A Multiplex Dot Hybridization Assay For Detection and Differentiation of *Acanthamoeba* and Herpes Keratitis

Ming-Tse Kuo,[1] Po-Chiung Fang,[1] Hun-Ju Yu,[1] Tsae-Ling Chao,[2] Chun-Chih Chien,[2] Shun-Hua Chen,[3] Jen-Ren Wang,[4] Shin-Ling Tseng,[1] Yu-Hsuan Lai,[1] Chang-Chun Hsiao,[5] and Tsung C. Chang[4]

[1]Department of Ophthalmology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
[2]Department of Laboratory Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
[3]Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[4]Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[5]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, and Center for Shockwave Medicine and Tissue Engineering, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan

Correspondence: Tsung C. Chang, Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan; tsungcha@mail.ncku.edu.tw. Chang-Chun Hsiao, Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, and Center for Shockwave Medicine and Tissue Engineering, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan; cchsiao@mail.cgu.edu.tw.

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Purpose. We verified a multiplex dot hybridization (MDH) assay for the rapid detection and differentiation of *Acanthamoeba* keratitis (AK) and herpes simplex keratitis (HSK).

Methods. Molecular detection of *Acanthamoeba* and herpes simplex virus in corneal scrapes was performed with the MDH assay and standard diagnostic methods. The experimental group included corneal scrapes ($n = 33$) from patients with culture- or pathology-confirmed AK ($n = 15$) and real-time PCR-confirmed HSK ($n = 16$). The control group included 50 samples from cases of bacterial keratitis ($n = 15$), fungal keratitis ($n = 15$), and initially presumed AK ($n = 5$) or HSK ($n = 17$) which finally were excluded by culture for *Acanthamoeba* or by real-time PCR for herpes simplex virus, respectively. Discrepant results between methods were resolved by DNA sequencing of the PCR amplicons.

Results. After discrepant analysis, the sensitivity for the diagnosis of AK and HSK was both 93.3% by the MDH assay, while the specificity was 100% for the two types of keratitis. The turnaround time of MDH assay was within a working day using an already prepared array. Two false-negatives (one AK case and one HSK case) were obtained by the MDH assay.

Conclusions. The MDH assay could effectively prevent missed or delayed diagnosis of AK and HSK and has a potential to be adopted in routine clinical practice if the test is commercialized.

Keywords: *Acanthamoeba* keratitis, herpes keratitis, dot hybridization assay, early diagnosis

Acanthamoeba keratitis (AK) is a severe corneal infection worldwide.[1] Despite improvement of clinical diagnosis and management, many AK patients still must undergo surgery and experience visual loss. The risk of surgery for an active AK patient is high, ranging from 17% to 55%.[2–5] Herpes simplex keratitis (HSK) is a highly recurrent disease with a high risk of eventual corneal scar potentially requiring keratoplasty.[6] Herpes simplex keratitis is caused by herpes simplex virus type 1 (HSV-1) or 2 (HSV-2), and is thought to be the leading cause of infectious blindness in developed countries.[7]

The early clinical presentation of AK is highly variable; there may be no perineural infiltrates and it often presents in a manner similar to the epitheliopathy, dendriform lesions of HSK.[3] In addition to the similarity between early AK and HSK in clinical pictures, some AK patients have decreased corneal sensation as in HSK due to contact lens wear. Consequently, many patients with early AK are misdiagnosed as having HSK and, therefore, are mismanaged with topical corticosteroids,[8] resulting in a delay of anti-acanthamoebic therapy leading to a more rapid exacerbation course.[8,9] The incidence rate of HSK is approximately 10 new cases per 100,000 people annually.[10,11] Over 60% of affected patients experience more than one recurrent episode within 10 years, and up to 10% of HSK patients eventually need surgery due to severe vision impairment.[7,11] Given this situation, early diagnosis and differentiation between AK and HSK are crucial to promptly initiate an appropriate treatment and minimize vision loss.

Culture using *Escherichia coli*-enriched nonnutritional agar is the standard laboratory method to diagnose AK; however, the incubation time is relatively long (at least 3–7 days).[12] Viral culture is gold standard for HSK diagnosis but it is time-consuming and has low sensitivity.[13–15] Besides, preparation of viral host cells is tedious, which largely hampers the clinical value of conventional HSK diagnosis.[16] Direct microscopic examination is fast and easy for AK diagnosis, either by light microscopy using stains of lactophenol-cotton blue or Giemsa,

or by fluorescence microscopy using stains of calcofluor white or acridine orange.[17,18] However, these techniques require large corneal scrapes and expertise. The sensitivity of multinucleated giant cells in Giemsa stains for HSK diagnosis is lower than that of a rapid HSV antigen detection using the immunofluorescence assay.[19,20]

A kit for HSV detection by immunofluorescence microscopy has been commercialized and had a good performance, but the technique requires pathologists to use a fluorescence microscope. An immunochromatographic assay kit using fluorescent silica nanoparticles for *Acanthamoeba* diagnosis has been developed but further evaluation of the technique is needed.[21] Furthermore, an immunochromatographic assay was developed for rapid diagnosis of HSK, but the sensitivity was not desirable (approximately 45.5% using clinical diagnosis as a gold standard).[22] In vivo confocal microscopy is a rapid technique for diagnosing AK by detection of highly reflective round-shaped, high-contrast particles at the light-transmissible area of the cornea.[23] Although this technique has a sensitivity of up to 90% for AK diagnosis, it is not suitable for HSV diagnosis because of its limited resolution.[24] Moreover, the use of in vivo confocal microscopy requires a well-trained technician.

In general, the aforementioned methods for AK and HSK diagnoses are not as sensitive as PCR-based techniques. Currently, real-time PCR is the fastest method for diagnosis of AK[25] and HSK.[26] However, the method requires a sophisticated instrument, a well-trained medical staff, and may require running two separate PCRs to diagnose the two diseases. A dot hybridization assay based on oligonucleotide probes immobilized on a synthetic membrane was sensitive and specific for diagnosing fungal keratitis[27] and *Toxoplasma* uveitis.[28] Based on the previous studies, the aim of this study was to develop a novel multiplex dot hybridization assay (MDH) using oligonucleotide probes immobilized on a nylon membrane to diagnose AK and HSK.

Materials and Methods

Participants

All procedures involving human subjects adhered to the Declaration of Helsinki. Institutional Review Board (IRB)/Ethics Committee approval was obtained from the Committee of Medical Ethics and Human Experiments of Chang Gung Memorial Hospital (CGMH, Taiwan). Informed consent was obtained from each subject at the CGMH. Corneal samples from clinically suspected patients were collected prospectively from February 1, 2011 to January 31, 2015. Some of the stored DNA samples were tested in 2013. Because of limited sample number, the other DNA samples collected and stored in the following years were tested in 2015.

Clinical Specimens

For clinically suspected cases of AK or HSK with a major presentation of epitheliitis, a standard scraping procedure for corneal debridement was performed for patients with lesion sizes $\geq$2 mm by using a #15 sterilized knife under biomicroscopy. Each corneal scrape was divided into two approximately equal parts; one part was sent to the Department of Laboratory Medicine of CGMH for *Acanthamoeba* culture or HSV real-time PCR, based on clinical impression. The other part of the scrape was washed into a 1.5-ml sterile Eppendorf tube containing 1 mL normal saline and was stored at −20°C before DNA extraction. For clinically suspected cases of AK or HSK with a major presentation of necrotizing ulceration, the same procedure mentioned above was performed for patients with lesion sizes between 2 and 3 mm, and the corneal scrape was divided into two approximate equivalents. However, for lesion sizes $\geq$3 mm, each corneal scrape was approximately divided into three parts; one part was sent for culture or HSV real-time PCR, the second part was sent for direct microscopy (Gram stain and acid fast stain), and the third part was washed into a 1.5-ml sterile Eppendorf tube containing 1 mL of normal saline and was stored at −20°C before DNA extraction. Patients with lesions <2 mm were excluded.

In the experimental group, 15 patients with culture- or pathology-confirmed AK and 16 with HSK diagnosed by real-time PCR[29] were consecutively collected. The control group included 52 scraped samples from 52 consecutively enrolled patients with suspected AK ($n = 5$) or HSK ($n = 17$), which finally were excluded by culture or real-time PCR, respectively, and from a subset of random patients with culture-confirmed bacterial ($n = 15$) and fungal ($n = 15$) keratitis obtained from our corneal ulcer tissue bank using an online random number calculator (available in the public domain at http://graphpad.com/quickcalcs/randomSelect1/. Accessed February 1, 2015).

DNA Extraction and Multiplex PCR

The thawed corneal scrape in normal saline was transferred to a 1.5-ml Eppendorf tube and centrifuged at 13,200*g* in a microfuge for 10 minutes. DNA in the precipitate was extracted using a commercial kit (DNeasy Blood & Tissue Kit; Qiagen, Valencia, CA, USA). The extracted DNA was amplified by a multiplex PCR using three pairs of newly designed primers: one was used to amplify the 18S rRNA gene of *Acanthamoeba* (forward, 5′-digoxigenin-GCTACCACTTC TAAGGAAGGC-3′; reverse, 5′-digoxigenin-CCAACTGAAAATAG GAGGACAG-3′), the second was used to amplify the long unique region 30 (UL30) of HSV-1 and HSV-2 (forward, 5′-digoxigenin-CTGTTCTTCGTCAAGGCTCAC-3′; reverse, 5′-digoxigenin-ATCGTCGTAAAACAGCAGGTC-3′), and the third one to amplify the open reading frame 28 (ORF28) gene of varicella zoster virus (VZV; forward, 5′-digoxigenin-CGGCTCTGTTTTGTCCTC-3′; reverse, 5′-digoxigenin-CTTCCCCACACCGTTTAC-3′). Each primer was labeled with a digoxigenin molecule at its 5′ end. The thermocycling number of PCR was set at 35, which was determined by the end point MDH assay for strains of *A. castellanii* ATCC50370, HSV-1 KCGMH1001, and VZV KCGMH3001.

The PCR mixture (25 μL) consisted of 2 μL template DNA, 0.4 μM each primer, and other necessary reagents from a PCR kit (JMR-THS5; JMR Holdings, Inc., St. Augustine, FL, USA). The cycling conditions were as follows: initial denaturation (95°C, 3 minutes); 35 cycles of denaturation (95°C, 45 seconds), annealing (55°C, 60 seconds), and extension (72°C, 60 seconds); and a final extension step at 72°C for 10 minutes. Positive controls were performed with each run by using template DNAs of *A. polyphaga* ATCC 30461, HSV-1 KCGMH1002, HSV-2 KCGMH2002, and VZV KCGMH3003, respectively. A negative control was performed with each run by replacing the template DNA with sterile water.

Immobilization of *Acanthamoeba*-, HSV-, and VZV-Specific Oligonucleotide Probes on a Nylon Membrane

The procedure for the immobilization of oligonucleotide probes on a nylon membrane is described elsewhere.[27] The universal *Acanthamoeba* probe (code ACA) was designed from a conserved sequence in the 18S rRNA gene (Table 1). The common probe (code HSV_{12}) for HSV (types 1 and 2) and the HSV-1–specific probe (code HSV_1) were designed from conserved sequences in the *UL30* gene. The VZV-specific probe

Table 1. Oligonucleotide Probes Used in the MDH Assay

Probe Code	Target Microorganism	Probe Sequence (5′–3′)*
ACA	*Acanthamoeba* spp.	AAAGCAGGCAGATCCAATTTTCTGCCAttttt†
HSV_{12}	Herpes simplex virus types 1 & 2	CCTCCTCAGCATCCTCCTGCGGGACTGG
HSV_1	Herpes simplex virus type 1	TCCCCAGAGCAGCCCCGAGGAGGCCGTG
VZV	Varicella zoster virus	ATTATGCCCGCAAACTTGTAGAACTG

* All probes were designed in this study.
† Multiple bases of thymine (t) were added to the 3′ end of the probe to increase hybridization signal.

(code VZV) was designed from a conserved sequence in the *ORF28* gene of the virus (Table 1). Each probe was diluted 1:1 (final concentration, 10 μM) with a tracking dye solution and spotted on a positively charged nylon membrane (Roche, Mannheim, Germany) using a spotter (SR-A300; EZlife Technology, Taipei, Taiwan) to form an array (0.6 × 0.4 cm; Fig. 1a).[27] A digoxigenin-labeled irrelevant probe (code M, digoxigenin-GCATATCAATAAGCGGAGGA) was used as a position marker. 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 probes on the membrane.

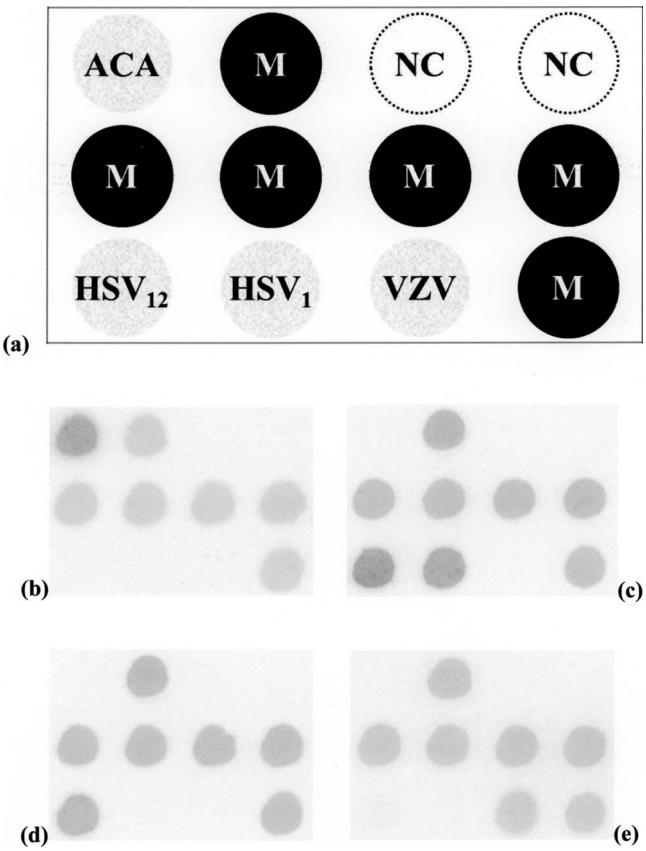

Figure 1. (**a**) Layout of oligonucleotide probes on the array (0.6 × 0.4 cm). The probe "ACA" was used to identify species of *Acanthamoeba*. The probes "HSV_1" and "VZV" were used to identify HSV-1 and VZV, respectively. The probe "HSV_{12}" was used to identify HSV-1 and HSV-2. The dot "NC" is a negative control (tracking dye only). The probe "M" is a position marker, that is, an irrelevant digoxigenin-labeled oligonucleotide probe (digoxigenin-GCATATCAATAAGCGGAGGA). The corresponding sequences of probes are listed in Table 1 and the concentration all probes was 10 μM. (**b–e**) Representative hybridization patterns of *A. polyphaga* ATCC 30461, HSV-1 KCGMH 1002, HSV-2 KCGMH 2002, and VZV KCGMH 3003, respectively. The hybridized spots (400 μm in diameter) could be read by the naked eye. Images of hybridized arrays were captured with a scanner (Perfection V600 Photo; Epson).

The MDH Assay

A 10-μl aliquot of the PCR product was used for the MDH assay. The reagents and procedures for prehybridization, hybridization (55°C for 60 minutes), and reaction with alkaline phosphatase-conjugated anti-digoxigenin antibodies followed by color development using phosphatase substrates have been described previously.[27] The hybridized spots (400 μm in diameter) could be read by the naked eye. Images of the hybridized arrays were captured with a scanner (Perfection V600 Photo; Epson, Suwa, Japan).

Detection Limit of the MDH Assay

The detection limit of the MDH assay for each type of organism was determined by hybridizations with 10-fold serial dilutions of the prenquantified DNA samples. *Acanthamoeba castellanii* ATCC 50370 was grown by culture, and the cell number in a diluted aliquot was determined by cell counting under a light microscope. Herpes simplex virus-1 and HSV-2 were prepared by viral cultures and then quantified by the plaque assay (plaque forming units per microliter; PFU/μL). A standard quantified VZV sample was prepared by construction of a plasmid containing the PCR amplicon of VZV (copies/μL). The plasmids were propagated in *E. coli* host cells, purified from the host cells, and the quantity of plasmid DNA was determined by the absorbance at 260 nm.

Sensitivity and Specificity Tests of the MDH Assay

Sixteen strains of *Acanthamoeba* (eight species), HSV (four clinical isolates), and VZV (one strain and three clinical isolates) were used for sensitivity test. In addition, 24 strains of bacteria (10 species), fungi (10 species), and microsporidia (four species) were used for specificity test of the array (Supplementary Table S1).

Discrepant Analysis

For a sample showing discrepant results between the MDH assay and standard diagnostic methods (culture or pathology for AK,[16] real-time PCR for HSK[29]), the result of DNA sequencing was used for discrepant analysis. The purified DNA from a clinical sample was amplified by PCR with primers being not labeled with digoxigenin molecules (Table 1), the amplicon was sequenced, and the determined sequence was used to search for homologous sequences of the infectious agent in GenBank using the BLASTN program (available in the public domain at http://blast.ncbi.nlm.nih.gov/, accessed Nov. 25, 2015).

Statistical Analysis

The performance indices, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value

Table 2. Results of Clinical Samples Analyzed by the MDH Assay and by Standard Diagnostic Methods

Results of the MDH Assay	Results of the Standard Diagnostic Methods*			
	AK		HSK	
	Positive	Negative	Positive	Negative
Positive	14	0	14	0
Negative	1	68	2 (1)†	67 (68)†

* The standard diagnostic methods: AK, culture or pathologic examination; HSK, real-time PCR.

† Values in parenthesis are results after discrepant analysis.

(NPV), for diagnosis of AK and HSK were calculated after discrepant analysis. The 95% confidence interval for a proportion of the sample was estimated with an online calculator (available in the public domain at http://www.causascientia.org/math_stat /ProportionCI.html, accessed Nov. 30, 2015). As the case number of VZV was very limited, the array performance for VZV detection was not calculated in this study.

Results

Detection Limits of the MDH Assay

The serial 10-fold diluted DNA samples of *Acanthamoeba*, HSV-1, HSV-2, and VZV were analyzed by the MDH assay to determine the detection limits of these pathogens. The detection limits for *A. castellanii*, HSV-1, HSV-2, and VZV were estimated to be 10 cells, 100 PFUs, 100 PFUs, and 1000 copies per test, respectively. The detection limits of the multiplex PCR were the same as a single-plex PCR for each category of pathogen.

Sensitivity and Specificity of the MDH Assay

All 8 species (eight strains) of *Acanthamoeba*, four clinical isolates of HSV, and four isolates VZV (Supplementary Table S1) were identified correctly, as they hybridized to their respective probes. No PCR product was amplified from any microorganisms among the 24 nontarget species belonging to bacteria, fungi, and microsporidia (Supplementary Table S1), and, hence, no cross-hybridization of the MDH assay was observed. In addition, the strains of *Acanthamoeba* did not cross-hybridize with the herpesvirus probes HSV_1, HSV_{12}, and VZV, and there was no cross-hybridization of strains of HSV-1, HSV-2, and VZV with the *Acanthamoeba* probe. The hybridization patterns of the MDH assay for *Acanthamoeba*, HSV-1, HSV-2, and VZV are shown in Figures 1b to 1e.

Detection of *Acanthamoeba* and HSV in Corneal Scrapes

Once the MDH assay was validated, the assay was used to analyze 83 corneal scrapes from the experimental and control groups. Concordant and discrepant results between the MDH assay and the standard diagnostic methods are shown in Table 2. For AK diagnosis (probe ACA) in the 83 clinical samples, 82 concordant (14 positives and 68 negatives) and one discordant (negative by the MDH assay but positive by the standard method) results were obtained (Table 2). For diagnosis of HSK (probe codes HSV_{12} and HSV_1), 81 concordant (14 positives and 67 negatives) and two discordant (negative by the MDH assay but positive by the standard method) results were obtained. All HSK cases identified by MDH assay and real-time PCR were caused by HSV-1.

Discrepant Analysis

One patient with AK was not diagnosed by the MDH assay (Table 2). This patient was not a contact lens-wearer and had no perineural infiltrate or ring infiltrate. Furthermore, the scrape from the patient was acid-fast stain positive, and a bacterium of nontuberculous mycobacteria was isolated by culture. The presence of *Acanthamoeba* in this patient could not be confirmed by gene sequencing; however, additional evidence from *Acanthamoeba* real-time PCR[25] demonstrated that the sample was weakly positive ($C_t = 31.3$).

For HSK diagnosis, two false-negatives by the MDH assay were observed (Table 2). One was weakly positive ($C_t = 32.6$) by real-time PCR, and the presence of HSV in this patient could not be confirmed by gene sequencing. The second sample was determined to be HSV-negative but VZV-positive by the MDH assay; the presence of VZV in this patient was further confirmed by DNA sequencing of the PCR amplicon. Therefore, this case was considered to be a true case of late VZV keratitis (VZK) and not a case of HSK (Fig. 2). There were two VZV-positive samples from clinically suspected HSK controls (HSV-negative by real-time PCR) by the MDH assay, and the two subjects were late VZK by real-time PCR for VZV[30] (data not shown).

Performance of the MDH Assay

The MDH assay had good performances for diagnoses of AK and HSK (Table 2). After discrepant analysis, the sensitivity, specificity, PPV, and NPV for AK and HSK diagnoses were 93.3%, 100%, 100%, and 98.4%, respectively (Table 3).

Discussion

To our knowledge, this is the first study to describe a molecular assay for the simultaneous diagnosis of AK and HSK. The MDH assay, based on specific oligonucleotide probes targeting the 18S rRNA gene of *Acanthamoeba* species, *UL30* gene of HSV-1 and HSV-2, and *ORF28* gene of VZV, had high sensitivity and specificity (Table 3). The assay requires a minimal instrumentation and can be completed within a standard workday. Therefore, this novel molecular technique has great potential to improve the diagnoses of AK and HSK. However, it is difficult for general users to prepare the array and all reagents for hybridization. Therefore, this assay has a routine diagnostic value only when the array is commercialized.

Table 3. Performance of the MDH Assay for Diagnosis of *Acanthamoeba* and HSK

Type of Keratitis, $n = 83$	Performance of the MDH Assay, % (95% Confidence Interval)			
	Sensitivity	Specificity	PPV	NPV
AK	93.3 (73.4–99.6)	100 (95.8–100)	100 (81.9–100)	98.4 (93.4–99.9)
HSK	93.3 (73.4–99.6)	100 (95.8–100)	100 (81.9–100)	98.4 (93.4–99.9)

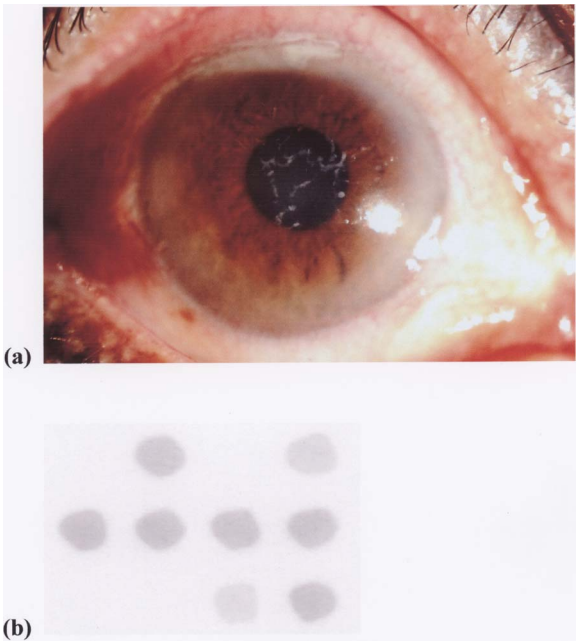

FIGURE 2. (**a**) A 69-year-old female patient had mucous plaque keratopathy with finely branching, migratory dendriform lesions over her right eye, but had no periocular skin rash. Her medical history included an episode of herpes zoster ophthalmicus that occurred approximately 3 months before her ocular symptoms. (**b**) Late VZV keratitis was diagnosed by the MDH assay, but the result was discordant with that obtained by real-time PCR (HSV-positive). However, the sequence of the PCR amplicon showed 99% identity with a reference sequence of VZV in GenBank (accession no. KF853225.1).

The false-negative of AK obtained by the MDH assay (Table 2) might have been caused by a low number of amebic cells in the specimen and/or coinfection with a bacterium of nontuberculous mycobacteria. Ikeda et al.[23] found that the *Acanthamoeba* DNA load is inversely proportional to the bacterial DNA load in clinical samples by real-time PCR. Although direct microscopy for *Acanthamoeba* can have a sensitivity up to 100%, a relatively large tissue sample is normally required for this technique.[25] Therefore, the priority for laboratory examination of AK should be a PCR-based technique, followed by culture, and smear staining for diagnosing AK at an early stage.

The MDH assay also produced a false-negative for HSK diagnosis (Table 2); this may suggest that the assay is not as sensitive as real-time PCR. However, the real-time PCR technique can detect the shed HSV-1 DNA in normal tears in 35% of cases.[31] Therefore, real-time PCR can produce false-positives in some cases in which asymptomatic individuals are HSV carriers. Indeed, real-time PCR produced one false-positive for HSV detection (Fig. 2; Table 2) in a late VZK case as detected by the MDH assay and confirmed by gene sequencing.

Since HSK is much more common than AK in our hospital, most cases with pathognomonic dendritic epithelial keratitis with terminal bulbs are clinically diagnosed and treated for HSK without laboratory proof. As the sensitivity and specificity of the MDH assay are high for diagnoses of AK and HSK (Table 3), currently a multicenter study is under conduction to assess the clinical impact of the array. As sampling order also can influence the results of laboratory tests, a priority of the MDH assay followed by culture and direct smear staining is recommended to avoid false-negatives, especially for the diagnosis of early AK.

There are some limitations for the MDH assay. The inherent drawback of DNA-based techniques is the chance of detecting nonviable or contaminating microorganisms. However, *Acanthamoeba*, HSV, and VZV are not airborne contaminants. Besides, positive and negative controls should be performed concomitantly when using the molecular technique. These procedures need well-trained staffs and sterile technique in the laboratory. In general, clinical samples were not tested by the MDH assay immediately after sample receiving. Therefore, this study cannot estimate the impact of this assay on the clinical outcomes of these patients.

In conclusion, the molecular assay developed here is a useful technique for diagnoses of AK and HSK. In addition to good performance, the method has a short turnaround time (a workday). The current assay can help minimize the erratic diagnosis of early AK as HSK. Although the MDH assay has a potential to detect late VZK, more cases are needed to assess the clinical performance of the assay for diagnosing late VZK.

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References

1. Kashiwabuchi RT, Carvalho FR, Khan YA, et al. Assessing efficacy of combined riboflavin and UV-A light (365 nm) treatment of *Acanthamoeba* trophozoites. *Invest Ophthalmol Vis Sci*. 2011;52:9333–9338.
2. Tu EY, Joslin CE, Sugar J, et al. Prognostic factors affecting visual outcome in *Acanthamoeba* keratitis. *Ophthalmology*. 2008;115:1998–2003.
3. Radford CF, Minassian DC, Dart JK. *Acanthamoeba* keratitis in England and Wales: incidence, outcome, and risk factors. *Br J Ophthalmol*. 2002;86:536–542.
4. Fong CF, Tseng CH, Hu FR, et al. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am J Ophthalmol*. 2004;137:329–336.
5. Fraser MN, Wong Q, Shah L, et al. Characteristics of an *Acanthamoeba* keratitis outbreak in British Columbia between 2003 and 2007. *Ophthalmology*. 2012;119:1120–115.
6. Al-Yousuf N, Mavrikakis I, Mavrikakis E, Daya SM. Penetrating keratoplasty: indications over a 10 year period. *Br J Ophthalmol*. 2004;88:998–1001.
7. Farooq AV, Shukla D. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. *Surv Ophthalmol*. 2012; 57:448–462.
8. Robaei D, Carnt N, Minassian DC, Dart JK. The impact of topical corticosteroid use before diagnosis on the outcome of *Acanthamoeba* keratitis. *Ophthalmology*. 2014;121:1383–1388.
9. Herz NL, Matoba AY, Wilhelmus KR. Rapidly progressive cataract and iris atrophy during treatment of *Acanthamoeba* keratitis. *Ophthalmology*. 2008;115:866–869.
10. Labetoulle M, Auquier P, Conrad H, et al. Incidence of herpes simplex virus keratitis in France. *Ophthalmology*. 2005;112: 888–895.

11. Baratz KH. The role of antiviral therapy after the resolution of acute herpes simplex keratitis or acute herpes zoster ophthalmicus. *Arch Ophthalmol*. 2012;130:108–110.
12. Maubon D, Dubosson M, Chiquet C, et al. A one-step multiplex PCR for *Acanthamoeba* keratitis diagnosis and quality samples control. *Invest Ophthalmology Vis Sci*. 2012;53:2866–2872.
13. Pramod NP, Gopalakrishnan V, Mohan R, et al. Enhanced detection of herpes simplex virus from ocular specimens of herpetic keratitis patients. *Indian J Pathol Microbiol*. 1998; 41:49–53.
14. El-Aal AM, El Sayed M, Mohammed E, Ahmed M, Fathy M. Evaluation of herpes simplex detection in corneal scrapings by three molecular methods. *Curr Microbiol*. 2006;52:379–82.
15. Athmanathan S, Bandlapally S, Rao GN. Comparison of the sensitivity of a 24 h-shell vial assay, and conventional tube culture, in the isolation of herpes simplex virus-1 from corneal scrapings. *BMC Clin Pathol*. 2002;2:1.
16. Byrne KA, Burd EM, Tabbara KF, Hyndiuk RA. *Diagnostic Microbiology and Cytology of the Eye*, 1 ed. Boston, MA: Butterworth-Heinemann, 1995.
17. Hammersmith KM. Diagnosis and management of *Acanthamoeba* keratitis. *Curr Opin Ophthalmol*. 2006;17:327–331.
18. Lorenzo-Morales J, Khan NA, Walochnik J. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite*. 2015;22:10.
19. Farhatullah S, Kaza S, Athmanathan S, Garg P, Reddy SB, Sharma S. Diagnosis of herpes simplex virus-1 keratitis using Giemsa stain, immunofluorescence assay, and polymerase chain reaction assay on corneal scrapings. *Br J Ophthalmol*. 2004;88:142–144.
20. Subhan S, Jose RJ, Duggirala A, et al. Diagnosis of herpes simplex virus-1 keratitis: comparison of Giemsa stain, immunofluorescence assay and polymerase chain reaction. *Curr Eye Res*. 2004;29:209–213.
21. Toriyama K, Suzuki T, Inoue T, et al. Development of an immunochromatographic assay kit using fluorescent silica nanoparticles for rapid diagnosis of *Acanthamoeba* keratitis. *J Clin Microbiol*. 2015;53:273–277.
22. Inoue Y, Shimomura Y, Fukuda M, et al. Multicentre clinical study of the herpes simplex virus immunochromatographic assay kit for the diagnosis of herpetic epithelial keratitis. *Br J Ophthalmol*. 2013;97:1108–1112.
23. Cruzat A, Witkin D, Baniasadi N, et al. Inflammation and the nervous system: the connection in the cornea in patients with infectious keratitis. *Invest Ophthalmol Vis Sci*. 2011;52:5136–5143.
24. Kumar RL, Cruzat A, Hamrah P. Current state of in vivo confocal microscopy in management of microbial keratitis. *Semin Ophthalmol*. 2010;25:166–170.
25. Ikeda Y, Miyazaki D, Yakura K, et al. Assessment of real-time polymerase chain reaction detection of *Acanthamoeba* and prognosis determinants of *Acanthamoeba* keratitis. *Ophthalmology*. 2012;119:1111–1119.
26. Inoue T, Ohashi Y. Utility of real-time PCR analysis for appropriate diagnosis for keratitis. *Cornea*. 2013;32(supp1): S71–S76.
27. Kuo MT, Chang HC, Cheng CK, et al. A highly sensitive method for molecular diagnosis of fungal keratitis: a dot hybridization assay. *Ophthalmology*. 2012;119:2434–2442.
28. Basu S, Sharma S, Kar S, Das T. DNA chip-assisted diagnosis of a previously unknown etiology of intermediate uveitis-*Toxoplasma gondii*. *Indian J Ophthalmol*. 2010;58:535–537.
29. Espy MJ, Uhl JR, Mitchell PS, et al. Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *J Clin Microbiol*. 2000;38:795–799.
30. Sugita S, Shimizu N, Watanabe K, et al. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. *Br J Ophthalmol*. 2008; 92:928–932.
31. Kaufman HE, Azcuy AM, Varnell ED, et al. HSV-1 DNA in tears and saliva of normal adults. *Invest Ophthalmol Vis Sci*. 2005; 46:241–247.

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