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Comparison of 16S rDNA Amplicon Sequencing With the Culture Method for Diagnosing Causative Pathogens in Bacterial Corneal Infections

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Purpose: The purpose of this study was to explore if 16S rDNA amplicon sequencing can improve the conventional diagnosis of causative pathogens for bacterial corneal infection.

Methods: Corneal scraping and conjunctiva and eyelid margin swab samples from infected eyes of patients diagnosed with "bacterial corneal infection" and conjunctiva and eyelid margin swab samples from a random eye of healthy participants were collected. Each swab was used for both aerobic and anaerobic cultures and 16S rDNA amplicon sequencing. The V3 to V4 region of the 16S rDNA was amplified using polymerase chain reaction (PCR) and sequenced on the Illumina HiSeq 2500 Sequencing Platform.

Results: The overall culture positivity rate for all 72 samples was 69% (72% in the bacterial keratitis group and 67% in the healthy control group), whereas 1719 operational taxonomic units in total were generated using 16S rDNA amplicon sequencing with each sample showing 123 to 337 different genera. *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Micrococcus* most frequently appeared in culture, whereas *Streptococcus, Acinetobacter*, and *Lactobacillus* were the most common genera, with large ratios in 16S rDNA amplicon sequencing. The causative pathogens detected by the two methods were inconsistent for most samples, except for several corneal samples.

Conclusions: We suggest that a combination of different techniques, such as clinical observation, microscopic analysis, culture, and next-generation sequencing techniques including 16S rDNA amplicon sequencing, should be used to comprehensively analyze pathogens in corneal and external ocular infections.

Translational Relevance: This paper uses a basic research methodology for studying the microbiome in ocular samples to help improve the diagnostic accuracy of corneal and external ocular infections.

Introduction

Corneal infection is one of the most severe ophthalmic diseases worldwide and can even lead to vision loss.[1] Thus, prompt and accurate diagnosis of the causative pathogen is essential to ensure appropriate and personalized treatment. In contrast to most other infectious diseases, the nidus of infection in the cornea is usually quite small, imposing substantial limitations on regular sample collection for conventional culture or microscopic examination. Even with the addition of in vivo confocal microscopy, it is difficult to identify the causative pathogen, with an overall positive rate of approximately 50% to 60%.[2,3] Although conventional culture shows a limited positive rate and is time-consuming, it is still relied upon by doctors and remains the only way to acquire pure strains of the pathogen to test for drug resistance.

For decades, polymerase chain reaction (PCR) has consistently been proven to be a more sensitive and faster technology to detect pathogens, and

it is often used to classify them into prokaryotes, eukaryotes, or viruses or to identify the cultured pure organisms in combination with Sanger sequencing.[4–6] The emergence of next-generation sequencing (NGS) technology has solved the problem of identification of pathogens in mixed bacterial samples, with metagenomic deep sequencing (MDS) showing the ability to detect all bacteria, fungi, viruses, and parasites in one micro-volume sample.[3,7,8] Although MDS can be considered to be the most powerful tool for the diagnosis of pathogens, its use is still associated with some obstacles, such as the high cost of its application in clinical practice even when replacing conventional culture. Especially for bacterial keratitis, the lower-cost 16S rDNA amplicon sequencing has been more widely used in studies of the microbiome of ocular samples, and the findings obtained in these studies have validated the presence of comparatively stable microbial commensals at the normal ocular surface.[9–14] However, the ability of 16S rDNA amplicon sequencing to improve the sensitivity and accuracy of the conventional methods used to detect pathogenic bacteria in bacterial keratitis remains unknown. Therefore, we aimed to explore this aspect by comparing the results of both 16S rDNA amplicon sequencing and conventional culture (both aerobic and anaerobic) of the same samples from patients diagnosed with bacterial keratitis.

Methods

Ethics

This pilot study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Xi'an No. 1 Hospital (2021-EA-5). The study was registered on the Chinese Clinical Trial Registry (ChiCTR2100042546), and written informed consent was obtained from all participants.

Recruitment of Participants

All participants were recruited from the Ophthalmic Department of Xi'an No. 1 Hospital between January and March 2021. A total of 40 patients with infectious keratitis were recruited in this study, of whom 12 were diagnosed with bacterial keratitis (10 men and 2 women, age 57 ± 19 years). The study participants were comprehensively evaluated on the basis of both classical symptoms and signs of infectious keratitis (ocular congestion, corneal edema, and corneal ulcer with a diameter of 3–7 mm and 1/3–4/5 depth of the front corneal stromal layer) and the results of in vivo confocal microscopy and microscopic examinations of corneal scrapings to exclude infections caused by fungi, viruses, or Acanthamoeba. In addition, 18 participants without any infection on the ocular surface were recruited as the healthy control group (3 men and 15 women, age 62 ± 13 years). The exclusion criteria were as follows: (1) patients who had used topical or systemic antibiotics or hormones in the past month; (2) patients with Sjögren's syndrome, diabetes, or other systemic diseases that may have affected the health of the ocular surface; (3) patients with a history of eye trauma or surgery within 1 year; and (4) pregnant or lactating women.

Sample Collection

After topical anesthesia with 0.4% oxybuprocaine hydrochloride eye drops (Santen, Osaka, Japan), corneal scrapings from the infected eye were wiped on the tip of a sterile dry cotton swab; another sterile dry cotton swab was used to wipe the lower conjunctival sac from the nasal to the temporal side and backward while rotating the swab. Two more sterile cotton swabs were used to squeeze the lower meibomian gland from the bottom to the top of both the inside and outside of the lower eyelid until the meibum was visible at the openings, and the lower eyelid margin including the squeezed meibum from the nasal to temporal side and backward. Conjunctival and eyelid margin samples for the control group were obtained using the same procedure for one random eye without obtaining a corneal scraping sample. Each sample swab was stored in a sterile tube immediately and carried to the laboratory for culture.

Culture

Each tube containing a sample was vortexed for 1 minute after adding 2 mL of sterilized phosphate-buffered saline; 200 µL of the resultant solution was used for aerobic and anaerobic culture, whereas the remaining 1800 µL was stored in an ultra-low temperature freezer at −80°C until DNA extraction. For the culture, 100 µL aliquots of the solution were separately transferred to two blood plates, and spread evenly using a sterile bar. One of the plates was then incubated in an incubator at 37°C while the other was sealed in an anaerobic bag. The plates were observed at intervals of 24 hours, and the colonies were classified according to their characteristics on the plates. The species or genus of each pure bacterium was identified using 16S rDNA amplicon sequencing (Sangon Biotech Co. Ltd., Shanghai, China).

DNA Extraction, PCR Amplification, Library Preparation, and Amplicon Sequencing

DNA extraction from all the samples was performed using the NucleoSpin 96 Soil Kit (Macherey-Nagel, Düren, Germany), and the V3 to V4 region of the 16S rDNA was amplified using PCR with universal primers (338F5′-ACTCCTACGGGAGGCAGCA-3′ and 806R5′-GGACTACHVGGGTWTCTAAT-3′) and sequenced on the Illumina HiSeq 2500 Sequencing Platform (Biomarker Technologies Corporation, Beijing, China) in the PE250 mode (2 × 250 bp paired ends). The analyses of microbiota were conducted on the BMKCloud Platform (www.biocloud.net).

Bioinformatics and Statistical Analysis

The bioinformatics was conducted on the BMK Cloud Platform (www.biocloud.net). The Raw Reads were filtered by Trimmomatic (version 0.33), and then Cutadapt (version 1.9.1), FLASH (version 1.2.7), and UCHIME (version 4.2) were used to obtain the Effective Reads. The Effective Reads with more than 97% identity were clustered into operational taxonomic units (OTUs) by USEARCH (version 10.0), whereas the OTUs whose proportions were less than 0. 005% of the total OTUs were removed. Taxonomy was assigned using the Silva as the reference database and the community composition of each sample can be counted at various levels (phylum, class, order, family, genus, and species). The alpha-diversity of all groups was obtained using the QIIME2 software. The richness and diversity of the microbiome of each group were calculated and evaluated based on Chao1 and Simpson index separately. The beta-diversity of all groups was obtained using the QIIME software; the results among different groups was shown by principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). The PCoA indicates the samples clustered with high community structure similarity, whereas PERMANOVA is used to test whether there are significant differences in beta-diversity among the samples in different groups. Linear discriminant analysis effect size (LEfSe) was used to identify bacterial biomarkers of each group. The linear discriminant analysis (LDA) score was used to classify the data and assess the influence of significantly different species. Differences and changes related to functional genes in the microbial community of different groups of samples in the metabolic pathway were observed through the composition and difference analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway using PICRUSt2 software.

Results

Culture Findings

We isolated 112 pure strains through aerobic and anaerobic cultures from 72 samples (including 36 samples from the cornea, eyelid margin, and conjunctiva of 12 patients in the bacterial keratitis group and 36 samples from the eyelid margin and conjunctiva of the 18 participants in the control group), yielding an average of 1.6 strains per sample. The numbers of isolated strains from different eye sites in each group are shown in Table 1. The detailed species of isolated strains in cultures are listed in Table 2. Only a limited group of bacteria were cultured from the control group, including *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Micrococcus*. These four common bacteria also appeared most frequently in the samples in the bacterial keratitis group; and some uncommon bacteria, such as *Klebsiella* and *Capnocytophaga*, were occasionally detected in this group.

The 16S rDNA Amplicon Sequencing

A total of 5,747,137 raw reads were obtained from the same 72 samples (average 79,821 raw reads per sample), and 4,650,975 effective reads (average 64,597 effective reads per sample) were generated after removing low-quality sequences, which were clustered into 1719 OTUs on a 97% similarity level. The flat ends of the rarefaction curves (Fig. 1A) or the Shannon

Table 1. The Numbers of Isolated Strains in Cultures

Group	Site	Sample Size	Culture Positive Samples	Positive Rate
Bacterial keratitis	Cornea	12	8	66.7%
	Conjunctiva	12	8	66.7%
	Eyelid margin	12	10	83.3%
Healthy control	Conjunctiva	18	11	61.1%
	Eyelid margin	18	13	72.2%

Table 2. The Detailed Species of Isolated Strains in Cultures

Group	Site	Sample Size	Isolated Strains	Strain Number	Percent
Bacterial keratitis	Cornea	12	*Staphylococcus epidermidis*	1	8.3% (1/12)
			Staphylococcus hominis	1	8.3% (1/12)
			Corynebacterium macginleyi	1	8.3% (1/12)
			Propionibacterium sp.	1	8.3% (1/12)
			Streptococcus pneumoniae	1	8.3% (1/12)
			Capnocytophaga sp.	1	8.3% (1/12)
			Micrococcus luteus	1	8.3% (1/12)
			Klebsiella oxytoca	1	8.3% (1/12)
			Moraxella osloensis	1	8.3% (1/12)
			Sphingomonas paucimobilis	1	8.3% (1/12)
			Microbacterium testaceum	1	8.3% (1/12)
				11 (total)	
	Conjunctiva	12	*Staphylococcus epidermidis*	1	8.3% (1/12)
			Staphylococcus warneri	1	8.3% (1/12)
			Staphylococcus haemolyticus	1	8.3% (1/12)
			Propionibacterium acnes	1	8.3% (1/12)
			Propionibacterium sp.	2	16.7% (1/12)
			Corynebacterium tuberculostearicum	1	8.3% (1/12)
			Corynebacterium sp.	1	8.3% (1/12)
			Streptococcus pneumoniae	1	8.3% (1/12)
			Capnocytophaga sp.	1	8.3% (1/12)
			Micrococcus luteus	1	8.3% (1/12)
			Klebsiella oxytoca	1	8.3% (1/12)
			Kocuria rosea	1	8.3% (1/12)
				13 (total)	
	Eyelid margin	12	*Staphylococcus epidermidis*	3	25% (1/12)
			Propionibacterium acnes	1	8.3% (1/12)
			Propionibacterium avidum	1	8.3% (1/12)
			Propionibacterium sp.	1	8.3% (1/12)
			Corynebacterium macginleyi	2	16.7% (1/12)
			Corynebacterium tuberculostearicum	1	8.3% (1/12)
			Corynebacterium mastitidis	1	8.3% (1/12)
			Streptococcus pneumoniae	1	8.3% (1/12)
			Bacillus simplex	1	8.3% (1/12)
			Micrococcus luteus	1	8.3% (1/12)
			Klebsiella oxytoca	1	8.3% (1/12)
			Moraxella osloensis	1	8.3% (1/12)
			Microbacterium testaceum	1	8.3% (1/12)
				16 (total)	
Healthy control	Conjunctiva	18	*Staphylococcus epidermidis*	10	55.6% (10/18)
			Staphylococcus aureus	2	11.1% (2/18)
			Staphylococcus lugdunensis	2	11.1% (2/18)
			Staphylococcus hominis	2	11.1% (2/18)
			Staphylococcus warneri	1	5.6% (1/18)
			Propionibacterium acnes	4	22.2% (4/18)
			Propionibacterium sp.	1	5.6% (1/18)
			Corynebacterium macginleyi	4	22.2% (4/18)
			Corynebacterium mastitidis	1	5.6% (1/18)
			Corynebacterium tuberculostearicum	1	5.6% (1/18)
			Corynebacterium segmentosum	1	5.6% (1/18)
			Corynebacterium sp.	5	27.8% (5/18)
			Micrococcus luteus	4	22.2% (4/18)
				38(total)	
	Eyelid margin	18	*Staphylococcus epidermidis*	9	50% (9/18)
			Staphylococcus aureus	2	11.1% (2/18)
			Staphylococcus lugdunensis	2	11.1% (2/18)
			Staphylococcus hominis	1	5.6% (1/18)
			Propionibacterium acnes	5	27.8% (5/18)
			Propionibacterium avidum	1	5.6% (1/18)
			Corynebacterium macginleyi	4	22.2% (4/18)
			Corynebacterium mastitidis	1	5.6% (1/18)
			Corynebacterium tuberculostearicum	1	5.6% (1/18)
			Corynebacterium segmentosum	1	5.6% (1/18)
			Corynebacterium sp.	5	27.8% (5/18)
			Micrococcus luteus	2	11.1% (2/18)
				34 (total)	

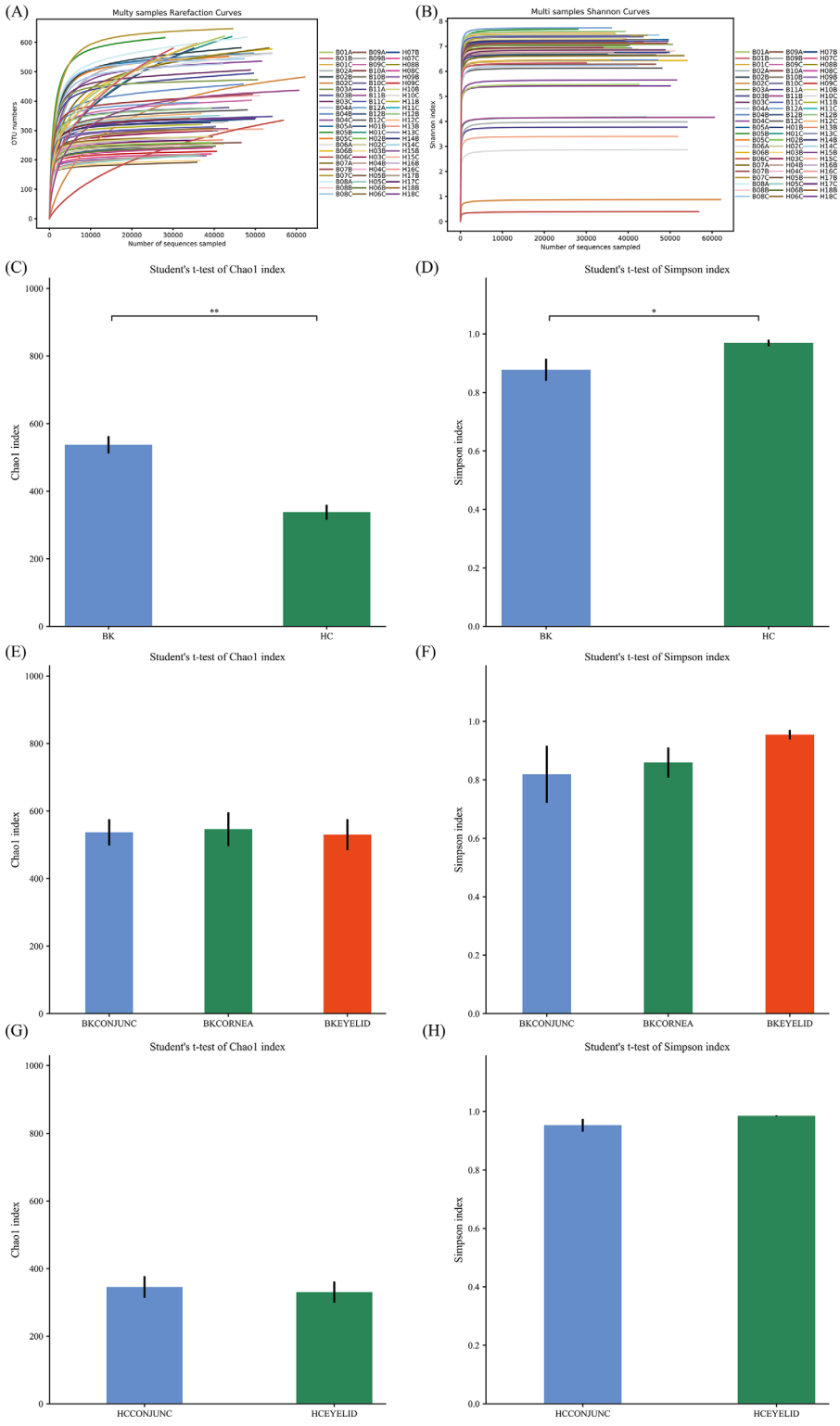

Figure 1. Alpha-diversity of the samples. (**A**) The multi-sample rarefaction curves. (**B**) The multi-sample Shannon curves. (**C**) Chao1 index of samples between the bacterial keratitis group (BK) and the healthy control group (HC). (**D**) Simpson index of samples between the bacterial keratitis group (BK) and the healthy control group (HC). (**E**) Chao1 index of samples among cornea (BKCORNEA), conjunctiva (BKCONJUNC), and eyelid margin (BKEYELID)of the bacterial keratitis group. (**F**) Simpson index of samples among cornea (BKCORNEA), conjunctiva (BKCONJUNC), and eyelid margin (BKEYELID) of the bacterial keratitis group. (**G**) Chao1 index of samples between conjunctiva (HCCONJUNC) and eyelid margin (HCEYELID) of the healthy control group. (**H**) Simpson index of samples between conjunctiva (HCCONJUNC) and eyelid margin (HCEYELID) of the healthy control group.

curves (Fig. 1B) indicate that the sequencing data volume was sufficient and the diversities discovered were adequate. The richness and diversity of the microbiome of each group were evaluated based on Chao1 and Simpson index separately. The results showed that the richness of the microbiome of the bacterial keratitis group was significantly higher than that of the healthy control group (Fig. 1C; $P < 0.01$, Mann-Whitney U test), but the diversity of the microbiome of the bacterial keratitis group was significantly lower (Fig. 1D; $P < 0.05$, Mann-Whitney U test). Among the different sites of the bacterial keratitis group (cornea, conjunctiva, and eyelid margin) or the healthy control group (conjunctiva and eyelid margin), there was no significant differences in the richness and diversity of the microbiome (Figs. 1E–H). Regarding beta-diversity among different groups, according to the results of PCoA and PERMANOVA analysis, most of the gathered plots of each group indicated a significant difference in the microbiome between the bacterial keratitis group and the healthy control group (Fig. 2A, $P = 0.001$). There was no significant difference among the different sites of either the bacterial keratitis or the healthy control group (Figs. 2B, 2C, $P > 0.05$). The top 15 genera/families of each group detected using 16S rDNA amplicon sequencing are shown in Figure 3. *Streptococcus*, *Acinetobacter*, and *Lactobacillus* were the most frequently identified bacteria in both the bacterial keratitis and control groups. LEfSe analysis of the potential bacterial biomarkers of each group (Supplementary Fig. S1) and information on predicted function of the samples (Supplementary Fig. S2) are shown in the supplementary materials.

Comparison of the Sensitivities of Detection Between Culture and 16S rDNA Amplicon Sequencing

All the 72 specimens gained positive results detected by 16S rDNA amplicon sequencing, 50 of them were culture positive and 22 were culture negative. The positive rate of 16S rDNA amplicon sequencing detection was 100% for these specimens from the ocular surface, whereas the positive rate of the culture method was 69.4% for the same specimens. The maximum number of bacterial strains isolated from a single sample was six (*Staphylococcus epidermidis, Staphylococcus lugdunensis, Staphylococcus warneri, Staphylococcus aureus, Micrococcus luteus*, and *Propionibacterium acnes*) from conjunctival sample H09. The samples in the bacterial keratitis group showed a maximum of 12 genera, whereas those in the healthy control group showed a maximum of only 4 genera (see Table 2). In contrast, the positive rate of 16S rDNA amplicon sequencing was 100%. The OTUs obtained for each sample ranged from 216 to 672, which were classified into 123 to 337 different genera.

Consistency of the Results Between Culture and 16S rDNA Amplicon Sequencing

For the 22 culture-negative specimens, the results of 16S rDNA amplicon sequencing were positive, which revealed that 16S rDNA amplicon sequencing detection was more sensitive than the conventional culture method and could detect more kinds of bacteria. The bacteria detected only by 16S rDNA amplicon sequencing included not only fastidious

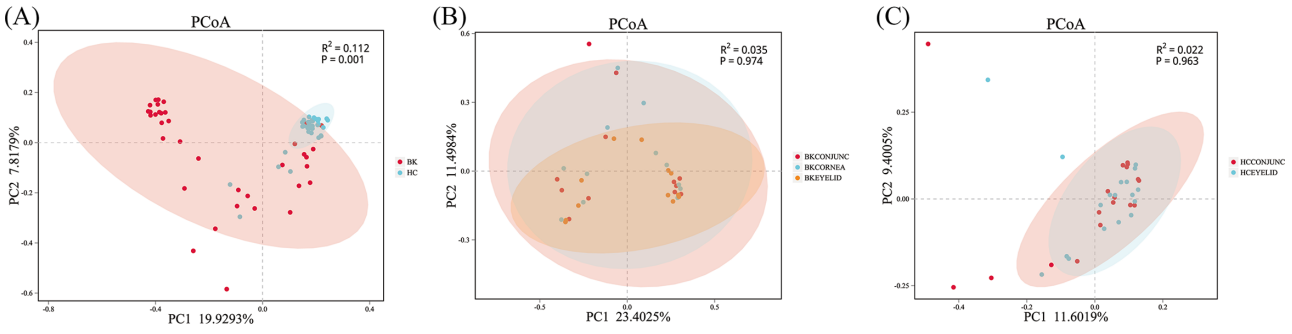

Figure 2. Beta-diversity of the samples expressed by principal coordinate analysis (PCoA) plots and PERMANOVA analysis to show the significant difference among the groups, X-axis label PC1 and Y-axis label PC2 separately represented the first and second principal axes that distinguished all samples the most and the percent of difference can be explained. (**A**) Dividing all the samples into the bacterial keratitis group (BK) and the healthy control group (HC). (**B**) Dividing all the samples of the bacterial keratitis group into the conjunctiva (BKCONJUNC), cornea (BKCORNEA), and eyelid margin (BKEYELID) of the bacterial keratitis group. (**C**) Dividing all the samples of the healthy control group into conjunctiva (HCCONJUNC) and eyelid margin (HCEYELID) of the healthy control group.

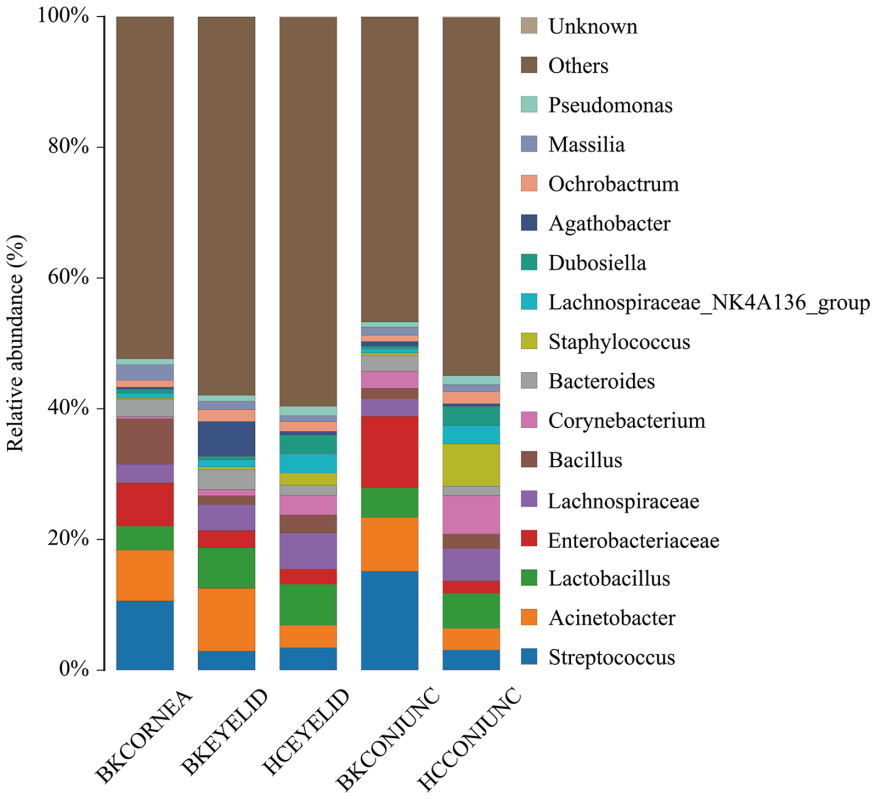

Figure 3. The top 15 bacteria detected using 16S rDNA amplicon sequencing. The top 15 bacterial genera or families in all the samples from each site in the two groups (BK-Bacterial Keratitis; HC-Healthy Control; CONJUNC-Conjunctiva; EYELID-Eyelid margin) are comprehensively analyzed by BMKCloud Platform (www.biocloud.net).

bacteria, such as *Lactobacillus* and *Bacteroides*, but also frequently cultured bacteria, such as *Propionibacterium*, *Corynebacterium*, and *Staphylococcus* (Table 3).

The results of microscopy, culture, and 16S rDNA amplicon sequencing for the patients of the bacterial keratitis group are listed in Table 3. The top 30 genera/families from each cornea sample in the bacterial keratitis group are shown in Figure 4. In the cases of samples B02, B04, and B06, the 16S rDNA amplicon sequencing results were consistent with the corneal scraping-based culture and microscopy findings. However, the consistency of these results was not always satisfactory. In the cases of samples B09 and B11, the results of 16S rDNA amplicon sequencing suggested pathogenic bacterium with a high ratio in the microbiome, which was consistent with the finding of Gram-positive bacteria on microscopy analysis, but

Table 3. Results of Bacteria Checks of the Patients in the Bacterial Keratitis Group

Patient	Corneal Scraping Microscopy(*)	Cause	Culture Results	Top Bacterium in 16S rDNA Amplicon Sequencing (Percent)
B01	G+ (2-9)	No obvious cause	*C. macginleyi*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (33.2%)*Acinetobacter* (2.4%)
B02	G– (7–25), G+ (2–11)	Foreign bodies	*K. Oxytoca*	*Enterobacteriaceae* (61.1%)
B03	G+ (1–7)	Contact lens related trauma	*P. acnes*	*Acinetobacter* (20.3%)
B04	G+ (2–7)	No obvious cause	*Capnocytophaga*	*Capnocytophaga* (25.6%)
B05	G+ (4–17)	Dirty gloves rubbing	*M. osloensisS. paucimobilis*	*Massilia* (14.6%)*Acinetobacter* (2.4%)
B06	G+ (2–30)	Plant trauma	*S. pneumonia*	*Streptococcus* (71.8%)
B07	G+ (3–15)	No obvious cause	Negative	*Bacteroides* (10.6%)
B08	G+ (2–7)	Splashed cement	Negative	*Acinetobacter* (17.2%)
B09	G+ (7–19)	No obvious cause	Negative	*Streptococcus* (25.6%)
B10	G+ (3–16)	No obvious cause	*M. testaceum*	*Lactobacillus* (12.2%)
B11	G+ (2–11)	Plant trauma	Negative	*Bacillus* (64.1%)
B12	G+ (9–30)	Plant trauma	*StaphylococcusM. luteus*	*Streptococcus* (6.6%)

*G+ stands for Gram-positive bacteria, G– stands for Gram-negative bacteria. The accompanying numbers in the brackets refer to the number of bacteria observed in each field of vision under the oil lens of the microscope (1000 ×).

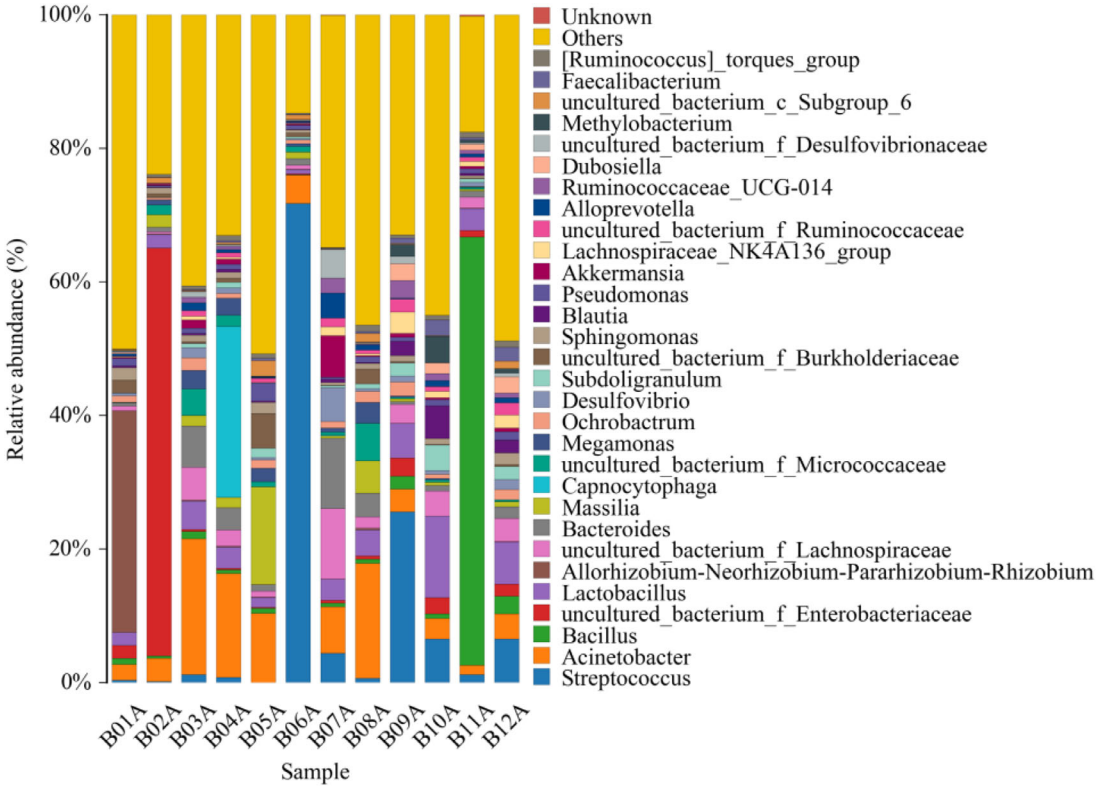

Figure 4. The top 30 bacteria of each cornea sample of the bacterial keratitis group detected using 16S rDNA amplicon sequencing. Bars B01A to B12A represent the 12 cornea samples of the bacterial keratitis group; the data were comprehensively analyzed by BMKCloud Platform (www.biocloud.net).

the culture method results were negative. In some cases, such as B01, B05, B07, and B10, the bacteria detected with the highest ratio using 16S rDNA amplicon sequencing were all culture-fastidious bacteria; therefore, the reliability of the culture results was low and we had to diagnose the pathogen based on the results of 16S rDNA amplicon sequencing and microscopy results. Samples B08 and B12 showed what we called "dysbacteriosis" — the results of 16S rDNA amplicon sequencing results showed average ratios of the microbiome, the culture results were negative or positive for possible commensals, and few bacteria were observed microscopically, the findings were also very common in the healthy control group; in such cases, it was difficult to diagnose the pathogenic bacteria.

Discussion

Culture-independent NGS techniques including 16S rDNA amplicon sequencing and MDS, undoubtedly possess the highest potential in improving diagnostic sensitivity and accuracy for detecting infectious pathogens.[15–17] Our results demonstrated that 16S rDNA amplicon sequencing offered an advantage over culture methods in detection sensitivity. Even though the consistency between the two methods was less than satisfactory except in a few cases (e.g. *K. oxytoca* in sample B02, *Capnocytophaga* in sample B04, or *S. pneumonia* in sample B06), 16S rDNA amplicon sequencing can improve the diagnosis of the pathogenic bacteria in ocular bacterial infection. When the culture results provide a negative result or yielded only possible commensals, the results of 16S rDNA amplicon sequencing could help to analyze the cases: if there are fastidious bacteria with a high ratio in the microbiome, they might be the pathogens; if the bacteria with the highest ratio in the microbiome are ocular commensals, they might be the opportunistic pathogens; if the ratios of most of the bacteria in the microbiome are average, it might be a case of "dysbacteriosis" and other possible effects should be analyzed for diagnosis and treatment. Of course, 16S rDNA amplicon sequencing is not a perfect technology that always provides accurate and satisfactory results: it can usually identify bacteria in the genus

level instead of species through the V3 to V4 sequences of 16S rDNA; several procedures of the process including DNA extraction, PCR amplification, matching in the databases for identification would introduce bias to influence the results.[18–21] Those defaults could be improved gradually through detecting more ocular samples. Conversely, our findings proved that the culture method is not always reliable to diagnose pathogenic bacteria even when samples show positive culture results: sometimes, bacteria from the environment may provide false-positive results; sometimes, the pathogenic bacteria that cannot grow easily under classical culture conditions may show false-negative findings; sometimes, the commensal bacteria that adapted well to the culture conditions may grow extremely vigorously and provide a false-positive result. For example, *S. aureus* and *P. aeruginosa* are reported to be the two most common organisms implicated in bacterial keratitis,[22–26] but, in our study, the *Staphylococcus* and *Pseudomonas* genera were not as abundant as in conventional experience. It is possible that they are commonly detected in bacterial keratitis samples because they are easily cultured under routine culture conditions. However, the culture method remains indispensable because it is one of the most important visible results for analyzing the causative pathogen; it is also the origin of pure strains for possible pathogenic bacteria to acquire drug resistance or for deriving more information. The accumulating data from the results of 16S rDNA amplicon sequencing of ocular surface samples may contribute to improving the technology for enhancing culture conditions in order to culture more fastidious but common bacteria on the ocular surface.

Therefore, neither the culture method nor 16S rDNA amplicon sequencing can identify the pathogenic bacteria accurately or be the sole method to diagnose the pathogenic bacteria for the majority of ocular surface samples, at present. A comprehensive approach using information from all the possible tests, including culture, microscopic observation of slides, confocal microscopy, and 16S rDNA amplicon sequencing, may be optimal, and there may currently be no "gold standard" test for the diagnosis of ocular surface infection.

Nevertheless, the application of NGS technology can improve the diagnosis of pathogenic bacteria in an ocular infectious specimen. For infectious specimens from sterile sites, like intraocular liquid, MDS is definitely the best method to diagnose the causative pathogen, because it allows detection of a large variety of pathogens, including bacteria, fungi, viruses, and parasites, for which there is drug-resistance information, which is especially useful for urgent and critical infections without credible pathogen information.[7,15] However, for bacterial corneal infections, 16S rDNA amplicon sequencing can complement the results of cultures and other microscopic assessments at a relatively lower cost. This approach could provide nearly 100% positive results when conventional tests show negative results.

Unfortunately, the sample size of this pilot study was limited because of the short study duration and strict exclusion criteria; however, these limited cases also yielded some reliable findings. The results of the limited cases have updated our recognition of both the diagnosis of pathogenic bacteria of ocular surface samples and the definition of bacterial keratitis. In fact, patients with a pure bacterial infection in the cornea represent the minority of infectious keratitis cases, similar to samples B02, B04, and B06; in such cases, we could easily diagnose the pathogenic bacteria and provide accurate treatment. Many more cases showed a comparatively higher ratio of fastidious or possible opportunistic bacteria that cannot be confirmed by the results of culture and microscopy. Furthermore, in some cases, the microbiome was detected with an average ratio of various bacteria, a condition we called "dysbacteriosis," where we could not diagnose the pathogen. For ease of analysis, we excluded the participants with fungal or amoeba or possible viral infectious keratitis from the bacterial keratitis group. Sometimes, the cornea is infected by multiple pathogens; we could infer that it would be more difficult to analyze the microbiome of those cases and provide accurate treatment. In the future, accumulating data from a larger sample size and more cases might improve our recognition of the ocular infectious disease and help decide the accurate treatment.

In reality, many factors can skew the results of microbiome analysis using the amplification technique; several steps of the 16S rDNA amplicon sequencing might lead to bias, including those involved in initial DNA sample preparation, such as DNA extraction, PCR amplification, amplicons computationally clustering criteria for taxonomy, and quantification error due to the different copies of 16S rDNA from various bacteria. As an example, the primers used in routine 16S rDNA amplicon sequencing are designed according to the sequences of conserved domains between V3 and V4 variable domains. However, variation in the conserved domain remains, which means that "universal primers" are not always universal. These primer sets might underperform when they encounter mismatches, which results in the under-amplification of certain organisms. The above factors may explain the

culture-positive but 16S rDNA amplicon sequencing negative cases (B05 and B10). MDS seems more reliable than 16S rDNA amplicon sequencing for avoiding bias and may identify the bacterial species and provide more information by reading longer sequences. However, 16S rDNA amplicon sequencing has its advantages, such as lower cost and higher sensitivity for ocular samples. We need to explore the characteristics of the microbiome of ocular samples from a wider range of sources and improve the procedures of the amplicon sequencing technique, such as screening DNA extraction kits, picking primers, or exploiting more species-specific sequences for identification, to make it suitable for our particular type of sample in the future. Conversely, the conventional culture method should be improved to increase the positive rate and accuracy; the fastidious bacteria discovered in the microbiome of ocular specimens would be an important reference.

Recent studies have reported that the environment can continuously influence the ocular surface microbiome.[27] Considering the complexity of this microbiome, further studies with larger sample sizes and scope are needed to evaluate this aspect. Moreover, new and improved approaches based on NGS will be explored with the accumulation of increasing discoveries from more parts of the world.[21]

Furthermore, identification of the causative pathogen and subsequent targeted treatment should be performed with caution. In cases of monocular corneal infection, the conjunctiva and eyelid margin samples from the contralateral eye can be used to eliminate the interference of background bacteria.

In conclusion, a comprehensive assessment of the causative pathogens of corneal and external infections using data from all the available methods may be advisable while considering the influence of the ocular surface microbiome.

Data Availability Statement

The datasets presented in this study can be found in the National Center for Biotechnology Information (accession number PRJNA744578).

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References

1. Flaxman SR, Bourne RRA, Resnikoff S, et al. Global causes of blindness and distance vision impairment 1990–2020: a systematic review and meta-analysis. *Lancet Glob Health*. 2017;5:e1221–e1234.
2. Aoki R, Fukuda K, Ogawa M, et al. Identification of causative pathogens in eyes with bacterial conjunctivitis by bacterial cell count and microbiota analysis. *Ophthalmology*. 2013;120:668–676.
3. Seitzman GD, Hinterwirth A, Zhong L, et al. Metagenomic Deep Sequencing for the Diagnosis of Corneal and External Disease Infections. *Ophthalmology*. 2019;126:1724–1726.
4. Rudolph T, Welinder-Olsson C, Lind-Brandberg L, Stenevi U. 16S rDNA PCR analysis of infectious keratitis: a case series. *Acta Ophthalmol Scand*. 2004;82:463–467.
5. Chiquet C, Cornut P-L, Benito Y, et al. Eubacterial PCR for Bacterial Detection and Identification in 100 Acute Postcataract Surgery Endophthalmitis. *Invest Opthalmol Vis Sci*. 2008;49:1971–1978.
6. Daroy ML, Lopez JS, Torres BC, Loy MJ, Tuano PM, Matias RR. Identification of unknown ocular pathogens in clinically suspected eye infections using ribosomal RNA gene sequence analysis. *Clin Microbiol Infect*. 2011;17:776–779.
7. Doan T, Acharya NR, Pinsky BA, et al. Metagenomic DNA Sequencing for the Diagnosis of Intraocular Infections. *Ophthalmology*. 2017;124:1247–1248.
8. Lalitha P, Seitzman GD, Kotecha R, et al. Unbiased Pathogen Detection and Host Gene Profiling for Conjunctivitis. *Ophthalmology*. 2019;126:1090–1094.
9. Shivaji S, Jayasudha R, Chakravarthy SK, et al. Alterations in the conjunctival surface bacterial microbiome in bacterial keratitis patients. *Exp Eye Res*. 2021;203:108418.
10. Zilliox MJ, Gange WS, Kuffel G, et al. Assessing the ocular surface microbiome in severe ocular surface diseases. *Ocul Surf*. 2020;18:706–712.
11. Suzuki T, Sutani T, Nakai H, Shirahige K, Kinoshita S. The Microbiome of the Meibum and

Ocular Surface in Healthy Subjects. *Invest Ophthalmol Vis Sci*. 2020;61:18.
12. Shin H, Price K, Albert L, Dodick J, Park L, Dominguez-Bello MG. Changes in the Eye Microbiota Associated with Contact Lens Wearing. *mBio*. 2016;7:e00198.
13. Zhang H, Zhao F, Hutchinson DS, et al. Conjunctival Microbiome Changes Associated With Soft Contact Lens and Orthokeratology Lens Wearing. *Invest Ophthalmol Vis Sci*. 2017;58:128–136.
14. Ozkan J, Nielsen S, Diez-Vives C, Coroneo M, Thomas T, Willcox M. Temporal Stability and Composition of the Ocular Surface Microbiome. *Sci Rep*. 2017;7:9880.
15. Doan T, Wilson MR, Crawford ED, et al. Illuminating uveitis: metagenomic deep sequencing identifies common and rare pathogens. *Genome Med*. 2016;8:90.
16. Ung L, Bispo PJM, Doan T, et al. Clinical metagenomics for infectious corneal ulcers: Rags to riches? *Ocul Surf*. 2020;18:1–12.
17. Lalitha P, Prajna NV, Sikha M, et al. Evaluation of Metagenomic Deep Sequencing as a Diagnostic Test for Infectious Keratitis. *Ophthalmology*. 2021;128:473–475.
18. Brooks JP, Edwards DJ, Harwich MD, Jr., et al. The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol*. 2015;15:66.
19. Boers SA, Hiltemann SD, Stubbs AP, Jansen R, Hays JP. Development and evaluation of a culture-free microbiota profiling platform (MYcrobiota) for clinical diagnostics. *Eur J Clin Microbiol Infect Dis*. 2018;37:1081–1089.
20. Eisenstein M. Microbiology: making the best of PCR bias. *Nature Methods*. 2018;15:317–320.
21. Kuo MT, Chao TL, Kuo SF, et al. A Genomic Approach to Investigating Ocular Surface Microorganisms: Monitoring Core Microbiota on Eyelid Margin with a Dot hybridization Assay. *Int J Mol Sci*. 2020;21:829.
22. Xu S, Guo D, Liu X, et al. Ocular pathogens and antibiotic resistance in microbial keratitis over three years in Harbin, Northeast China. *Acta Ophthalmol*. 2021;8:909–915.
23. Urwin L, Okurowska K, Crowther G, et al. Corneal Infection Models: Tools to Investigate the Role of Biofilms in Bacterial Keratitis. *Cells*. 2020;11:2450.
24. Shrestha GS, Vijay AK, Stapleton F, Henriquez FL, Carnt N. Understanding clinical and immunological features associated with Pseudomonas and Staphylococcus keratitis. *Cont Lens Anterior Eye*. 2021;1:3–13.
25. Nishida T, Sugioka K, Fukuda K, Murakami J. Pivotal Role of Corneal Fibroblasts in Progression to Corneal Ulcer in Bacterial Keratitis. *Int J Mol Sci*. 2021;16:8979.
26. Puig M, Weiss M, Salinas R, Johnson DA, Kheirkhah A. Etiology and Risk Factors for Infectious Keratitis in South Texas. *J Ophthalmic Vis Res*. 2020;2:128–137.
27. Deng Y, Wen X, Hu X, et al. Geographic Difference Shaped Human Ocular Surface Metagenome of Young Han Chinese From Beijing, Wenzhou, and Guangzhou Cities. *Invest Ophthalmol Vis Sci*. 2020;61:47.