

PCR Assessment of HSV-1 Corneal Infection in Animals Treated with Rose Bengal and Lissamine Green B

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PURPOSE. In vivo, the ophthalmic dye rose bengal displays profound antiviral effects against herpes simplex virus (HSV)-1, thus limiting its utility in diagnosis of epithelial keratitis when used before viral culture is performed. In contrast, lissamine green B does not possess significant antiviral activity in vivo. To determine whether polymerase chain reaction (PCR) could successfully detect HSV-1 DNA in ocular samples that have been exposed to ophthalmic dyes, animal models were used to observe the presence of infectious HSV-1 and viral DNA in eyes treated with rose bengal or lissamine green B.

METHODS. Animals were bilaterally infected with HSV-1 strain H129, and at daily intervals up to 16 days post infection (dpi) rose bengal or lissamine green B was instilled in the left eyes. The right eyes were not treated with dyes. Swabs of the dye-treated and untreated eyes were assayed by PCR for viral infectivity by culture and the presence of DNA specific for a fragment of the HSV-1 DNA polymerase gene.

RESULTS. A statistically equivalent number of samples from lissamine green B-treated and untreated eyes were positive by both viral culture and PCR. In contrast, rose bengal significantly decreased the infectious virus present in ocular secretions. A total of 44% and 78% of the rose bengal-treated and untreated eye samples, respectively, were positive by culture from 1 through 16 dpi. PCR was more sensitive than culture for detection of HSV-1 in rose bengal-treated eyes, in that 74% of rose bengal-treated samples were positive by PCR compared with 44% that were positive by culture during the 16-day period studied. It was also noted that both rose bengal and lissamine green B treatments slightly prolonged the period during which viral DNA was detectable in ocular secretions by PCR, possibly because the singlet oxygen produced by these photoreactive dyes compromised ocular cellular, humoral, and nonspecific immune factors allowing viral DNA to persist for slightly longer periods.

CONCLUSIONS. PCR can successfully detect HSV-1 DNA in ocular samples that are culture negative and contain rose bengal or lissamine green B. Visualization of ocular epithelial defects with lissamine green B does not interfere with detection of infectious virus or HSV-1 DNA. (*Invest Ophthalmol Vis Sci.* 2000;41:2096-2102)

Herpes simplex virus type 1 (HSV-1) is an enveloped, double-stranded DNA virus capable of causing sight-threatening corneal infections.¹ Diagnosis of herpetic corneal infection is often facilitated by the use of ophthalmic

dyes. Fluorescein and rose bengal are the two most commonly used dyes in the United States, but each has diagnostic value for different conditions affecting the cornea and conjunctiva.²⁻⁴ Fluorescein and rose bengal highlight different aspects of HSV-1-induced corneal epithelial injury. Rose bengal stains both healthy and cytopathic cells, such as those infected with HSV-1, in vitro, but is prevented from staining living corneal epithelial cells in vivo because of the overlying protective mucin.^{5,6} In contrast, fluorescein indicates areas of epithelial cell injury by flowing between cell-to-cell junctions and quickly staining the underlying stroma.⁶ Many clinicians have preferred rose bengal to visualize HSV-1-induced corneal defects, because it provides high contrast between areas of infected and uninfected epithelium and does not require visualization with cobalt blue-filtered light. However, rose bengal is profoundly antiviral in vitro and in vivo,^{5,6} most likely because it generates singlet oxygen when exposed to 550-nm wavelength light, and singlet oxygen inactivates enzymes and damages single-stranded DNA and cell membranes. The photoreactive properties of rose bengal limit its usefulness in the diagnosis of herpetic epithelial keratitis, because it inactivates infectious HSV.⁵⁻⁷

In our search for an ophthalmic dye that had high specificity for epithelial keratitis and low antiviral activity, we previously compared the staining characteristics and antiviral

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A preliminary report of these findings was presented at the 10th Anniversary of the Arkansas Center for Eye Research at the Jones Eye Institute, Little Rock, October 4, 1997.

Supported by Department of Veterans Affairs Medical Research Service, National Institute of Deafness and Other Communication Disorders Grant CD01706 and an unrestricted grant to the University of Arkansas for Medical Sciences Harvey and Bernice Jones Eye Institute from Research to Prevent Blindness. JC is a recipient of a Research to Prevent Blindness Career Development Award, and WGS was a Jules and Doris Stein RPB Professor at the Baylor College of Medicine Cullen Eye Institute during the performance of part of this work.

Submitted for publication June 15, 1999; revised October 28, 1999, and January 7, 2000; accepted February 7, 2000.

Commercial relationships policy: N.

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properties of lissamine green B and sulforhodamine B with rose bengal in vitro and in an animal model of herpetic keratitis.^{5,6} In those studies, we demonstrated that rose bengal and lissamine green B displays little cellular toxicity in vitro or in rabbit eyes. Although both dyes exhibited anti-herpes virus activity in tissue culture systems, only rose bengal reduced viral shedding in vivo. After infection with 10^6 tissue culture doses of HSV-1, instillation of 1% rose bengal solution on days 1 through 5 and 7 through 10 after infection virtually eliminated the shedding of infectious virus through 11 days post infection (dpi) except for days 2 and 3. In contrast, untreated eyes shed between 10^2 and 10^6 tissue culture infectious doses per milliliter of infectious virus during the same 11-dpi period.

Because rose bengal markedly reduced or completely eliminated the ability to recover infectious HSV-1 in ocular samples, an unresolved question in our previous studies was whether rose bengal thoroughly "sterilizes" ocular secretions, rendering them free of all HSV-1 particles. In the present investigation, we sought to determine whether polymerase chain reaction (PCR) could detect HSV-1 DNA in ocular samples rendered noninfectious due to instillation of rose bengal. Parallel experiments were conducted with lissamine green B. The ability to recover infectious virus and to detect viral DNA in dye-treated eyes was compared with untreated eyes in a rabbit model of herpetic keratitis.

MATERIALS AND METHODS

Animal Infections and Virus Titrations

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. Four 5-lb specific pathogen-free (SPF) New Zealand White (NZW) rabbits were obtained from Myrtle Rabbitry (Thompson Station, TN). Six rabbits were bilaterally inoculated with HSV-1, strain H129 by placing 100 μ l containing 2×10^6 plaque forming units (pfu) onto the corneas and gently massaging the eyelids. These six rabbits were divided into two groups. Beginning at 1 dpi, the left eyes of three rabbits in one group received 10 μ l of 1% lissamine green B (Sigma, St. Louis, MO) diluted in calcium- and magnesium-free phosphate-buffered saline, and the left eyes of the rabbits in the second group received 10 μ l 1% rose bengal dye (Akorn, Abita Springs, LA). Instillation of dyes occurred at 1, 2, 3, 4, 7, 9, 11, and 16 dpi. The right eyes of the rabbits in both groups were not treated with any dye. External eye photography was performed on each rabbit in this study at 4 and 7 dpi, immediately after the swabbing procedure.

After instillation of the dyes, the left and right eyes of the virus-infected animal were separately swabbed with sterile cotton applicators. Each swab was separately placed into 1 ml transport medium (minimal essential medium containing 2% fetal calf serum) in a 15-ml conical tube and shaken several times. The excess fluid was expressed from the swab, and the swab was discarded. The medium was kept on ice and then frozen at -70°C . The medium was thawed, and 0.4 ml was removed for titration on VERO cells in triplicate by a tissue culture infectious dose (TCID) assay. To ensure that no cross-contamination occurred, individually wrapped 1-ml pipets were used for all titrations. Also, the samples taken from the dye-treated left eyes were titered separately from the non-treated right eye samples, and samples from the rose bengal-

treated rabbits were titered separately from the lissamine green B-treated eye samples. Within 1 hour, the remainder of the eye swab medium was returned to -70°C , to await DNA extraction.

PCR Analysis

The eye swab medium was thawed, and 125 μ l of the liquid was added to 125 μ l minimal essential tissue culture medium plus 2% fetal calf serum. To this mixture was added an equal volume of 0.02 M Tris, 0.01 M EDTA, and 1% sodium dodecyl sulfate (SDS), to which 100 μ g/ml proteinase K was added. The solution was incubated at 37°C for 90 minutes, and the DNA was separated by two extractions in phenol-chloroform-isoamyl alcohol (25:24:1) followed by one extraction in chloroform-isoamyl alcohol. The DNA was precipitated at room temperature for 15 minutes by addition of a 0.1 volume of 3 M sodium acetate (pH 5.2) and a 0.6 volume of isopropyl alcohol. The DNA was concentrated by centrifugation at 12,000g for 20 minutes and the DNA pellet washed with 70% cold ethanol, dried in a vacuum, and dissolved in 50 μ l of water. To 25 μ l of the DNA sample was added 10 mM Tris-HCl (pH 8.3); 0.75 mM Mg Cl₂; 50 mM KCl; 2.5 U *Taq* DNA polymerase; 250 μ M each of dATP, dCTP, dTTP, and dGTP; and 25 picomoles of each primer nucleotide in a final volume of 50 μ l. The primers used were based on the polymerase gene of the 17syn + HSV-1 strain.⁸ The 23-base 5'-primer (sense) oligonucleotide (CGGACTCCATCTTTGTGCTGTGC) was from nucleotides 65,466 to 65,489 in the unique long segment of the DNA sequence, and the 23-base 3'-primer (anti-sense) oligonucleotide (AAAGTCCTGGATGTCCCTCTCCG) was from nucleotides 65,877 to 65,900⁹; both primers were synthesized by Integrated DNA Technology (Coralville, IA).

The reaction volume was overlaid with 2 to 3 drops of mineral oil, and the DNA was amplified using a thermal cycler (model 480; Perkin-Elmer, Foster City, CA) programmed for 35 1-minute cycles of denaturation at 96°C , a 2-minute 67°C annealing step, and a 2-minute 72°C primer extension step followed by a 7-minute 72°C delay step. Controls for the DNA amplification included 100 ng HSV-1 strain 17syn + DNA (type-specific positive control) and distilled water (negative control). Fifteen microliters of the amplified reaction material was applied to a 2.5% agarose gel, and the amplified DNA was visualized by staining in 0.5 μ g/ml of ethidium bromide in water. DNA of known size (base pairs) were run in parallel to confirm the size of the amplified product.

The sensitivity of the PCR reaction was determined in two ways. First, serial dilutions of infectious HSV-1 were prepared in transport medium, and the DNA from each was extracted and the PCR reaction performed identically with the procedure used for the actual eye swab cultures. This showed that the extraction process coupled with the PCR could detect 200 pfu of HSV-1 (data not shown). Second, the sensitivity of the PCR reaction was determined by adding known quantities of purified DNA to the PCR reaction cocktail. This showed that the PCR itself could detect 100 fg of viral DNA after ethidium bromide staining and visual inspection of an agarose gel (data not shown). Because PCR is notoriously sensitive to contaminants, in preliminary experiments, we added serial 10-fold dilutions of rose bengal or lissamine green B from 1% to 0.00001% to mock swab preparations before extracting the DNA. In separate experiments, we added serial 10-fold dilutions of the dyes to known amounts of HSV-1 strain 17syn + DNA in the reaction mixture before thermocycling the prepa-

rations. Under both circumstances, neither dye diminished detection of viral DNA (data not shown).

The specificity of the PCR reaction for detection of HSV-1 was confirmed by using the described primers in separate reactions containing DNAs from HSV-2 strains HG52pl8, MS, and 186; herpesviruses EBV, VZV, and herpes saimiri; and adenovirus types 2, 5, 7, 8, 9, 10, 11, 13, 19, and 37. No product of the expected 434-bp size was detected in agarose gels or by Southern blot hybridization using a ^{32}P -dATP-labeled HSV-1-specific DNA probe (data not shown). However, when the primers were used in reactions containing HSV-1 DNA from strains 17syn +, KOS-63, KOS-79, + GC, and H129, the predicted 434-bp fragment was seen in agarose gels and by Southern blot hybridization.

Statistical Analysis

Analyses of viral shedding and detection of HSV-1 DNA by PCR between groups of animals were conducted by computer (SigmaStat ver. 1.0; Jandel Scientific, San Rafael, CA). Fisher exact test or χ^2 analysis was performed as appropriate.

RESULTS

In designing these experiments, an animal model was chosen to reasonably mimic clinical findings in humans with ocular infections. Animals were infected bilaterally with HSV-1 strain H129 and then on days 1, 2, 3, 4, 7, 9, 11, 14, and 16 dpi, 10 μl 1% rose bengal solution was instilled in the left eye of each animal. A second group of animals was similarly infected and treated with freshly prepared 1% lissamine green B. The right eyes of animals in both groups were not treated with dyes and served as virus-infected (positive), non-dye-treated control eyes. After instillation of the dyes, the eyes were visually examined. We did not examine uninfected eyes treated with rose bengal or lissamine green B, because we have previously shown that neither dye causes any clinically detectable lesions in the eyes of rabbits.⁶ Just before examination and photography, the eyes were separately swabbed with cotton-tipped applicators, and each swab was immersed in transport medium and shaken several times to elute virus from the applicator. The liquid was expressed from the swabs by pressing them on the inside of the transport tubes, and the swabs were discarded. Samples were placed on ice for transport to the laboratory and then frozen at -70°C for subsequent analysis.

Clinical Outcome of Infection

The clinical effect of infection was assessed by examination of the dye-treated left eyes in the animals in each group. By 4 dpi, two of three animals and three of three in the rose bengal- and the lissamine green B-treated groups, respectively, had moderate conjunctivitis and were photophobic. One of three rabbits in each group had geographic corneal lesions at 4 dpi. By 7 dpi, corneal lesions were found in all animals. The geographic lesions observed in the lissamine green B-treated group were larger and accompanied by peripheral dendritic and punctate lesions (Fig. 1). The smaller geographic lesions in the rose bengal-treated group only rarely displayed punctate or dendritic satellite lesions. Beginning at approximately 7 dpi, the geographic lesions in both groups began to decrease in size, and by 16 dpi, all lesions were nearly healed. The eyelids of rabbits in both groups were swollen at 7 dpi, and this

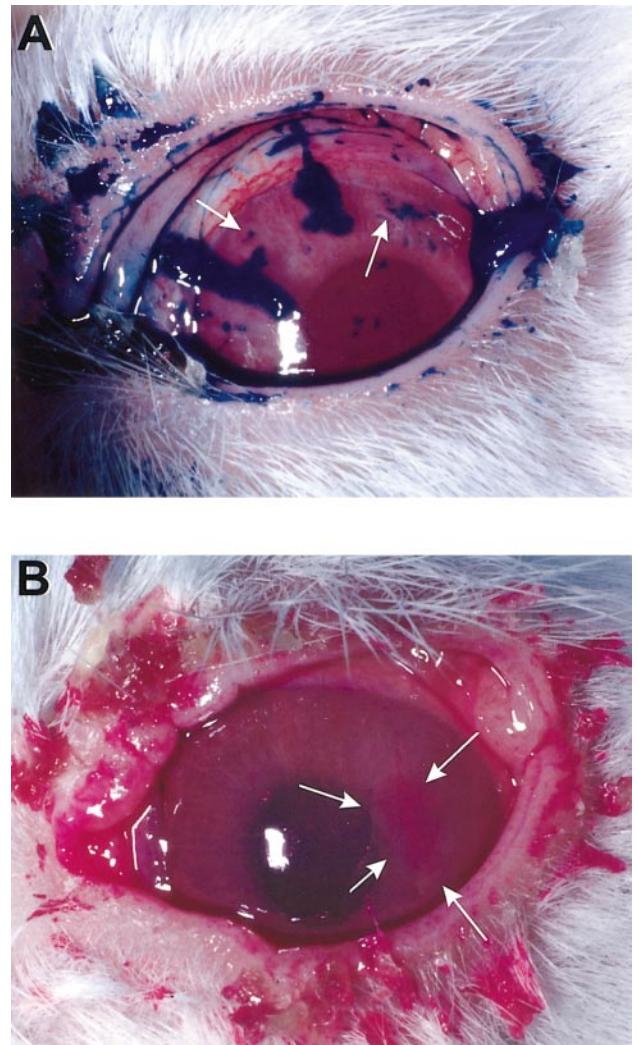


FIGURE 1. Keratitis induced by HSV-1 strain H129 at 7 dpi stained with lissamine green B (A) or rose bengal (B) ophthalmic dyes. Lissamine green B (A) stains the large geographic lesions as well as the punctate and dendritic lesions (arrows). Rose bengal (B) stained the periphery of the geographic lesion shown (bounded by arrows), but only poorly stained the center area. Note that the corneal lesion in the rose bengal-treated eye (B) is smaller and less conspicuous than in the lissamine green B-treated eye (A).

condition continued through 11 dpi but was reduced by 14 to 16 dpi.

Quantitation of Virus in Ocular Secretions

Swabs from both the dye-treated left and nontreated right eyes were separately titered for viral infection. As expected, from 2 through 9 dpi, rose bengal significantly reduced the amount of titratable infectious virus in ocular secretions compared with the non-dye-treated contralateral eyes ($P < 0.001$ on each day from 2 through 9 dpi; Fig. 2A). This correlates with the clinical impression that the lesions in the rose bengal-treated eyes were smaller than those in the lissamine green B-treated group. As expected, lissamine green B did not affect the recovery of titratable virus from ocular secretions (Fig. 2B), and no statistically meaningful difference in the titers recovered from the three lissamine green B-treated left eyes or the three non-dye-treated right eyes was found on any day after infec-

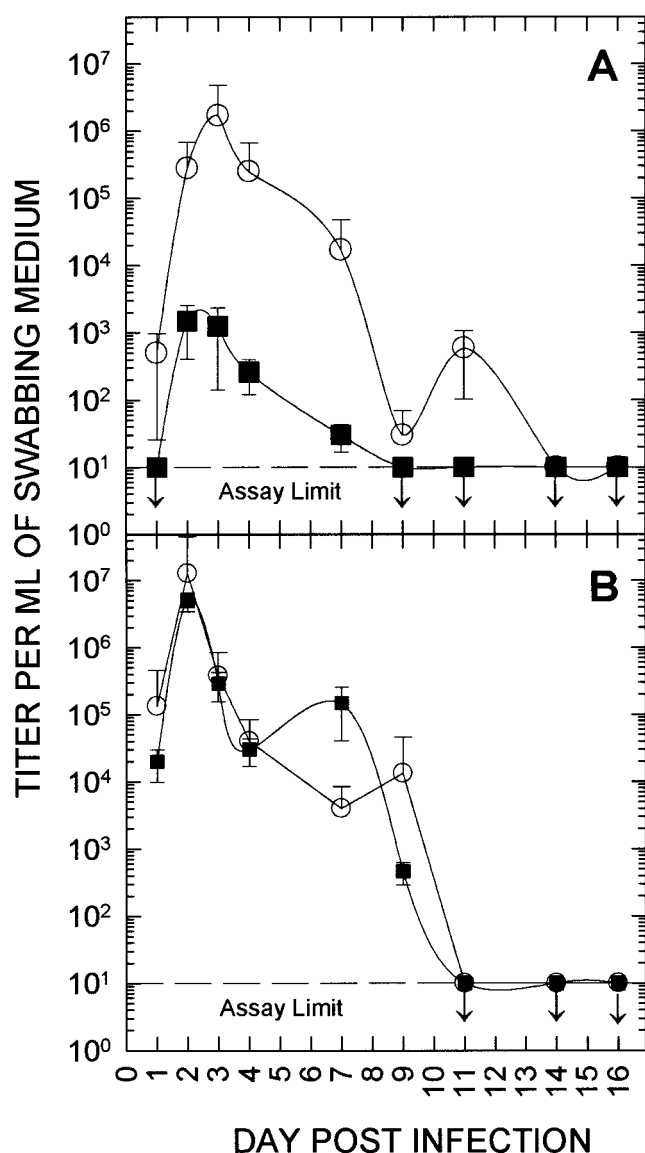


FIGURE 2. Recovery of HSV-1 strain H129 from the ocular secretions of NZW rabbits treated with and without rose bengal (A), or lissamine green B (B). Animals were bilaterally infected at 0 dpi, and on 1, 2, 3, 4, 7, 9, 11, and 16 dpi, 10 μ l of 1% solutions of the dyes were added to the left eyes of three rabbits in each group. The right eyes were not treated. Swabs were taken separately from each eye immediately after instillation of the dyes. The figures show the titer of virus recovered from swabs of the dye-treated left (■) and untreated right eyes (○) at each time point. Error bars: SEM. Downward arrows: titer below the lower limit of the assay (<10 TCID₅₀/ml).

tion. In the non-dye-treated eyes of both groups, the viral titers began to decrease rapidly beginning from 4 through 7 dpi, and by 14 dpi no infectious virus was detectable (Fig. 2). Because infectious virus was detected in rose bengal-treated animals from 1 through 7 dpi, but not at 9 dpi or beyond, we grouped the results from 1 through 7 dpi together for comparative purposes (Tables 1 and 2). Similarly, data from 14 and 16 dpi were grouped together, because all animals in both dye-treated groups were virus-free at these time points. Nine and 11 dpi were accordingly grouped together, because this was a transitional period during which virus titers were decreasing to undetectable levels in both treatment groups.

PCR Analysis of Virus Shedding

PCR was performed on aliquots from both eyes in each group of animals. Extreme care was taken to avoid cross-contamination between dye-treated and nontreated ocular samples, as well as between the rose bengal and lissamine green B groups of animals. In addition, in performing the initial swab of the animals, the cotton-tipped applicator was mixed with the transport medium, the excess liquid expressed from it, and then discarded. This is an important step, because we have found that leaving the swab in the transport medium is detrimental to detection of HSV-1 DNA.

The specific DNA fragment of the 434-bp polymerase gene HSV-1 was amplified from the ocular samples taken from one or more of the three rose bengal-treated eyes on all days sampled (Table 1; Figs. 3A, 3B, 3C). In total, 20 (74%) of 27 rose bengal-treated samples were positive for viral DNA. As expected, most of the swabs were positive for HSV-1 DNA by PCR during the acute phase of virus replication from 1 through 7 dpi, regardless of rose bengal treatment (Tables 1 and 2; Figs. 3A, 3B, 3C). As the infection proceeded, infectious virus was not detectable in any sample taken from rose bengal-treated eyes at 9, 11, 14, or 16 dpi (Tables 1 and 2; Fig. 2A), yet viral DNA was detected in 10 (83%) of 12 of these samples (Table 1). In the non-rose bengal-treated contralateral right eyes, 21 (78%) of 27 samples were positive. At 14 and 16 dpi, when no infectious virus was detected in the samples of either eye in the rose bengal-treated group (Tables 1 and 2; Fig. 2A), viral DNA was detected in two (33%) of six of the infectious virus-negative, nontreated eye swabs and in five (83%) of six of the rose bengal-treated eye swabs (Tables 1 and 2). Although this difference between the rose bengal-treated and nontreated eyes was not significant ($P = 0.24$, Fisher exact test), a trend was apparent in the detection of HSV-1 by culture versus PCR. During the acute phase of infection (1-7 dpi), PCR and culture were approximately equal to each other in sensitivity (Tables 1 and 2). However, at times when virus was no longer detectable by viral culture (14-16 dpi), PCR was able to amplify HSV-1 DNA in approximately one third of the eyes examined (Table 2).

TABLE 1. Summary of HSV-1 Identified in NZW Rabbits by Culture and PCR

	Rose Bengal-Treated		Lissamine Green B-Treated	
	Culture Positive	PCR Positive	Culture Positive	PCR Positive
Days 1-7 ($n = 15$)				
Treated left eyes	12 (80)	10 (67)	15 (100)	14 (93)
Nontreated right eyes	15 (100)	13 (87)	15 (100)	15 (100)
Days 9-11 ($n = 6$)				
Treated left eyes	0 (0)	5 (83)	3 (50)	6 (100)
Nontreated right eyes	6 (100)	6 (100)	1 (17)	3 (50)
Days 14-16 ($n = 6$)				
Treated left eyes	0 (0)	5 (83)	0 (0)	4 (66)
Nontreated right eyes	0 (0)	2 (33)	0 (0)	2 (33)
Totals (Days 1-16; $n = 27$)				
Treated left eyes	12 (44)	20 (74)	18 (67)	24 (89)
Nontreated right eyes	21 (78)	21 (78)	16 (59)	20 (74)

Data are number of samples (percentage of total). Refer to Figures 2 and 3.

TABLE 2. Number of Individual Samples Positive for HSV-1 by Culture and PCR as a Function of Days after Infection

Rabbit	1, 2, 3, 4 and 7 dpi		9 and 11 dpi		14 and 16 dpi	
	Culture	PCR	Culture	PCR	Culture	PCR
Rose bengal-treated left eyes						
368	2/5	1/5	0/2	1/2	0/2	2/2
369	1/5	4/5	0/2	2/2	0/2	2/2
370	4/5	5/5	0/2	2/2	0/2	1/2
Untreated right eyes						
368	5/5	5/5	2/2	2/2	0/2	0/2
369	5/5	4/5	2/2	2/2	0/2	0/2
370	5/5	4/5	2/2	2/2	0/2	2/2
Lissamine green B-treated left eyes						
371	5/5	5/5	1/2	2/2	0/2	1/2
372	5/5	5/5	1/2	2/2	0/2	1/2
373	5/5	4/5	1/2	2/2	0/2	2/2
Untreated right eyes						
371	5/5	5/5	0/2	0/2	0/2	1/2
372	5/5	5/5	1/2	2/2	0/2	0/2
373	5/5	5/5	0/2	1/2	0/2	1/2

Negative isolation refers to the absence of detectable virus at less than 10 TCID₅₀/ml of swabbing medium or the absence of a visible PCR product in ethidium bromide-stained agarose gels of PCR-amplified DNA extracted from 125 μ l of swabbing medium. Data are positive samples/total samples.

In general, the results in the lissamine green B group were similar to those in the rose bengal group. In total, 18 (67%) of 27 and 24 (89%) of 27 of the treated eyes were positive by culture and by PCR, respectively (Figs. 3D, 3E, 3F; Table 1). Similar results were seen in the untreated eyes (Table 1). During the first 7 days of infection, all eyes, both treated and nontreated, were culture positive (Fig. 1, Table 1), and all but one sample was positive by PCR (Fig. 2D, Table 1). From 9 through 11 dpi, viral DNA was detected in six of six of the lissamine green B-treated eyes, but in three of six of the nontreated eyes; however, this was not statistically significant ($P = 0.09$, Fisher exact test). From 14 through 16 dpi, when virus was no longer detectable by culture (Fig. 1; Tables 1 and 2), four of six eyes in the lissamine green B-treated group and two of six eyes in the nontreated group were positive for virus by PCR (Tables 1 and 2). This difference was not significant ($P = 0.342$, Fisher exact test).

DISCUSSION

To our knowledge, this study is the first that demonstrates the time course of HSV-1 detection by culture and by PCR in dye-treated and untreated ocular secretions in an animal model of herpetic keratitis. We show that PCR can be used to detect very low levels of viral DNA in ocular samples that are culture negative. Data from the non-dye-treated eyes show the sensitivity of PCR compared with viral culture for detection of HSV-1. During the early phases of infection (1-7 dpi) when relatively high titers of virus were easily recovered from the non-dye-treated eyes, the majority of the animals were positive by PCR, as one would expect. The recovery of virus by culture was statistically comparable to the detection of HSV-1 DNA by PCR. Similarly, during the period when viral titers were waning (9-11 dpi), infectious virus was recovered from between 50% and 100% of animals (Tables 1 and 2), and between 50% and 100% were positive by PCR. When virus was not present in extraocular secretions (14 and 16 dpi), 33% of eyes were positive by PCR (Tables 1 and 2). Because latency is well established in rabbits infected with HSV-1 strain H129 by

approximately 18 dpi,^{10,11} the HSV-1 DNA detected from 14 through 16 dpi could either represent infectious virus present at a level below the 10 TCID₅₀/ml limit of detection of the titration assay or viral DNA from noninfectious virus. The latter is possible, because the particle-to-infectious unit ratio for herpesviruses is on the order of 50. It should be noted that we have found that by using a hot-start PCR method we are able to increase the detection limit of PCR to 0.02 pfu of HSV-1 (data not shown).

Although lissamine green B binds to membrane-damaged cells, binds to HSV-1-infected cells late in the infectious cycle, and can inhibit HSV-1 plaque formation in vitro,⁶ it does not inhibit HSV-1 replication in vivo (Reference 6 and current results). When the lissamine green B-treated and untreated eyes were compared, nearly the same percentages of eyes were positive for virus by culture and for viral DNA by PCR from 1 through 7 dpi (Table 1). From 9 through 11 dpi, a seemingly higher percentage of eyes in the dye-treated group contained HSV-1 DNA by PCR (100% versus 50%), however this difference was not statistically significant ($P = 0.09$, Fisher exact test), because of the small groups of animals examined, a difference of 67% is required to demonstrate statistical significance. From 14 through 16 dpi, the percentage of samples positive for HSV-1 DNA in treated eyes was higher (66%) than in nontreated eyes (33%) but this difference was also not statistically significant ($P = 0.342$, Fisher exact test). These results show that lissamine green B does not adversely affect virus recovery in cell culture or hamper the detection of HSV-1 DNA by PCR techniques.

It was of interest to compare the untreated eyes in the rose bengal and the lissamine green B groups, because theoretically, the shedding and PCR detection frequencies between these groups should have been equivalent. Although there were numerical differences in the percentages of PCR positive samples between these groups, they were not statistically significant. For example, from 9 through 11 dpi 100% of the untreated eyes in the rose bengal group were positive by PCR, whereas 50% were positive in the lissamine green B group ($P = 0.09$, Fisher exact test). From 14 through 16 dpi, a nearly

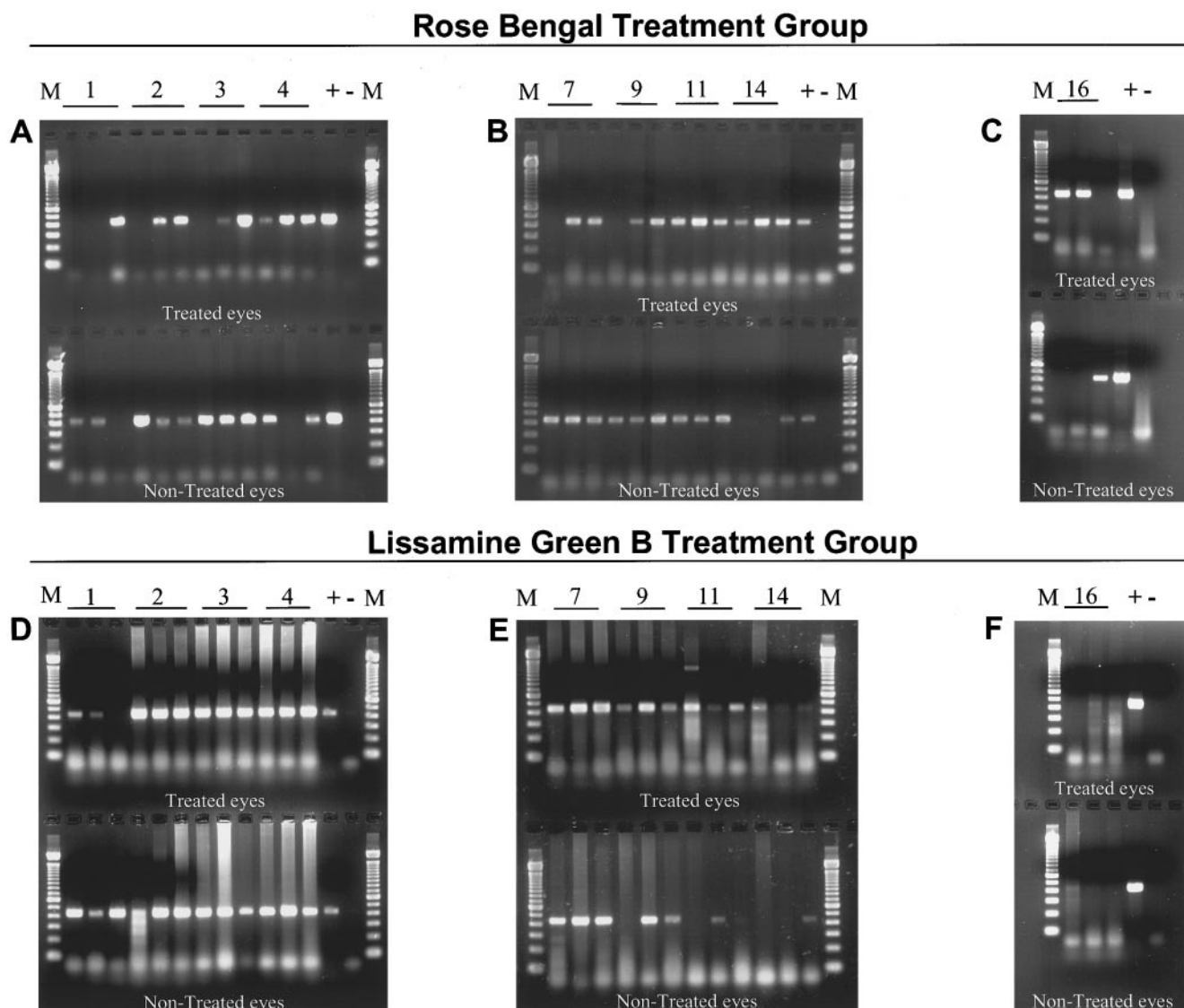


FIGURE 3. Detection of HSV-1 DNA in ocular swabs by PCR. Two groups of three rabbits each were bilaterally infected with HSV-1 strain H129. At the day after infection indicated by the numbers above the lanes of each gel shown, 10 μ l rose bengal (A, B, and C) or lissamine green B (D, E, and F) was instilled in the left eyes. Swabs of both eyes (three dye-treated eyes and three untreated eyes per day) were taken and PCR performed on the DNA extracted from 125 μ l of each sample from each rabbit at each day after infection. Each *lane* under each day represents the DNA amplified from a separate eye. The positive control (+) consisted of purified HSV-1 DNA from HSV-1 strain 17syn + run in parallel. The negative control (-) consisted of water substituted for the DNA. *Lane M*: 1-kb DNA ladder.

equivalent number of eyes untreated with dyes were positive by culture and by PCR in the rose bengal and lissamine green B groups (Tables 1 and 2). These findings suggest that at the late stages of active herpetic keratitis, when the lesions are healing, the likelihood of detecting HSV-1 DNA by PCR is approximately 33% if no dye is used. Furthermore, our data suggest that the likelihood of detecting HSV-1 is greater during the later stages of herpetic keratitis if PCR is used instead of culture.

Treatment of eyes with rose bengal decreased shedding of infectious HSV-1 over time in a manner similar to that in our previous report.⁶ In our previous studies, the maximum titer and the duration of shedding were higher and longer, respectively, than that reported here. However, in our previous study we used 50 μ l rose bengal on each treatment day, and in the present study we used 10 μ l. We used a smaller volume in the present investigation, because we thought less of the dye

would escape from the corneal surface during dye application. However, it is possible that the five-fold lower dose of rose bengal is responsible for the difference between our previous data and the current data.

Treatment of eyes with either dye slightly, but not significantly, enhanced detection of virus by PCR at later times after infection. From 14 through 16 dpi, five (83%) of six of the rose bengal-treated eyes were positive by PCR (Table 1) whereas only 33% of the nontreated eyes were positive (Table 1). Similarly in the lissamine green B group, four (66%) of six of the treated eyes and two (33%) of six of the untreated eyes were positive by PCR (Table 2). Although the percentage of positives was higher in both treatment groups than the percentage of positives in the non-dye-treated eyes, the differences were not significant. We believe that this slight elevation in positivity may have occurred because both dyes produce singlet oxygen in response to light. Singlet oxygen is a reactive

molecule that can indiscriminately attack all neighboring proteins and lipids, including cell membranes. Because specific neutralizing antibodies, other nonspecific antiviral molecules (i.e., interferon), and inflammatory cells probably would all have been present, especially at late times after infection, it is possible that the dyes inactivated these immune system components, which in turn significantly incapacitated clearance of virus from the eye and allowed viral DNA to persist. In other words, the dyes may have interfered with the components needed for efficient elimination of virus and virus-infected cellular debris from the ocular surface. This conclusion is strengthened by the fact that the percentage of PCR-positive samples between the nontreated eyes in the rose bengal and lissamine green B groups were identical (33%). Therefore, the higher rate of HSV-1 DNA detection in the rose bengal-treated eyes was not due to inherent differences between the groups of animals, but was more likely due to a nonspecific effect of the dyes in dampening the ocular immune response through the production of singlet oxygen.

An important aspect of this finding is that PCR is capable of detecting HSV-1 DNA in ocular samples that contain rose bengal. Thus, if the clinician examines the eye and uses rose bengal before viral cultures are taken, it is still possible to use PCR to detect the presence of HSV-1 DNA even though the sample is most likely devoid of infectious viral particles. Use of lissamine green B, however, has the advantage of allowing good visualization of corneal epithelial keratitis and not interfering with either virus culture or PCR. Finally, our results suggest that PCR is a better technique to use for the detection of HSV-1 under conditions in which active virus replication is expected to be low, such as at the later stages of acute keratitis or during recurrent infections.

Acknowledgments

The authors thank Gail C. Wright and James Norman for technical assistance and Kirk Wilhelmus for helpful discussions.

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