Exploratory Use of Fluorescent SmartProbes for the Rapid Detection of Microbial Isolates Causing Corneal Ulcer

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• PURPOSE: To explore the use of optical SmartProbes for the rapid evaluation of corneal scrapes from patients with suspected microbial keratitis, as a clinical alternative to Gram stain.

• DESIGN: Experimental study with evaluation of a diagnostic technology.

• METHODS: Corneal scrapes were collected from 267 patients presenting with microbial keratitis at a referral cornea clinic in South India. Corneal scrapes were flooded with SmartProbes (BAC One or BAC Two) and evaluated by fluorescence microscopy (without the need for sample washing or further processing). The SmartProbe-labeled samples were scored as bacteria/fungi/none (BAC One) or gram-negative bacteria/none (BAC Two) and compared to Gram stain results.

• RESULTS: Compared to Gram stain, BAC One demonstrated sensitivity and specificity of 80.0% and 87.5%, respectively, positive and negative predictive values (PPV, NPV) of 93.8% and 65.1%, and an accuracy of 82.2. BAC Two demonstrated sensitivity and specificity of 93.3% and 84.8%, respectively, an NPV of 99.2%, and an accuracy of 85.6%. When the corresponding culture results were compared to the Gram stain result, the sensitivity and specificity were 73.4% and 70.7%, the PPV and NPVs were 86.5% and 51.0%, and overall accuracy was 72.6.

• CONCLUSIONS: Fluorescent SmartProbes offer a comparative method to Gram stain for delineating gram-positive or gram-negative bacteria or fungi within corneal scrapes. We demonstrate equivalent or higher sensitivity, specificity, PPV and NPVs, and accuracy than culture to Gram stain. Our approach has scope for point-of-care clinical application to aid in the diagnosis of microbial keratitis. (Am J Ophthalmol 2020;219:341–350. © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).)

MICROBIAL KERATITIS (MK) IS AN OCULAR EMERgency.[1,2] Without microbiological confirmation of the infecting microbe, appropriate management cannot be effectively instigated, particularly so in regions that experience multiple MK etiologies (bacteria, fungi, or protozoa).[3–6] Without rapid and adequate treatment, corneal infection leads to permanent visual loss through corneal scarring, corneal perforations, extrusion of intraocular contents, or intractable secondary glaucoma.[7–11] This diagnostic requirement is particularly pertinent in high-incidence regions such as South and Southeast Asia where ~50% of cases are fungal and ~50% are bacterial, and thus require differential antimicrobial treatment.[8,12–15] Current diagnostic methods (direct smear microscopic examination and/or culture of scraped corneal material) for MK are somewhat dated, require dedicated microbiology laboratory infrastructure, and suffer from a wide variation in their sensitivity and specificity.[16] Although culture of microorganisms is considered to be the gold standard, it may take 2-14 days to yield a diagnosis, which is positive only 50.5% of the time[16,17]—valuable time that cannot be afforded by MK patients. On the other hand, immediate microscopic evaluation of smears provides information on the Gram status of bacteria or the presence of fungi, but again, staining has been shown to be effective in MK diagnosis only 27.3%-61.6% of the time.[16]

An overhaul in the diagnostic approach for MK is required. The purpose of this exploratory study was to assess whether fluorescence microscopy and optical molecular imaging approaches with microbe-specific SmartProbes[18–21] could be an alternative and additive approach to conventional direct microscopy and/or microbial culture for cases of suspected MK. Optical molecular imaging of infection is an emerging field, and here we have investigated the utility of 2 activatable peptide-based fluorescent imaging probes that have been previously developed and evaluated for in situ point-of-care pulmonary applications: BAC One is able to report on the presence of microbes (bacterial and fungal) through an

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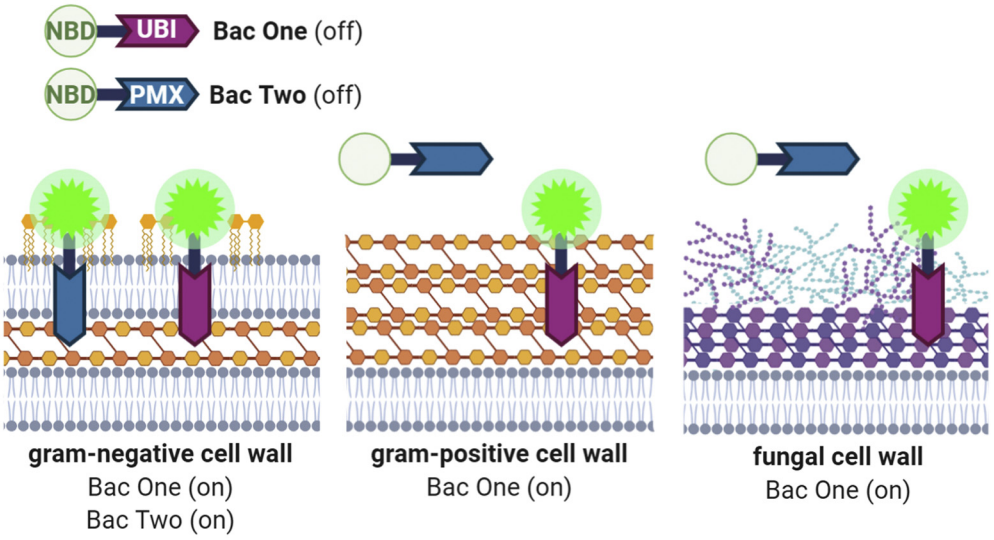

FIGURE 1. Schematic of BAC One and BAC Two binding to target microbes and activating fluorescence signal. NBD = fluorophore; PMX = modified polymyxin B binding domain; UBI = ubiquicidin$_{29\text{-}41}$ binding domain. Not to scale. Created with BioRender.com.

antimicrobial peptide binding fragment, ubiquicidin ($UBI_{29\text{-}41}$)[22,23]; and BAC Two is able to report on the presence of gram-negative bacteria; utilizing a derivative of polymyxin B to bind to lipid A of the gram-negative cell wall.[24] In both cases, the fluorophore incorporated into the compound is 7-nitrobenz-2-oxa-1,3-diazole (NBD), an environmentally sensitive dye that self-quenches in aqueous environments but fluoresces strongly in hydrophobic environments,[25] such as the microbial membrane, thus enabling wash-free detection of positive samples, providing ease of sample preparation and a high signal-to-noise ratio (Figure 1).

We assessed BAC One and BAC Two on 267 clinical corneal scrapes and evaluated their accuracy to detect gram-positive, gram-negative, or fungal isolates compared to clinical gold standards at a tertiary referral eye care center in South India.

METHODS

• **STUDY DESIGN:** The primary objective of this study was to determine the efficacy of the SmartProbes, BAC One and BAC Two, toward the identification of bacteria or fungi within clinical corneal scraping samples by fluorescence microscopy. These SmartProbes are not available commercially. A prospective experimental laboratory study was conducted on 267 patients clinically diagnosed with corneal ulcer during the study period from March 2018 to July 2018. Approval was obtained from the Institutional Review Board of Aravind Eye Hospital, Madurai, India, for this study, and the research adhered to the tenets of the Declaration of Helsinki. After informed consent was obtained from all patients, the corneal material was collected from the infected eye by standard clinical procedure under topical anesthesia (0.5% proparacaine). Specimens included 3 scrapings for smear examination (1 each for Gram stain, 10% KOH wet mount, and labeling with BAC probes [BAC One or BAC Two]). A final scrape was collected for culture on blood agar and potato dextrose agar. Gram stain, KOH staining, and culture were performed as per standard clinical practice. Identification of the bacteria or fungi was based on the colony morphology and standard biochemical procedures. Information regarding demographic details, predisposing factors, clinical course, treatment, and visual outcome was also collected during the study period. The results obtained by fluorescence microscopy with the SmartProbes did not inform any clinical decisions or patient management.

• **IN VITRO VALIDATION OF BAC ONE AND BAC TWO WITH OCULAR PATHOGENS:** BAC One and BAC Two have previously been evaluated against a panel of lung pathogens.[23,24] Here they were evaluated against clinically isolated ocular pathogens; bacterial isolates *Staphylococcus epidermidis* (gram-positive) and *Pseudomonas aeruginosa* (gram-negative); and fungal isolates *Aspergillus flavus* and *Fusarium spp*. Colonies were sampled from solid media with a toothpick and smeared onto a glass slide within 20 μL saline. The slides were allowed to dry and underwent staining with BAC One or BAC Two, either immediately or after 5 hours storage on the bench (to mimic clinical scenarios). A total of 20 μL of each SmartProbe (5 μM in saline) was added directly to the slide, as appropriate, and the sample was mounted with a coverslip and sealed with nail

polish. The samples were immediately interrogated by fluorescence microscopy (Zeiss Axio Scope A1 (Carl Zeiss Microscopy GmbH, Jena, Germany), FITC filters, 1 s integration time).

• **PREPARATION OF BAC ONE AND BAC TWO CLINICAL SPECIMENS:** Corneal scrapes were collected under standard clinical conditions. A total of 20 μL of 5 μM BAC One (for patient samples collected between March 9, 2018, and April 20, 2018) or BAC Two (for patient samples collected between April 23, 2018, and July 31, 2018) was added directly to the specimen on the same day following collection. A coverslip was added directly to the sample and sealed with nail polish. Clinical-grade BAC One and BAC Two were synthesized and characterized in-house at the University of Edinburgh as previously described.[23,24]

ASSESSMENT OF BAC ONE AND BAC TWO: TRAINING SET

THE MICROBIOLOGIST SCORING THE LABELED CLINICAL samples trained by examining 50 samples with prior knowledge of the Gram stain result. Slides were examined with a Nikon Eclipse 50i fluorescence microscope, with Nikon Mercury lamp and FITC filters (Nikon, Tokyo, Japan). Visualization of the sample was performed using a 40× objective.

ASSESSMENT OF BAC ONE AND BAC TWO: CLINICAL SAMPLES

THE EXAMINING MICROBIOLOGIST WAS THEN MASKED TO the Gram stain and culture result when scoring the BAC One (n = 107) and BAC Two (n = 160) samples by fluorescence microscopy as outlined above. Representative images, for display purposes, were captured using a smartphone (Huawei P-smart, model FIG-LX1 (Huawei Technologies Co., Ltd, Shenzhen, China)) held over the eye-piece. Image capture was performed with fixed manual settings (including ISO, shutter, and exposure valuation) to enable comparison between samples, followed by brightness and contrast enhancement with Fiji_ImageJ for presentation. Following BAC One or BAC Two bacterial/fungal/negative scoring, the sample was compared to both Gram stain result and microbiological culture result, which had been captured according to the standard clinical procedure.

• **STATISTICAL ANALYSIS:** Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy scores, including 95% confidence intervals were calculated using Prism 8.4 (GraphPad Software, San Diego, CA, USA). The same software was used for plotting the graphical representations of the data.

RESULTS

• **CLINICAL DIAGNOSIS OF CORNEAL SAMPLES:** Following informed consent, corneal scrapes were collected from 267 patients presenting with suspected MK within the corneal clinic at Aravind Eye Hospital, Madurai, South India between the months of March-April 2018 (Cohort 1, BAC One) and April-July 2018 (Cohort 2, BAC Two). Scrapes were collected for standard clinical diagnosis by direct microscopy (Gram stain) and culture. Although direct observation of stained corneal smears provides a rapid preliminary indication of causative microbial etiology, culture remains the clinical gold standard. A summary of Gram stain results compared to the clinical gold standard attained within this study is shown in Table 1.

Of the 267 corneal samples collected, following microbial culture, 112 (41.9%) had fungal growth alone and 47 (17.6%) had bacterial growth alone. One eye (0.4%) had *Acanthamoeba* only and the remaining 104 (39%) had no growth. Three corneal samples cultured 2 organisms; all 3 grew gram-negative bacteria plus fungi (in 2 cases) and *Acanthamoeba* (in 1 case).

Supplemental Table 1 (Supplemental Material at AJO.com) presents the spectrum of microbial isolates isolated from the 163 corneal samples with culture-confirmed MK. A total of 166 microbial isolates were isolated, of which 30% were bacterial and 69% were fungal. The most common bacterial isolate was *Pseudomonas aeruginosa* (25, 15.1%), followed by *Streptococcus pneumoniae* (8, 4.8%). *Fusarium* species (39, 23.5%) and *Aspergillus flavus* (22, 13.3%) were the most commonly isolated fungal pathogens.

When the same 267 clinically retrieved corneal samples were examined by direct observation (Gram stain and microscopy), 200 (75%) were positive for microbial pathogens, compared to 61% that were culture positive. A total of 146 (54.7%) were positive for fungi, 49 (18.3%) were positive for bacteria, and 4 (1.5%) smears had mixed organisms. One sample (0.4%) was positive for *Acanthamoeba* and the remaining 67 (25.1%) had no discernable microbe identified.

• **IN VITRO VALIDATION OF BAC ONE AND BAC TWO ON MICROBIAL KERATITIS PATHOGENS:** We have previously demonstrated that BAC One is able to fluorescently label gram-positive and gram-negative bacteria, and *Aspergillus fumigatus*[23] in vitro, without the requirement of removal of unbound, excess probe prior to visualization by confocal fluorescence microscopy. Similarly, BAC Two was shown to be selective to gram-negative bacteria in our previous work.[24] Here we validated the selectivity of both BAC One and BAC Two on clinically isolated ocular pathogens from patients with microbial keratitis, which were smeared directly onto slides (mimic of clinical specimens) to optimize our labeling procedure, determining that a concentration of 5 μM was optimal for specific microbe labeling and

TABLE 1. Correlation Between Direct Microscopy (Gram Stain)–Based and Microbial Culture–Based Diagnosis of Microbial Keratitis

	Direct Microscopy						
	Gram-positive Bacteria	Gram-negative Bacteria	Fungi	Amoeba	Mixed species	Negative	Total
Culture							
Gram-positive bacteria	13 (72.2%)*	0	1 (5.6%)	0	1 (5.6%)	3 (16.7%)	18 (6.7%)
Gram-negative bacteria	1 (3.4%)	23 (79.3%)*	0	0	0	5 (17.2%)	29 (10.9%)
Fungi	1 (0.9%)	1 (0.9%)	103 (92%)*	0	1 (0.9%)	6 (5.4%)	112 (41.9%)
Amoeba	0	0	0	1 (100%)*	0	0	1 (0.4%)
Mixed species	0	0	2 (66.7%)	0	1 (33.3%)*	0	3 (1.1%)
Negative	3 (2.9%)	7 (6.7%)	40 (38.5%)	0	1 (1%)	53 (51%)*	104 (39.0%)
Total	18 (6.7%)	31 (11.6%)	146 (54.7%)	1 (0.4%)	4 (1.5%)	67 (25.1%)	267

Asterisk indicates corroborating results between the 2 compared methodologies.

imaging using a wide-field fluorescent microscope. BAC One was able to label all bacterial and fungal strains (Figure 2A), while BAC Two enabled specific labeling of the gram-negative bacterium *P. aeruginosa* ocular isolate, and did not demonstrate any off-target labelling of gram-positive *S. epidermidis* or *Fusarium* (Figure 2B). One small *A. flavus* filament did appear to produce a positive BAC Two signal (Figure 2B, white arrow); however, the morphology was clearly distinct from that of bacteria.

• **BAC ONE AND BAC TWO OFFER EQUIVALENT OR BETTER SENSITIVITY AND SPECIFICITY COMPARED TO GOLD-STANDARD ASSESSMENTS:** Each patient specimen (n = 267) underwent direct microscopy (Gram stain) and microbial culture as part of standard clinical practice, the results of which are outlined above (Table 1 and Supplemental Table 1). BAC One (n = 107) or BAC Two (n = 160) was added to 1 additional clinical smear as appropriate, and the scoring microbiologist was masked to the clinical diagnosis while assessing the BAC One/BAC Two–labeled slide by fluorescence microscopy.

Table 2 and Supplemental Figure 1 (Supplemental Material at AJO.com) present the summary comparison of sensitivity, specificity, PPV and NPV, and the overall accuracy of BAC One, BAC Two, and culture compared to direct microscopy following Gram stain. Comparisons to the microbial culture are reported in Supplemental Table 2 and Supplemental Figure 2 (Supplemental Material at AJO.com). Both BAC One and BAC Two demonstrated equivalent or increased sensitivity, specificity, and accuracy toward the Gram stain result compared to microbial culture, as determined by overlapping (equivalent) or nonoverlapping (improved or reduced performance) 95% confidence intervals. The low PPV for BAC Two (second row of Table 2; based on fluorescence only) toward identifying gram-negative bacteria was attributed to the small proportion (19.5%) of fungal isolates that were recorded as fluorescent. The morphology of the BAC Two–labeled fungi did enable a classification that was distinct from bacteria, and when morphology was considered (third row of Table 2) the PPV increased from 38.9% to 88.9%. Although BAC Two labeling of fungal isolates was not anticipated, we were able to demonstrate that smeared fungal isolates left on the bench for 4-5 hours prior to addition of BAC Two did exhibit some fluorescence (Supplemental Figure 3, Supplemental Material at AJO.com) that was not observed when BAC Two was added within an hour of culture smear (Figure 2). Moreover, it is widely reported that fungi exhibit some autofluorescence at this wavelength, and that may be a contributing factor.

• **COHORT 1: BAC ONE:** BAC One fluorescence is an indicator of microbial presence. With the addition of morphologic validation (ie, size and shape), it was possible for the operator to score the slide as "Bacteria," "Fungi," or "Negative," but it was not possible to delineate the Gram status. The results were compared to the immediate Gram stain result and the microbial culture result (Table 3). In summary, 87 (81.3%) of BAC One scored samples matched the smear microscopy (Gram stain) result, and 81 (75.7%) matched the microbial culture result. By comparison, 72 (67.3%) of smear microscopy samples matched the microbial culture results (Supplemental Table 3, Supplemental Material at AJO.com). Representative images from both the Gram stain (obtained with a 100× objective under oil immersion) and fluorescence imaging (obtained with a 40× objective) are shown in Figure 3. In all instances, the images were captured via a smartphone camera down the eye-piece and therefore the size of the field-of-view (FOV) within the image cannot be deduced and varies between images.

BAC One vs Direct Microscopy. A total of 45 of 54 (83.3%) of the clinical samples were positive for fungi based on both direct microscopy (Gram stain) and fluorescence microscopy (BAC One). The remaining 9, which were

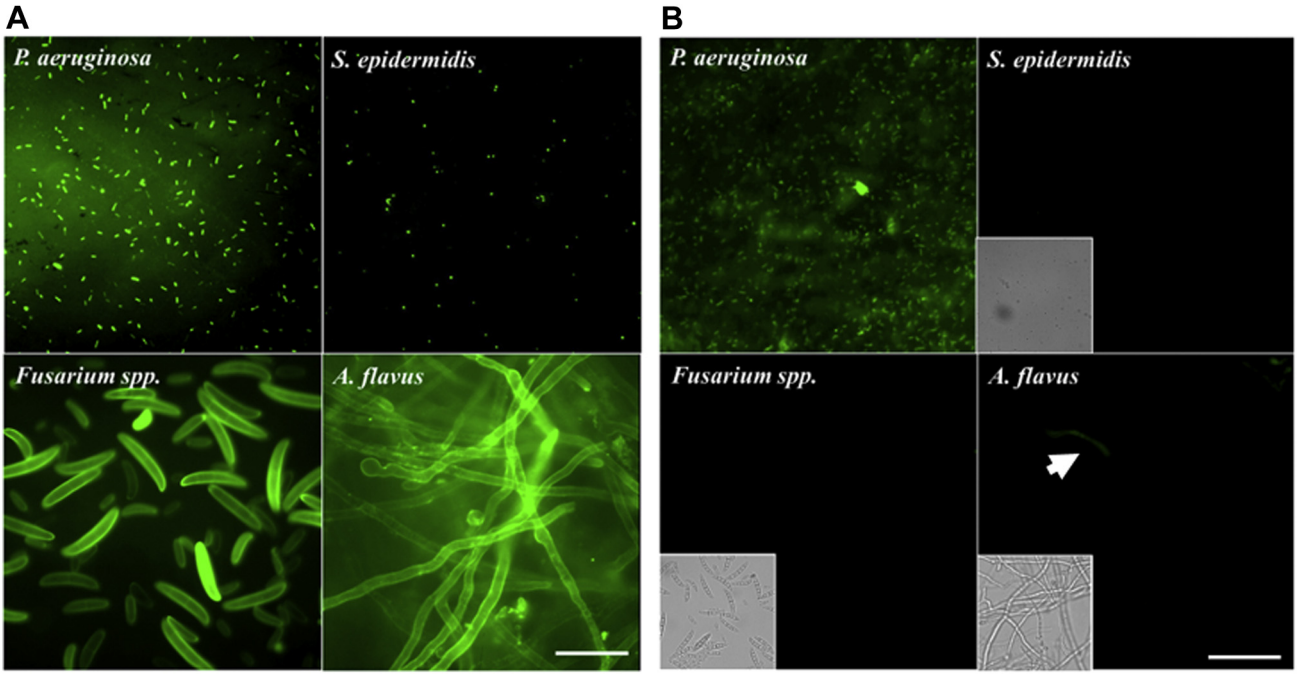

FIGURE 2. BAC One labels all microbes, and BAC Two is selective for gram-negative bacteria. BAC One (A) (5 μM) and BAC Two (B) (5 μM) were added to smears of ocular clinical isolates (*Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Fusarium*, and *Aspergillus flavus*) and imaged by wide-fluorescence microscopy using 488 nm excitation and FITC filters. White arrow shows fluorescent fungal filament. Scale bar = 50 μM.

fungal positive by direct microscopy, were BAC One negative. There was also excellent similarity in results for microbe-negative samples between the 2 modalities, with 28 of 32 (87.5%) of negative direct microscopy samples also being scored as fluorescence negative. The largest discrepancy lay in the reporting of bacterial samples. Only 14 of the 21 (66.7%) bacterial specimens identified by direct microscopy were recorded as positive by fluorescence microscopy, 6 (28.6%) were recorded as negative (5 of 6 were culture negative), and 1 was positive for fungus (and culture positive for fungus). This could be attributed to scarcity of bacteria within the sample or be due to the comparatively smaller size of bacterial cells compared to fungi, which may be difficult to visualize at 40× magnification, particularly if the sample had a large amount of autofluorescent tissue present.

BAC One vs Culture. A total of 14 of 17 (82.4%) bacterial and 32 of 37 (86.5%) fungal specimens determined by culture were also BAC One positive. The remaining culture-positive samples were recorded as BAC One negative. The major discrepancy between the microbial culture and BAC One scoring was for culture-negative samples. Although they matched in two-thirds of cases (35/53), 16 samples (30.2%) that were culture negative were fungal positive by fluorescence imaging (and also Gram stain), with the remaining 2 positive for bacteria (by fluorescence imaging and Gram stain).

• **COHORT TWO: BAC TWO:** BAC Two should enable the selective identification of gram-negative bacteria based on fluorescence (ie, without requiring morphologic data to be observed). BAC Two was validated against 160 patient samples. While BAC Two offered ≥90% identification of gram-negative specimens within this study when compared to both the Gram stain result and the microbiological culture result (Table 4), there was fluorescent signal recorded for ~20% of the fungal samples, thus accounting for the relatively low PPV reported in Table 2 (second row). Despite this, sensitivity and specificity values of 90% and 87.1%, respectively, were achieved by using BAC Two as a diagnostic tool for gram-negative bacteria, based on fluorescence only. When the morphology information was added alongside the fluorescence information the PPV was increased to 97.2% (vs 50.0% without morphology data) (Table 2, third row). The addition of morphologic information further increased the sensitivity and specificity compared to the Gram stain with respect to the microbial culture results.

In summary, 137 (85.6%) of BAC Two scored samples matched the smear microscopy (Gram stain) result, (96.9% when fungal-positive isolates were included), and 141 (88.1%) matched the microbial culture result (98.8% when fungal-positive isolates were included). By comparison, 121 (75.6%) of smear microscopy samples matched the microbial culture results for these patients (Supplemental Table 4, Supplemental Material at AJO.com). Representative images from both the Gram stain (obtained under oil immersion

TABLE 2. Comparison of BAC One, BAC Two, and Microbial Culture to Gram Stain Scoring

	Vs Direct Microscopy					
	Sensitivity (95 % CI)	Specificity (95 % CI)	PPV (95 % CI)	NPV (95 % CI)	Accuracy (95 % CI)	
BAC One	80.0 (69.6-87.5)	87.5 (71.9-95.0)	93.8 (85.0-97.5)	65.1 (50.2-77.6)	82.2 (73.7-89.0)	n = 107
BAC Two[a]	93.3 (70.2-99.7)	84.8 (78.1-89.8)	38.9** (24.8-55.1)	99.2* (95.6-100)	85.6* (79.2-90.7)	n = 160
BAC Two[b]	97.0* (84.7-99.8)	96.9* (92.2-98.8)	88.9 (74.7-95.6)	99.2* (95.6-100)	96.9* (92.9-99.0)	n = 160
Culture	73.4 (66.8-79.2)	70.7 (59.6-79.8)	86.5 (80.4-90.9)	51.0 (44.5-60.4)	72.6 (66.9-77.9)	n = 267

CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value.

All values presented as %. Value with no asterisk indicates "equivalence," single asterisk indicates "superior," and double asterisk indicates "inferior," determined by overlapping/separated CIs with Culture.

[a]BAC Two–labeled fungi samples classified as false-positive (no morphology consideration).

[b]Fungi-positive samples classified as true-positives based on fluorescence plus morphology.

TABLE 3. Correlation Between Direct Microscopy (Gram Stain)–, Culture–, and Optical Image Probe Assay (BAC One)–Based Diagnosis of Microbial Keratitis

	BAC One			
	Bacteria	Fungi	Negative	Total
Direct microscopy				
Bacteria	14 (66.7%)*	1 (4.7%)	6 (28.6%)	21 (19.6%)
Fungi	0	45 (83.3%)*	9 (16.7%)	54 (50.5%)
Negative	2 (6.25%)	2 (6.25%)	28 (87.5%)*	32 (29.9%)
Total	16 (15%)	48 (44.8%)	43 (40.2%)	107
Culture				
Bacteria	14 (82.4%)*	0	3 (17.6%)	17 (15.9%)
Fungi	0	32 (86.5%)*	5 (13.5%)	37 (34.6%)
Negative	2 (3.8%)	16 (30.2%)	35 (66%)*	53 (49.5%)
Total	16 (15%)	48 (44.8%)	43 (40.2%)	107

Asterisk indicates corroborating results between the 2 compared methodologies.

with a 100× objective) and fluorescence imaging (obtained with a 40× objective) are shown in Figure 4. In all instances, the images were captured via the smartphone camera down the eye-piece, and therefore the size of FOV within the image cannot be deduced and varies between images.

BAC Two vs Smear Microscopy. Fourteen of the 160 patient samples were recorded as gram-negative based on direct smear microscopy. Thirteen of these 14 (92.9%) were also identified as gram-negative bacteria based on BAC Two fluorescence imaging and morphology. Eighteen of 92 (19.6%) fungal specimens identified by direct microscopy were also positive for fluorescence. These could readily be distinguished from bacterial samples based on the size and morphology of the organism. As expected, it is noteworthy that BAC Two did not identify any gram-positive bacteria, as determined by Gram stain.

BAC Two vs Culture. Twenty of 160 specimens were recorded as gram-negative following microbial culture; 18 of these (90%) were also BAC Two positive. In concordance with the direct microscopy results, 17 of the 18 fungal-positive fluorescent samples were also cultured as fungal positive, and BAC Two did not enable any identification of gram-positive bacteria, as determined by microbial culture. Of the fungal isolates that were fluorescent, 9 were *A. flavus*, 4 were *Fusarium spp.*, 1 was *Pythium insidiosum*, 2 were unidentified hyaline fungi, and 1 was unidentified dematiaceous fungi.

DISCUSSION

THE IDEAL TREATMENT FOR A CORNEAL ULCER SHOULD BE tailored based on microbiological guidance rather than

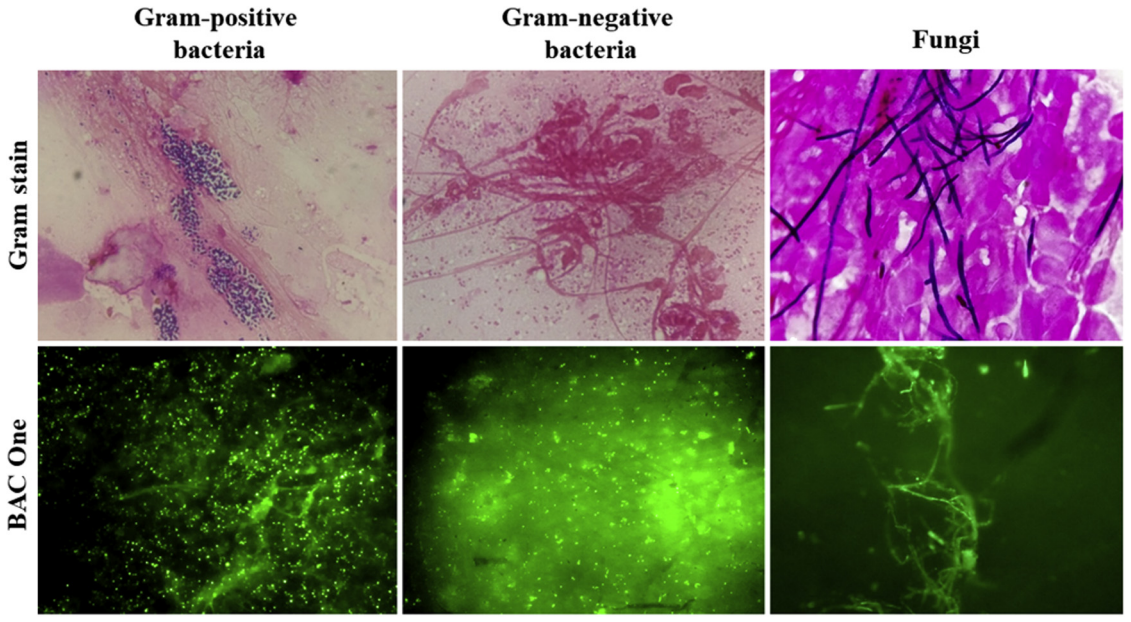

FIGURE 3. BAC One allows identification of all microbes. Representative bright-field and fluorescent images of corneal scrapes following Gram stain and BAC One treatment. Bright-field images were viewed with 100× objective under oil immersion; fluorescence images were viewed with 40× objective using 488 nm excitation and FITC filters. A smartphone camera with fixed settings was used to capture images via the microscope eye-pieces.

clinical presentation, where visual delineation between bacteria and fungi is known to be challenging.[5,6] However, except for tertiary care facilities with dedicated microbiological setup, anecdotal experiences suggest that empirical treatment is being initiated based on clinical appearance of MK. The reasons for this may be nonavailability of trained microbiologists and less-than-optimum results obtained by current smear and culture,[3,4,16] the reasons for which can include prior antimicrobial use, low microbe numbers within the smear, and inexperienced microbiologists. It is evident that alternative, cost-effective approaches to MK diagnosis are required.

Indeed, many of the drawbacks of direct smear microscopy from MK patient smears are similar to those encountered by operators examining sputum smear samples from suspected tuberculosis patients that have been stained with colored dyes.[26] Vast improvements have been achieved in the field of tuberculosis sputum smear microscopy through the incorporation of fluorescence microscopy and *Mycobacteria*-specific fluorescent stains (such as auramine and rhodamine), leading to increased sensitivity, particularly with low-grade positives, while retaining specificity. Fluorescence dyes also enable slides to be examined at a lower magnification compared to nonfluorescent colored dyes, increasing the FOV, thus reducing both operator time and fatigue.

Our ambition was to evaluate the potential of using fluorescent SmartProbes to label ocular pathogens within corneal scrapes, and to compare the sensitivity and specificity of our technique to direct microscopy and culture. To this end, we selected 2 optical SmartProbes (BAC One and BAC Two) for incorporation into this study, which we have previously evaluated clinically for in situ, point-of-care pulmonology applications.[23,24] However, their efficacy toward MK pathogens, the sample type (human corneal scrape, which will likely include tissue debris and dead microbes), and the sample handling chain were unknown at the outset of this study. We were able to demonstrate wash-free labeling of bacteria and fungi by BAC One (using a combination of fluorescence and morphology to delineate between the two) with an accuracy of 82.2% compared to direct microscopy (Gram stain) and 75.2% compared to culture (there was a 72.6% accuracy between direct microscopy and culture).

BAC Two was only anticipated to identify gram-negative bacteria; however, ~20 percent of fungal samples were also recorded as fluorescent within the BAC Two cohort. While this could be a demonstration of off-target labeling of samples that had been left on the bench throughout the work day (as we were able to show in Supplemental Figure 3), it may also be a product of fungal autofluorescence, which has been reported within the literature for UV and blue-light excitation, as used within this study.[27] Further work using fluorescence intensity thresholding is warranted to fully characterize the underlying contribution of off-target labeling with these fungal samples. Importantly, those BAC Two–positive fungal specimens were all characterized as fungi based on the

TABLE 4. Correlation Between Direct Microscopy (Gram Stain)–, Culture–, and Optical Image Probe Assay (BAC Two)–Based Diagnosis of Microbial Keratitis

	BAC Two					
	Gram-positive Bacteria	Gram-negative Bacteria	Fungi	Amoeba	Negative	Total
Direct microscopy						
Gram-positive bacteria	0	0	0	0	15 (100%)	15 (9.4%)
Gram-negative bacteria	0	13 (92.9%)*	0	0	1 (7.1%)	
Fungi	0	1 (1.1%)	18 (19.6%)**	0	73 (79.3%)	92 (57.5%)
Amoeba	0	0	0	0*	1 (100%)	1 (0.6%)
Mixed organisms	0	1[a] (33.3%)*	0	0	2 (66.7%)	3 (1.9%)
Negative	0	3 (8.6%)	0	0	32 (91.4%)*	35 (21.9%)
Total	0	18 (11.25%)	18 (11.25%)	0	124 (77.5%)	160
Culture						
Gram-positive bacteria	0	0	0	0	13 (100%)	13 (8.1%)
Gram-negative bacteria	0	16 (94.1%)*	0	0	1 (5.9%)	17 (10.6%)
Fungi	0	0	17 (22.7%)**	0	58 (77.3%)	75 (46.9%)
Amoeba	0	0	0	0*	1 (100%)	1 (0.6%)
Mixed organisms	0	2[a] (66.7%)*	0	0	1 (33.3%)	3 (1.9%)
Negative	0	0	1 (2%)	0	50 (98%)*	51 (31.2%)
Total	0	18 (11.25%)	18 (11.25%)	0	124 (77.5%)	160

Single asterisk indicates corroborating results between the 2 compared methodologies. Double asterisk indicates corroborating results, but unexpected for BAC Two.

[a]Positive for gram-negative bacteria by direct microscopy and/or culture.

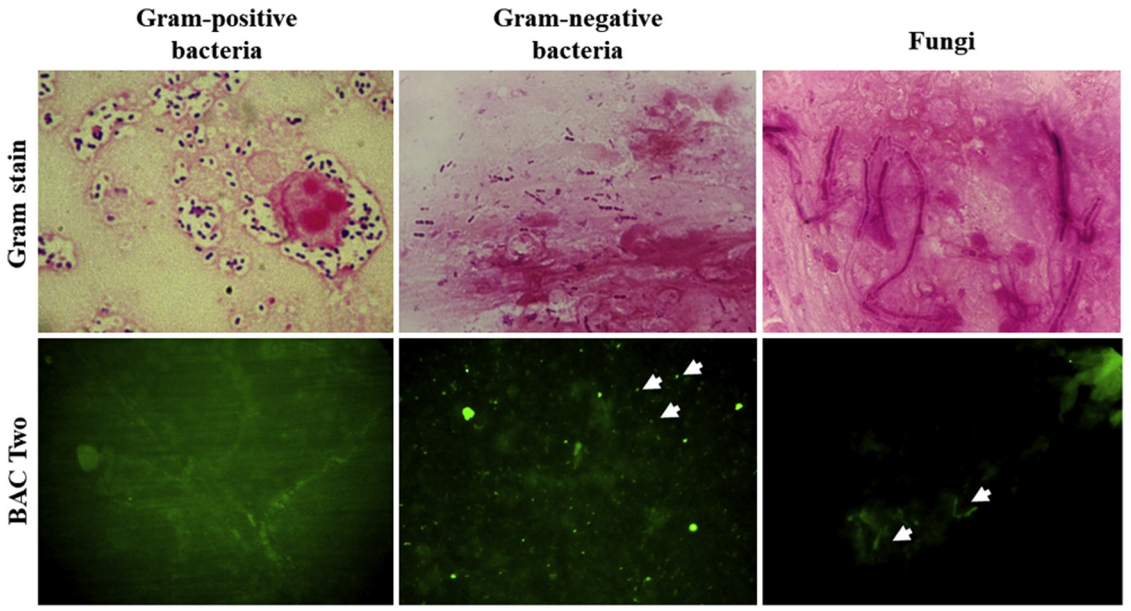

FIGURE 4. BAC Two selectively labels gram-negative bacteria over gram-positive. Representative bright-field and fluorescent images of corneal scrapes following Gram stain and BAC Two incubation. Bright-field images were viewed with 100× objective under oil immersion; fluorescence images were viewed with 40× objective using 488 nm excitation and FITC filters. A smartphone camera with fixed settings was used to capture images via the microscope eye-pieces. White arrow shows fluorescently labeled microorganism.

morphology of the cell. Vitally, no off-target labeling of gram-positive bacteria was observed, which is the key motivation for using BAC Two, as BAC One is unable to differentiate bacteria based on Gram status. Thus when signal and morphology were taken into consideration, an accuracy score of 96.9% was reached, with sensitivity,

specificity, and NPV all above 95% compared to direct smear microscopy and microbial culture.

In all cases, we found that BAC One and BAC Two examination provided equivalent, if not better, corroboration to the direct microscopy (Gram stain) and culture results than direct microscopy did to culture (as demonstrated by overlapping [for equivalence] or nonoverlapping [for improved] 95% confidence intervals). Additionally, our approach enabled simple sample processing, with the SmartProbe added directly to the slide and coverslip added without any fixation or wash procedure, and the fluorescence microscopy technique was readily learned by the microbiologist. We were also able to demonstrate that a 40× microscope objective was sufficient for screening the SmartProbe-labeled slides, compared to the use of a 100× objective for direct microscopy, thus enabling a larger FOV to be examined, making slides quicker and easier to examine. It should also be noted that 75% of the patients within our study were microbe positive based on Gram stain. This is in contrast to 27.3%-61.6% reported within the literature.[16] Microbial culturing was only slightly better than reported within the literature, thus suggesting that the microscopist conducting the Gram stain procedure and imaging within this study was highly skilled; and thus the value of our proposed BAC One and BAC Two approach, with its simplified processing steps and ease of imaging, may indeed be increased in other laboratory scenarios, although this warrants further investigation.

Although in the past, fluorescent microscopes have been prohibitively expensive and considered specialist equipment, there has been a recent concerted effort to innovate low-cost optical systems that can be brought to the point-of-care, rather than located within centralized core laboratories.[28–31] We believe that such systems could be used for our optical molecular imaging approach, and may be particularly useful for centers that do not have in-house microbiology laboratories or facilities for preparing samples for Gram stain or growing culture.[32] We anticipate that such devices could eventually enable automation of fluorescence and morphology detection, negating the need for highly skilled on-site microbiologists to examine each sample.

Here we have taken the first step in demonstrating efficacy of 2 well-characterized "lung" SmartProbes on a large (n = 267) ophthalmic clinical sample set and determined that performance is at least equivalent to clinical direct microscopy (Gram stain) when compared to culture (the clinical gold standard). Preparation and examination of the samples fit well within usual laboratory procedure. Additionally, performance may be further enhanced with the development and incorporation of specific fungal SmartProbes,[33] particularly if developed in a shifted spectral window to BAC One or Two, which will enable wash-free multiplexing.

In conclusion, we have shown that fluorescent SmartProbes offer a comparative method to direct microscopy (Gram stain) for delineating gram-positive or gram-negative bacteria or fungi within corneal scrapes, demonstrating equivalent or higher levels of sensitivity, specificity, PPV and NPV, and accuracy than culture to Gram stain, without the need for sample fixation or washing. We believe that this work opens an exciting avenue for modernizing the diagnosis of microbial keratitis and has true clinical tractability, with the potential to bring user-friendly, cost-effective microbial keratitis diagnosis to the bedside for the first time.

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Data Availability: All raw data is available via the Edinburgh DataShare (https://doi.org/10.7488/ds/2812).

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