and fungi could be captured, along with their functional genomic capacity and phylogeny (eg, the presence of antibiotic resistance genes or factors affecting pathogenicity). This could have clinical relevance for treatment, particularly in immunocompromised patients and endogenous endophthalmitis cases. To illustrate, in this study, the bacteriophage *Staphylococcus* phietavirus was identified in two cases of *Staphylococcus* endophthalmitis and the bacteriophage *Streptococcus* virus 9874 was identified in one case of *Streptococcus* endophthalmitis through WGS but not on 16S amplicon sequencing.

Two of the five culture-negative samples in this study revealed potential organisms on molecular sequencing. The 16S nanopore sequencing results agreed with Illumina short-read sequencing, suggesting an identification of a putative organism in the context of a false negative culture. The cause of endophthalmitis in the three culture-negative and sequencing-negative cases is unknown and could either be due to clearance of the organism by the host immune system prior to biopsy, extra-ocular source of infection not present in the aqueous or vitreous humor, or pathogen undetected due to potential bias in the study laboratory or bioinformatics workflow such as RNA virus or parasite.[10,28] Lee and associates pioneered the application of deep DNA sequencing (Biome Representational in Silico Karyotyping) to intraocular biopsies of patients with endophthalmitis and confirmed that culture-negative cases of presumed infectious endophthalmitis were either devoid of or have limited bacterial loads.[18] Additionally, they reported an unexpected finding of anellovirus (torque teno virus) in culture-negative endophthalmitis samples.[18] Seminal work by Lee and associates, on 50 endophthalmitis patients (24 culture-positive and 26 culture-negative) enrolled prospectively, demonstrated that the detection of pathogens and their bacterial load by Illumina WGS and directed PCR had prognostic significance for clinical outcomes in post-procedural endophthalmitis, where the presence of torque teno virus was associated with higher rates of retinal detachment and secondary intraocular surgery.[10]

Molecular sequencing technology has the potential to advance diagnostics for ocular infections.[10,17-19,40] Using metagenomic deep sequencing, Doan and associates were able to detect the presence of RV virus (*Rubivirus* genus) in the intraocular fluid sample of a patient with a 16-year history of idiopathic uveitis. By performing phylogenetic analysis of the genomic sequence in comparison with other RV strains deposited in public repositories (GenBank), along with estimates of the nucleotide substitution rate, they were able to approximate the time and place of when the patient might have been exposed to the virus.[17] Whole genome sequencing has shown a positive impact on clinical care in other medical specialties such as neurology and critical care: Wilson and associates showed that metagenomic sequencing of cerebrospinal fluid improved diagnosis and guided treatment in seven of 13 cases of infectious meningitis and encephalitis in a 1-year multi-center prospective clinical trial.[41]

In comparison with the large and expensive Illumina sequencing platforms (estimated cost for Illumina MiSeq sequencer is USD100,000), the nanopore MINion sequencer is pocket-sized, weighs < 450 g, costs < USD1000, and is able to provide rapid sequencing results, which would make it ideal to deliver point-of-care diagnostics.[21,42] The estimated cost of sequencing reagents (excluding labor) for Illumina and nanopore sequencing was USD170 and USD150 per sample, respectively,[43] whilst the median cost of conventional microbiology is between USD128 to 242.[44] A major advantage of nanopore sequencing is the rapid turnover time from sample collection to diagnosis, with a median of 6 to 8 hours compared with 48 hours to 5 days for full culture and sensitivities.[43] More recently, nanopore sequencing has provided genetic diagnosis in critically ill patients within 8 hours of sample collection.[45] The portability and ease of use of the nanopore MINion sequencing system was first demonstrated by Quick and associates, who successfully developed a portable Ebola virus genome surveillance system in Guinea, using just instruments, reagent, and disposable consumables that fit in an aircraft bag,[24] and could potentially be replicated in a community hospital setting.

Genomic sequencing of ocular clinical samples is extremely challenging, given the low volumes and high background of host DNA, which can lower the sensitivities for microbial detection.[17,31,46] Therefore, differential centrifugation and saponification methods have been used to enrich for microbial DNA.[28] The current study showed that there

was excellent genus level agreement between nanopore WGS and culture (15 of 15 in culture-positive cases; 3 of 3 in culture-negative cases). In clinical cases, genus level identification may be sufficient to guide long-term clinical therapy.[47] Reduced sensitivities at species level taxonomic identification may be attributed to the relatively higher error rate in nanopore compared with Illumina sequencing. The read accuracy for nanopore sequencing is lower, ranging from 80% to 98% compared with > 99.9% in Illumina sequencing; however, there is ongoing effort to overcome this, with improvements in pore chemistry and development of newer bioinformatics software.[42] Implementation of the Scalable Metagenomics Alignment Research Tool (SMART) metagenomic algorithm, which only considers 30-mer matches for speciation and incorporates human and mammalian sequence filtering, resulted in higher specificity for non-host taxonomic assignments.[48] In terms of taxa classification, Pearman and associates reported that longer reads improved the accuracy in taxa classification compared with short reads, albeit having higher sequencing error rates.[49] This might explain the higher agreement rates between culture and nanopore WGS compared with Illumina WGS seen in this study.

There were no observable differences in the identification of microbial sequences between aqueous or vitreous fluid biopsy samples using nanopore sequencing in the current study, which is in concordance with the previously published studies using Illumina sequencing.[10,17,19] The current study has also shown that nanopore sequencing could be directly applied to other clinical ocular samples with low biomass, such as conjunctival and corneal swabs.[31]

In the present study, nanopore WGS was able to reliably detect the pathogen and its bacterial load directly from intraocular fluid biopsy samples. This suggests that with further optimization, future use of the portable, real-time nanopore sequencing technology as point-of-care testing for pathogen identification and quantification may be beneficial in the initial management of endophthalmitis.[10]

There were several limitations to this study. First, there was limited sample size and limited amounts of ocular samples available for molecular sequencing. Ocular samples from the USA cohort were included that have previously been processed for Illumina WGS and other molecular testing; therefore, there was insufficient DNA leftover to perform both 16S and whole genome nanopore sequencing in certain cases. There could be potential impact from the saponification process or different DNA extraction kits used for both cohorts. However, the nanopore and Illumina sequencing protocol were completed in a single center to minimize any potential sequencing bias.

In summary, this study demonstrated the utility of nanopore sequencing in identifying potential pathogens and its bacterial load in endophthalmitis. Further optimization through the use of internal spike-in controls to determine the limit of detection and accuracy, single-use flowcell (Nanopore Flongle) to reduce cost and minimize risk of contamination between samples, miniaturization of laboratory steps through microfluidic devices, and automation of bioinformatic workflow (cloud-based, secure, and encrypted) may facilitate the adoption of nanopore sequencing as point-of-care testing in a clinical environment.[20] The combination of transcriptomic analyses of host immune responses and pathogenic virulence may elucidate the pathophysiologic mechanisms underpinning poor clinical outcomes and provide personalized, targeted treatment in endophthalmitis.

Funding/support: This study was funded through the Royal College of Ophthalmologists and Fight for Sight Ophthalmology Trainee Research Network Award (Ref. 24CO3), the National Institute for Health Research (NIHR) Surgical Reconstruction and Microbiology Research Centre (SRMRC)/Royal Centre for Defence Medicine, Ministry of Defence (UK) grant (Ref. RCDM—ADMST0003: 'Rapid Diagnosis of Corneal Infections'), and the Sandwell and West Birmingham Hospitals 2021 Ophthalmology Research and Development fund. Dr Liying Low was funded by a Fight for Sight Clinical Research Fellowship (Ref. 1840/41) and is a recipient of the National Institute for Health Research (NIHR) Clinical Lectureship. Dr Cecilia Lee is supported by an National Institutes of Health (NIH) Research Career Development Award (Ref. K23EY02492). Dr Aaron Lee is supported by grants from the NIH/National Eye Institute (NEI) Research Career Development Award (Ref. K23EY029246), National Institute on Ageing (NIA)/NIH (Ref. U19AG066567) and Research to Prevent Blindness, Alcon Research Institute, and Latham Vision Funds. Professor Russell N Van Gelder is supported by an unrestricted grant from Research to Prevent Blindness and the Mark J. Daily, MD Research Fund. Miss Saaeha Rauz is supported by the Medical Research Council Developmental Pathway Funding Scheme "Development of a synthetic biomembrane dressing that prevents corneal scarring" (Ref. MR/N019016/1). **Financial disclosures:** Dr Aaron Lee has received grants/contracts from Lowy Medical Research Institute, Carl Zeiss Meditec, Microsoft, and Santen with payments made to the University of Washington, Seattle. Dr Aaron Lee has received payments or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Bascom Palmer Eye Institute, Harvard Children's Hospital, iVista Medical Education, Inc, Topcon and Tufts Medical Center. Dr Aaron Lee has leadership roles in the Collaborative Community on Ophthalmic Imaging (unpaid member of the Executive Committee), and American Academy of Ophthalmology (unpaid chair of Information Technology). Dr Aaron Lee has received payments from the University of Washington (Latham Vision Research Innovation Award), US Food and Drug Administration, and Verana Health. Professor Philip I. Murray receives royalties from the Oxford University Press (Oxford Handbook of Ophthalmology 4th Edition), and has received payment or honoraria for presentation from Scope Eyecare. All authors attest that they meet the current ICMJE criteria for authorship. **Other acknowledgements:** We would like to thank Angela F. Sandt, Radoslaw Poplawski, and Zahira Maqsood for their contribution to this project. Dr Cecilia Lee is supported by grants from the National Eye Institute (NEI) Research Career Development Award (Ref. K23EY02492), National Institute on Ageing (NIA) (Ref. R01AG060942, U19AG066567) and Latham Vision Funds.

The West Midlands Collaborative Ophthalmology Network for Clinical Effectiveness & Research by Trainees (WM CONCERT) collaborators: George Moussa, Pavitra Garala, Li Jiang, Sam Yuen Sum Lee, Ian de Silva, Aaron Ng, William Fusi-Rubiano, Yu Jeat Chong, Jesse Panthagani, Xiao Li Chen, Maninee Purohit, Hetvi Bhatt, Hemalatha Kolli, Usama Kanj, Richard Blanch, and Sreekanth Sreekantam.

Data Availability: Data are available upon request.

REFERENCES

1. Relhan N, Forster RK, Flynn HW. Endophthalmitis: then and now. *Am J Ophthalmol*. 2018;187 xx-xxvii. doi:10.1016/j.ajo.2017.11.021.
2. Callegan MC, Engelbert M, Parke DW, Jett BD, Gilmore MS. Bacterial endophthalmitis: Epidemiology, therapeutics, and bacterium-host interactions. *Clin Microbiol Rev*. 2002;15(1):111–124. doi:10.1128/CMR.15.1.111-124.2002.
3. Moussa G, Bhatt H, Reekie I, et al. Using the West Midlands CONCERT to characterise regional incidence of acute-onset post cataract surgery endophthalmitis. *Eye*. 2020:1–11. doi:10.1038/s41433-020-01158-6.
4. Barry P, Gettinby G, Lees F, et al. Prophylaxis of postoperative endophthalmitis following cataract surgery: results of the ESCRS multicenter study and identification of risk factors. *J Cataract Refract Surg*. 2007. doi:10.1016/j.jcrs.2007.02.032.
5. Coleman AL. How big data informs us about cataract surgery: the LXXII Edward Jackson memorial lecture. *Am J Ophthalmol*. 2015. doi:10.1016/j.ajo.2015.09.028.
6. Diago T, McCannel CA, Bakri SJ, Pulido JS, Edwards AO, Pach JM. Infectious endophthalmitis after intravitreal injection of antiangiogenic agents. *Retina*. 2009. doi:10.1097/IAE.0b013e31819d2591.
7. Klein KS, Walsh MK, Hassan TS, et al. Endophthalmitis after anti-VEGF injections. *Ophthalmology*. 2009. doi:10.1016/j.ophtha.2009.02.031.
8. Yannuzzi NA, Si N, Relhan N, et al. Endophthalmitis after clear corneal cataract surgery: outcomes over two decades. *Am J Ophthalmol*. 2017;174:155–159. doi:10.1016/j.ajo.2016.11.006.
9. Johnson MW, Doft BH, Kelsey SF, et al. The endophthalmitis vitrectomy study: Relationship between clinical presentation and microbiologic spectrum. *Ophthalmology*. 1997;104(2):261–272. doi:10.1016/S0161-6420(97)30326-1.
10. Lee CS, Hong B, Kasi SK, et al. Prognostic utility of whole-genome sequencing and polymerase chain reaction tests of ocular fluids in postprocedural endophthalmitis. *Am J Ophthalmol*. 2020. doi:10.1016/j.ajo.2020.03.008.
11. Taravati P, Lam D, Van Gelder RN. Role of molecular diagnostics in ocular microbiology. *Curr Ophthalmol Rep*. 2013;1(4):181–189. doi:10.1007/s40135-013-0025-1.
12. Kim E, Chidambaram JD, Srinivasan M, et al. Prospective comparison of microbial culture and polymerase chain reaction in the diagnosis of corneal ulcer. *Am J Ophthalmol*. 2008;146(5):714–723 e1. doi:10.1016/j.ajo.2008.06.009.
13. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev*. 2004;17(4):840–862. doi:10.1128/CMR.17.4.840-862.2004.
14. Johnson JS, Spakowicz DJ, Hong BY, et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun*. 2019;10(1):1–11. doi:10.1038/s41467-019-13036-1.
15. Winand R, Bogaerts B, Hoffman S, et al. Targeting the 16s rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (illumina) and third (Oxford nanopore technologies) generation sequencing technologies. *Int J Mol Sci*. 2020. doi:10.3390/ijms21010298.
16. Nygaard AB, Tunsjø HS, Meisal R, Charnock C. A preliminary study on the potential of Nanopore MinION and Illumina MiSeq 16S rRNA gene sequencing to characterize building-dust microbiomes. *Sci Rep*. 2020;10(1):3209. doi:10.1038/s41598-020-59771-0.
17. Doan T, Wilson MR, Crawford ED, et al. Illuminating uveitis: metagenomic deep sequencing identifies common and rare pathogens. *Genome Med*. 2016. doi:10.1186/s13073-016-0344-6.
18. Lee AY, Akileswaran L, Tibbetts MD, Garg SJ, Van Gelder RN. Identification of torque teno virus in culture-negative endophthalmitis by representational deep DNA sequencing. *Ophthalmology*. 2015;122(3):524–530. doi:10.1016/j.ophtha.2014.09.001.
19. Kirstahler P, Bjerrum SS, Friis-Møller A, et al. Genomics-based identification of microorganisms in human ocular body fluid. *Sci Rep*. 2018;8(1):1–14. doi:10.1038/s41598-018-22416-4.
20. Chiu CY, Miller SA Nature Publishing Group; 2019:341–355.
21. MinION | Oxford Nanopore Technologies. https://nanoporetech.com/products/minion. [Accessed October 30, 2020].
22. Jain M, Koren S, Miga KH, et al. Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nat Biotechnol*. 2018;36(4):338–345. doi:10.1038/nbt.4060.
23. Amarasinghe SL, Su S, Dong X, Zappia L, Ritchie ME, Gouil Q. Opportunities and challenges in long-read sequencing data analysis. *Genome Biol*. 2020;21(1):1–16. doi:10.1186/s13059-020-1935-5.
24. Quick J, Loman NJ, Duraffour S, et al. Real-time, portable genome sequencing for Ebola surveillance. *Nature*. 2016;530(7589):228–232. doi:10.1038/nature16996.
25. Faria NR, Quick J, Claro IM, et al. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. *Nature*. 2017;546(7658):406–410. doi:10.1038/nature22401.
26. Roll-out of 2 new rapid coronavirus tests ahead of winter - GOV.UK. https://www.gov.uk/government/news/roll-out-of-2-new-rapid-coronavirus-tests-ahead-of-winter. [Accessed October 30, 2020].
27. Schmidt K, Mwaigwisya S, Crossman LC, et al. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. *J Antimicrob Chemother*. 2017. doi:10.1093/jac/dkw397.
28. Charalampous T, Kay GL, Richardson H, et al. Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol*. 2019;37(7):783–792. doi:10.1038/s41587-019-0156-5.
29. Akram A, Maley M, Gosbell I, Nguyen T, Chavada R. Utility of 16S rRNA PCR performed on clinical specimens in patient management. *Int J Infect Dis*. 2017;57:144–149. doi:10.1016/j.ijid.2017.02.006.
30. Leggett RM, Alcon-Giner C, Heavens D, et al. Rapid MinION profiling of preterm microbiota and antimicrobial-resistant pathogens. *Nat Microbiol*. 2019. doi:10.1038/s41564-019-0626-z.
31. Low L, Fuentes-Utrilla P, Hodson J, et al. Evaluation of full-length nanopore 16S sequencing for detection of pathogens in microbial keratitis. *Peer J*. 2021;9:e10778. doi:10.7717/peerj.10778.
32. Public Health England. *Investigation of Bacterial Eye Infections*; 2017.

33. Seitzman GD, Hinterwirth A, Zhong L, et al. Metagenomic deep sequencing for the diagnosis of corneal and external disease infections. *Ophthalmology*. 2019;126(12):1724–1726. doi:10.1016/j.ophtha.2019.06.013.
34. Il Jun K, BL Oh, Kim N, Shin JY, Moon J. Microbial diagnosis of endophthalmitis using nanopore amplicon sequencing. *Int J Med Microbiol*. 2021;311(4):151505. doi:10.1016/J.IJMM.2021.151505.
35. Huang Q, Fu A, Wang Y, Zhang J, Zhao W, Cheng Y. Microbiological diagnosis of endophthalmitis using nanopore targeted sequencing. *Clin Experiment Ophthalmol*. 2021;49(9):1060–1068. doi:10.1111/CEO.13992.
36. Stoddard SF, Smith BJ, Hein R, Roller BRK, Schmidt TM. rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res*. 2015;43(D1):D593–D598. doi:10.1093/nar/gku1201.
37. Haas BJ, Gevers D, Earl AM, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res*. 2011;21(3):494. doi:10.1101/GR.112730.110.
38. Doan T, Akileswaran L, Andersen D, et al. Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. *Investig Ophthalmol Vis Sci*. 2016;57(13):5116–5126. doi:10.1167/iovs.16-19803.
39. Cuscó A, Catozzi C, Viñes J, Sanchez A, Francino O. Microbiota profiling with long amplicons using Nanopore sequencing: full-length 16S rRNA gene and the 16S-ITS-23S of the rrn operon. *F1000Research*. 2019;7:1755. doi:10.12688/f1000research.16817.2.
40. Lalitha P, Seitzman GD, Kotecha R, et al. Unbiased pathogen detection and host gene profiling for conjunctivitis. *Ophthalmology*. 2019. doi:10.1016/j.ophtha.2019.03.039.
41. Wilson MR, Sample HA, Zorn KC, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med*. 2019;380(24):2327–2340. doi:10.1056/NEJMOA1803396/SUPPL_FILE/NEJMOA1803396_DISCLOSURES.PDF.
42. Logsdon GA, Vollger MR, Eichler EE. Long-read human genome sequencing and its applications. *Nat Rev Genet*. 2020. doi:10.1038/s41576-020-0236-x.
43. Gu W, Deng X, Lee M, et al. Rapid pathogen detection by metagenomic next-generation sequencing of infected body fluids. doi:10.1038/s41591-020-1105-z
44. Dempsey C, Skoglund E, Muldrew KL, Garey KW. Economic health care costs of blood culture contamination: A systematic review. *Am J Infect Control*. 2019;47(8):963–967. doi:10.1016/J.AJIC.2018.12.020.
45. Gorzynski JE, Goenka SD, Shafin K, et al. Ultrarapid nanopore genome sequencing in a critical care setting. January 2022. doi:10.1056/NEJMC2112090.
46. Ung L, Bispo PJM, Doan T, et al. Clinical metagenomics for infectious corneal ulcers: rags to riches? *Ocul Surf*. 2020;18(1):1–12. doi:10.1016/j.jtos.2019.10.007.
47. Chen KJ, Sun MH, Hou CH, et al. Susceptibility of bacterial endophthalmitis isolates to vancomycin, ceftazidime, and amikacin. *Sci Reports 2021 111*. 2021;11(1):1–8. doi:10.1038/s41598-021-95458-w.
48. Lee AY, Lee CS, Van Gelder RN. Scalable metagenomics alignment research tool (SMART): A scalable, rapid, and complete search heuristic for the classification of metagenomic sequences from complex sequence populations. *BMC Bioinformatics*. 2016;17(1):1–12. doi:10.1186/s12859-016-1159-6.
49. Pearman WS, Freed NE, Silander OK. Testing the advantages and disadvantages of short- And long- read eukaryotic metagenomics using simulated reads. *BMC Bioinformatics*. 2020;21(1):220. doi:10.1186/s12859-020-3528-4.