Evaluation of the SmartCycler II System for Real-Time Detection of Viruses and Chlamydia From Ocular Specimens

Regis P. Kowalski, MS, M(ASCP); Paul P. Thompson, BMedSc; Paul R. Kinchington, PhD; Y. Jerold Gordon, MD

Objective: To compare the SmartCycler II system (Cepheid, Sunnyvale, Calif) results with those of standard cell culture, to compare the SmartCycler II system results with those of a dedicated polymerase chain reaction facility, and to establish the SmartCycler II system as a polymerase chain reaction method for detecting viral and chlamydial DNA from ocular specimens.

Methods: True-positive samples (test-positive specimens based on standard testing) and true-negative samples (test-negative specimens based on standard testing) were processed for polymerase chain reaction using the SmartCycler II system for adenovirus, herpes simplex virus type 1, varicella-zoster virus, and Chlamydia trachomatis. Sensitivity, specificity, positive predictive value, negative predictive value, and efficiency were based on the testing of true-positive and true-negative specimens.

Results: The descriptive statistics for adenovirus, herpes simplex virus type 1, varicella-zoster virus, and C trachomatis were, respectively, as follows: sensitivity, 85%, 98%, 100%, and 94%; specificity, 98%, 100%, 100%, and 100%; positive predictive value, 98%, 100%, 100%, and 100%; negative predictive value, 85%, 91%, 100%, and 98%; and efficiency, 92%, 95%, 100%, and 99%. Test sensitivity for the SmartCycler II system was equivalent to that from a central molecular laboratory.

Conclusion: The descriptive statistics of the SmartCycler II system obtained in a small laboratory were comparable to those of a central molecular laboratory for detecting viruses and Chlamydia species.

Clinical Relevance: Polymerase chain reaction has great potential in the routine diagnosis of ocular infections in any conventional laboratory.

Arch Ophthalmol. 2006;124:1135-1139

POLYMERASE CHAIN REACTION (PCR) has become a powerful tool in the laboratory diagnosis of many infections and has evolved from a complex test to a routine diagnostic tool. Although widely used, PCR is still somewhat confined to larger central laboratories, where special room requirements necessitate appropriate ventilation with positive and negative airflow to eliminate testing contamination. These space precautions are necessary because of the open system, which involves separate steps of amplification and detection. After amplification in a sealed tube is completed in the first step, the sealed tube is opened to remove amplified product for confirmation with gel electrophoresis or an enzyme immunoassay technique. Removed amplified product can create aerosols that have the potential to contaminate clinical samples and control samples, creating false test results.

Laboratories that use these open PCR systems are highly scrutinized by certifying agencies (eg, College of American Pathologists) and must have adequate facilities to minimize possible aerosol contamination to maximize PCR testing.

Recent developments have advanced the molecular field to closed systems for processing PCR testing. In a closed system, amplified product is contained in a sealed environment in which the detection process is accomplished concurrently with amplification using fluorescent probes. The amount of fluorescence is measured using unique photodetector instrumentation and software. Because there is no threat of aerosol contamination, it is conceivable that more laboratories with limited space could process PCR for routine diagnostic testing.

We evaluated the SmartCycler II system (Cepheid, Sunnyvale, Calif) as a closed system of real-time PCR for detecting vi-
ral and chlamydial DNA from ocular specimens. Protocols were developed using sequence-specific primers and fluorescent probes to detect common causative agents of conjunctivitis and keratitis, including adenovirus, herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), and Chlamydia trachomatis. The objectives of the study were to compare the SmartCycler II system results with those of standard cell culture, to compare the SmartCycler II system results with those of a dedicated PCR facility, and to establish the SmartCycler II system as a PCR method for detecting viral and chlamydial DNA from ocular specimens. Success of the SmartCycler II system in this study would indicate that PCR is no longer confined to the dedicated molecular laboratory and that PCR should be recognized as an important tool for routinely diagnosing ocular infections in any conventional laboratory.

**METHODS**

**KEY INSTRUMENTATION**

The SmartCycler II system contains a computer, processing block, 25-µL reaction tubes, and a small centrifuge. A biologic cabinet with type 2 containment, a refrigerated microcentrifuge (10 000 rpm; Fisher Scientific, Pittsburgh, Pa), and a heating block (98°C) were also necessary to process PCR reactions.

**SmartCycler II SYSTEM OVERVIEW**

The SmartCycler II system is a closed PCR system by which amplification and detection are accomplished concurrently with TaqMan technology (Applied Biosystems, Foster City, Calif) using fluorescent probes to detect amplification after each replicating cycle. To start the process, a probe is created by conjugating carboxyfluorescein fluorescent dye (FAM) and a fluorescent quencher, tetramethylrhodamine (TAMRA), to a single-stranded DNA (ssDNA) oligonucleotide that is complementary to a conserved region of the target genome or plasmid. No fluorescence is emitted so long as the probe is intact, with the TAMRA quenching the FAM. The probe anneals to a conserved region within the ssDNA sequence, and specific complementary ssDNA primers also anneal to the end of the ssDNA sequence. Taq polymerase adds nucleotides to the 3’ end of the ssDNA primer. As it extends, the Taq polymerase, which has exonuclease activity, will come into contact with the downstream probe and cleave it, releasing the FAM into the reaction solution away from the TAMRA quencher. After each PCR cycle, increasing amounts of FAM are released and the emitted fluorescence is detected with the system’s optical unit using SmartCycler II software and is visualized on a computer monitor (SmartCycler II Operator Manual).

**STUDY SAMPLES FOR TESTING**

**SmartCycler II SYSTEM**

**Specimen Collection**

All clinical specimens were obtained from the conjunctiva or cornea of patients with a differential diagnosis of viral or chlamydial infection. The specimens were collected with a soft-tipped applicator or by scraping the area of infection with a Kimura spatula. All patient samples were placed in 2.0 mL of chlamydial transport medium (Bartels Immunodiagnostic Supplies, Inc, Bellevue, Wash) and frozen at −80°C.

**Routine Laboratory Clinical Studies: Standard Testing**

For routine clinical laboratory studies, chlamydial transport medium was divided for testing that included cell culture isolation (0.5 mL), shell vial testing (0.2 mL), and PCR testing (0.5 mL). Remaining sample was saved and stocked at −80°C for retrospective PCR studies using the SmartCycler II system (University of Pittsburgh Institutional Review Board No. 0506122).

A clinical sample for routine DNA detection (using the LightCycler System; Roche Diagnostic Corp, Indianapolis, Ind) of adenovirus, HSV-1, or VZV was transported to the Molecular Diagnostics Division of the University of Pittsburgh Medical Center, Pittsburgh, Pa. A clinical sample for the DNA detection (using the Amplicor System; Roche Diagnostic Corp, Indianapolis, Ind) of Chlamydia trachomatis was transported to the Virology Department of the University of Pittsburgh Medical Center. Polymerase chain reaction detection from both of these testing areas represent state-of-the-art molecular laboratory diagnosis from a tertiary care medical center.

**SmartCycler II System Testing**

All viral and chlamydial PCR protocols were tested against batteries of true-positive and true-negative samples to determine sensitivity, specificity, positive predictive value, negative predictive value, and efficiency of testing. True-positive samples were specimens that contained targeted DNA (adenovirus, HSV-1, VZV, or Chlamydia trachomatis) determined from other testing, and true-negative samples contained nontargeted DNA as determined from other testing. The descriptive statistics were calculated as follows: sensitivity = TP/(TP + FN); specificity = TN/(TN + FP); positive predictive value = TP/(TP + FP); negative predictive value = TN/(TN + FN); and efficiency = (TP + TN)/(TP + FP + TN + FN), where TP are true-positive samples that test positive, TN are true-negative samples that test negative, FN are true-positive samples that test negative, and FP are true-negative samples that test positive (http://www.musc.edu/dc/lcrebm/sensitivity.html [accessed April 22, 2006]).

The true-positive samples (n = 53) for adenovirus PCR testing were direct conjunctival samples from patients with conjunctivitis or keratitis as confirmed by positive cell culture for adenovirus. The true-negative samples (n = 45) consisted of 15 adenovirus test-negative clinical samples and 3 samples each of spiked Pseudomonas aeruginosa, Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Chlamydia trachomatis, and 5 direct patient samples with culture-positive HSV.

The bacterial samples used for true-negative testing were prepared by suspending overnight bacterial growth from solid agar medium (5% sheep blood or chocolate agar) to a 0.5 McFarland standard in tryptic soy broth and pipetting 50 µL of the suspension in 0.5 mL of chlamydial transport medium. All direct samples used for true-negative testing were extracted clinical specimens that were PCR positive for adenovirus, HSV-1, VZV, or Chlamydia trachomatis.

The true-positive specimens for HSV-1 PCR testing (n = 64) were direct conjunctival or corneal samples from patients with keratitis or conjunctivitis as confirmed with positive cell culture for HSV. The true-negative samples (n = 58) consisted of 23 HSV test-negative clinical samples and 3 samples each of spiked P aeruginosa, S pneumoniae, H influenzae, S aureus, Chlamydia trachomatis, and VZV and 5 direct patient samples with culture-positive adenovirus. Polymerase chain reaction testing was not developed for HSV-2 because of a lack of available ocular specimens submitted for HSV-2 infection. Ocular HSV-2 infection occurs rarely. The true-positive specimens for VZV PCR testing (n = 11) were direct conjunctival or corneal samples from patients with keratitis as confirmed with positive PCR for VZV. The true-
negative samples (n = 46) consisted of 11 VZV test-negative clinical samples and 5 samples each of spiked P aeruginosa, S pneumoniae, H influenzae, S aureus, and C trachomatis, 5 direct patient samples with culture-positive adenovirus, and 5 direct patient samples with culture-positive HSV.

The true-positive specimens for C trachomatis PCR testing (n = 18) were direct conjunctival or corneal samples from patients with conjunctivitis or keratitis as confirmed by positive culture for C trachomatis. The true-negative samples (n = 36) consisted of 26 C trachomatis test-negative clinical samples and 5 samples each of spiked P aeruginosa, S pneumoniae, H influenzae, and S aureus, 5 direct patient samples with culture-positive adenovirus, and 5 direct patient samples with culture-positive HSV.

An appropriate positive control (target DNA) and negative control (Tris-EDTA buffer without target DNA) were processed with each PCR batch run to ensure proper testing conditions. False-negative specimens were spiked with DNA and reamplified to determine whether PCR inhibitors were present in the samples.

SAMPLE PREPARATION FOR REAL-TIME PCR BY THE SmartCycler II SYSTEM

Protein and DNA Extraction and Fluorescein Elimination

Thawed specimens (from −80°C stock) for PCR processing were prepared by heating 0.3 mL of sample for 10 minutes at 98°C, then placing it on ice. (Heating helps to lyse cells to expose DNA.) The protein was extracted from boiled samples with 0.15 mL of Master Pure Complete protein precipitation reagent (Epicentre Technologies, Madison, Wis). The mixture was vortexed for 10 seconds and centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet containing the DNA was washed twice with 75% ethanol (0.5 mL) using centrifugation at 10 000 rpm for 5 minutes at 4°C. The remaining pellets were dried under vacuum to eliminate any residual ethanol, which would inhibit the PCR. The final DNA pellet was suspended in 35 µL of Tris-EDTA buffer (10 mmol/L Tris hydrochloride [pH 8.0] and 1 mmol/L EDTA; Epicentre Technologies).

Primers and Probes

Table 1 lists the primers and probes for detecting adenovirus, HSV-1, VZV, and C trachomatis using PCR. The primers and probes were selected from the literature and adapted for the SmartCycler II system. The efficacy of the primers and probes for HSV-1, VZV, and C trachomatis PCR was validated using cloned PCR product prepared using the pGEM-T Easy Vector system (Promega Corp, Madison, Wis). The DNA was linearized using the restriction enzyme EcoRI (New England Biolabs Inc, Ipswich, Mass) and purified using the Wizard Plus Miniprep DNA Purification System (Promega Corp). The efficacy of the primers and probe for adenovirus PCR was evaluated using adenovirus type 2 DNA purchased from Invitrogen Corp (Carlsbad, Calif). Amplification efficiencies (E) for adenovirus, HSV-1, VZV, and C trachomatis were calculated using the following equation: 

\[ E = 10^{(1.0/A - 1)} \]

The slopes were determined from regression plots (cycle threshold vs dilution of target DNA) using known amounts of target DNA. The cycle threshold is the cycle that denotes a significant accumulation of target DNA over the background threshold. From the line equation 

\[ Y = b + aX \]

a is the slope of the regression line.

SmartCycler II Reaction Mix

All PCR reactions contained 15 µL of master mix and 10 µL of patient (or control) sample placed in 25-µL SmartCycler II tubes. The master mix for 2 reactions was composed of a forward primer, reverse primer, probe, DNase-free water, and an OmnMix "hot-start" bead (3 units of Taq DNA Polymerase, Hot-Start Version [Takara Bio Inc, Otsu, Shiga, Japan], 200 µmol/L deoxyribonucleoside triphosphate, and 4 µmol/L magnesium chloride in 25 mmol/L HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid] buffer [pH 8.0±0.1]). The respective concentrations of forward primer, reverse primer, and probe in the final reaction tube for adenovirus were 0.5 µmol/L,
**RESULTS**

Efficiency was determined from regression plots (cycle threshold vs dilution of target DNA) of PCR for adenovirus, HSV-1, VZV, and *C. trachomatis*. From the slopes of the regression plots, the calculations of the amplification efficiency for adenovirus, HSV-1, VZV, and *C. trachomatis* were 0.914, 0.986, 0.923, and 1.09, respectively.

Table 2 gives the descriptive statistics of SmartCycler II real-time PCR for detecting adenovirus, HSV-1, VZV, and *C. trachomatis*. The sensitivity based on positive cell culture (83%; 45/53) was lower (*P* = .04) than for positive PCR testing (100%; 32/32) for adenovirus. For the 8 tests that were PCR negative, cell culture positive for adenovirus, additional testing of the false-negative samples determined that no PCR inhibitors were present. For these clinical samples, the cell cultures required longer than normal incubation to achieve an adenovirus cytopathic effect (mean, 14.4 days; range, 10-20 days), indicating an initial low load of viable virus. In addition, 5 patients had herpetic disease. These 8 negative results could be due to sampling error (0.5 mL of sample for culture vs 0.01 mL of sample for PCR), loss of DNA during extraction, or possible contamination with external adenovirus during the incubation period that produced a false-positive result in cell culture. Only 1 true-negative specimen, an *H. influenzae* sample, tested positive for adenovirus DNA. It can be speculated with high suspicion that the *H. influenzae* was isolated from a patient who was co-infected with adenovirus. No specimen for viral culture was submitted with the initial examination to prove this speculation.

There were no statistical differences between sensitivity based on positive cell culture vs positive PCR for HSV-1 (*P* < .99), VZV (*P* < .99), and *Chlamydia* (*P* = .27) (Monte-Carlo randomization test; True Epistat, Richardson, Tex.). Only 2 patient samples with *Chlamydia* tested negative with SmartCycler II real-time PCR compared with samples that tested positive from the virology laboratory. This may also have been due to sampling error or loss of DNA with our extraction method. No true-negative samples tested positive for HSV-1, VZV, or *C. trachomatis*.

**COMMENT**

An optimal laboratory test for the diagnosis of viral and chlamydial infections must be highly sensitive and specific, cost-effective, and timely to provide a definitive di-

---

Table 2. Descriptive Statistics of SmartCycler Real-Time PCR for Detecting Ocular Pathogens*

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Adenovirus</th>
<th>HSV-1</th>
<th>VZV</th>
<th><em>Chlamydia trachomatis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>True-positive sample †</td>
<td>53</td>
<td>64</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>True-negative sample</td>
<td>45</td>
<td>58</td>
<td>46</td>
<td>56</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>85 (45/53)</td>
<td>98 (63/64)</td>
<td>100 (11/11)</td>
<td>94 (17/18)</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>98 (44/45)</td>
<td>100 (58/58)</td>
<td>100 (46/46)</td>
<td>100 (56/56)</td>
</tr>
<tr>
<td>Positive predictive value, %</td>
<td>98 (45/46)</td>
<td>100 (58/58)</td>
<td>100 (46/46)</td>
<td>98 (56/57)</td>
</tr>
<tr>
<td>Negative predictive value, %</td>
<td>85 (45/53)</td>
<td>91 (58/64)</td>
<td>98 (46/46)</td>
<td>96 (58/56)</td>
</tr>
<tr>
<td>Efficiency, %</td>
<td>92 (90/98)</td>
<td>95 (116/122)</td>
<td>100 (57/57)</td>
<td>99 (73/74)</td>
</tr>
<tr>
<td>Minimum titer (DNA) per 10 µL, No. of copies ‡</td>
<td>1</td>
<td>17</td>
<td>11</td>
<td>496</td>
</tr>
<tr>
<td>Minimum titer (mass) per 10 µL</td>
<td>34 ag</td>
<td>55.5 ag</td>
<td>39 ag</td>
<td>1.7 fg</td>
</tr>
<tr>
<td>Sensitivity, based on PCR results from a central molecular laboratory, %</td>
<td>100 (32/32)</td>
<td>100 (9/9)</td>
<td>100 (11/11)</td>
<td>86 (12/14)</td>
</tr>
</tbody>
</table>

Abbreviations: ag, attogram; fg, femtogram; HSV-1, herpes simplex virus type 1; PCR, polymerase chain reaction; VZV, varicella-zoster virus.

*The descriptive statistics were calculated as follows: sensitivity = TP/(TP + FN); specificity = TN/(TN + FP); positive predictive value = TP/(TP + FP); negative predictive value = TN/(TN + FN); efficiency = (TP + TN)/(TP + FP + TN + FN); TP are true-positive samples that tested positive; TN are true-negative samples that tested negative; FN are true-positive samples that tested negative; and FP are true-negative samples that tested positive.

†True-positive results for adenovirus, HSV-1, and *C. trachomatis* were based on positive cell culture isolation. True-positive results for VZV were based on positive PCR results from the central molecular laboratory.

‡Titer detected is based on 10 µL of sample tested for each PCR reaction.

§Sensitivity based on PCR for *Chlamydia* was not calculated from the exact sample population of sensitivity based on positive cell culture.
agnosis. Tests that fail to provide definitive answers or to yield a high rate of false-positive or false-negative results are of little use to the clinician and may be detrimental to the patient. In this age of managed care, tests must not only be cost-effective for the patient but also for the laboratory providing the tests. High technical costs and manpower issues create time problems for hospitals and health care centers. Despite these boundaries, laboratories must still be accountable for providing 24- to 48-hour turnaround for essential tests.

Many laboratories are entering highly specialized areas of testing but still depend on older, established procedures to validate these new rapid laboratory tests. At our ophthalmic microbiology laboratory, we still use standard cell culture testing to detect viruses that cause conjunctivitis, keratitis, and intraocular infections, but we have included PCR, through a dedicated central molecular laboratory, to our testing battery. We are using PCR to detect adenovirus, HSV, VZV, cytomegalovirus, Epstein-Barr virus, and C. trachomatis from ocular specimens.

In this study, we evaluated the SmartCycler II real-time PCR system, a compact, closed system apparatus that can be used in any laboratory space as an alternative to a central molecular laboratory. The SmartCycler II system demonstrated its wide potential for detecting adenovirus, HSV-1, VZV, and C. trachomatis in a small laboratory setting in comparison with a large central molecular laboratory. Another advantage of the SmartCycler II system is that it can process from 1 to 16 samples with different PCR protocols for each reaction. New tests can be designed for different targets by modifying the protocols for established tests. This system could establish batteries of tests for the diagnosis of infectious uveitis, retinitis, and infectious keratitis in any laboratory. Multiplex PCR (detection of 2 or more targets in 1 reaction tube) is also a possibility, and a future refinement in our laboratory will include more streamlined DNA extraction that will eliminate fluorescein with less chance of contamination.

This study emphasizes the importance of proper design for evaluating diagnostic tests. True-positive and true-negative samples based on standard testing are mandated to evaluate the overall potential of a new test. The results from cell culture isolation (the gold standard) and PCR testing from a central molecular laboratory were each used independently to determine the sensitivity of the SmartCycler II real-time PCR system. For adenovirus and HSV-1, cell culture isolation provided more positive results than did PCR testing, indicating that cell culture may still be the more sensitive test. A recent report for detecting adenovirus with a rapid molecular technique used clinical diagnosis as the standard. Clinical diagnosis alone is an unreliable measure for the early diagnosis of adenoviral conjunctivitis (Y. J. G., oral communication, February 20, 2005). Of the 52 samples from patients with a clinical diagnosis of possible adenovirus infection, only 15 patients tested positive by PCR and the remaining 37 tested negative. Some of the 37 PCR-negative samples may have tested positive by cell culture, had this established test been used. Consequently, the important parameter of sensitivity could not be calculated in the absence of an acceptable, established laboratory standard. True-negative samples were not obtained for determining specificity and other pertinent descriptive statistics.

The SmartCycler II real-time PCR system is comparable to the dedicated central molecular laboratory for detecting adenovirus, HSV-1, VZV, and C. trachomatis in ocular samples. Testing is highly sensitive and specific, and it can provide timely results within 24 hours of specimen collection. Further laboratory and clinical studies for detecting other ocular pathogens will determine the full potential of the SmartCycler II real-time PCR system.

Submitted for Publication: October 4, 2005; final revision received January 10, 2006; accepted January 26, 2006.

Correspondence: Regis P. Kowalski, MS, M(ASCP), Eye and Ear Institute, Ophthalmic Microbiology, Room 642, 203 Lothrop St, Pittsburgh, PA 15213.

Author Contribution: Mr Kowalski assumes full responsibility for the accuracy and data analysis of the manuscript.

Financial Disclosure: None reported.

Funding/Sponsor: The Charles T. Campbell Foundation financed the purchase of equipment and essential materials. The Eye and Ear Foundation of Pittsburgh, Pittsburgh, Pa, provided salary support. Core grant EY 08098 for vision research from the National Eye Institute, Bethesda, Md, provided expertise within the molecular module, and Research to Prevent Blindness, New York, NY, has provided continued support of the ophthalmology department.

REFERENCES