A DNA Dot Hybridization Model for Assessment of Bacterial Bioburden in Orthokeratology Lens Storage Cases

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Purpose. The aim of this study was to evaluate a DNA dot hybridization assay (DHA) for assessing bacterial bioburden in orthokeratology lens (OK) storage cases.

Methods. Forty-one OK wearers participated in this study. The dot hybridization assay was used to assess the bacterial bioburden of OK after removal and 6-hour soaking in a storage case. Signals of the DHA were standardized after gray image transformation. The correlations between the hybridization intensities of three universal bacteria probes (BP1, BP2, and BP3) and bacterial bioburden determined by culture (colony forming units per milliliter) was analyzed by Pearson's correlation coefficient and receiver operating characteristic plots. In addition, three genus-specific probes for *Pseudomonas*, *Acinetobacter*, and *Klebsiella* were used to detect potentially hazardous bacterial contamination regardless of bacterial viability status.

Results. Among the three universal probes, there were good correlations between probe BP2 ($r^2 = 0.31$, $P = 9.5 \times 10^{-5}$) and probe BP3 ($r^2 = 0.35$, $P = 3.1 \times 10^{-5}$) with bacterial bioburden, but no correlation was found between probe BP1 and bacterial bioburden ($r^2 = 0.04$, $P = 0.11$). In 41 samples, one was *Pseudomonas*-positive by both DHA and culture, while 10 were *Pseudomonas*-positive by DHA but negative by culture. Furthermore, nine samples tested positive for *Acinetobacter* ($n = 7$) and *Klebsiella* ($n = 2$) by DHA only.

Conclusions. The dot hybridization assay provides a novel way to assess the bacterial bioburden of OK storage cases. Lens care quality can be assessed with universal bacteria probes, while potentially hazardous bacterial contamination can be traced with genus-specific probes.

Keywords: dot hybridization assay, bacterial bioburden, orthokeratology, lens storage case

Over 100 million people wear contact lenses (CLs) worldwide.[1] The wearers are generally young (average age: 32 years) and most often female (67% female versus 33% male).[2] The distribution of CLs varies by country, depending on availability, wearer income, culture, and perceptions of eye care practitioners.[3] Contact lenses are convenient optical aids for correction of visual acuity and provide cosmetic benefits and convenience during exercise or outdoor activities. Orthokeratology lenses (OKs) are a reverse-geometry rigid gas-permeable CL developed to retard myopia progression and reduce astigmatism by temporarily flattening the central cornea.[4,5] Contact lenses have become one of the most important and popular methods in visual rehabilitation, complicated majorly by the incidence of microbial keratitis in addition to other minor complications, such as ocular allergy, noninfectious corneal infiltrates and dry eye.[6]

Regardless of type, CL-wearing is the most important risk factor for microbial keratitis in several countries and regions.[7] The incidence of microbial keratitis per 10,000 CL-wearers annually is approximately 1.5 for rigid gas permeable CLs, three for daily-wear hydrogel CLs, 15 for extended-wear hydrogel CLs, and 20 for extended-wear silicone hydrogel CLs.[8] Contact lenses–related microbial keratitis and inflammation is preceded by transfer of microorganisms from the lens to the ocular surface.[9] Testing of CL care equipment identified the same microorganism as the corneal culture in up to 90% individuals with CL-related microbial keratitis.[10,11] Therefore, identification, enumeration, and eradication of microorganisms in and on CL accessories during the course of normal wear are important to identify and prevent the risks of microbial keratitis.[9,11–14]

Culture is the standard method for assessing the microbial bioburden of CL accessories. Because bacterial contamination is much more common than fungal or *Acanthamoeba*

contamination, bacteria—especially *Pseudomonas*—are more frequently associated with CL-related microbial keratitis.[7] Most contaminated lens accessories were found to have multiple species of bacteria,[15] although not all bacteria isolated can cause keratitis.[9] Contaminating bacteria may cause issues other than infection; for instance, toxins released by nonviable bacteria can cause ocular inflammation.[7] Cultures are time-consuming and fail to detect fastidious microorganisms and nonviable bacteria. Assessment of microbial contamination of OK care systems is important since OK is intended for overnight wear in children,[16,17] a vulnerable group at high risk in the event of microbial keratitis. Lens storage cases are the major microbial contamination sources in the lens care system[9,11] and assessing case contamination may provide insight into the microorganisms that may transfer to the lens surface and then to the ocular surface.[10,11]

Here, we proposed a DNA dot hybridization assay (DHA) to assess bacterial bioburden in OK storage cases. The assay is a rapid, specific, and quantifiable molecular technique, and can detect potentially hazardous bacteria, such as *Pseudomonas*, *Acinetobacter*, and *Klebsiella*.

Methods

Subjects

Subjects were enrolled in this prospective study if they had undergone successful OK fitting (Euclid Systems Orthokeratology; Euclid Systems Corporation, Herndon, VA, USA) and treatment for over 1 year in Kaohsiung Chang Gung Memorial Hospital (CGMH). Subjects were excluded if they were aged younger than 8 or older than 18 years, had renewed their lens cases within 3 days before sample collection, or were unable to complete scheduled follow-up. Informed consent was obtained from all subjects after the aims and procedures were explained to the subjects and their responsible parents/guardians. All procedures involving human subjects adhered to the tenets of the Declaration of Helsinki. Institutional review board/ethics committee approval was obtained from the committee of medical ethics and human experiments of CGMH.

Sample Collection

Between January and March 2013, subjects were asked to remove their OK after overnight wear; the lenses were then rinsed, cleaned, and soaked in their cases for approximately 6 hours per each subject's usual habit. Thirty-nine subjects used multipurpose solutions and two subjects used hydrogen peroxidase products for OK disinfection. The entire OK cases were brought to our outpatient department by the study subjects or their parents/guardians on the same day for assessment. After removal of the OK from the case, the inner surface of each case was rubbed with an aseptic cotton swab. The resulting case fluid was collected with a pipette and 1 mL was transferred to a microcentrifuge tube.

Culture-Based Methods

The case fluid (1 mL) was centrifuged at 12,000 rpm for 10 minutes in a microfuge and the precipitate was resuspended in 100 μL phosphate-buffered saline. Each 10 μL of the suspension was spread on blood agar, chocolate agar, and eosin methylene blue agar (all media from BBL, Becton Dickinson and Company, Sparks, MD, USA) for cultivation of bacteria in the fluid. After incubation at 35°C for 72 hours in 5% CO_2, bacteria were identified by Gram staining, followed by standard biochemical tests, including catalase, coagulase, oxidase, and carbohydrate utilization, routinely used at CGMH to identify clinically relevant bacteria. The number of isolated strains and bacterial bioburden (colony forming unit per milliliter [CFU/mL]) were used to characterize bacterial contamination in each lens storage case.

DNA Extraction and Amplification by PCR

An aliquot (30 μL) of case fluid was transferred to a 1.5-mL Eppendorf tube after thawing and centrifuged at 12,000 rpm for 10 minutes. We extracted DNA in the precipitate using a commercial kit (DNeasy Blood & Tissue Kit; Qiagen, Valencia, CA, USA). The microbial DNA was stored at −70°C. Bacteria-specific universal primers 13BF (5′-digoxigenin-GTGAATACGT TCCCGGGCCT-3′) and 6R (5′-digoxigenin-GGGTTYCCCCRTT CRGAAAT-3′; Y = C or T; R = A or G) were used to amplify a DNA fragment that encompassed a portion of the 16S rRNA gene, 16S-23S rRNA spacer region, and a portion of the 23S rRNA gene.[18] Each primer was labeled with a digoxigenin molecule at its 5′ end.

The polymerase chain reaction mixture (25 μL) consisted of 2.5 μL template DNA, 0.4 μM each primer, and other necessary reagents obtained from the PCR kit (JMR-THS5, JMR Holdings, Inc., St. Augustine, FL, USA). Cycling conditions were as follows: initial denaturation (95°C, 3 minutes); 35 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 45 seconds), and extension (72°C, 45 seconds); followed by a final extension at 72°C for 10 minutes. A negative control was performed with each run by replacing the template DNA with sterile water.

Immobilization of Oligonucleotide Probes on Nylon Membrane

Immobilization of oligonucleotide probes on a nylon membrane was described elsewhere.[19] The universal bacteria probes were designed from conserved sequences at the 3′ end of the 16S rRNA gene, while the genus-specific probes were designed from the 16S-23S rRNA spacer regions described previously (Table 1).[20–22] Each probe was diluted 1:1 (final concentration, 10 μM) with a tracking dye solution and spotted on a positively charged nylon membrane (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) using a spotter (SR-A300; EZlife Technology, Taipei, Taiwan).[19] A digoxigenin-labeled irrelevant probe (code M) was used as a marker and spotted on the membrane to form a cross (0.6 × 0.4 cm; Fig. 1). Once all probes had been spotted, the membrane was exposed to a shortwave UV (Stratalinker 1800; Stratagene, La Jolla, CA, USA) for 30 seconds to fix the probes on the membrane.

The Dot Hybridization Assay

A 10-μl aliquot of the PCR product was used for DHA. The reagents and procedures for prehybridization; hybridization (50°C for 60 minutes); reaction with alkaline phosphatase-conjugated antidigoxigenin antibodies and phosphatase substrates were described elsewhere.[21] The hybridized spots (400 μm in diameter) could be read by the naked eye. Images of hybridized patterns were captured with a scanner (ImageScanner III; GE Healthcare, UK).

The hybridization signal intensity was quantified with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). In brief, each captured image was adjusted to a fixed size (300 × 200 pixels) and transformed to gray scale. The gray level of each hybridized dot was detected and recorded. Gray levels in the image background were estimated by averaging the negative controls (NC). Mean gray levels of the markers were calculated by averaging the levels of all marker dots. Corrected intensity of

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Table 1. Oligonucleotide Probes Used in the DHA

Probe	Target Microorganisms	Probe Sequence(s) (5′–3′)
PB1	All bacteria	TGTCGTAACAAGGTAGCCGTA
PB2	All bacteria	GGGGCTAAGTCGTAACAAGGTAGCCGTAttttttttttt†
PB3	All bacteria	GGGGTGAAGTCGTAACAAGGTAGCCGTAttttttttttt
Psu*	*Pseudomonas* spp.	GTTCTTTAAAAATTTGGGTATGTGATAGAA; CAGTGACCAGATTGCTTGGGGTTATATtttttttt
Aci	*Acinetobacter* spp.	GAATCGAGCGTTTTGGTATATGAATTtttttttt
Klb*	*Klebsiella* spp.	TGTGTTCGAGTCTCTCAAATTTTCGC; CTTAAAGAACCTGCCTTTGTAGTGCTC

* Two probes of equal concentration were used in the same dot.
† Multiple bases of thymine (t) were added to the 3′ end of the probe.

markers was obtained by subtraction of the background level from the mean gray level of markers. Similarly, the corrected intensity of a bacterial probe was obtained by subtraction of the background level from the gray level of a bacterial probe. Standardized intensity of a bacterial probe was defined as the corrected intensity of the bacterial probe divided by the corrected intensity of markers in the same image.

Detection Limit and Specificity of the DHA

Serial 10-fold dilutions of genomic DNA extracted from *Pseudomonas aeruginosa* ATCC 10944, *Acinetobacter baumannii* ATCC 15884, and *Klebsiella pneumoniae* ATCC 11644 were analyzed to determine the detection limit of the DHA. Twenty-four species including bacteria (nine species), fungi (10 species), and *Acanthamoeba* (5 species) of pure microorganisms that may cause microbial keratitis were used for specificity testing of the three genus-specific probes (Supplementary Table S1).

Statistical Analysis

A general linear model was used to analyze the correlation between the universal bacteria probes and culture-based bacterial bioburden. Pearson's correlation coefficient was calculated with spreadsheet software (Excel 2010; Microsoft Corporation, Redmond, WA, USA). Receiver operating characteristic (ROC) analysis was used to determine signal thresholds of universal bacteria probes at the predetermined cutoff values of bacterial bioburden. The predetermined cutoff values (200 CFU/mL, 2000 CFU/mL, and 12,000 CFU/mL) were used to classify the levels of bacterial bioburden as rare (≤200 CFU/mL), light (>200 but ≤2000 CFU/mL), moderate (>2000 but ≤12,000), and heavy (>12,000 CFU/mL) contaminations.

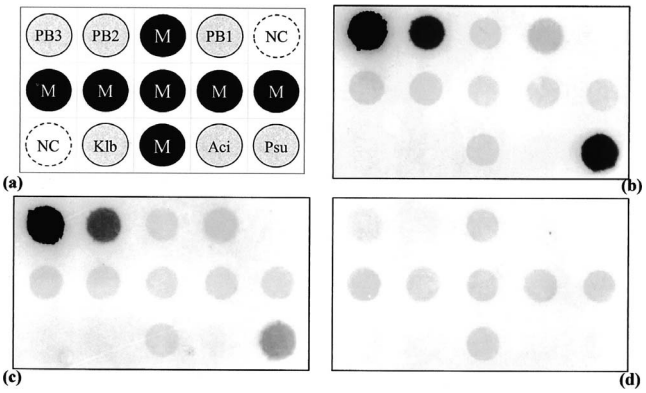


Figure 1. (**a**) Layout of oligonucleotide probes on the array (0.6 × 0.4 cm). The universal bacteria probes PB1, PB2, and PB3 were designed from a conserved region at the 3′ end of the 16S rRNA gene. *Dots* labeled "NC" were negative controls (tracking dye only). *Dots* labeled "M" were position markers (i.e., an irrelevant digoxigenin-labeled oligonucleotide). *Dots* labeled "Psu," "Aci," and "Klb" were genus-specific probes used to identify species of *Pseudomonas*, *Acinetobacter*, and *Klebsiella*, respectively. Probe sequences for all dots are listed in Table 1. (**b**–**d**) Representative hybridization patterns of 10 ng, 10 pg, and 10 fg DNAs of *P. aeruginosa*.

Results

Participants

A total of 41 OK wearers, including 20 females and 21 males, participated in this study. Mean ages were 12.6 ± 2.5 (females, 8–18) and 12.6 ± 2.7 (males, 8–18) years, respectively. There was no significant difference in age between girls and boys.

Bacterial Bioburden of OK Storage Case Determined by Culture

Of the 41 samples, 38 (92.7%) were contaminated with at least one microbe. Eighty-six microbial strains were isolated (Fig. 2a), with nonidentified Gram-positive bacilli being predominant (17 strains; 19.8%), followed by coagulase-negative

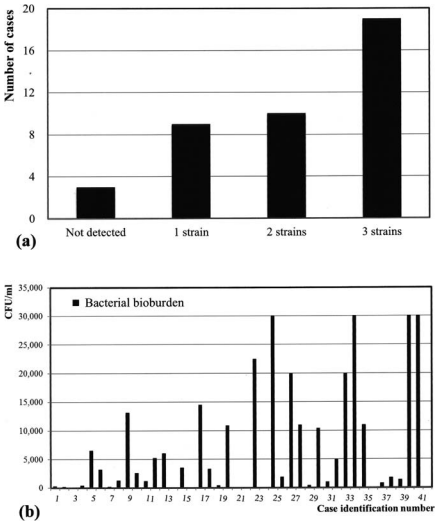


Figure 2. (**a**) Number of bacterial strains isolated from different patients. A total of 86 strains were isolated from 38 of 41 samples. (**b**) Bacterial bioburden (CFU/mL) for each sample.

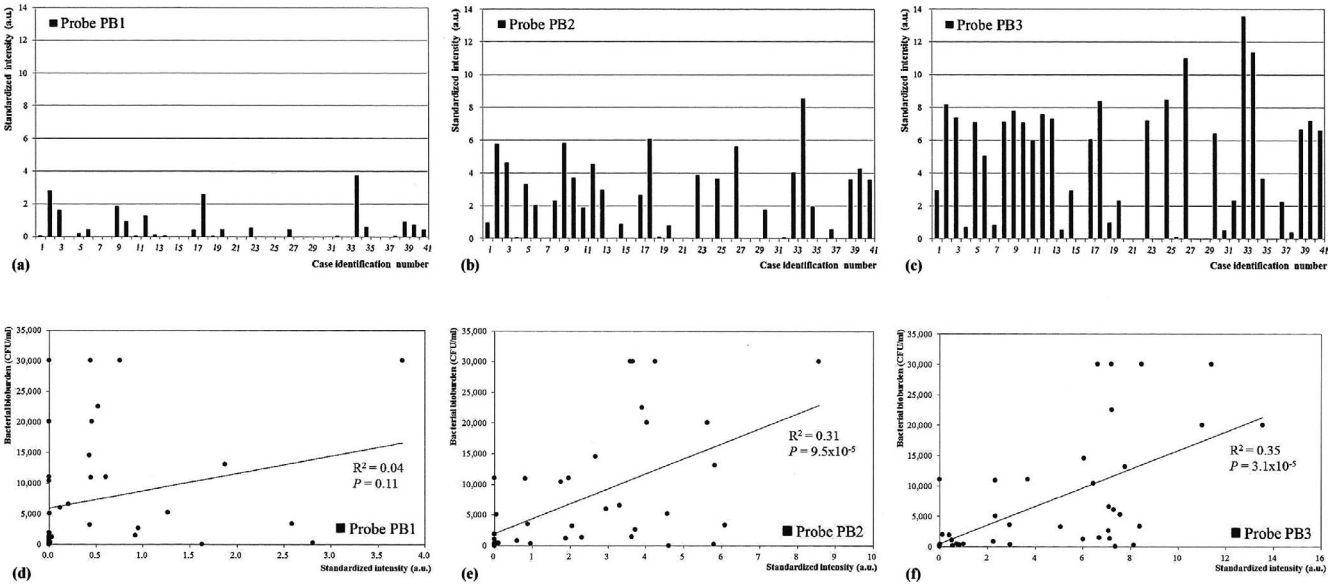


Figure 3. (**a–c**) Standardized hybridization intensities of different samples with each of the three universal bacteria probes (PB1, PB2, and PB3). (**d–f**). Correlation between the hybridization intensities of the three probes and the bacterial bioburden of each sample.

Staphylococcus spp. (15 strains; 17.4%), and Gram-negative glucose nonfermenters (10 strains; 11.6%). Several strains of known pathogens in contact lens–related microbial keratitis were isolated, including *Serratia marcescens* (4 strains; 4.7%), *Pseudomonas aeruginosa* (1 strain; 1.2%), and *Staphylococcus aureus* (1 strain; 1.2%).

The bacterial bioburden of each sample was determined by culture (Fig. 2b) with an average of 7326 ± 9670 CFU/mL (range, 0–30,000/mL). Subjects with three isolated strains had a significantly higher ($P < 0.001$) bacterial bioburden ($n = 19$; 12,863 ± 10,872 CFU/mL) than those with one or two isolated strains ($n = 22$; 2545 ± 5013 CFU/mL). Boys (61.9%, 13/21) were more likely than girls (30%, 6/20) to have three isolated strains ($P < 0.05$). Boys had a significantly higher bacterial bioburden than girls did (10,738 ± 11,661 CFU/mL versus 3745 ± 5230 CFU/mL, $P < 0.05$), although there was no significant difference between age groups (13–18 years, $n = 20$ versus 8–12 years, $n = 21$) in the context of bacterial bioburden (7333 ± 9042 CFU/mL versus 7405 ± 10,502 CFU/mL, $P = 0.98$).

Detection Limit and Specificity of the DHA

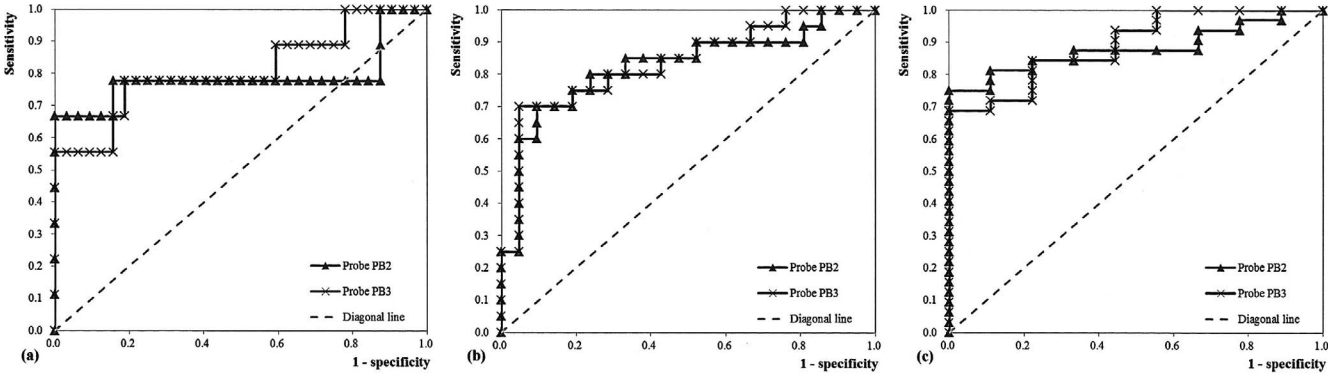
The detection limits (μg DNA/μL) of the three universal bacteria probes and three genus-specific probes were obtained by serial 10-fold dilutions of bacterial genomic DNA (Supplementary Table S2). The detection limits were transformed into bacterial cell numbers based on the estimation that each bacterial cell contains approximately 8.4 fg genomic DNA.[23] Of the three universal probes, PB3 had the lowest detection limit (10 fg, approximately 2 cells), followed by PB2 (100 fg, approximately 12 cells) and PB1 (1 pg, approximately 120 cells). The detection limits of the three genus-specific probes were Psu (100 fg), Aci (1 pg), and Klb (1 pg). A total of 15 species of fungi and *Acanthamoeba* (Supplementary Table S1) did not cross-hybridize with any of the three bacteria universal probes, and species not belonging to the same genus or belonging to different categories of microorganisms (fungi and *Acanthamoeba*) did not cross-hybridize with the three genus-specific probes (Supplementary Table S1).

Bacterial Bioburden of OK Storage Case Assessed by the DHA

Among the universal bacteria probes, PB1 had a relatively higher detection limits (1–10 pg; Supplementary Table S2) and hence had lower sensitivities for assessing contaminating bacteria (Fig. 3a). The probe PB1 correlated poorly with bacterial bioburden (Fig. 3d), but probes PB2 and PB3 provided relatively higher sensitivities (Figs. 3b, 3c) and significantly correlated with bacterial bioburdens (Figs. 3e, 3f). The probe PB3 showed stronger hybridization intensities than PB2, and the results were consistent with its lower detection limits (Supplementary Table S2). According to the areas under curves of ROC plots (Fig. 4), probes PB2 and PB3 showed better grading performance at the highest bacterial bioburden (>12,000 CFU/mL, heavy bioburden); the grading of bacterial contamination may be useful in clinical practice. Some cases were culture negative but DHA positive (Table 2). Of the 41 samples, one was *Pseudomonas*-positive by both culture and DHA, while 10 were *Pseudomonas*-positive by DHA but negative by culture. Furthermore, seven and two samples were *Acinetobacter*- and *Klebsiella*-positive, respectively, by DHA only. Based on the results of culture and DHA, it was demonstrated that the three genus-specific probes (Psu, Aci, and Klb) were able to detect potentially hazardous bacteria, regardless of microbial viability status.

Discussion

It is valuable to assess microbial contamination for CL accessories, not only to identify pathogens in clinically suspected CL-related microbial keratitis, but also as a warning strategy to identify CL wearers with inappropriate lens care habits. Cultures of corneal scrapings and the CL accessories in CL-related keratitis are correlated, and cultures of the CL accessories may provide additional microbiological information in negative corneal scraping cases.[24,25] The issue of lens care quality has become increasingly important for the growing population of OK wearers in areas with a high prevalence of pediatric myopia.[26–29] Conventional cultures for bacteria are



Bacterial bioburden	Probe PB2 Cutoff	%Sensitivity	%Specificity	AUC (95% C.I.)	Probe PB3 Cutoff	%Sensitivity	%Specificity	AUC (95% C.I.)
200	0.03	78	84	0.77 (0.74 - 0.80)	0.92	78	81	0.78 (0.75 - 0.81)
2,000	1.92	80	76	0.83 (0.80 - 0.86)	3.33	75	81	0.84 (0.81 - 0.87)
12,000	3.50	81	89	0.88 (0.85 - 0.91)	7.17	84	78	0.89 (0.87 - 0.91)

Figure 4. Determination of signal cutoff values of the three universal bacteria probes (PB1, PB2, and PB3) based on bacterial bioburdens determined by culture. The cutoff values were obtained by ROC analysis. The cutoff values corresponding to 200 (**a**), 2000 (**b**), and 12,000 (**c**) CFU/mL of bacteria were determined. The inserted table summarized the performance of the cutoff values of probes PB2 and PB3. AUC, area under curve; CI, confidence interval.

time-consuming and may give negative results due to improper transportation of samples, inappropriate media used, or the presence of fastidious and nonviable microorganisms. Here we developed a novel approach to assess bacterial bioburden by a molecular dot assay. The current approach may be a good technique for promoting lens care quality at a cost estimated to be less than $15 USD per test.

According to a comprehensive review by Szczotka-Flynn et al.,[9] microbial bioburden of the lens care system is consistently associated with CL-related corneal inflammation and infection. Among CL accessories, the lens case is the most frequently contaminated site, with a contamination rate over 50% (46%–81%).[30–33] A previous study indicated that most contaminated lens cases were found to contain multiple strains of bacteria.[15] In this study, we determined bacterial bioburden by culture and found the bioburden well correlated with hybridization signals of the two universal bacteria probes (PB2 and PB3; Fig. 3). However, the three genus-specific probes (Psu, Aci, and Klb) were able to detect potentially hazardous microorganisms regardless of bacterial viability (Table 2). If the results of DHA and culture were integrated, the efficacy of the OK disinfection systems was estimated to be 90.9% (10/11), 58.3% (7/12), and 40% (2/5), respectively, for *Pseudomonas* spp., *Acinetobacter* spp., and *Klebsiella* spp. (Table 2).

Table 2. Positive Samples Determined by the Three Genus-Specific Probes and Culture

Genus-Specific Probe	Culture and DHA Positive	Culture and DHA Negative	Culture Positive, DHA Negative	Culture Negative, DHA Positive
Psu	1	30	0	10
Aci	5	29	0	7
Klb	3	36	0	2

There were some confounding factors that might influence the estimation of bacterial bioburden in this study. First, we did not use anaerobic culture for isolation of anaerobes such as *Propionibacterium* sp. Second, bacterial DNA from nonviable bacteria could not be differentiated from viable bacteria by the DHA. Third, PCR efficiency might not be equal for different microorganisms due to different DNA extraction efficiency. These factors may create a selection bias and lead to good (but not excellent) correlations between the universal bacteria probes (PB2 and PB3) and bioburden determined by culture (Figs. 3e, 3f). Regardless of different targets between DHA (bacterial DNA) and culture (viable bacteria), ROC analysis enabled us to establish cutoff signals for universal probes (PB2 and PB3) to grade bacterial bioburden as rare, light, moderate, or heavy (Table 3).

In conclusion, the universal bacteria probes (PB2 and PB3) can be used to assess lens care quality, while the genus-specific probes may forewarn CL wearers of the presence of potentially hazardous microorganisms in the lens storage cases. The novel DHA can be a valuable tool to prevent CL-related microbial keratitis in the increasing population of CL wearers.

Table 3. Establishment of Bacterial Bioburden Using Hybridization Signal Intensities of Universal Bacteria Probes

Grade*	Bacterial Bioburden, CFU/mL	Standardized Intensity (au) of Universal Bacteria Probe PB2	PB3
Rare	≤200	≤0.03	≤0.92
Light	201–2000	0.04–1.92	0.93–3.33
Moderate	2001–12,000	1.92–3.50	3.34–7.17
Heavy	≥12,000	≥3.51	≥7.18

au, arbitrary unit.
* Grade of bacterial contamination.

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