The Relative Value of Confocal Microscopy and Superficial Corneal Scrapings in the Diagnosis of *Acanthamoeba* Keratitis

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Purpose: To compare the relative diagnostic value of confocal microscopy and superficial corneal cultures in the diagnosis of *Acanthamoeba* keratitis by using clinical and microbiologic definitions of disease.

Methods: Results of confocal microscopy, superficial corneal smear, and superficial corneal culture were analyzed for validity against 2 different microbiologic and a clinical composite standard for *Acanthamoeba* keratitis.

Results: In patients with both clinical characteristics and objective evidence of *Acanthamoeba* keratitis, confocal microscopy exhibited a sensitivity of 90.6% (95% confidence interval [CI]: 79.3%–96.9%) and a specificity of 100% (95% CI: 95.0%–100%). In patients with either positive culture or smear evidence of *Acanthamoeba* keratitis, confocal microscopy showed a sensitivity of 90.9% (95% CI: 78.3%–97.5%) and specificity of 90.1% (95% CI: 81.5%–95.6%). In strictly culture-positive patients, confocal microscopy showed a sensitivity of 92.9% (95% CI: 76.5%–99.1%) and a specificity of 77.3% (95% CI: 67.7%–85.2%). Of the 53 patients with *Acanthamoeba* keratitis, confocal microscopy was positive in 48 patients, whereas corneal smears and cultures were positive in 30 of 41 and 23 of 42 patients, respectively. Sensitivity of *Acanthamoeba* culture was 52.8% (95% CI: 38.6%–66.7%) in patients with a clinical diagnosis of *Acanthamoeba* keratitis. Simultaneous testing of smear and superficial corneal scraping resulted in a sensitivity of 83.0% (95% CI: 70.2%–91.9%), independent of the results of confocal microscopy.

Conclusions: As confocal microscopy comes into wider clinical use, it remains in need of clinical and pathologic correlation. When performed and interpreted by an experienced operator, confocal microscopy is both sensitive and specific in the diagnosis of *Acanthamoeba* keratitis. Contemporaneous corneal scrapings are independently sensitive in the detection of *Acanthamoeba* keratitis, and a combination of both diagnostic modalities offers the highest likelihood of rapidly and accurately diagnosing *Acanthamoeba* keratitis in patients with atypical keratitis.

Key Words: *Acanthamoeba*, keratitis, confocal microscopy, culture, histology

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First described in the 1970s as an ocular pathogen, *Acanthamoeba* causes a rare, chronic keratitis refractory to traditional antibiotic therapy. Early reports suggested a poor prognosis for *Acanthamoeba* keratitis (AK) primarily because of a lack of effective medical therapy and, secondarily, because of significant delays in diagnosis. With the emergence of more effective agents, clinical studies suggest improved treatment success with earlier diagnosis and, correspondingly, an earlier stage of corneal involvement.[1,2] Therefore, accurate and early diagnosis is integral to the prognosis of AK; however, AK diagnosis has and continues to be heavily dependent on the clinical features of AK, some of which may be nonspecific to AK and seen in unrelated disorders.[3–5]

Diagnostic tests for AK include microbiologic identification of *Acanthamoeba* from various sources, including epithelial scrapings, corneal biopsy, and corneal pathology, as well as contact lens solutions and cases.[6–13] The relative value of these various microbiologic methods is not well understood, and although they are generally considered to have high specificity, they are often perceived as having low sensitivity and, therefore, marginal use because special techniques are needed to isolate acanthamoebae.[6] These microbiologic hurdles, combined with a low index of clinical suspicion caused by the rarity of the infection, represent a significant barrier in AK diagnosis.

Confocal microscopy use in AK diagnosis is attractive because it can provide a noninvasive method to image corneal *Acanthamoeba* cysts. Confocal imaging of corneas with suspected AK show bright white opacities similar in size and shape to *Acanthamoeba* cysts[14]; however, most series using confocal microscopy have low microbiologic isolation rates, raising the possibility of confocal microscopy either having a substantially higher sensitivity or producing substantially more false-positive test results, making a definitive diagnosis uncertain.[6,15,16] Difficulties in evaluation of newer diagnostic modalities with enhanced sensitivities parallel challenges

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faced in evaluation of diagnostic tests in systemic disease (eg, *Chlamydia trachomatis*), in which novel, non–culture-based technologies compared against the less sensitive, historical "gold standard" of isolation by culture.[17] Although various study methods have been used and criticized in comparison to an outdated reference or gold standard in systemic disease diagnosis, multitest reference standards have been applied in the validation of newer technologies.[17–20] The purpose of this study is to evaluate the relative diagnostic value of confocal microscopy and superficial corneal scrapings in AK diagnosis by using a similar, multitest reference standard consisting of clinical and microbiologic definitions of disease in a cohort of subjects undergoing confocal microscopy for atypical keratitis.[21]

MATERIALS AND METHODS

The University of Illinois at Chicago (UIC) institutional review board reviewed and approved this research. A retrospective cohort study including all UIC patients undergoing confocal microscopy corneal imaging for atypical keratitis was conducted to evaluate the sensitivity, specificity, and positive and negative predictive value (PPV, NPV) of clinical criteria and laboratory tests used in AK diagnosis in patients with an atypical keratitis presentation. An atypical keratitis cohort was chosen to allow evaluation of a population most representative of congruent diagnostic applications because AK is often nonspecific and clinically indistinguishable from other types of keratitis.

Assemblage of the Confocal Microscopy Cohort

All patients (N = 125) evaluated for potential AK with confocal microscopy at the UIC Cornea Service between June 1, 2003, and September 1, 2006, were identified and included in the cohort. Patients evaluated with confocal microscopy for potential AK were identified for analysis by searching the following confocal microscopy diagnostic codes: *Acanthamoeba*, possible *Acanthamoeba,* rule out *Acanthamoeba*, chronic keratitis, contact lens keratitis, corneal ulcer, deep keratitis, fungal infection, fungal keratitis, *Fusarium*, herpes simplex virus, immune keratitis, immune keratopathy, keratitis, keratitis atypical, keratitis immune. Scan results for patients without a diagnostic code were evaluated, and patients with epithelial and stromal scans were included in analysis (scans of the endothelium only were excluded as endothelial cell counts).

UIC Cornea Service patients were considered for confocal microscopy if they had an atypical keratitis, which was defined by the presence of any the following general

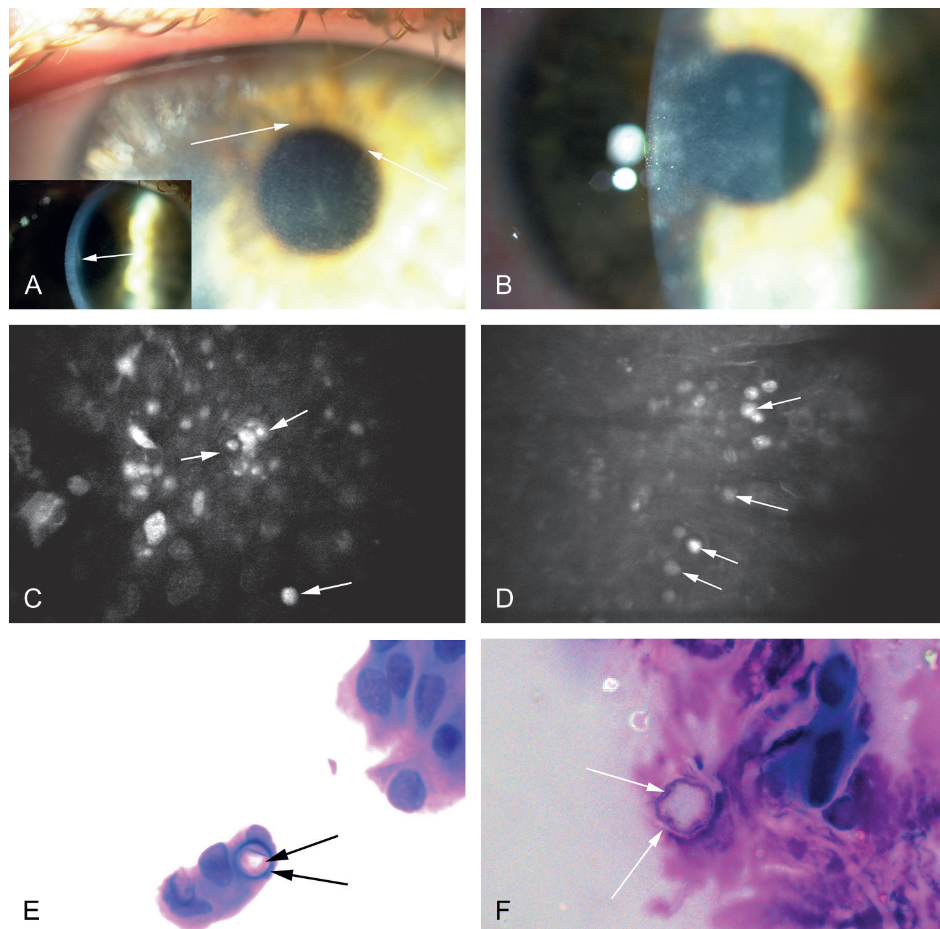

FIGURE 1. Case 1 (top left). A 14-year-old contact lens wearer with chronic *Acanthamoeba* epitheliitis: Arrows show sharp demarcation line between normal (above) and infested (below) corneal epithelium. Inset, slit-lamp beam showing central radial neuritis. Case 1 (top right). Slit-lamp photomicrograph 2 months later showing resolving lesions with residual scarring. Case 1 (middle right). Confocal microscopy with the Confoscan 2.0 showing multiple forms throughout the epithelium with white and dark centered targets and dense reflective ovoid forms. (Middle left) Confocal microscopy of another patient with epitheliitis by using the Confoscan 3.0; note the improved detail of the nuclear centers and better imaging of background cysts and structures. Case 1 (bottom left). Diff-Quik stain of superficial corneal smear showing a double-walled cyst (arrows) with a polygonal inner wall. (Bottom right) Diff-Quik corneal smear of another patient showing a characteristic starlike configuration of the inner cyst wall made up of 5 cogs or pores.

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criteria: (1) an atypical appearance (Fig. 1A), the presence of keratoneuritis, ring infiltrate, geographic or diffuse granular epitheliopathy, nummular infiltrates, or stromal keratitis; (2) an atypical history, chronic keratitis, keratitis refractory to conventional medications, routine culture-negative keratitis, contact lens wear with risk factors of poor hygiene or water exposure; or (3) an unusually painful keratitis. Patients were evaluated at the slit lamp by 1 clinician (E.Y.T.) to determine the applicability of confocal microscopy and the position and depth of corneal involvement.

Confocal Microscopy

Full-thickness corneal imaging with confocal microscopy was performed by 1 operator (E.Y.T.) over the central portion of densest inflammation and in areas just peripheral to the area of keratitis. The Confoscan 2.0 (NIDEK, Gamagori, Japan) was used until November 2005, after which a Confoscan 3.0 (NIDEK) was used on subsequent patients; both instruments use identical slit-scanning technologies. Three to 7 scans were performed until the areas of regard were adequately imaged. Transmitted light intensity was adjusted during the scan to improve resolution and minimize scatter in areas of high reflectivity. The semiautomatic setting, which allows 1 trigger to start a full-thickness scan, was used for all but the smallest ulcers, which were imaged by using the manual mode to maximize optical sections within a small area. Scans were read immediately for signs of infectious forms.

Confocal microscopy was treated as positive with the identification of forms consistent with *Acanthamoeba* cysts or trophozoites (Figs. 1C, D). These included classically described round or ovoid dense highly refractile bodies measuring 10–25 μm; it also included round or ovoid forms of similar size with a dense bright white center and dark surrounding cyst wall (target form), a bright surrounding cyst wall with dark nuclear center, or a paired reflection from the cyst interior wall resembling a pair of kidney beans or coffee beans with concave surfaces facing each other (Nakano EM et al. IOVS 2006;47:ARVO E-Abstract 1352). For purposes of analysis, scans were categorized as positive only when multiple forms suggestive of AK were noted. Negative scans were interpretable and showed no signs suggestive of AK. Scans that were uninterpretable secondary to corneal haze, patient movement, or exuberant cellular response were defined as equivocal and treated as negative for purposes of analysis. Scans that contained 1 suggestive form in a scan series were also graded as equivocal and treated as negative for analysis; only 1 subject exhibited 1 suggestive form.

Superficial Corneal Sampling

Corneal scraping for smear and culture was recommended to all patients with positive confocal microscopy results and all patients with clinical features or a history suggestive of AK. In patients with primarily epithelial disease, the entire affected epithelium was removed with a sterile Kimura spatula both for diagnostic confirmation and therapeutic debridement. In patients with deeper disease, the ulcer base and surrounding epithelium were included. A small portion of the uninoculated specimen was smeared directly onto 1 or 2 glass slides and stained by using the Diff-Quik system (DIFCO, Detroit, MI), a modified Romanowski stain consisting of methanol fixation with methylene blue and eosin. These were examined by 1 clinician (E.Y.T.) for signs of *Acanthamoeba* cysts or trophozoites (Figs. 1E, F). Most of the sample was dislodged into and transported in sterile Page saline. *Acanthamoeba* cultures were grown by using nonnutrient agar plates with an *Escherichia coli* overlay incubated at 37°C, plated by the UIC laboratory. Beginning in July 2005, corneal scrapings from all presumed AK cases were also sent to the Ohio State University (OSU) for culturing and genetic typing of *Acanthamoeba* organisms. Corneal scrapings received at OSU were cultured in amoeba saline with *Enterobacter aerogenes* or nonnutrient amoeba saline agar with *E. aerogenes* prey and incubated at 30°C. When needed, additional cultures were performed by using media appropriate for naked amoebae. When scant material was available, preference was given to culture samples. Five patients diagnosed with AK refused scrapings at UIC for various reasons including the need to drive or insurance restrictions, as well as the referring physician's preferences. Subjects with a positive *Acanthamoeba* culture at either UIC or OSU were treated as positive *Acanthamoeba* cultures in the analysis.

AK Disease–positive Subjects

Patients were defined as AK cases if they presented with an atypical keratitis with at least 1 of the following conditions: (1) identification of trophozoites or cysts on confocal microscopy; (2) identification of trophozoites or cysts through smears when specimens were stained with Diff-Quik stain; (3) positive *Acanthamoeba* cultures; or (4) pathology identification of AK on keratoplasty specimens. Only incident cases diagnosed at UIC were included. Ancillary culture sources (eg, contact lens cases, solutions, and household water) were not used in diagnosis because they represent indeterminate evidence of disease.[22,23] Diagnosis of definite AK disease was on the basis of clinical, microbiologic, and confocal evidence of disease along with appropriate response to anti-*Acanthamoeba* therapy as outlined in the following. The 5 patients who refused corneal scrapings and were diagnosed as AK and successfully managed were treated as positive for each definition. Patients with bilateral disease were analyzed at the subject level by using data from the first symptomatically presenting eye only. Subjects without evidence of disease by confocal microscopy who were monitored and recovered without treatment known as effective against *Acanthamoeba* were judged as free of AK. Follow-up was performed by the authors or confirmed with referring physicians to identify AK disease state.

Analysis

The accuracy and validity of AK diagnostic and screening tests were evaluated in regard to 3 main questions: (1) can confocal microscopy provide accurate diagnostic results; (2) what is the sensitivity of cultures when used as a stand-alone test; and (3) are diagnostic results improved with the initial use of multiple, simultaneous microbial tests (ie, does use of cultures and smears improve sensitivity compared with use of cultures alone).

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Reference Standards: Definition of AK Disease

Because of the inadequacies of 1 reference standard in AK disease identification, analysis included multiple reference standards in which 3 disease definitions were identified for comparison purposes: culture AK, microbial AK positive, and definite AK disease, as defined in the following. Culture AK is the most rigorous disease definition, whereas definite AK disease is the least rigorous and is primarily from clinical findings with secondary objective support.

Culture AK is on the basis of *Acanthamoeba* culture results and defined as positive if the *Acanthamoeba* culture result is positive ($n_{positive}$ = 25; $n_{negative}$ = 100).

Microbial AK positive is on the basis of a simultaneous testing protocol and defined as positive if any result for *Acanthamoeba* cultures, smear, or pathology is positive; it is defined as negative if results from smear, *Acanthamoeba* cultures, and pathology are all negative ($n_{positive}$ = 41; $n_{negative}$ = 84).

Definite AK disease is a clinical definition on the basis of disease resolution with anti-*Acanthamoeba* treatment in which disease is defined as positive on the basis of a keratitis with a positive result for at least 1 of the following tests: confocal microscopy, *Acanthamoeba* cultures, smear, or pathology of keratoplasty specimens ($n_{positive}$ = 50; $n_{negative}$ = 75).

To determine whether confocal microscopy can provide accurate diagnostic results (question 1), we compared confocal microscopy against each of the 3 different reference standards. To establish the accuracy of cultures in AK when used as a stand-alone test (question 2), we assumed the definition of definite AK disease, although not perfect in identifying AK disease, in secondary analyses as the reference or gold standard. Finally, to determine whether the initial use of multiple simultaneous tests improves diagnostic results (question 3), we used the same definite AK disease definition as the reference standard against which the simultaneous testing protocol was compared.

Analyses were performed by using SAS version 9.1.3 (SAS Institute, Cary, NC) with estimation of exact binomial-based 95% confidence intervals (CIs). The 2-tailed Student *t* test was used to compare continuous variables, and the χ^2 test was used to compare categorical variables (Fisher exact test for comparisons between rigid contact lens use and contact lens use in general) in Table 1.

RESULTS

Fifty-three patients in the cohort were diagnosed with AK. Descriptive statistics for subjects with and without AK are provided in Table 1. Five of 53 AK patients had bilateral evidence of disease. Forty-eight (91%) of 53 subjects had findings characteristic of AK on confocal microscopy. Cultures were obtained on 42 of 53 patients, of which 23 (55%) were positive. Histologic smears were performed on 41 patients and positive in 30 (73%). Five patients needed corneal transplantation for either persistent disease or optical reasons, and positive pathologic evidence of disease was present in 4 corneal buttons. Summary statistics of diagnostic test results and AK disease definitions are presented in Table 2; among subjects classified with positive disease, positive smears and cultures were possible in both early and late disease.

Confocal microscopy diagnostic test sensitivity ranged between 90.6% and 92.9% (95% CI: 76.5%–99.1%), and specificity ranged from 77.3% to 100% (95% CI: 67.7%–100%) depending on the reference standard used in comparison (Table 3). Highest sensitivity and lowest specificity were seen with the most rigorous definition (culture AK), whereas lowest sensitivity and highest specificity were seen with the most relaxed definition (definite AK disease). Negative confocal microscopy findings were highly predictive of AK disease absence regardless of disease definition (NPV: 93.5%–97.4%; 95% CI: 85.5%–99.7%); however, predictive values differed by disease prevalence and may vary in different populations. Statistically significant likelihood ratios, which estimate the probability of a positive test result among AK subjects versus the same positive result in subjects without AK independent of disease prevalence, indicate the strong predictive significance of a positive result with confocal microscopy, regardless of disease definition (Table 3).

Sensitivity of stand-alone cultures in AK diagnosis (culture AK) approximated chance (52.8%; 95% CI: 38.6%–66.7%), whereas specificity was high (100%; 95% CI: 95.0%–100%; Table 4). The rate of culture positivity was poorer early in the series than later: Only 3 of the first 10 cultures were

TABLE 1. Descriptive Demographic Information for the Confocal Microscopy Cohort (N = 125)

Demographic	*Acanthamoeba* Keratitis Positive	*Acanthamoeba* Keratitis Negative
Number	53	72
Age (y)		
Mean ± SD (range)	28.9 ± 16.7 (13–70)	35.0 ± 15.5 (10–73)
Male sex [n (%)]	29 (54.7)	28 (38.9)
Current contact lens use [n (%)]		
Contact lens use	51 (96.2)	53 (79.1)
Contact lens type		
Soft contact lens use; *P* = 0.16	47	53
Rigid contact lens use; *P* = 0.04	4	0
Missing data	0	5

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TABLE 2. AK Diagnostic Test Results and AK Disease Definitions (Italics) Used in Diagnostic Test Accuracy Analysis for All Patients in the Confocal Microscopy Cohort

AK Diagnostic Test	AK Disease Positive Total* (n)	AK Disease Negative (n)	Test Result AK Negative† (n)	Followed Up and Judged Free of AK (n)	Total (N)
Confocal	48	77	*77*	*0*	125
Positive AK culture	23	102	*35*	*67*	125
Disease onset ≤6 wks‡	14 of 18	—	—	—	—
Disease onset >6 wks§	4 of 8	—	—	—	—
Disease onset missing	5	—	—	—	—
Positive smear	30	95	*8*	*87*	125
Disease onset ≤6 wks¶	22 of 28	—	—	—	—
Disease onset >6 wks**	7 of 8	—	—	—	—
Disease onset missing	1	—	—	—	—
Pathology	4	121	*1*	*120*	125
AK disease definition††					
Culture AK	28	97	*35*	*62*	125
Microbial AK positive	44	81	*24*	*57*	125
Definite AK disease‡‡	53	72	*72*	*0*	125

*AK disease positive/negative columns represent the final disease classification used in analysis.
†Test result AK negative and followed up and judged free of AK represent how the AK disease–negative status was decided.
‡Among AK disease–positive subjects, 14 reporting a disease onset ≤6 weeks had a positive AK culture, whereas the remaining 4 subjects reporting a disease onset ≤6 weeks had a negative AK culture.
§Among AK disease–positive subjects, 4 reporting a disease onset >6 weeks had a positive AK culture, whereas the remaining 4 subjects reporting a disease onset >6 weeks had a negative AK culture.
¶Among AK disease–positive subjects, 22 reporting a disease onset ≤6 weeks had a positive smear, whereas the remaining 6 reporting a disease onset ≤6 weeks had a negative smear.
**Among AK disease–positive subjects, 7 reporting a disease onset >6 weeks had a positive smear, whereas the remaining 1 reporting a disease onset >6 weeks had a negative smear.
††Five AK cases refused corneal scrapings but were considered AK positive, which is reflected in the AK disease definitions.
‡‡Actual disease status is defined through the clinical definition "definite AK disease."

positive compared with 8 of the last 10 cultures (data not shown). Sensitivity increased (83.0%; 95% CI: 70.2%–91.9%) with a simultaneous protocol when smears were performed in addition to cultures without compromising specificity (100%; 95% CI: 95.0%–100%; Table 5).

DISCUSSION

Our results showed the value of noninvasive confocal microscopy in diagnostic testing for patients with suspected AK. Because clinical features of AK are nonspecific in early disease, such as epithelial, superficial stromal, and nummular keratitis, cases may go unrecognized and undiagnosed until the disease exhibits more characteristic signs and symptoms of advanced disease, worsening prognosis.[24–26] An advanced understanding of confocal microscopy validity may improve utilization and interpretation, leading to earlier diagnosis and easier treatment, presumably before the organism is more deeply established in the corneal stroma.[1,27]

Validation and accuracy of infectious disease diagnostic tests is determined by comparison to a reference standard, which historically is culture and identification. No established definition of AK exists within the ophthalmic literature because the disease definition in outbreak analyses varies by center on the basis of available technology and laboratory expertise; as a result, cultures, confocal microscopy, and clinical findings have each independently been used in disease diagnosis and case definition, further bringing into question the equivalence of study populations.[2,6,16,28,29] Strict use of a reference criterion with frequent false-negative test results, such as *Acanthamoeba* cultures in which culture rates approximate 50%, overestimates diagnostic test sensitivity and underestimates specificity in diagnostic test validation.[17,28] Therefore, we chose to use a multitest reference standard approach similar to diagnostic testing in systemic disease in evaluating confocal microscopy accuracy and validity, with 3 reference standard definitions of disease on the basis of increasing objective evidence supporting AK diagnosis.[17] Regardless of reference standard, confocal microscopy sensitivity exceeded 90%; specificity ranged from 77% to 100% depending on reference standard. This outcome supports the role of corneal imaging with confocal microscopy as a diagnostic procedure in establishing an AK diagnosis.

Our cohort results suggest a superficial culture sensitivity of 53% compared against a disease definition incorporating objective confocal and/or histologic confirmation of disease (definite AK disease). Patients were offered cultures regardless of perceived level of stromal or epithelial involvement, and whereas superficial corneal cultures in most forms of infectious keratitis may have lower yields in progressive stromal disease because of empiric topical treatment, superficial necrosis, or stromal migration, it has not been established that acanthamoebae completely abandon the ocular surface in favor of a less accessible stromal bed in the untreated eye. Furthermore, our data are not supportive of this perception because cultures were routinely positive regardless of disease presentation (Table 2). In addition, we previously reported differences in culture isolation rates

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TABLE 3. Confocal Microscopy Diagnostic Test Validity and Reliability Compared Against Three Different Criterion Standards, or Gold Standards

Confocal Microscopy Versus Culture AK

		Gold Standard Culture AK				
		Positive	**Negative**	**Total**		**Value (95% CI)**
Test	Positive	26	22	48	Sensitivity (%)	92.9 (76.5–99.1)
Confocal microscopy	Negative	2	75	77	Specificity (%)	77.3 (67.7–85.2)
	Total	28	97	125	PPV (%)	54.2 (39.2–68.6)
					NPV (%)	97.4 (90.0–99.7)
					Positive likelihood ratio	4.1 (3.0–4.6)

Confocal Microscopy Versus Microbial AK Positive

		Gold Standard Microbial AK Positive				
		Positive	**Negative**	**Total**		**Value (95% CI)**
Test	Positive	40	8	48	Sensitivity (%)	90.9 (78.3–97.5)
Confocal microscopy	Negative	4	73	77	Specificity (%)	90.1 (81.5–95.6)
	Total	44	81	125	PPV (%)	83.3 (69.8–92.5)
					NPV (%)	94.8 (87.2–98.6)
					Positive likelihood ratio	9.2 (5.6–13.5)

Confocal Microscopy Versus Definite AK Disease

		Gold Standard Definite AK Disease				
		Positive	**Negative**	**Total**		**Value (95% CI)**
Test	Positive	48	0	48	Sensitivity (%)	90.6 (79.3–96.9)
Confocal microscopy	Negative	5	72	77	Specificity (%)	100.0 (95.0–100.0)
	Total	53	72	125	PPV (%)	100.0 (92.6–100.0)
					NPV (%)	93.5 (85.5–97.9)
					Positive likelihood ratio	∞ (23.2–∞)

Likelihood ratios predict likelihood of AK diagnosis from positive test results.
Five AK cases refused corneal scrapings but were considered AK positive, which is reflected in the AK disease definitions.

between 19 "in common" samples that were sent to both a hospital-based and university research-based laboratory, in which only 8 (42%) of 19 hospital-based samples compared with 18 (94%) of 19 research-based samples were positive per the same disease definition as currently described (definite AK disease; Tu et al. IOVS 2007;48:ARVO E-Abstract 753). This finding suggests the perception that superficial cultures are of marginal use in AK, especially in deeper disease is more likely a failure of culture methods rather than inadequate sampling.

Acanthamoeba trophozoites or cysts were identified in 30 (78.9%) of 38 smears performed in patients with suspected AK, with positive yields possible in both early and late disease (Table 2). Diff-Quik staining was selected over other histologic methods in *Acanthamoeba* identification[7,8] because of its availability, characteristics similar to Giemsa cytopathologic examination methods, and rapid preparation that allows contemporaneous disease confirmation, providing support beyond confocal microscopy for initiating AK treatment at the primary visit. These results are comparable to those of other

TABLE 4. Culture Test Validity and Reliability Comparing *Acanthamoeba* Cultures Against "Definite AK Disease" as the Criterion Standard, or Gold Standard

		Gold Standard Definite AK Disease				
		Positive	**Negative**	**Total**		**Value (95% CI)**
Test	Positive	28	0	28	Sensitivity (%)	52.8 (38.6–66.7)
Culture AK	Negative	25	72	97	Specificity (%)	100.0 (95.0–100.0)
	Total	53	72	126	PPV (%)	100.0 (88.7–100.0)
					Positive likelihood ratio	∞ (11.2–∞)

Likelihood ratios predict likelihood of AK diagnosis from positive test results.
Five AK cases refused corneal scrapings but were considered AK positive, which is reflected in the AK disease definitions.

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TABLE 5. Simultaneous Testing (*Acanthamoeba* Cultures and Smear Only) With Microbial AK Positive Compared Against Definite AK Disease as the Criterion Standard, or Gold Standard

Microbial AK-Positive Versus Definite AK Disease						
		Gold Standard Definite AK Disease				
		Positive	Negative	Total		Value (95% CI)
Test	Positive	44	0	44	Sensitivity (%)	83.0 (70.2–91.9)
Microbial AK positive	Negative	9	72	81	Specificity (%)	100.0 (95.0–100.0)
	Total	53	72	125	PPV (%)	100.0 (92.0–100.0)
					NPV (%)	88.9 (80.0–94.8)
					Positive likelihood ratio	∞ (19.7–∞)

Likelihood ratios predict likelihood of AK diagnosis from positive test results.

microbiologic methods: Bharathi et al[7] reported a 92% identification rate with KOH preparations in culture-positive patients and 60% and 46% identification rates similarly with Gram and Giemsa stains, respectively, supporting this use of histologic preparations in AK; Mathers et al[9] described a method of epithelial biopsy stained with hematoxylin–eosin read by an ocular pathologist with 100% sensitivity in suspected AK patients on the basis of confocal microscopy. Other laboratory-based diagnostic tests such as polymerase chain reaction, cytospin concentration, and impression cytology show similar success; however, Diff-Quik staining offers a significant logistical advantage because it provides nearly immediate, in-office confirmatory results.[8,9,16,30,31] Simultaneous testing protocols when using both Diff-Quik smears and *Acanthamoeba* cultures improved sensitivity to 83% and specificity to 100% in our cohort, supporting the value of superficial corneal scrapings in rapid confirmation of corneal imaging and clinical diagnosis.

Despite clinical reports describing the application of confocal microscopy in successful AK management in which *Acanthamoeba* diagnosis was supported by histology, the rate of culture and isolation has been low and, therefore, inconsistent with that of larger international studies, leading to skepticism over the validity of confocal microscopy.[6,9,16] As an example, of the 56 AK cases reported by Parmar et al[6] over a 10-year period, only 9 (26%) of 35 cultures taken from an unidentified collection of corneal specimens and contact lens paraphernalia were positive. Suggested explanations for inconsistencies and uncertainties of previous studies include technical challenges with *Acanthamoeba* cultures resulting in a lack of consistent culture positivity or that the confocal optical system may allow organism identification in the deep stroma not obtainable through cultures. In contrast to previous studies, our results with sensitivity exceeding 90% and specificity ranging from 77% to 100%, depending on the reference standard, support the use of confocal microscopy in patients with suspected AK, regardless of layer of corneal involvement. However, correct interpretation of a confocal microscopy examination requires experience to suitably image the affected area and expertise in identifying *Acanthamoeba* presence. For our AK outbreak,[21] we attempted a regimented approach by using as many of the 3 ancillary diagnostics tests as possible per patient both to validate disease and to evaluate our own interpretation of disease.

Accuracy of diagnostic test assessment is compounded by potential bias introduction if patients are differentially referred for verification against the gold standard according to whether diagnostic tests results are positive or negative. This bias, known as workup or verification bias, is common if further testing is invasive and makes establishing a retrospective cohort of subjects having undergone identical testing protocols difficult.[32–36] If patients without further diagnostic testing can be monitored to demonstrate disease absence, they can be judged as disease free (or true negatives) for analysis purposes without subsequent verification.[37] In our case, not all subjects underwent corneal scrapings for disease verification; however, because symptomatic AK is not considered a silent disease that resolves without specific, effective anti-*Acanthamoeba* treatment, patients who did not receive corneal scrapings and were followed up until resolution without antiacanthamoebal drugs can be confirmed as true AK negatives for analysis purposes.

Potential sources of bias within the study include a lack of masking among results from the clinical examination, confocal microscopy, and histology. Although knowledge of previous test results could bias subsequent interpretations, it may be unavoidable for several reasons. For instance, effective confocal microscopy application, because of its small 330 × 440-μm imaging window, requires knowledge of the precise location and depth of corneal involvement to more accurately determine *Acanthamoeba* presence. Similarly, we examined an in-office histologic test that provides the benefits of simultaneous test results, which would not be possible if readings were performed by an outside certified laboratory, and diagnostic delays would be similar to delays with cultures. Although both may contribute real biases, we believe our masked culture results, which not only are consistent with generally accepted culture isolation rates but improve when we restrict results to a research-based laboratory, generally support outbreak validity and suggest that it is reasonable to conclude that these potential biases would not significantly alter study conclusions.

In addition, although independence between the test under evaluation and the gold standard existed when the reference criterion was either cultures or the simultaneous testing protocol of cultures and smears (microbial AK positive), it did not exist for the composite definition (definite

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AK disease), which violates methodologic standards in evaluation of diagnostic tests.[35] However, we chose to include our composite definition as a reference criterion because we believe that it best represents clinical disease and hence is most consistent with previous studies evaluating diagnostic test validity by using clinical disease as the reference criterion, allowing comparison against previous experiences.[2,6,9,16,28] Alternative biases include our treatment of 5 patients diagnosed with AK who refused corneal scrapings as disease positive, which would underestimate culture, simultaneous culture, and smear test sensitivity, as well as underestimate confocal microscopy specificity if patients were instead disease negative. In addition, confocal microscopy scans interpreted as equivocal were considered negative for analysis purposes, which may underestimate confocal microscopy sensitivity if results were falsely negative.

Absent a uniformly accepted reference standard in AK, our microbiologic identification rate's consistency with international studies not using confocal microscopy provides validation to our cohort and supports its potential for broad predictive value. However, as with any imaging modality, confocal microscopy interpretation requires validation most reliably gained through clinical and pathologic correlation, even for its primary application, AK. Given the often varied appearance of confocal appearance of acanthamoebae, individual centers and operators would benefit from confirming confocal findings with the objective tests described.

The goal for any disorder is a highly accurate, rapid, and noninvasive diagnostic method, and high sensitivity is preferred in screening purposes. This factor is especially important in AK, where a primary determinant of successful treatment is early diagnosis. Although 2 simultaneous studies have recently identified a specific contact lens solution as independently associated with AK, a substantial percentage of cases in both studies used alternative solutions, suggesting the AK outbreak both locally and nationally may continue despite the recent voluntary solution recall.[38,39] Validated, rapid, in-office diagnostic methods in AK detection are vital to the identification, treatment, and investigation of the outbreak. When used and interpreted properly, confocal microscopy has excellent sensitivity and reasonable specificity in AK diagnosis. Confocal microscopy, in combination with corneal scrapings for smears and culture, cofirms early findings and offers the clinician the best likelihood of rapidly and accurately diagnosing AK.

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