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Identification of causative pathogens in mouse eyes with bacterial keratitis by sequence analysis of 16S rDNA libraries

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Abstract: The clone library method using PCR amplification of the 16S ribosomal RNA (rRNA) gene was used to identify pathogens from corneal scrapings of C57BL/6-corneal opacity (B6-Co) mice with bacterial keratitis. All 10 samples from the eyes with bacterial keratitis showed positive PCR results. All 10 samples from the normal cornea showed negative PCR results. In all 10 PCR-positive samples, the predominant and second most predominant species accounted for 20.9 to 40.6% and 14.7 to 26.1%, respectively, of each clone library. The predominant species were *Staphylococcus lentus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. The microbiota analysis detected a diverse group of microbiota in the eyes of B6-Co mice with bacterial keratitis and showed that the causative pathogens could be determined based on percentages of bacterial species in the clone libraries. The bacterial species detected in this study were mostly in accordance with results of studies on clinical bacterial keratitis in human eyes. Based on the results of our previous studies and this study, the B6-Co mouse should be considered a favorable model for studying bacterial keratitis.

Key words : bacteria, keratitis, mice, 16S rDNA

Introduction

Bacterial keratitis is a disease of the cornea characterized by pain, redness, inflammation, and opacity and is a major cause of blindness, particularly in the developing world [15]. The predisposing factors, infecting microorganisms, and therapeutic choices can affect the course and outcome [34]. If appropriate therapy is not promptly initiated, these bacteria can proliferate rapidly and cause a rapidly destructive infection that can lead to loss of the entire eye [24]. A key element in successful treatment of this condition is timely identification of the pathogen. The majority of the early studies of ocular

bacterial infections were focused on clinical cases and treatment [1, 6]. In recent years, studies have expanded to address the mechanisms of pathogenesis and the inflammatory response [9, 35]. The eye is a unique organ that is virtually impermeable to most environmental agents [27]. During normal ocular development in mammals, the eyelids grow across the eye, fuse, and subsequently reopen [31]. An intact corneal epithelium provides an effective barrier against most microorganisms, and therefore, bacterial keratitis rarely occurs in the normal eye [10]. However, predisposing factors such as corneal injury, corneal abnormalities, and overuse of contact lenses may alter the defence mechanisms of the

ocular surface and permit bacteria to invade the cornea through epithelial defects that occur [5].

Infectious diseases of the eye not only involve the host responses to the pathogen but also the effects of bacterial colonization and virulence factors [22]. This interplay between bacterium and host often necessitates the use of live animal models for the study of ocular infections. The mouse eye has many similarities to the human eye, and so mice carrying mutations causing eye defects provide useful models of human eye disease [32]. In addition, the advantage of mice as a model for bacterial keratitis is that mice are easier to operate on and, less expensive to keep in large numbers, and specific and abundant antibodies are available for them, which is beneficial to further research [4]. Mouse models of ocular diseases have been used in therapy efficacy studies of antibiotics and other potential antimicrobial compounds [20].

In mice, defects in embryonic eyelid closure lead to an obvious eye-open-at-birth (EOB) phenotype, in clear contrast to normal mice born with closed eyelids [16]. Without the protection of eyelids, severe corneal inflammation arises in mutant mice shortly after birth, and the affected eyes develop corneal opacity [33]. In our previous study, a heritable corneal opacity mutant mouse of the C57BL/6 strain (B6-Co) was obtained by N-ethyl-N-nitrosourea (ENU) mutagenesis, and the mutant mice exhibited and EOB phenotype and keratitis [28]. Single nucleotide polymorphism (SNP) markers were used to map the mutant gene of B6-Co mice, and the results showed that the mutant gene was located between 112 546 283 and 113 397 654 bp on chromosome 13 [14]. B6-Co is an ENU-induced mutation conveying either a unilateral or bilateral corneal opacity phenotype. The founder B6-Co mouse, which was the progeny of an ENU-treated B6 mouse and an untreated B6 mouse, had a corneal opacity phenotype. After mating the mutant with B6 mice, approximately 43.1% of the progeny were recorded to have the corneal opacity phenotype. The keratitis mice model with genetic deficiency was evaluated clinically and histologically and the results showed that the development process of this phenotype was similar to that of the relevant disease in human eyes [30, 36]. To further evaluate the advantages of this keratitis mice model, this study aimed to identify the causative pathogens in eyes of B6-Co mice with keratitis. To accomplish this, the authors designed a convenient clone library method to analyze 96 clones from each sample and evaluated the dominated bacterial composition.

Materials and Methods

Animals

Twenty C57BL/6 and B6-Co mice were used at 10 days of age (half male (samples 1, 2, 3, 5, and 9) and half female (samples 4, 6, 7, 8, and 10)). B6-Co mice were the progeny of a mutant mouse and B6 mice. All mice were obtained from the Laboratory Animal Center of Nantong University (Nantong, Jiangsu, P.R.China). Mice were raised and maintained under SPF conditions (temperature $21 \pm 1°C$, relative humidity $60 \pm 10\%$) with a 12-h light/12-h dark cycle and provided with food and water *ad libitum*. All samples were collected randomly from different cages to avoid potential litter and cage effects. Cages and supplies were autoclaved. Caretakers entering the barrier were required to shower and change into autoclaved clothes and to put on gloves and mouth and hair covers provided inside the barrier. All animal handling was performed in a laminar air-flow cabinet. All animal experiments were performed in compliance with the Medicine Institutional Animal Care and Use Committee of Nantong University and national regulations and policies.

Sampling and DNA extraction

Ten samples each from the edges of the corneal ulcers of B6-Co mice were obtained with a knife and placed in a sterile tube containing 500 μl of NaCl 0.9%, and ten samples each from the eyes of C57BL/6 mice with normal corneas were examined as controls. For DNA extraction, corneal scrapings were mixed with 500 μl DNA extraction buffer (100 mM Tris-Cl pH 8.0, 150 mM EDTA pH 8.0, 100 mM NaCl, and 1% SDS) and 50 μl lysozyme (20 mg/ml). After incubation at 37°C for 30 mins, 4 μl RNase A (100 mg/ml) and 20 μl proteinase K (20 mg/ml) were added. Total genomic DNA was subsequently extracted using a QIAGEN TIANamp Bacteria DNA Kit (Qiagen, Beijing, P.R.China) according to the protocol of the manufacturer. DNA was eluted with 60 μl ddH$_2$O, and DNA quality was assessed using agarose (1% w/v) gel electrophoresis and staining with SYBR Green 1 Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA). DNA obtained by this treatment was used for PCR.

Amplification of 16S rRNA gene

Bacterial 16S rDNA was amplified from total DNA using a TaKaRa 16S rDNA Bacteria Identification PCR

Kit (TaKaRa, Dalian, P.R.China). All reactions were carried out in 50 μl (total volume) mixtures containing approximately 100 ng of genomic DNA extract, 25 μl PCR Premix, 0.15 mM of each primer, and PCR-grade water to adjust the volume. PCR reactions were performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) set to the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min; and then final extension at 72°C for 5 min. A negative control containing no DNA template was also retained every time. Expected PCR products (approximately 1.5 kb) were visualized on 1% TBE agarose gel stained with SYBR Green 1 Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA). PCR operations were carried out under sterile conditions, and all disposable plasticware was autoclaved and UV treated prior to use. A MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, P.R.China) was used to elute the PCR product from the gel.

Clone library construction

Clone libraries from PCR products were constructed with the pGEM-T Vector System (Promega, Mannheim, Germany), according to the manufacturer's instructions. Ligation reactions (20 μl final, 15 h at 16°C) contained 50 ng of vector pGEM-T, 50 ng of PCR product, and 6U of T4 DNA ligase in supplied buffer. The ligation mixtures were used for heat-shock transformation of 200 μl of *Escherichia coli* DH5α competent cells (Qiagen, Beijing, P.R.China). Cells were grown in Luria-Bertani (LB) agar medium containing 100 μg/ml ampicillin, 240 μg/ml IPTG, and 80 μg/ml X-Gal at 37°C for 16 h, which allows blue-white screening. Selection of positive clones with a 1.5-kb PCR insert was based on the expressed blue-white phenotype. White clones were randomly selected, resuspended in 4 ml of LB-ampicillin (100 μg/ml), and grown under continuous shaking (140 rpm, 15 h, 37°C).

Sequence analyses

A total of 96 white colonies from each clone library were selected randomly for sequencing analysis by Sangon Biotech, P.R.China. The resulting sequences were compared to the known sequences in the database using the NCBI Basic Local Alignments Search Tool (BLAST) algorithm. Clone sequences were checked for chimeras using Chimera Check from Ribosomal Database Project II (Bellerophon (version 3)) to detect and omit chimeric DNA [21]; these chimeras were excluded from further analysis.

Nomenclature and nucleotide sequence accession numbers

For the first library (sample 1), clone names begin with the letters COA (e.g., COA1). For the second library (sample 2), clone names begin with COB (e.g., COB1); the clone names for the other libraries were formed in the same manner. The partial nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers KJ910348 to KJ910369.

Results

Bacterial composition of clone libraries of 10 bacterial corneal samples

The predominant and second most predominant bacterial species and the number of detected species in the clone libraries of the 10 bacterial corneal scrapings with PCR amplification of the 16S rRNA gene are shown in Table 1. Although the bacterial florae were slightly different from each other, the dominant bacterial genera were similar to each other. In 4 of the 10 bacterial corneal samples (samples 3, 4, 6, and 10), the predominant bacterial species was *Staphylococcus lentus* in each sample. Of the remaining 6 samples, the predominant species were *Pseudomonas aeruginosa* (samples 1, 7, and 8) and *Staphylococcus epidermidis* (samples 2, 5, and 9). The percentage of the predominant bacterial species ranged from 20.9 to 40.6% in each sample. The second most predominant species were *Staphylococcus lentus* and *Pseudomonas aeruginosa*, and the percentage ranged from 14.7 to 26.1%.

The detected bacterial species and the respective percentages in each of the clone libraries are shown in Fig. 1. The three dominant species, *Staphylococcus lentus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*, were simultaneously detected in 6 samples (samples 1, 2, 3, 4, 9, and 10), and the total sum of the three species accounted for more than 50% of all species. Of the remaining samples (samples 5, 6, 7, and 8), the total sum of the predominant and second most predominant species accounted for about 50% of all species. Other species such as *Staphylococcus aureus*, *Corynebacterium ammoniagenes*, and *Micrococcus luteus* were also

Table 1. Analysis results of the dominant bacteria in the corneal scrapings

| Phenotype	Sample number	Clone Library Analysis of *16S Ribosomal RNA* Gene	
		Predominant (clones/clones, %†)	Second most dominant (clones/clones, %)
Corneal opacity	1	*Pseudomonas aeruginosa* (20/80, 25.0%)	*Staphylococcus lentus* (15/80, 18.8%)
	2	*Staphylococcus epidermidis* (18/86, 20.9%)	*Pseudomonas aeruginosa* (15/86, 17.4%)
	3	*Staphylococcus lentus* (25/81, 30.9%)	*Pseudomonas aeruginosa* (18/81, 22.2%)
	4	*Staphylococcus lentus* (20/75, 26.7%)	*Pseudomonas aeruginosa* (11/75, 14.7%)
	5	*Staphylococcus epidermidis* (21/71, 29.6%)	*Pseudomonas aeruginosa* (13/71, 18.3%)
	6	*Staphylococcus lentus* (19/69, 27.5%)	*Pseudomonas aeruginosa* (15/69, 21.7%)
	7	*Pseudomonas aeruginosa* (20/61, 32.8%)	*Staphylococcus lentus* (12/61, 19.7%)
	8	*Pseudomonas aeruginosa* (22/68, 32.4%)	*Staphylococcus lentus* (11/68, 16.2%)
	9	*Staphylococcus epidermidis* (28/69, 40.6%)	*Staphylococcus lentus* (18/69, 26.1%)
	10	*Staphylococcus lentus* (20/72, 27.8%)	*Pseudomonas aeruginosa* (12/72, 16.7%)
Normal cornea (control)	11–20	(−)	(−)

†Positive clones/tested clones.
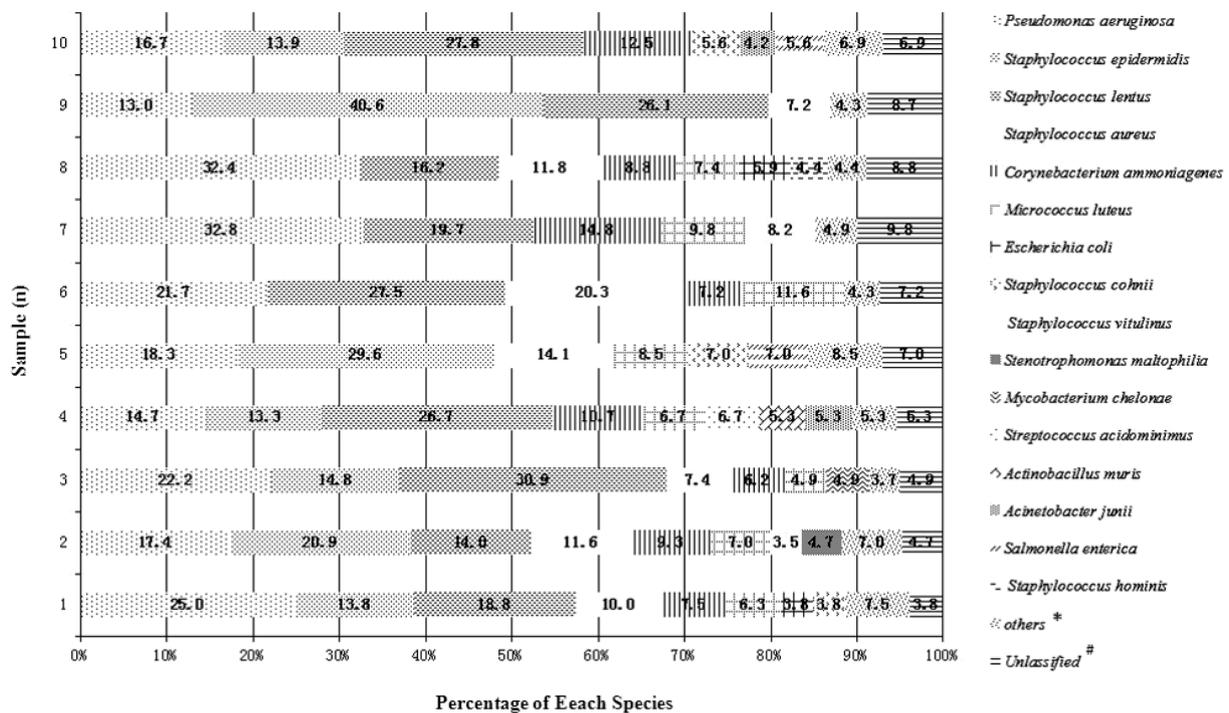
Fig. 1. Graph showing the percentage of bacterial species in each clone library of 10 polymerase chain reaction (PCR)-positive bacterial corneal scrapings (samples 1 through 10).
The stacked bars represent the bacterial composition of the clone libraries of 10 PCR-positive bacterial corneal scrapings. The x- and y-axes represent the percentage of each species and sample number, respectively. * The species with 2 clones or less in each of the clone libraries appear as "others". # The sequences with under 97% similarity to the reference type strain were defined as "unclassified" bacteria.

detected in the sequencing results but at much lower frequencies.

The species with 2 clones or less in each of the clone libraries were designated as "others" in Table 2, and the species shown as "others" made up less than 10% of the species in any of the samples. The sequences that showed less than 97% homology to the reference type strain were grouped as "unclassified bacteria." The numbers of other species and unclassified bacteria in each clone library are shown in Table 2. Other species, such as *Streptococcus sanguinis*, *Arthrobacter tumbae*, and *Rothia nasimurium*, were also detected, and the homology was equal to or more than 97%; however, the clone number for these species were only 1 or 2 in each of the clone

Table 2. Analysis results of the other species and unclassified bacteria in the corneal scrapings

Sample number	Clone Library Analysis of *16S Ribosomal RNA* Gene	
	Other species (≥ 97% homology) (clones/clones, %[†])	Unclassified bacteria (83-96% homology) (clones/clones, %[†])
1	*Acinetobacter junii* (2/80, 2.5%) *Mycobacterium chelonae* (2/80, 2.5%) *Streptococcus sanguinis* (2/80, 2.5%)	*Dechloromonas agitate* (1/80, 1.3%) *Mannheimia glucosida* (1/80, 1.3%) *Staphylococcus sciuri* (1/80, 1.3%)
2	*Acinetobacter junii* (2/86, 2.3%) *Salmonella enteric* (2/86, 2.3%) *Streptococcus acidominimus* (2/86, 2.3%)	*Bacillus carboniphilus* (1/86, 1.2%) *Clostridium algidixylanolyticum* (2/86, 2.3%) *Ruminococcus] gnavus* (1/86, 1.2%)
3	*Staphylococcus cohnii* (1/81, 1.2%) *Actinobacillus muris* (1/81, 1.2%) *Stenotrophomonas maltophilia* (1/81, 1.2%)	*Natranaerovirga pectinivora* (2/81, 1.2%) *Roseburia intestinalis* (2/81, 1.2%)
4	*Arthrobacter tumbae* (2/75, 2.6%) *Mycobacterium chelonae* (2/75, 2.6%)	*Barnesiella intestinihominis* (2/75, 2.6%) *Chryseobacterium hominis* (2/75, 2.6%)
5	*Acinetobacter junii* (2/71, 2.8%) *Actinobacillus muris* (2/71, 2.8%) *Stenotrophomonas maltophilia* (2/71, 2.8%)	*Bacillus firmus* (2/71, 2.8%) *Gordonia effusa* (2/71, 2.8%) *Haemophilus ducreyi* (1/71, 1.4%)
6	*Mycobacterium chelonae* (1/69, 1.4%) *Rothia nasimurium* (1/69, 1.4%) *Staphylococcus cohnii* (1/69, 1.4%)	*Blautia coccoides* (1/69, 1.4%) *Klebsiella oxytoca* (1/69, 1.4%) *Roseburia intestinalis* (1/69, 1.4%) *Stanieria cyanosphaera* (1/69, 1.4%) *Staphylococcus sciuri* (1/69, 1.4%)
7	*Escherichia coli* (1/61, 1.6%) *Mannheimia glucosida* (1/61, 1.6%) *Stenotrophomonas maltophilia* (1/61, 1.6%)	*Blautia coccoides* (1/61, 1.6%) *Clostridium saccharolyticum* (1/61, 1.6%) *Trichodesmium erythraeum* (2/61, 3.2%) *Virgibacillus kekensis* (1/61, 1.6%) *Virgibacillus marismortui* (1/61, 1.6%)
8	*Acinetobacter junii* (1/68, 1.5%) *Actinobacillus muris* (1/68, 1.5%) *Stenotrophomonas maltophilia* (1/68, 1.5%)	*Blautia coccoides* (1/68, 1.5%) *Clostridium saccharolyticum* (2/68, 3.0%) *Eubacterium contortum* (1/68, 1.5%) *Mycobacterium abscessus* (2/68, 3.0%)
9	*Actinobacillus muris* (1/69, 1.4%) *Escherichia coli* (1/69, 1.4%) *Mycobacterium chelonae* (1/69, 1.4%)	*Bacillus firmus* (2/69, 2.8%) *Chryseobacterium hominis* (2/69, 2.8%) *Desulfovibrio desulfuricans* (2/69, 2.8%)
10	*Bacteroides uniformis* (2/72, 2.7%) *Escherichia coli* (1/72, 1.4%) *Stenotrophomonas maltophilia* (2/72, 2.7%)	*Stanieria cyanosphaera* (2/72, 2.7%) *Trichodesmium erythraeum* (1/72, 1.4%) *Virgibacillus marismortui* (2/72, 2.7%)

[†]Positive clones/tested clones.

libraries. The percentage similarity of the 16S rRNA sequences of unclassified bacteria ranged from 83 to 96% compared with reference sequences in the GenBank database.

Discussion

In the present study, the causative pathogens in eyes of the B6-Co mice with bacterial keratitis were investigated by 16S rDNA sequence analyses. Working with corneal scrapings, only very little bacterial material is available. Polymerase chain reaction and sequencing-based detection are suitable for detecting bacteria in ocular samples containing few bacteria [7, 8]. Bacterial ocular infections occasionally have been reported to be polymicrobial by cultures and denaturing gradient gel electrophoresis [19, 26]. Several studies of the detection of bacteria by the clone library method using the 16S rRNA gene have reported on the microbiota of clinical samples [2, 3, 17]. The amplification of 16S rDNA is therefore a useful method for the investigation of such samples. The clone library method can detect the ratios

of detected bacteria in clone libraries, and therefore, this method was applied to the microbiota analysis of bacterial keratitis in this study.

In total, eubacterial DNA could be amplified from investigated corneal scrapings of mice. PCR-positive results were obtained from all specimens taken from corneal scrapings of B6-Co mice. Negative PCR results from control eyes of C57BL/6 mice had been expected. Comparison of sequences with sequences listed in the NCBI database revealed that most of them had sequence similarities of 98 to 100% to sequences of known genera. Analyses of 10 bacterial corneal samples (samples 1 through 10) showed that the percentages of the predominant and second most predominant bacterial species were 20.9 to 40.6% and 14.7 to 26.1% in each sample, respectively. The results showed that the predominant species were *Staphylococcus lentus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. There are no standard criteria for evaluating the pathogenicities of bacterial species detected by the clone library method. However, all normal samples demonstrated negative PCR results, and based on the percentages of bacterial species in clone libraries, it was most likely that all samples were bacterial keratitis caused by the predominant species detected in our study.

Multiple organisms have been reported from bacterial keratitis in human eyes all over the world [5, 15]. Different species of *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus* have been found to play roles as pathogens in different ocular diseases [10, 23, 26]. In this study, corneal scrapings of B6-Co mice were analyzed and the results revealed that *Staphylococcus epidermidis*, *Staphylococcus lentus*, and *Pseudomonas aeruginosa* were the predominant bacteria. Most of the bacteria frequently observed in this study were expected pathogenic organisms causing infections in human eyes. Other detected bacterial species in our study, such as *Actinobacillus spp.*, *Escherichia coli*, *Micrococcus spp.*, *Mycobacterium spp.*, *Salmonella enteric*, *Acinetobacter spp.*, and *Stenotrophomonas maltophilia*, were also reported in other previous studies on microbial keratitis in human eyes [8, 19]. The obtained results in this study are mostly in accordance with results of previous studies on bacterial keratitis in human eyes, which suggests that the B6-Co mouse could be a favorable model for studying bacterial keratitis in human eyes.

The different bacterial species were detected preferentially in each sample. The reasons for this are not completely clear, and similar results were obtained by other researchers [3, 11, 18]. We considered that there may be several possible reasons. Firstly, previous studies showed that sex could affect the composition of the fecal microbiota in mice [11]. The results in this study showed that sex did seem to affect the composition of the microbiota in mouse eyes. Another possible reason for this result was interindividual variation. Microbiologic examination of cecal samples of rats has revealed a substantial interindividual variation [12]. Therefore, it was proposed that a higher inbreeding coefficient could still be achieved in future laboratory mice and that doing so would decrease interindividual variation between the microbiota of mice of the same strain [13]. Kibe *et al.* [18] found that cecal microbiota in mice changed drastically at different ages and that there were also some differences between mice of the same age; that is, an individual specificity difference existed in mice of the same age. According to the results of our study, we supposed that the composition of the microbiota in mouse eyes changed dynamically. Thus, different bacterial species were detected in each sample even though the samples were obtained from mice at the same age. However, the reasons for this need to be further studied.

In our previous studies, pathological analysis of the cornea in B6-Co mice with a genetic deficiency was carried out at different ages [30, 36]. The B6-Co mice were shown to exhibit a gradual development of corneal pathology. The results showed that a serious inflammatory reaction, such as proliferation of fibroblasts, inflammatory cell infiltration, and tissue necrosis, could be observed in the corneas of B6-Co mice on the 10th day after birth under transmission electron microscope (TEM) [30]. Therefore, 10-day-old B6-Co mice with acute bacterial keratitis were chosen for analysis in this study, and we expected to identify causative pathogens in the eyes of the B6-Co mice. The previous studies examining clinical and histological symptoms focused on the dynamic changes in the cornea in B6-Co mice at different stages. Whether differences exist in mice at the same age and between the sexes needs to be explored in subsequent studies given that different bacterial species were detected in each sample in this study.

It has been reported that microbial keratitis could result from infection with viruses, fungi, yeast, and amoebae or from immune-related complications besides bacteria [25, 29]. Although previous investigations reported that the pathogen associated with microbial

keratitis was frequently a bacterium, it is necessary to develop studies of other pathogens associated with microbial keratitis considering their importance [29]. Some researchers are trying to study the pathogens of keratitis. For instance, Ren *et al.* [25] reported that they established rat and mouse models of amoeba keratitis. Other pathogens associated with keratitis in B6-Co mice, such as amoebae and fungi, need to be evaluated in the future.

The B6-Co model of bacterial keratitis established in our laboratory is essential for detailed study of the mechanisms of pathogenesis and immunology of bacterial keratitis. The bacterial species detected in this study were mostly in accordance with results of other studies on clinical bacterial keratitis in human eyes. The results of the present study suggested that the B6-Co mouse could be a favorable model for studying bacterial keratitis in human eyes.

Acknowledgments

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