• Clinical Research •

Nanopore targeted sequencing identifies pathogens in patients with postoperative endophthalmitis

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Abstract

● **AIM:** To estimate if nanopore targeted sequencing (NTS) could identify pathogens causing postoperative endophthalmitis and further determine the feasibility of clinical application of NTS.

● **METHODS:** A total of 55 patients (55 eyes) with postoperative endophthalmitis were retrospectively included in this study with their medical records. Intraocular fluid samples were examined by NTS and microbial culture. All included patients had undergone examinations including measurement of best corrected visual acuity (BCVA) and intraocular pressure (IOP), slit-lamp biomicroscopy, and indirect ophthalmoscopy; additionally, they underwent B-ultrasound, anterior segment photography, and fundus photography if necessary.

● **RESULTS:** Among 55 patients with postoperative endophthalmitis, the age was 65.25±15.04y and there were 30 female (54.54%) patients. Forty-one (74.54%) vitreous humor samples and fourteen (25.45%) aqueous humor samples were sent for both NTS and microbial culture. NTS had a notable higher detection rate than microbial culture in detecting pathogens (90.91% *vs* 38.18%, χ^2=33.409, *P*<0.001). NTS exhibited high sensitivity of pathogen detection in both microbial culture positive and negative samples (100% and 85.29%, respectively). In 16 of 21 (76.19%) patients who showed culture-positivity, their results corresponded with those of NTS. Moreover, in two patients (9.52%), NTS showed a better species resolution than microbial culture; in three patients (14.28%), NTS identified additional pathogens. As for fungus, the positive detection rate of NTS was significantly higher than that of microbial culture (20% *vs* 3.64%, χ^2=7.066, *P*=0.008). Also, NTS could detect multi-infection by bacteria and fungi than microbial culture (32.73% *vs* 0, χ^2=21.522, *P*<0.001). NTS could detect bacteria as well as fungi simultaneously within 48h in all patients. Meanwhile, NTS had a shorter detection time than microbial culture (1.13±0.34 *vs* 2.67±0.55d, *Z*=-9.218, *P*<0.001). After the NTS results were obtained, 15 patients received additional intravitreal/intracameral anti-infection treatment. At follow-up, there was a statistically significant improvement in the visual acuity relative to the baseline (*Z*=−5.222, *P*<0.001).

● **CONCLUSION:** NTS can provide rapid identification and highly sensitive detection of pathogens among patients with postoperative endophthalmitis, which can guide anti-infection treatment and improve visual prognosis.

● **KEYWORDS:** postoperative endophthalmitis; nanopore targeted sequencing; third-generation sequencing; microbial culture

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INTRODUCTION

Endophthalmitis, a rare (0.02%–0.3%) but extremely devastating disease, is mainly caused by the infection of bacteria and fungi, and it often leads to irreversible vision loss[1-3]. Most cases of endophthalmitis are those of exogenous endophthalmitis, which involves the introduction of pathogens on the ocular surface or in the environment into the eye[1]. Depending on the origin of risk factors, exogenous

endophthalmitis is further divided into three categories: postoperative endophthalmitis, post-traumatic endophthalmitis, and keratitis-related endophthalmitis. Postoperative endophthalmitis is a major type of endophthalmitis with incidence rate varying from 40% to 80%[1]. Although the incidence of post-cataract endophthalmitis is 0.14%, it will lead to vision loss that impairs patient's quality of life[3]. Timely and accurate diagnosis and identification of microbial etiology is critical for the treatment and improvement of outcomes. Currently, the diagnosis of postoperative endophthalmitis is made according to microbial culture using aqueous humor (AH) or vitreous humor (VH). However, due to the varying positive rate of microbial culture (40% to 70%), negative culture results do not rule out the diagnosis[1,4]. Meanwhile, because of the limited volume of intraocular samples and the lack of confirmatory tests for rare pathogens, it remains a challenge to identify pathogens using traditional tests such as microbial culture and polymerase chain reaction (PCR) in patients with postoperative endophthalmitis[5-6].

With the advancement of sequencing technology and micro-sampling technology, it becomes feasible to identify ocular pathogens using a small quantity of intraocular fluid[7-8]. Metagenomic sequencing is able to detect all pathogens in samples without any bias and identify novel pathogens that are not currently associated with endophthalmitis. Therefore, it can efficiently facilitate the diagnosis and determine the pathogen of endophthalmitis. Nanopore sequencing technology is one of the most advanced sequencing technologies with high cost-effectiveness, fast turn-around time, low difficulty of bioinformatics analysis, and convenient portability[9-10]. It can not only identify microbial species but also provide information on virulence, drug resistance, metabolism, *etc*[9,11]. Previous studies have advocated the feasibility of nanopore sequencing technology for the diagnosis of infectious diseases in other systems[12-17]. Using bacterial 16S rRNA and fungal internal transcribed spacers (ITS) as target genes, we creatively combined a nanopore sequencer and targeted sequencing technique, namely nanopore targeted sequencing (NTS), to identify bacteria or fungi in AH or VH samples collected from 55 patients with postoperative endophthalmitis. By comparing the sensitivity and accuracy of NTS and microbial culture, this study aimed to evaluate the feasibility of NTS for identifying pathogens causing postoperative endophthalmitis.

PARTICIPANTS AND METHODS

Ethical Approval This study was conducted in compliance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Renmin Hospital of Wuhan University (WDRY2021-KS054). Informed consent form and surgical consent form were obtained from each participant.

Enrollment of Patients This retrospective study included patients who were clinically diagnosed with postoperative endophthalmitis from January 2019 to June 2022 in the Department of Ophthalmology, Renmin Hospital of Wuhan University, Wuhan, China.

Patients were included according to the following criteria: 1) having a history of intraocular surgery and clinical diagnosis of postoperative endophthalmitis; 2) receiving NTS and microbiology culture for identifying pathogens; 3) having complete medical records; 4) regularly followed up for at least 3mo. Patients with viral or parasitic infection, endogenous endophthalmitis, post-traumatic endophthalmitis, keratitis-related endophthalmitis, incomplete medical records and insufficient follow-up time were excluded. All included patients had undergone examinations including measurement of best corrected visual acuity (BCVA) and intraocular pressure (IOP), slit-lamp biomicroscopy, and indirect ophthalmoscopy; additionally, they underwent B-ultrasound, anterior segment photography, and fundus photography if necessary.

Vision Assessment The Snellen visual acuity chart was used to evaluate the BCVA, and the obtained results were converted into logarithmic minimal angle of resolution (logMAR) for statistical analysis. Non-Snellen vision acuities were recorded as follows: patients whose visual acuities were at the levels of counting fingers, hand movement, light perception and no light perception were assigned the values of 2.0, 2.3, 2.6, and 2.9, respectively[18]. Regarding follow-up BCVA and IOP, due to the impact of the coronavirus, some patients could not return to our hospital on time, so they received examinations in the local hospital, and the relevant data were obtained through phone calls.

Biological Sample Collection Intraocular specimens were collected under strictly sterile conditions. AH or VH samples were obtained using vitrectomy and other surgical procedures in the operating room or by anterior chamber paracentesis conducted under a slit-lamp microscope. Specimens were stored in sterile EP tubes at a low temperature (2℃-8℃).

NTS and Contamination Control The NTS procedure was performed as previously described[19]. All samples were centrifuged at 20 000×g for 10min, and the supernatant was discarded. The precipitate was reserved for nucleic acid extraction. Microbial DNA was extracted using a Sanure DNA extraction kit (Sansure Biotech Inc., Changsha, Hunan) and stored at -20℃. Amplification products of 16S rRNA and ITS1/2 were mixed at a mass ratio of 10:3 using a 1D Ligation kit (SQK-LSK109, Oxford Nanopore Technologies, Oxford, UK). The library was sequenced using the Oxford Nanopore MinION or GridION sequencer (Oxford Nanopore Technologies, Oxford, UK).

Albacore v2.3.1 software was used to analyze basecall and demultiplex Fast5 files generated by MinION or GridION, and

reads with a quality of <7 was filtered out. Porechop (v.0.2.4) was also used to trim barcodes and adapter sequences from raw data. The filtered sequenced reads were then mapped, using Blast (v.2.9.0+), to the reference database downloaded by NCBI FTP (ftp://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci) 16S rDNA/ITS reference database for comparison, so as to classify each read.

The requirements for the "Contamination Control" section were detailed in our previous article[20].

Statistical Analysis Statistical analysis was performed using Statistical Package for the Social Sciences 26.0 software (SPSS Inc., Chicago, Illinois, USA). Data were analyzed using the Mann-Whitney *U* test, Wilcoxon signed rank test, spearman correlation analysis, and Chi-squared test. Two-tailed $P<0.05$ was considered statistically significant.

RESULTS

Demographic Characteristics of Patients Fifty-five eyes of 55 consecutive patients with postoperative endophthalmitis were included in this single-center study. Clinical characteristics of patients were listed in Table 1. Among them, 32 and 23 were right and left eyes, respectively; the average age was 65.25±15.04y; 25 (45.45%) were men and 30 (54.54%) were women. Most patients (43 of 55, 78.18%) had short-term visual improvement, whereas 8 (14.54%) and 3 (5.45%) patients showed no change and deterioration, respectively, and 1 (1.82%) patient could not cooperate with the visual examination. In one case (Patient 49), eyeball exenteration was performed due to uncontrolled ocular infection. At follow-up, there was a statistically significant improvement in the BCVA (logMAR 1.51±0.76) relative to the baseline BCVA (logMAR 2.11±0.60, $Z=-5.222$, $P<0.001$), and they had a significant correlation ($r_s=0.706$, $P<0.001$).

Twelve eyes (21.82%) had ocular hypertension with an IOP of >21 mm Hg at baseline. All patients had normal IOP at follow-up, except for one patient (Patient 6) whose IOP was still higher than the normal value. For this patient, ophthalmologists recommended pars plana vitrectomy (PPV) surgery, however, the patient rejected PPV because of economic constraints, and received intracameral and intravitreal injections to alleviate eye pain. This patient was discharged from the hospital after her ocular pain had subsided.

Ocular manifestations were determined mainly based on intraoperative findings, especially during PPV. If PPV was not performed, preoperative fundus appearance and results of B-ultrasound examination prevailed. Inflammatory exudate was detected in the anterior chamber in all 55 eyes, and hypopyon was detected in 25 eyes (45.45%). All but three patients (94.54%) had exudate or purulence in the vitreous cavity, suggesting vitritis. Therefore, the clinical diagnosis of postoperative endophthalmitis was made for these patients.

Identification of Pathogens by Microbial Culture and NTS To identify pathogens causing postoperative endophthalmitis, 55 samples were sent for microbial culture and NTS, including 41 (74.54%) VH samples and 14 (25.45%) AH samples. The results of microbial culture (smear microscopy data attached) were shown in Table 2. The positivity rate of microbial culture was 38.18% (21/55). In 21 positive samples, only one pathogen was identified per sample, including 19 bacteria (90.48%) and 2 fungi (9.52%). The bacteria included *Streptococcus sanguis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Cutibacterium acnes*, *Escherichia coli* and Gram-negative bacilli as revealed by smear microscopy and the fungi included *Candida albicans* and *Candida parapsilosis*. However, we could not simultaneously isolate two or more pathogens from one sample (0). Fungi were identified as etiological agents in 3.64% (2/55) of these patients with postoperative endophthalmitis.

In contrast, NTS identified pathogens in 50 of 55 specimens, which included 21 culture-positive and 29 culture-negative specimens (Table 2). Hence, NTS had a significantly higher detection rate than microbial culture (90.91% *vs* 38.18%, $\chi^2=33.409$, $P<0.001$). In 50 positive samples, 32 cases had single bacterial infection, whereas 7 cases had multiple bacterial infection and 11 cases had a mixed bacterial and fungal infection. No sample showed single or multiple fungal infection. The detection rate of fungus by NTS was notably higher than microbial culture (20% *vs* 3.64%, $\chi^2=7.066$, $P=0.008$). Moreover, NTS was able to identify multiple infection, which could not be obtained by microbial culture (32.73% *vs* 0, $\chi^2=21.522$, $P<0.001$). For the remaining 5 negative samples, they had the same results as microbial culture, indicating a true negative result.

Additionally, since the cultivation time for bacteria is 2-4d and that for fungi is 7-10d, several test reports might be acquired for microbial culture over time. In this study, the time required for obtaining microbial culture results was dependent on the first report. The average report time for microbial culture was 2.67±0.55d, which was significantly longer than that of NTS (1.13±0.34d, $Z=-9.218$, $P<0.001$). Therefore, NTS was faster and more sensitive for identifying pathogens of postoperative endophthalmitis compared to microbial culture.

NTS for Culture-positive Pathogens Among the 21 culture-positive samples, NTS successfully detected pathogens in all samples that were highly consistent with microbial culture. The results of 16 samples revealed by NTS were completely the same as microbial culture (76.19%), and in two samples (9.52%), NTS showed a better species resolution than did the traditional microbial culture-based testing, further confirmed the Gram-negative bacilli (Patients 18 and 55) as

Table 1 Basic demographics and clinical characteristics in postoperative endophthalmitis patients

No.	Sex	Age	Eye	Inciting event	Interval days	Systemic diseases	Initial BCVA	Initial IOP/mm Hg	Anterior chamber	Vitreous cavity	Surgery	Follow-up BCVA	Follow-up IOP/mm Hg
1	F	70	OD	Postcataract	60	-	LP	17	Fibrinous exudate	Diffuse exudates	IVI	HM	18
2	M	70	OD	Postcataract	2	HTN	LP	18	Hypopyon	Purulent	ILE+PPV+SOT+IVI	CF	15
3	M	73	OD	Postcataract	5	HTN, RC	CF	8	Hypopyon	Purulent	ILE+PPV+SOT+IVI	0.1	16
4	F	79	OD	Postcataract	7	HTN	0.4	18	Fibrinous exudate	Negative	ACW+ICI	0.4	12
5	M	54	OD	Postcataract	2	DM	HM	46	Hypopyon	Diffuse exudates	ILE+PPV+SOT+IVI	0.05	11
6	F	67	OD	Postcataract	7	HTN	LP	54	Fibrinous exudate	Diffuse exudates	ICI+IVI	HM	32
7	M	67	OD	Postcataract	4	HTN	HM	13	Fibrinous exudate	Diffuse exudates	ACW+PPV+SOT+IVI	0.12	10
8	M	87	OS	Postcataract	1	-	CF	14	Hypopyon	Purulent	ILE+PPV+IVI	CF	12
9	F	64	OD	Postcataract	1	-	NLP	21	Hypopyon	Purulent	ILE+PPV+SOT+IVI	NLP	15
10	F	78	OS	Postcataract	5	HTN, DM	0.1	15	Fibrinous exudate	Diffuse exudates	PPV+MP+SOT+IVI	HM	15
11	M	67	OS	Postcataract	2	DM	0.05	23	Fibrinous exudate	Diffuse exudates	IVI	0.4	18
12	F	51	OS	Postcataract	3	HTN, DM	HM	23	Fibrinous exudate	Diffuse exudates	ACW+PPV+SOT+IVI	0.05	16
13	F	58	OD	Postcataract	75	HTN, CI	0.05	12	Fibrinous exudate	Focal exudates	PPV+IVI	0.2	11
14	M	60	OD	Postcataract	2	-	HM	7	Fibrinous exudate	Purulent	ACW+ILE+PPV+IVI	CF	13
15	F	78	OD	Postcataract	2	HTN	LP	14	Hypopyon	Purulent	ACW+ILE+PPV+MP+SOT+IVI	HM	17
16	F	80	OD	Postcataract	1	-	LP	11	Hypopyon	Diffuse exudates	ILE+PPV+SOT+IVI	HM	7
17	M	61	OD	Postcataract	9	HTN	HM	6	Fibrinous exudate	Focal exudates	ACW+IVI	0.08	13
18	M	72	OS	Postcataract	7	HTN	HM	15	Hypopyon	Diffuse exudates	ILE+PPV+SOT+IVI	CF	14
19	F	69	OS	Postcataract	15	-	LP	37	Fibrinous exudate	Diffuse exudates	PPV+IVI	0.1	12
20	F	62	OD	Postcataract	3	HTN	HM	19	Hypopyon	Diffuse exudates	ACW+ILE+PPV+SOT+IVI	NLP	8
21	F	68	OD	Postcataract	2	-	HM	17	Fibrinous exudate	Diffuse exudates	ILE+PPV+IVI	CF	14
22	M	57	OD	Postcataract	10	HTN, DM, CKD	LP	13	Fibrinous exudate	Diffuse exudates	ACW+PPV+IVI	HM	12
23	F	66	OS	Postcataract	5	CAD	CF	11	Fibrinous exudate	Diffuse exudates	ACW+PPV+IVI	0.12	11
24	F	44	OD	Postcataract	1	BA, SLE	HM	26	Hypopyon	Purulent	ACW+PPV+IVI	0.3	14
25	F	69	OS	Postcataract	5	HTN	HM	18	Hypopyon	Diffuse exudates	ACW+PPV+SOT+IVI	0.3	19
26	M	46	OS	Postcataract	2	KT, HTN	HM	26	Fibrinous exudate	Diffuse exudates	ICI+IVI	0.1	21
27	M	70	OD	Postcataract	3	-	CF	18	Fibrinous exudate	Diffuse exudates	PPV+SOT+IVI	0.2	9
28	M	58	OD	Postcataract	3	-	CF	10	Hypopyon	Diffuse exudates	ACW+PPV+SOT+IVI	0.15	14
29	F	72	OS	Postcataract	2	-	HM	13	Fibrinous exudate	Purulent	ILE+PPV+SOT+IVI	CF	8
30	M	72	OS	Postcataract	2	CI	0.1	20	Fibrinous exudate	Diffuse exudates	ACW+ILE+PPV+SOT+IVI	0.25	12
31	F	86	OD	Postcataract	1	HTN	HM	17	Hypopyon	Diffuse exudates	ACW+ILE+PPV+SOT+IVI	CF	9
32	F	79	OD	Postcataract	7	HTN	0.5	14	Fibrinous exudate	Negative	ACW+ICI	0.5	10
33	F	67	OD	Postcataract	1	-	HM	21	Hypopyon	Diffuse exudates	ACW+PPV+MP+SOT+IVI	CF	19
34	F	65	OS	Postcataract	20	HTN	HM	6	Fibrinous exudate	Diffuse exudates	ILE+PPV+SOT+IVI	CF	19
35	F	61	OS	Postcataract	10	HTN	0.08	10	Fibrinous exudate	Diffuse exudates	ACW+ICI+IVI	0.12	16
36	M	76	OS	Postcataract	2	HTN	LP	28	Hypopyon	Purulent	ACW+PPV+SOT+ICI+IVI	LP	18
37	M	70	OD	Postcataract	2	HTN, ROS	CF	15	Hypopyon	Purulent	ACW+PPV+SOT+ICI+IVI	0.08	17
38	M	79	OD	Postcataract	3	-	HM	14	Hypopyon	Diffuse exudates	ACW+ILE+PPV+SOT+IVI	CF	14
39	F	62	OD	Postcataract	6	-	0.1	11	Fibrinous exudate	Diffuse exudates	ACW+ICI+IVI	0.1	8
40	M	94	OS	Postcataract	30	HTN, CAD	HM	19	Hypopyon	Diffuse exudates	ACW+ICI+IVI	CF	17
41	F	61	OS	Postcataract	4	-	LP	28	Fibrinous exudate	Diffuse exudates	ILE+PPV+SOT+IVI	HM	14
42	M	71	OS	Postcataract	3	-	CF	16	Fibrinous exudate	Diffuse exudates	ACW+ILE+PPV+SOT+IVI	0.05	10
43	M	62	OS	Post-PPV	2	-	HM	12	Hypopyon	Diffuse exudates	ACW+PPV+SOT+IVI	0.2	10
44	F	67	OD	Post-PPV	2	HTN, DM	LP	17	Fibrinous exudate	Diffuse exudates	ACW+ICI+IVI	LP	17
45	F	31	OS	Post-PPV	3	-	CF	15	Fibrinous exudate	Diffuse exudates	ACW+PPV+IVI	0.1	15
46	M	72	OD	Post-PPV	3	-	CF	14	Fibrinous exudate	Diffuse exudates	PPV+IVI	0.15	19
47	M	25	OS	Post ICL implantation	1	-	HM	23	Hypopyon	Diffuse exudates	ACW+ILE+ICI+IVI	0.12	13
48	F	67	OD	Bleb-related	720	HTN	LP	7	Fibrinous exudate	Purulent	Phaco+PPV+IVI	HM	15
49	M	72	OD	Bleb-related	180	HTN	NLP	39	Hypopyon	Purulent	EXE	—	—
50	F	78	OD	Bleb-related	15	HF	HM	9	Hypopyon	Purulent	ACW+PPV+SOT+IVI	0.12	20
51	M	55	OD	Postinjection	1	HTN, DM	HM	24	Hypopyon	Diffuse exudates	Phaco+PPV+SOT+ICI+IVI	0.12	16
52	F	1	OS	Postinjection	1	PD	Uncooperative	Uncooperative	Hypopyon	Purulent	PPL+PPV+IVI+SLR	Uncooperative	Uncooperative
53	M	65	OS	Postkeratoplasty	60	-	HM	20	Fibrinous exudate	Diffuse exudates	PPV+SOT+IVI	CF	13
54	F	76	OS	Post-ACP	4	HTN, DM	0.12	15	Hypopyon	Negative	ACW+ICI	0.2	15
55	F	58	OD	Post-PPL	5	HTN	NLP	18	Fibrinous exudate	Purulent	PPV+SOT+IVI	NLP	19

F: Female; M: Male; OD: Right eye; OS: Left eye; PPV: Pars plana vitrectomy; ICL: Implantable collamer lens; ACP: Anterior chamber paracentesis; PPL: Pars plana lensectomy; BA: Bronchial asthma; CAD: Coronary arterial disease; CI: Cerebral infarction; CKD: Chronic kidney disease; DM: Diabetes mellitus; HTN: Hypertension; KT: Kidney transplantation; PD: Premature delivery; RC: Renal cyst; ROS: Ringworm of scalp; SLE: Systemic lupus erythematosus; BCVA: Best corrected visual acuity; CF: Counting fingers; HM: Hand movements; LP: Light perception; NLP: No light perception; IOP: Intraocular pressure; ACW: Anterior chamber wash; EXE: Exenteration of eyeball; ICI: Intracameral injection; ILE: Intraocular lens extraction; IVI: Intravitreal injection; MP: Membrane peeling; Phaco: Phacoemulsification; SLR: Sclera laceration repair; SOT: Silicone oil tamponade; Interval: From the time of intraocular surgery to the onset of endophthalmitis symptoms; HF: Heart failure.

Table 2 Intravitreal antimicrobial drugs and etiological microorganisms identified using NTS and microbiology culture

No.	Sample	Time required for pathogen culture/days	Culture	Time required for NTS/days	NTS	Reads	Coverage	Empirical antimicrobials	Additional antimicrobials
1	AH	3	N	1	*Dialister micraerophilus*	69	NA	Van+Cef	Vor+Amp-B
					Parvimonas micra	45			
					Cladosporium halotolerans	43			
2	VH	3	*Streptococcus sanguis*	1	*Streptococcus sanguis*	155	NA	Cef	-
3	VH	3	N	1	*Streptococcus gordonii*	175	NA	Van	-
					Paraburkholderia dipogonis	20			
4	AH	3	N	1	*Deinococcus ficus*	160	96.70%	Van+Cef	-
5	VH	3	N	1	*Haemophilus influenzae*	560	NA	Van	Vor+Cef
					Yarrowia lipolytica	92			
6	AH	2	N	2	*Stenotrophomonas rhizophila*	142	97.3%; 94.7%	Van+Cef	-
					Staphylococcus hominis	66			
7	VH	2	N	2	*Staphylococcus epidermidis*	593	97.30%	Cef	-
8	VH	3	N	2	*Staphylococcus aureus*	41	98.1%; 97%	Cef	Vor
					Botrytis californica	245			
9	VH	3	*Staphylococcus aureus*	1	*Staphylococcus aureus*	106	98.30%	Van	-
10	VH	3	N	1	N	-	-	Van	-
11	AH	3	*Staphylococcus epidermidis*	1	*Staphylococcus epidermidis*	2572	95.60%	Van+Cef	-
12	VH	3	N		*Streptococcus gordonii*	67	98.6%; 98.6%; 91.9%	Van	Vor+Cef
				1	*Enterobacter cancerogenus*	54			
					Meyerozyma guilliermondii	2633			
13	VH	2	N	1	N	-	-	Van	-
14	VH	3	N	1	*Staphylococcus epidermidis*	9595	96.70%	Van+Cef	-
15	VH	3	*Enterococcus faecalis*	2	*Enterococcus faecalis*	38175	95.70%	Van+Cef	-
16	VH	4	*Enterococcus faecalis*	1	*Enterococcus faecalis*	47620	97.80%	Van+Cef	-
17	AH	2	N	1	*Corynebacterium jeikeium*	163	98.5%; 94.7%	Van	Vor
					Aspergillus gracilis	1170			
18	VH	3	Gram-negative bacilli	1	*Escherichia coli*	102	96.70%	Van+Cef	-
19	VH	3	N	2	N	-	-	Van	-
20	VH	4	*Pseudomonas aeruginosa*	1	*Pseudomonas aeruginosa*	94365	98.3%; 94.3%	Van+Cef	Vor
					Yarrowia lipolytica	7575			
21	VH	3	*Streptococcus pneumoniae*	1	*Streptococcus pneumoniae*	2074	98.30%	Van	-
22	VH	3	N	1	*Acinetobacter baumannii*	1366	98.4%; 96.4%	Van+Cef	Vor+Amp-B
					Cryptococcus magnus	143			
23	VH	3	*Staphylococcus epidermidis*	1	*Staphylococcus epidermidis*	2585	98.40%	Van	-
24	VH	3	*Candida parapsilosis*	1	*Staphylococcus aureus*	561	98.8%; 96.7%	Van+Cef	Vor
					Candida parapsilosis	7196			
25	VH	3	N	1	N	-	-	Van+Cef	-
26	AH	2	N	1	*Aerococcus viridans*	317	96.5%; 98.3%	Van+Cef	-
					Pseudomonas stutzeri	231			
27	VH	3	N		*Staphylococcus saccharolyticus*	1114	99%; 94.2%; 88.1%	Van	Vor
				1	*Lactococcus piscium*	757			
					Yarrowia lipolytica	13925			
28	VH	3	N	1	*Corynebacterium tuberculostearicum*	1178	98.30%	Van+Cef	-
29	VH	2	*Pseudomonas aeruginosa*	1	*Pseudomonas aeruginosa*	11973	97.80%	Van	Cef
30	VH	3	*Staphylococcus epidermidis*	1	*Staphylococcus epidermidis*	427	98.40%	Van+Cef	-
31	VH	3	N	1	*Streptococcus mitis*	84570	98.20%	Van+Cef	-
32	AH	2	*Staphylococcus epidermidis*	1	*Staphylococcus epidermidis*	1577	94.20%	Cef	-
33	VH	2	*Cutibacterium acnes*	1	*Cutibacterium acnes*	5273	92.70%	Van+Cef	-
34	VH	2	N	1	*Enterobacter asburiae*	479	98.60%	Van+Cef	-
35	AH	2	N	1	N	-	-	Cef	-
36	VH	3	*Streptococcus pneumoniae*	1	*Streptococcus pneumoniae*	179500	95.10%	Van+Cef	-
37	VH	2	N	1	*Staphylococcus aureus*	109	97.28%; 96.23%	Van+Cef	Vor
					Aspergillus penicillioides	3232			
38	AH	2	N	1	*Streptococcus mitis*	2653	85.41%	Van	-
39	AH	2	*Cutibacterium acnes*	1	*Cutibacterium acnes*	205	98.70%	Cef	-
40	AH	2	N	1	*Porphyromonas bennonis*	346	98.71%	Van	Cef
41	VH	2	*Staphylococcus aureus*	1	*Staphylococcus aureus*	2053	91.20%	Van+Cef	-
42	VH	2	*Staphylococcus epidermidis*	1	*Staphylococcus epidermidis*	2109	96.70%	Cef	-
43	VH	3	N	1	*Enterobacter sp.*	1210	NA	Van	-
44	AH	3	N	1	*Acidovorax delafieldii*	138	NA	Van	-
45	VH	2	N	1	*Bacillus stratosphericus*	44	96.70%	Van+Cef	-
46	VH	2	N	1	*Anaerococcus prevotii*	59	99.30%	Van	Cef
47	AH	3	N	2	*Pseudomonas moraviensis*	47	97.7%; 97.7%	Van+Cef	-
					Stenotrophomonas rhizophila	30			
48	VH	3	N	1	*Streptococcus pneumoniae*	67770	98.8%; 98.1%	Van	-
					Lactobacillus iners	649			
49	VH	3	*Escherichia coli*	2	*Escherichia coli*	5389	97.10%	-	-
50	VH	2	N		*Staphylococcus epidermidis*	12417	97.79%; 98.17%; 98.53%	Van+Cef	-
				1	*Bacteroides coagulans*	1061			
					Haemophilus parainfluenzae	191			
51	VH	3	N	1	*Staphylococcus epidermidis*	831	97.20%	Van	-
52	VH	3	N	1	*Streptococcus pneumoniae*	88363	98.80%	Van+Cef	-
53	VH	3	*Candida albicans*	1	*Staphylococcus epidermidis*	113	NA	Van	Vor
					Candida albicans	2214			
54	AH	3	N	1	*Pseudomonas stutzeri*	318	99.02%	Vor	Van+Cef
55	VH	2	Gram-negative bacilli		*Escherichia coli*	3505	97.19%; 98.89%; 98.85%	Van+Cef	-
				1	*Corynebacterium tuberculostearicum*	1411			
					Aggregatibacter segnis	364			

AH: Aqueous humor; VH: Vitreous humor; N: Negative; NTS: Nanopore targeted sequencing; NA: Not available (Some data was missing because partial reports were earlier records); Van: Vancomycin; Cef: Ceftazidime; Vor: Voriconazole; Amp-B: Amphotericin B.

Escherichia coli, and found additional pathogens in Patient 55, *Corynebacterium Tuberculostearicum* and *Aggregatibacter segnis*. Moreover, NTS helped identify additional species apart from the cultured pathogens in three samples (14.28%), namely *Yarrowia lipolytica* in Patient 20, *Staphylococcus aureus* in Patient 24, and *Staphylococcus epidermidis* in Patient 53, that were not identified by microbial culture. Interestingly, there was a magnitude difference between the reads of additional pathogens and cultured pathogens.

NTS for Culture-negative Pathogens In all 34 culture-negative samples, NTS revealed pathogens in 29 samples (85.29%). A single pathogen was detected in 15 samples and two or more pathogens were detected in 14 samples. Notably, we only detected single bacterial infection and no sample had single fungus infection. In 14 samples with mixed pathogens, 8 samples (57.14%) had mixed bacteria and fungi and 6 samples (42.86%) had multiple bacteria. Although we detected multiple pathogens, there existed a main pathogen, whose sequencing read was 17.27 times higher than that of the other pathogen, on average. Reads of highly abundant causative pathogens were 5 times more relative than those of the species ranked second in 10 communities (71.43%).

Administration of Intravitreal Antimicrobial Agents All patients except patient 49 were administered intravitreal and/or intracameral injections with antimicrobial agents before we obtained NTS results. It should be noted that three eyes with no posterior ocular inflammation did not receive intravitreal injection, and they received intracameral injection instead. Antimicrobial agents, such as vancomycin (1 mg/0.1 mL), ceftazidime (2.25 mg/0.1 mL), voriconazole (100 μg/0.1 mL) were empirically administered based on patient's condition. Vancomycin (n=46) and ceftazidime (n=33) were the most commonly used drugs, either in combination or as single drug regimes, followed by voriconazole (n=1). Voriconazole was administered in patient 54 that showed a high possibility of fungi-associated endophthalmitis because of the presence of clumps lesion in the anterior chamber.

After the NTS results were obtained, 15 patients received additional intravitreal/intracameral antimicrobials agents that differed from previously listed ones. Amongst them, 1, 6, 11, and 2 patients received supplemental vancomycin, ceftazidime, voriconazole, and amphotericin B (10 μg/0.1 mL), respectively. There was no need to further adjust the type of antimicrobials injected into the eyeball after results of the microbial culture analysis were acquired because microbiology culture provided less information with longer testing period.

Special Cases In Patient 16, we detected *Enterococcus faecalis*, a bacteria naturally found in the intestines, by NTS and pathogen culture in the intraocular fluid and the wound exudation fluid of the right forearm and the bandaged gauze, which supported the test results. Patient 37 had fungal skin disease for decades, which supported the identification of fungi in the intraocular fluid by NTS. Patient 54 developed endophthalmitis after anterior chamber paracentesis and had a clump lesion that indicated fungal infection, therefore, voriconazole was empirically administered. However, the ocular infection continued to progress. When we obtained NTS results that suggested bacterial infection, antibacterial drugs were injected into the anterior chamber, which successfully controlled the infection. This case further highlighted the significance of NTS in identifying pathogens, which might be missed by microbial culture or clinical experience. The prompt and accurate identification of pathogen by NTS could significantly improve the patient's outcome.

DISCUSSION

Current diagnostic standard of postoperative endophthalmitis is microbial culture, which is limited by its low sensitivity since some fastidious pathogens may not grow on routine media and patients may have received antimicrobial therapy before the test[9]. Extensive variation in culture positivity from 40% to 70% in samples collected from patients with ocular infection is known and reported[5-6], and the results of our study are far from promising. Molecular diagnostic tools such as PCR have the potential to improve the detection sensitivity and shorten the turnaround time[6]. However, multiplex PCR is associated with a technical challenge; owing to differences in amplification efficiencies of different primers and the availability of a limited number of fluorescent labels, the number of fungi and/or bacteria that can be concurrently detected is limited[6]. More importantly, the emergence of new pathogens and the mutation of known pathogens increase the difficulty in identifying pathogens[11]. Besides, most of the causative pathogens in patients with postoperative endophthalmitis are bacteria, and bacterial endophthalmitis usually presents acutely[1]. Thus, there is an urgent need for developing an efficient and accurate technique to identify pathogens causing postoperative endophthalmitis.

Sequencing techniques have revolutionized the profiling of clinical microbial communities as they facilitate hypothesis-free and high throughput screening. Oxford nanopore technology (ONT), a representative novel third-generation sequencing technique, uses special channel proteins called nanopores, which have a nanoscale diameter allowing single-stranded nucleic acids to pass through. Different charge properties of ATCG bases, when passing through nanopores, cause different current changes, which will be recorded as electric signals in real time and the type of bases can be identified through these distinctive signals, thereby realizing the sequencing[21]. Nanopore sequencing has the advantages of end-to-end sequencing, high throughput, low cost, real-time

data acquisition, and absence of GC bias[9,14,21]. Moreover, the compact MinION sequencer launched by ONT is affordable and portable; therefore, it enables quick sequencing in harsh locations and conditions[9]. Sanderson *et al*[22] used ONT and illumina metagenomic sequencing to detect 7 infected samples simultaneously, and two sequencing results corresponded with each other, however, ONT allowed species level identification in one sample, whereas short-read illumina sequencing can only detected *Bacillus spp*. In addition to better species resolution, ONT reflected species abundance more accurately in samples proven to be polymicrobial by PCR. Meanwhile, no pathogen was found in 2 negative controls by ONT, confirming its specificity[22]. Compared with metagenomics next-generation sequencing, ONT can span most repetitive sequences and fully assemble complex microbial genomes structure as a result of ultralong reads[11]. Taking the advantage of nanopore sequencing that could accurately differentiate species with highly similar genome and the advantage of targeted amplification that could reduce the interference of host background DNA[14,22-25], we creatively combined nanopore sequencing and targeted sequencing to achieve the NTS scheme, where 16S rRNA and ITS were used as target genes to identify bacteria or fungi in samples from postoperative endophthalmitis patients.

A previous study showed that nanopore sequencing based on 16S rRNA and ITS could successfully identify bacteria or fungi in clinical blood samples, which was confirmed by Sanger sequencing, and the agreement rate of nucleotide sequences between them was up to 99%[26]. Nanopore sequencing was performed on samples collected from patients with suspected bacterial lower-respiratory-tract infections, and a 96.6% sensitivity was reported for microorganism identification relative to culture-based methods[12]. Similarly, Yang *et al*[13] reported that nanopore sequencing identified all culture-confirmed pathogens with high abundance. Among 8 culture-positive bacterial pneumonia samples, the culture-isolated pathogens had the highest abundance among nanopore-identified pathogens, which was 90 fold change of second-ranked pathogen. Moreover, nanopore sequencing revealed extra bacteria with high abundance in 2 samples, which was not detected by microbial culture[13]. NTS can also detect pathogens in culture-negative samples. In 7 culture-negative heart valve tissue samples collected from patients with infective endocarditis, nanopore sequencing identified the pathogens *Coxiella burnetii* and *Bartonella Quintana*, which was completely consistent with next-generation sequencing results[27]. In our study, NTS successfully uncovered the presence of pathogens in 50 cases (90.91%) and detected additional pathogens other than culture-positive pathogens. In 21 culture-positive specimens, all cultured pathogens were detected by NTS, and additional microorganisms were discovered in 3 samples. Interestingly, the sequence reads of culture-positive pathogens were more than ten times that of the additionally identified pathogens in NTS, indicating that microbial culture could detect the main pathogen, however, there still existed other pathogens contributing to the infection. Moreover, NTS achieved a better species resolution in two samples than microbial culture, which merely revealed the presence of Gram-negative bacilli but failed to report the bacterial species. Among the 34 culture-negative samples, NTS successfully detected pathogens in 29 samples (85.29%). Therefore, NTS is a promising diagnostic tool for identifying pathogens causing postoperative endophthalmitis.

The short turnaround time is another advantage of nanopore sequencing. Fu *et al*[14] showed that the turnaround time of NTS from sample preparation to final report was approximately 8-14h. In our study, 48 reports (87.27%) were obtained within 24h and the remaining ones were obtained within 48h (it should be noted that some of them were earlier reports, and the detection time was not standardized to hours, but only recorded by days); all of them are shorter than the time required for obtaining microbial culture results, which varies according to the type of sample and pathogen. In most cases, the culture time of bacteria was shorter than that of fungi, however, it still took days to get the report. Hence, NTS can provide a prompt identification of both bacteria and fungi.

Before we got results from microbial culture or NTS, we chose antimicrobial drugs based on patient's clinical manifestation and clinician's experience. Fungal endophthalmitis is remarkably rarer than bacterial one. The highest proportion of patients with fungal endophthalmitis secondary to endogenous sources (81%) has been reported in Australian[28]. Fungal postoperative endophthalmitis is rarely observed in regions other than tropical regions such as India, where 10%–20% of patients show fungal etiology[1,29]. Hence, antifungal drugs were not routinely used in our study, where antibiotics such as vancomycin and ceftazidime were initially administered. Only when the patient had typical manifestations of fungal infections such as "clumped" appearance, were antifungal drugs used. Behera *et al*[29] demonstrated that waiting for a long time for microbial culture results without antifungal treatment may lead to poor results. Kim *et al*[30] reported an outbreak of fungal endophthalmitis after cataract surgery in Republic of Korea where early detection and prompt antifungal therapy conferred favorable visual outcome. The positive rate of fungus culture is low[31]. In contrast, NTS can detect bacteria and fungi simultaneously in a short time to facilitate timely antifungal treatment. In our study, NTS identified 11

patients with mixed bacterial and fungal infections, and all of them received additional intravitreal antifungal drugs in time. Especially, Patient 54 exhibited fungal infection initially, whereas antifungal drugs did not alleviate the infection. After NTS identified bacterial infection, we added antibacterial drugs, which efficiently attenuated the infection. At the time of discharge, most patients had good visual prognosis with ocular infection under control.

Our study showed that a certain part of patients had mixed infection as shown by NTS, whereas microbial culture failed to reveal multiple pathogens, which might be relative to the inherent characteristics of sequencing technology. NTS has no bias in the discovery of pathogenic microorganisms[9]. Nonetheless, a single organism without quantification was isolated and cultured through the conventional approach in this research. In an 11-year retrospective study in Taiwan, China, mixed infections had a low proportion (5%) in patients with culture-positive bacterial endophthalmitis[18]. We proposed that it might be due to the existence of a dominant pathogen that affected the detection of other pathogens; some pathogens were fastidious of culture media; and patients might receive antibiotic treatment before sample collection. These will reduce the detection rate of microbial culture[9].

The major limitation of NTS technology is the detection of microbial contamination in samples due to its high sensitivity[24]. To reduce contamination during sample collection and analysis, we have made detailed protocols (in the "Contamination Control" of methods section) to diminish the false positive rate of NTS results. However, due to the limited volume of intraocular fluid samples, especially AH sample, we could not conduct Sanger or metagenomic next-generation sequencing to verify the NTS results. Meanwhile, due to the impact of coronavirus, long-term follow-up results were not available for most patients, so only short-term follow-up data were collected. Thirdly, samples from healthy individuals were not obtained as a control because intraocular fluid sampling is an invasive procedure.

In summary, NTS can rapidly and accurately identify pathogens and facilitate the design of treatment regimen in patients with postoperative endophthalmitis. It can serve as an efficient supplemental diagnostic tool with significant clinical application value.

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