Establishment of Multiplex Solid-Phase Strip PCR Test for Detection of 24 Ocular Infectious Disease Pathogens

Satoko Nakano,[1] Sunao Sugita,[2] Yasuhiro Tomaru,[3] Ayumi Hono,[2] Takako Nakamuro,[1] Toshiaki Kubota,[1] Hiroshi Takase,[4] Manabu Mochizuki,[4,5] Masayo Takahashi,[2] and Norio Shimizu[3]

[1]Department of Ophthalmology, Oita University, Oita, Japan
[2]Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, Kobe, Japan
[3]Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University, Tokyo, Japan
[4]Department of Ophthalmology & Visual Science, Tokyo Medical and Dental University, Graduate School of Medicine and Dental Sciences, Tokyo, Japan
[5]Miyata Eye Hospital, Miyakonojo, Japan

Correspondence: Sunao Sugita, Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan; sunaoph@cdb.riken.jp.

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Purpose. To establish and evaluate a new multiplex solid-phase strip polymerase chain reaction (strip PCR) for concurrent detection of common ocular infectious disease pathogens.

Methods. A new multiplex strip PCR was established to detect 24 common ocular infectious disease pathogens: herpes simplex virus (HSV) 1, HSV2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus (HHV) 6, HHV7, HHV8, human T-cell lymphotropic virus (HTLV)-1, adenovirus, *Mycobacterium tuberculosis*, *Treponema pallidum*, *Propionibacterium acnes* (*P. acnes*), bacterial 16S ribosomal RNA (rRNA), *Candida* species (*Candida* sp.), *C. glabrata*, *C. krusei*, *Aspergillus*, *Fusarium*, fungal 28S rRNA, *Toxoplasma* (*T. gondii*), *Toxocara*, *Chlamydia trachomatis* (*C. trachomatis*), and *Acanthamoeba*. Strip PCR was tested with a negative control (distilled water) and standard positive control DNA. Cutoffs of quantification cycle (Cq) values were determined with noninfectious ocular samples to avoid false-positives caused by contamination with *P. acnes*, bacterial 16S, and fungal 28S from reagents and ocular surfaces. A pilot study to evaluate the strip PCR was performed using infectious ocular samples (aqueous humor, vitreous, cornea, and tears) by strip PCR and previously developed capillary-type multiplex PCR and quantitative real-time PCR (qPCR).

Results. Strip PCR was verified with negative and positive controls. Strip PCR rapidly detected HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HTLV-1, adenovirus, *P. acnes*, bacterial 16S, *Candida* sp., *C. glabrata*, *Aspergillus*, fungal 28S, *T. gondii*, *C. trachomatis*, and *Acanthamoeba* in patient samples. The sensitivity was comparable to that of qPCR.

Conclusions. Our novel strip PCR assay is a simple, rapid, and high-sensitivity method for detecting ocular infectious disease pathogens.

Keywords: PCR, ocular infection, ocular samples

Ocular infectious diseases including infectious uveitis, endophthalmitis, and ocular surface diseases, such as conjunctivitis, keratitis, and corneal endotheliitis, are caused by a variety of infectious pathogens that are often difficult to identify. Treatment depends on the detection and identification of the causative pathogen, which can include viruses (e.g., herpes group viruses[1–5]), bacteria, fungi, and protozoa, alone or in combination. Treatments without an accurate diagnosis of pathogens can worsen the infection, potentially resulting in blindness.

Traditional laboratory tests can identify causative pathogens, but have various limitations. For instance, culture-based methods require time, especially for fungal cultures, and cannot detect viruses. These limitations are shared by microscopy-based tests, while antibody-based tests require large amounts of sample and have low specificity and sensitivity, potentially leading to a delay of diagnosis or misdiagnosis. Polymerase chain reaction (PCR), a technology originally reported by Saiki et al.[6] in 1985, amplifies specific target DNA segments many millions of times in a short time period. It provides highly sensitive, specific, rapid, and reliable detection of infectious disease pathogens from a small amount of sample.[7] The requirement for only a small sample volume is particularly useful for the examination of ocular fluid samples, such as aqueous humor (Aqh; 50–100 μL), vitreous fluid (VF; 200–500 μL), tissues (e.g., a small piece of cornea or conjunctiva, 0.5–9 mm^3), and tears (2–4 μL). Although a standard single-target PCR provides rapid and accurate molecular diagnosis of ocular infectious diseases using Aqh or VF samples, only a few target DNAs are detected by a single-target PCR assay; therefore, many PCR assays are necessary if the suspected pathogen is unknown, or if many pathogens are suspected as candidates. This is particularly difficult when samples with limited amounts, such as Aqh, are used for the PCR assay. To overcome this limitation of single-target PCR, we previously developed a two-step PCR system combining a capillary-type multiplex PCR (capillary PCR) and broad-range quantitative PCR to detect all eight types of human herpes

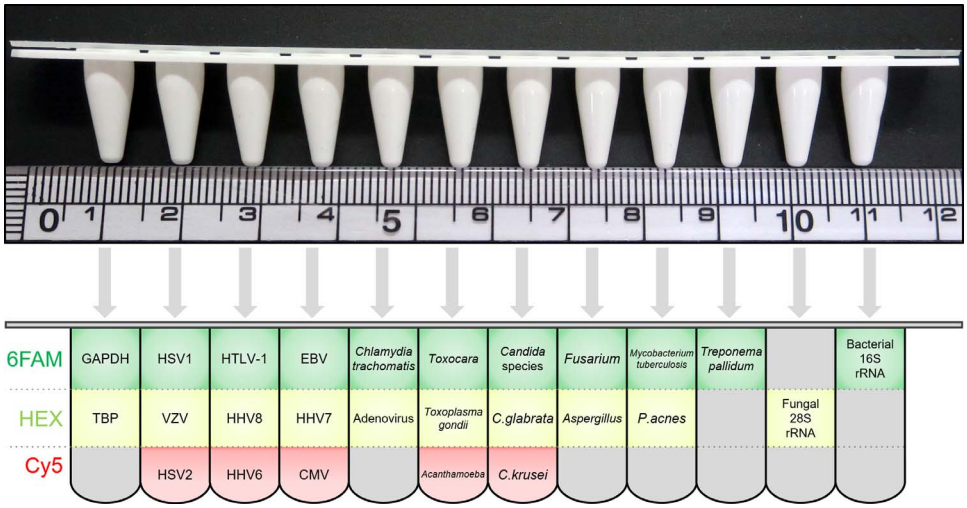


Figure 1. The strip PCR assay uses a 12-well multiplex PCR strip tube targeting the following 24 ocular infectious disease pathogens: HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HHV8, HTLV-1, adenovirus, *M. tuberculosis*, *T. pallidum*, *P. acnes*, bacterial 16S rRNA, *Candida* sp., *C. glabrata*, *C. krusei*, *Aspergillus*, *Fusarium*, fungal 28S rRNA, *T. gondii*, *Toxocara*, *C. trachomatis*, and *Acanthamoeba*. The internal controls were GAPDH and TBP. Each well targeted one to three types of pathogens and was coated with primers (forward and reverse) and multiple probes (6FAM, HEX, and/or Cy5) adapted for these target pathogens.

viruses,[5,8] bacterial 16S ribosomal RNA (rRNA), and fungal 28S rRNA sequences.[9–12] The two-step PCR[13] can simultaneously detect pathogens including herpes simplex virus (HSV) type 1 (HSV1),[5] HSV2,[5] varicella-zoster virus (VZV),[2,5] Epstein-Barr virus (EBV),[5,14] cytomegalovirus (CMV),[4,5,15] human herpes virus type 6 (HHV6),[3,5] HHV7,[5] HHV8,[5] human T-cell lymphotropic virus (HTLV)-1, bacterial 16S rRNA,[9,10] fungal 18S/28S rRNA,[11] and *Toxoplasma* (*T. gondii*),[16] all of which can cause ocular infectious disease.[13] However, this system has not been widely adopted because it requires two costly PCR instruments, and the procedure is complicated and difficult to perform without the requisite skills.

Here, we describe a new multiplex solid-phase strip PCR assay (referred to as strip PCR) that detects pathogens in a shorter time (around 90 minutes) than the former capillary PCR. The procedure is simpler than conventional multiplex PCR methods since the primer and probe coat the solid phase of the strip well. In the present study, we tested the ability of strip PCR to simultaneously detect 24 common pathogens in previously collected ocular samples[13] and compared the results with our previously developed capillary PCR and quantitative real-time PCR (qPCR) methods.

Materials and Methods

Subjects and Experimental Design

The protocols of the present study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from patients after they had been told of the nature and possible consequences of the study. The research was approved by the human experimentation committee or review board of RIKEN, Oita University, and Tokyo Medical and Dental University.

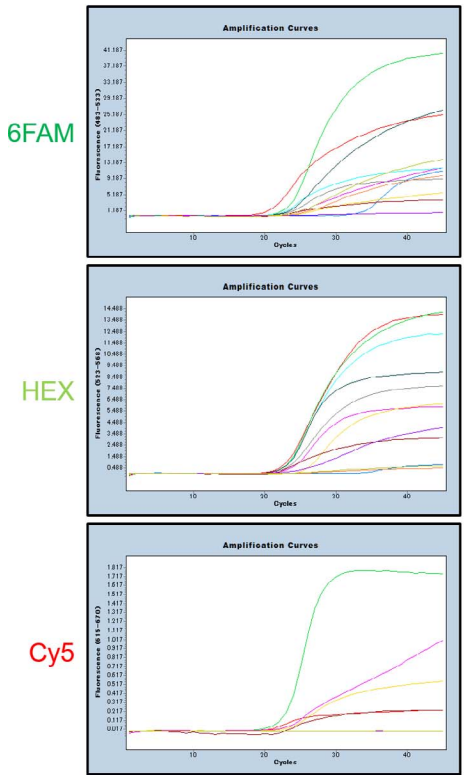
Infectious patient samples ($n = 23$), consisting of Aqh, VFs, smear samples of the cornea, and tears (diluted with physiological saline), had been previously collected from patients who were clinically diagnosed as having active ocular infectious diseases (i.e., uveitis, endophthalmitis, corneal endotheliitis, keratitis, and conjunctivitis). These samples were stored in separate cryotubes at –80°C for 1 to 3 months prior to being used for the strip PCR assay and a previously developed capillary PCR. The results were validated by qPCR. Noninfectious patient samples were collected from patients with cataract, glaucoma, retinal detachment, and macular hole who had no previous history of ocular infectious and inflammatory diseases (Aqh and VF samples, $n = 10$). Aqueous humor was aspirated with a 30G needle, and VF samples were collected from patients during pars-plana vitrectomy.[5] The ocular surface of the patients was sterilized with polyvinyl alcohol iodine (Nitten, Nagoya, Japan) before Aqh and VF collection.

DNA Extraction

DNA was manually extracted with the QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany) and automatically extracted with the EZ1 Advanced XL Virus Mini and DNA Tissue kits (Qiagen). The DNA was eluted with 40 to 100 μL elution buffer and quantified with NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA extraction and amplification were performed in separate laboratories to avoid sample contamination.[9]

Strip PCR Assay

The strip PCR tube (Fig. 1) was a multiplex PCR strip tube targeting the following 24 ocular infectious disease pathogens: HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HHV8, HTLV-1, adenovirus, *Mycobacterium tuberculosis* (*M. tuberculosis*), *Treponema pallidum* (*T. pallidum*), *Propionibacterium acnes* (*P. acnes*), bacterial 16SrRNA, *Candida* species (*Candida* sp.), *C. glabrata*, *C. krusei*, *Aspergillus*, *Fusarium*, fungal 28S rRNA, *T. gondii*, *Toxocara*, *Chlamydia trachomatis* (*C. trachomatis*), and *Acanthamoeba*, as well as internal controls such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TA-TA-binding protein (TBP). These viruses are common viruses that were assessed in a multicenter epidemiologic survey of uveitis.[17] Bacterial 16S rRNA primers can be synthesized for a wide variety of bacteria,[18] while fungal 28S rRNA covered *Candida*, *Aspergillus*, *Cryptococcus*, *Trichophyton*, *Mucor*, *Penicillium*, and *Pichia*.[19] Strip PCR consisted of 12 wells, which each targeted one to three types of pathogens. The wells were coated with primers (forward and reverse) and multiple



Pathogen	Cq value
HSV1	18.14
HTLV-1	20.42
EBV	22.52
Chlamydia trachomatis	21.09
Toxocara	23.69
Candida species	21.74
Fusarium	20.57
Mycobacterium tuberculosis	20.75
Treponema pallidum	22.77
Bacterial 16S rRNA	20.65

Pathogen	Cq value
VZV	19.51
HHV8	19.59
HHV7	20.13
Adenovirus	19.57
Toxoplasma gondii	23.78
C.glabrata	20.75
Aspergillus	19.91
P.acnes	19.83
Fungal 28S rRNA	20.89

Pathogen	Cq value
HSV2	20.64
HHV6	19.21
CMV	21.87
Acanthamoeba	21.86
C.krusei	22.00

Figure 2. Strip PCR results with standard DNA (24 infectious agents, 10^5 copies/mL). The Cq values of ocular infectious disease pathogens ($n = 24$) ranged from 18.14 to 23.78.

probes (6FAM, HEX, and/or Cy5) adapted for the targets (Fig. 1; Supplementary Table S1). We mixed 120 µL buffer, 93.6 µL PCR-grade water, 24 µL extracted nucleic acids, and 2.4 µL enzyme, and then we dispensed the mixture (20 µL) into the wells of a 12-well strip that had been coated with primers and probes. Moreover, GAPDH was also used as a PCR monitoring control. The strip PCR reaction conditions were 95°C for 10 seconds, followed by 45 cycles at 95°C for 5 seconds, and at 60°C for 60 seconds. A multiplex PCR LightCycler 480 system I and a LightCycler 480 system II were used (Roche Diagnostics, Basel, Switzerland). Semiquantitative measurement with quantification cycle (Cq) values after strip PCR provided an indication of the approximate amount of pathogen.

Validation

To validate the strip PCR assay, 2 µL/well of distilled water (negative DNA control), standard DNA (10^5 copies/mL) of each pathogen (positive DNA control), or noninfectious patient samples was applied to strip PCR without the DNA extraction procedure.

To evaluate the precision of strip PCR by assaying the intra- and interassay variability, repeatability tests were performed. Negative and positive controls (each target pathogen DNA of well, all target pathogen DNA of strip) were added to each well and tested in triplicate over 5 days of testing. For the limit of dilution study, we set a limited measurable goal of pathogen concentration for strip PCR as 50 copies, and we used different DNA dilutions for each pathogen (10^6 copies, 10^4 copies, 10^2 copies, and 50 copies) to calculate amplification efficiency and correlation coefficient. We performed analytical specificity measurements with high concentrations of nontargeted pathogens in each well to truly assess specificity. We used the following pathogens: HSV1 (strain F), HSV2 (strain G), VZV (Oka strain), EBV (B95-8), CMV (Towne), HHV6 (U1102), HHV7 (SB strain), HHV8 (BCBL-1), HTLV-1 (MT-4 cells), adenovirus (HAdV-3), *T. pallidum* (patient Aqh sample), *P. acnes* (JCM6425), bacterial 16S rRNA (*Streptococcus pneumoniae*, NBRC102642), *Candida* sp. (*C. albicans*, ATCC60193), *C. glabrata* (NBRC0005), *C. krusei* (NBRC0201), *Aspergillus* (NBRC9455), *Fusarium* (IFO5232), fungal 28S rRNA (ATCC60193, NBRC0005, NBRC0201, NBRC9455), *T. gondii* (patient Aqh sample), *Toxocara* (patient VF sample), *C. trachomatis* (patient tear sample), and *Acanthamoeba* (patient cornea sample). *M. tuberculosis* was not available.

A cutoff Cq value was determined with noninfectious patient samples to avoid positive results caused by contamination with *P. acnes*, bacterial 16S rRNA, or fungal 28S rRNA. Minute amounts of bacterial 16S and fungal 28S rRNA are often present in the enzyme used for PCR amplification and the DNA isolation reagent, respectively, while *P. acnes* is a resident microorganism of the ocular surface.

Subsequently, infectious patient samples were evaluated by strip PCR with the cutoff Cq value and previously developed capillary PCR and qPCR assays. A capillary PCR assay was performed on a LightCycler 2.0 system (Roche Diagnostics), and qPCR was performed on a LightCycler 480 II system and a LightCycler 2.0 system.

Results

Validation of the Strip PCR

Strip PCR results with distilled water were negative for all items except GAPDH (negative DNA control, Supplementary Fig. S1). To assess whether all 24 infectious agents were detected by this PCR assay with reliable results, standard control DNA (10^5 copies/mL) of each pathogen was applied to the strip PCR, and all control DNAs were detected (positive DNA control, Fig. 2).

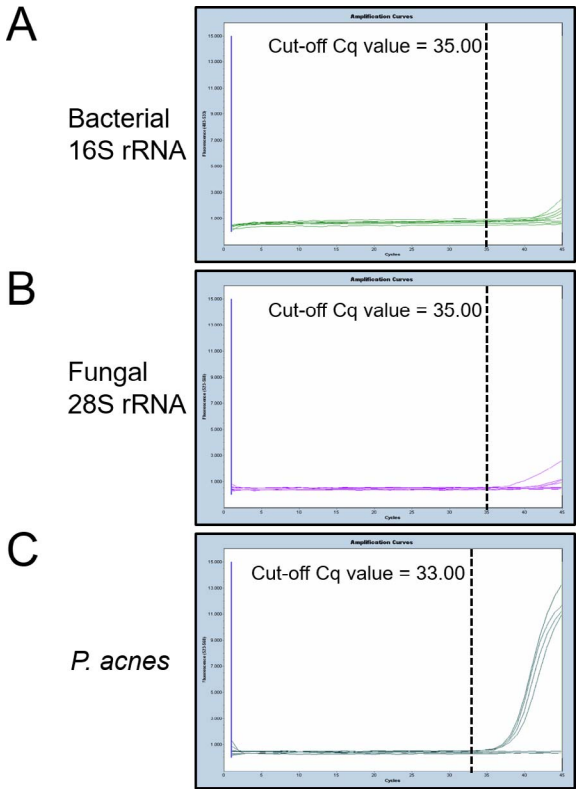


Figure 3. Collected control samples were positive for (**A**) bacterial 16S rRNA (5/10 samples, 39.77 ± 0.51 cycles) and (**B**) fungal 28S rRNA (3/10 samples, 40.55 ± 0.96 cycles) present in the PCR amplification reagents, as well as for (**C**) *P. acnes* (5/10 samples, 37.10 ± 1.52 cycles), a resident microorganism in ocular fluids and tissues. The cut-off of Cq values (a *dotted line* in the graph) was set to avoid false-positive results as follows: 35.00 for bacterial 16S and fungal 28S rRNA, and 33.00 for *P. acnes*.

The intra- and interassay variability was low, and the assay was thus highly reproducible (except for occasional contamination with *P. acnes* and bacterial 16S from the samples or reagents) based on the low standard deviation (<2.44) and low coefficient of variation (0.0%–4.2%) determined by repeatability tests (Supplementary Tables S2–S4).

The limit of dilution study showed a linear relationship with a good correlation coefficient (1.00) between Cq values in the 50 to 10^6 copies of DNA range. We believe that strip PCR will make semiquantitative analysis possible in this range (Supplementary Table S5).

In the analytical specificity measurements, none of the nontargeted pathogens gave a positive signal, except for *P. acnes*, bacterial 16S, and fungal 28S rDNA, which were contaminants in the reagents or on the ocular surface (Supplementary Table S6).

Cutoff Cq Values of the Strip PCR

We determined the cutoff Cq values of the strip PCR, because minute amounts of bacterial 16S and fungal 28S rRNA are often present in the enzyme used for PCR amplification and the DNA isolation reagent, respectively, while *P. acnes* is a resident microorganism of the ocular surface. Some noninfectious patient samples were positive for bacterial 16S (5/10 samples, 39.77 ± 0.51 cycles), fungal 28S (3/10 samples, 40.55 ± 0.96 cycles), and *P. acnes* (5/10 samples, 37.10 ± 1.52 cycles) DNA, in addition to GAPDH and TBP (Fig. 3; Supplementary Table S7). In all positive cases, the contaminating DNA was detected at the end of the PCR reaction at low concentrations by qPCR. We set the cutoff Cq values to 35.00 for bacterial 16S and fungal 28S rRNA and to 33.00 for *P. acnes* by using the control samples to avoid false-positive results. When using these cutoff Cq values, none of the 10 control samples was positive for any of the 24 pathogens by the strip PCR assay.

Comparison of Strip PCR, qPCR, and Capillary PCR

To confirm the sensitivity of this new strip PCR assay, we compared the results of strip PCR to those of previously developed capillary PCR and qPCR. The Table summarizes the results. Strip PCR detected HSV1 (samples 8, 12), HSV2 (sample 2), VZV (samples 10, 13, 21), EBV (samples 14, 23), CMV (samples 11, 22, 23), HHV6 (sample 16), HHV7 (sample 18), HTLV-1 (sample 17), adenovirus (sample 14), *P. acnes* (samples 14, 18), bacterial 16S rRNA (samples 7, 12, 15), *Candida* sp. (samples 5, 9), *C. glabrata* (sample 1), *Aspergillus* (sample 19), fungal 28S rRNA (samples 1, 5, 6, 9, 19), *T. gondii* (sample 4), *C. trachomatis* (sample 20), and *Acanthamoeba* (sample 3). All positive and negative results were validated by qPCR, and we summarized the copy numbers in the sample by qPCR. All of the clinical samples were positive for GAPDH and TBP. Samples 2, 4, 8, 10, 11, 12, 13, 14, 16, 18, and 23 in the Table were representative positive samples detected by both strip PCR and capillary PCR. Samples 21 and 22 were representative cases where strip PCR but not capillary PCR detected viral DNA. These positive results were verified by qPCR: 4.9×10^2 copies/mL of VZV-DNA in sample 21 and 1.3×10^2 copies/mL of CMV-DNA in sample 22. Thus, strip PCR, as well as qPCR, was more sensitive than capillary PCR. In addition, kinds of pathogens were newly detected by strip PCR, including adenovirus, *P. acnes*, *Candida* sp., *C. glabrata*, *Aspergillus*, *Fusarium*, *C. trachomatis*, and *Acanthamoeba*.

A Case Report

A 66-year-old man developed hyperemia in the left eye and was treated with subtenon triamcinolone acetonide injection after a diagnosis of idiopathic iridocyclitis by a local ophthalmologist. The inflammation worsened soon after the injection, and the patient was immediately referred to Oita University Hospital. Ophthalmic examination revealed necrotizing retinal lesions in the peripheral fundus of the left eye (Fig. 4A), and the patient was clinically diagnosed as having acute retinal necrosis (ARN). A pars plana vitrectomy was performed, and the VF (100 μL) was processed for PCR assays. VZV and EBV-DNA were detected by strip PCR and confirmed by qPCR (VZV, 1.3×10^8 copies/mL; EBV, 3.4×10 copies/mL; Fig. 4B). Quantification cycle values of bacterial 16S and *P. acnes* were lower than the cutoff values. None of the other 22 pathogens was detected by PCR examination.

Discussion

The present study showed that a newly established multiplex solid-phase strip PCR assay was able to detect 24 different genomic DNAs of common pathogens of ocular infectious diseases from ocular samples (tears, corneal smear, Aqh, and VF) comprehensively in one assay in a very short amount of time. The 24 pathogens consisted of all eight types of HHVs, HTLV-1, adenovirus, *M. tuberculosis*, *T. pallidum*, *P. acnes*, bacterial 16S rRNA, *Cansida* sp., *C. glabrata*, *C. krusei*, *Aspergillus*, *Fusarium*, fungal 28S rRNA, *T. gondii*, *Toxocara*, *C. trachomatis*, and *Acanthamoeba*. Our validation study of the strip PCR assay gave proof of repeatability and specificity. The reliability of the strip PCR assay to detect these genomic

Table. Results of Strip PCR, qPCR, and Capillary PCR

No.	Patient	Clinical Diagnosis	Sample	Strip PCR (Cq Values*)	qPCR†	Capillary PCR‡
1	74F	Fungal endophthalmitis	Aqh	*C. glabrata* (28.12) Fungal 28S (29.68)	*Candida* 18S (5.6×10^5) Fungal 28S (4.4×10^5)	nt
2	52F	Acute retinal necrosis	Aqh	HSV2 (35.82)	HSV2 (4.9×10^4)	HSV2
3	81F	Acanthamoeba keratitis	Cornea	*Acanthamoeba* (28.26)	*Acanthamoeba* (4.1×10^4)	nt
4	75M	Toxoplasmosis	Aqh	*T. gondii* (33.47)	*T. gondii* (2.9×10^5)	*T. gondii*
5	65F	Fungal endophthalmitis	VF	*Candida sp*. (34.23) Fungal 28S (33.00)	*Candida* 18S (1.9×10^6) Fungal 28S (1.5×10^6)	nt
6	35M	Fungal keratitis	Tear	Fungal 28S (33.95)	Fungal 28S (3.9×10^3)	nt
7	76F	Bacterial endophthalmitis (post OPE)	VF	Bacterial 16S (29.88)	Bacterial 16S (4.6×10^3)	nt
8	72M	Acute retinal necrosis	Aqh	HSV1 (30.66)	HSV1 (1.9×10^5)	HSV1
9	76F	Fungal endophthalmitis	VF	*Candida sp*. (25.63) Fungal 28S (26.90)	*Candida* 18S (8.5×10^6) Fungal 28S (4.7×10^6)	nt
10	69F	Anterior uveitis and secondary glaucoma	Aqh	VZV (30.71)	VZV (3.4×10^5)	VZV
11	64M	Corneal endotheliitis	Aqh	CMV (38.64)	CMV (3.3×10^3)	CMV
12	58M	Herpetic keratitis	Cornea	HSV1 (32.69) Bacterial 16S (30.22)	HSV1 (3.3×10^2) Bacterial 16S (1.6×10^3)	HSV1
13	57M	Acute retinal necrosis	Aqh	VZV (29.02)	VZV (1.7×10^6)	VZV
14	33M	Infectious conjunctivitis	Conjunctiva	Adenovirus (31.44) *P. acnes* (30.00) EBV (38.09)	Adenovirus (4.9×10^2) *P. acnes* (2.7×10^3) EBV (1.9×10^2)	EBV
15	74F	Bacterial endophthalmitis	VF	Bacterial 16S (23.53)	Bacterial 16S (9.8×10^6)	nt
16	46M	Bullous keratopathy	Cornea	HHV6 (26.53)	HHV6 (1.2×10^6)	HHV6
17	40F	HTLV-1 associated uveitis	Aqh	HTLV-1 (32.57)	HTLV-1 (2.72×10^4)	nt
18	36F	Conjunctivitis & dry eye	Tear	HHV7 (34.51) *P. acnes* (32.37)	HHV7 (3.2×10) *P. acnes* (8.8×10^2)	HHV7
19	57M	Fungal keratitis	Cornea	*Aspergillus* (34.73) Fungal 28S (34.26)	*Aspergillus*18S (3.6×10^2) Fungal 28S (5.1×10^3)	nt
20	24F	Chlamydial conjunctivitis	Tear	*C. trachomatis* (30.56)	*C. trachomatis* (4.6×10)	nt
21	62M	Anterior uveitis	Aqh	VZV (30.71)	VZV (4.9×10^2)	N.D. (VZV[−])
22	56M	Anterior uveitis and secondary glaucoma	Aqh	CMV (38.64)	CMV (1.3×10^2)	N.D. (CMV[−])
23	59F	CMV retinitis	Aqh	CMV (23.94) EBV (30.05)	CMV (4.1×10^6) EBV (3.8×10^5)	CMV EBV

N.D., not detected; nt, not tested.

* Cutoff Cq values: bacterial 16S and fungal 28S, < 35.00; *P. acnes*, < 33.00; others, < 40.00.

† Copy number in qPCR – Aqh or VF sample – copies/mL, and cornea, tear or conjunctiva sample – copies/μg·DNA.

‡ Capillary PCR: Our former capillary-type multiplex PCR.

DNAs was tested by standard positive DNA controls for each pathogen, as well as negative controls. Among 23 samples from patients highly suspected to have various ocular infectious diseases, genomic DNAs of infectious pathogens were detected in all samples by the strip PCR assay, and the positive results were confirmed by qPCR. Although our previously developed capillary PCR method has high sensitivity, specificity, and positive and negative predictive values,[13] the strip PCR assay but not the capillary PCR assay detected VZV and CMV DNA in two cases, and these results were confirmed by qPCR. This new multiplex strip PCR had higher sensitivity than the capillary PCR. Because small amounts of *P. acnes*, bacterial 16S, and fungal 28S rRNA are often present in the enzyme used for PCR amplification and the DNA isolation reagent, respectively, it is essential to determine an appropriate cutoff Cq to avoid false-positive results.

Our previously developed two-step PCR[12] assays, consisting of capillary PCR[5] and broad-range PCR,[9] detected a number of pathogens causing ocular infectious disease and have several advantages over single-target PCR. These advantages include not requiring gel analysis, detection of pathogens in a small sample, applicability to complex cases such as immunosuppressed patients and unexpected infections, semiquantitation based on amplification curves, and completion within a relatively short time (around 190 minutes). However, the PCR requires two costly PCR instruments and complicated processes with considerable skill to adjust reagent concentrations and reaction time to achieve optimal detection sensitivity. In contrast, the current strip PCR procedure is markedly simpler and more convenient because all reagents are in the solid phase. The present study demonstrated that it is possible to simultaneously detect 24 common ocular pathogens in only 90 minutes by strip PCR. The strip PCR can be performed with standard PCR instruments available in many laboratories.

The ocular samples tested in the present study were positive for the following 18 pathogens: HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HTLV-1, adenovirus, *P. acnes*, bacterial 16S rRNA, *Candida* sp., *C. glabrata*, *Aspergillus*, fungal 28S rRNA, *T. gondii*, *C. trachomatis*, and *Acanthamoeba*. Importantly, strip PCR had high sensitivity and specificity comparable to qPCR, and included many pathogens not covered by previously two-step PCR.

Viral load determination in infectious diseases is essential to determine the pathogenic role of viruses by PCR. The viral load is correlated with the severity of herpes virus–related infectious diseases such as ARN and CMV-associated corneal endotheliitis and iridocyclitis in the Aqh.[2,4,20] A high DNA load of *T. gondii* DNA in ocular fluids is also correlated with intraocular inflammation in uveitis patients with ocular toxoplasmosis.[16] Although strip PCR is qualitative, not quantitative, strip PCR estimates the amount of pathogen by semiquantitative analysis based on the Cq value (= acquisition of cycle). It can also be used during treatment to determine the treatment efficacy, if repeated sampling is possible. In the case

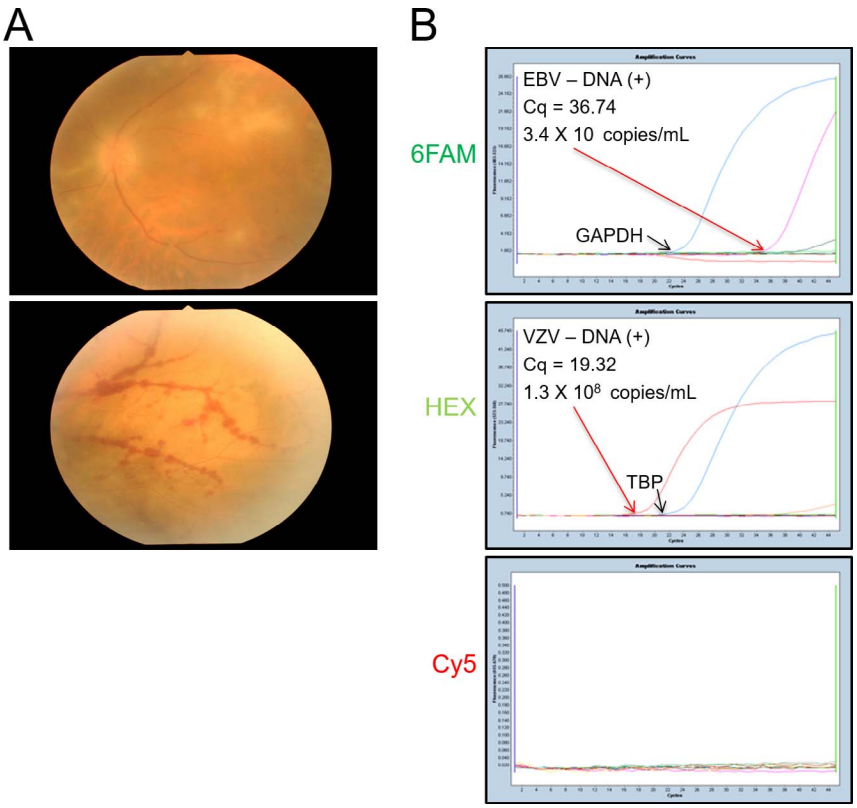


Figure 4. A case report: (**A**) A 66-year-old man with ARN presenting with retinal necrosis, vitreous opacity (*upper picture*), and retinal vasculitis (*lower picture*). (**B**) Varicella-zoster virus-DNA was detected in VF by strip PCR (*middle graph*), consistent with the clinical diagnosis. Epstein-Barr virus-DNA (*upper graph*), GAPDH (internal control: *upper graph*), and TBP (internal control: *middle graph*) were also detected in the sample.

report of the present study, both VZV-DNA (Cq = 19.32) and EBV-DNA (Cq = 36.74) were detected in the vitreous sample by strip PCR. The copy numbers determined by qPCR were 1.3×10^8 copies/mL for VZV-DNA and 3.4×10 copies/mL for EBV-DNA. Thus, we diagnosed this case as VZV-associated ARN.

There were some problems associated with strip PCR. Bacterial 16S rRNA, fungal 28S rRNA, and *P. acnes* in the PCR reaction and in the ocular surface are often detected at the end of the PCR cycles, although they are also present at low concentrations in real-time qPCR. This problem can be resolved by setting cutoff points for Cq values (Fig. 3; Supplementary Table S7). It is also critical to minimize contamination during sample collection and processing. We intend to validate the strip PCR method in a prospective study using a large number of patient samples and noninfectious control samples.

In conclusion, we developed a new multiplex strip PCR assay for simultaneous detection of 24 ocular infectious disease pathogens that is quicker and simpler than previously developed capillary-type multiplex PCR assays. Strip PCR has a sensitivity that is similar to that of qPCR and superior to that of conventional capillary-type multiplex PCR, making it a reliable and accurate diagnostic tool for patients who are immunocompromised due to immunosuppressive drug treatment or human immunodeficiency virus and may therefore acquire unexpected infectious diseases. We plan to conduct a prospective large multicenter study of this PCR assay.

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