# Polymerase chain reaction in the diagnosis of herpetic keratitis: experience in a developing country

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## ABSTRACT • RÉSUMÉ

**Background:** Herpetic ocular disease is a major cause of blindness. Rapid and accurate diagnosis is essential for prompt, proper treatment. We evaluated the usefulness of detection of herpes simplex virus (HSV) DNA by polymerase chain reaction (PCR) in the laboratory diagnosis of herpetic keratitis.

**Methods:** A retrospective study was conducted involving 234 patients who attended the cornea clinic at the Regional Ophthalmic Institute, Chennai, India, between March 1995 and September 1997. Inclusion in the study was based on clinical diagnosis of herpetic keratitis. Oligonucleotide primers directed against the HSV-1 thymidine kinase gene were used, yielding a 110 base pair amplicon. The utility of PCR analysis was assessed against other diagnostic markers: HSV isolation on cell culture, HSV antigen detection by indirect immunofluorescence, detection of anti-HSV IgG by enzyme-linked immunosorbent assay (ELISA) and detection of HSV-specific tear secretory IgA (sIgA) by ELISA. These tests showed overall sensitivity values of 22.4%, 39.8%, 30.4% and 20.3% respectively.

**Results:** In epithelial keratitis all 35 specimens from which virus was cultured were positive by PCR. PCR gave a positive result in 23 (82.1%) of the 28 specimens in which HSV antigen was detected and in 4 (57.1%) of the 7 specimens that showed HSV-specific IgG. In addition, PCR detected HSV DNA in 5 of the 30 cases in which these three tests gave a negative result. PCR of two pooled tear samples (collected 1 week apart from the same patient) from 40 patients with stromal keratitis gave a positive result in 12 cases (30%). In stromal keratitis the sensitivity of PCR in detecting HSV DNA in tear samples was 85.7% with culture, indirect immunofluorescence and detection of anti-HSV IgG as the gold standard, and 80% with detection of sIgA as the gold standard.

**Interpretation: The results confirm the good correlation with the clinical picture that can be obtained with PCR analysis. They also highlight the diagnostic utility of PCR in detecting HSV DNA in tear samples. This is particularly important in herpetic stromal keratitis, in which collection of corneal scrapings is not advised and, hence, conventional techniques such as virus isolation and antigen detection become difficult.**

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**Contexte :** Les maladies oculaires herpétiques constituent une cause importante de cécité. Un diagnostic rapide et exact est essentiel à un traitement prompt et approprié. Nous avons évalué l'utilité de la détection de l'ADN du virus herpès simplex (HSV) par la réaction en chaîne à la polymérase (PCR) dans le diagnostic en laboratoire de la kératite herpétique.

**Méthodes :** Nous avons réalisé une étude rétrospective portant sur 234 patients traités à une clinique de la cornée de l'institut ophtalmique régional de Chennai, en Inde, entre mars 1995 et septembre 1997. L'inclusion dans l'étude reposait sur le diagnostic clinique de la kératite herpétique. Des amorces dirigées contre le gène de la kinase thymidine HSV-1 ont été utilisées pour produire un amplicon de 110 paires de base. L'utilité de l'analyse au moyen de la PCR a été évaluée par rapport à d'autres marqueurs de diagnostic : isolation de l'HSV par culture cellulaire, détection de l'antigène anti-HSV par immunofluorescence indirecte, détection de l'IgG anti-HSV par titrage immuno-adsorbant lié à une enzyme (technique ELISA) et détection de l'IgA sécréteur de larmes (sIgA) spécifique à l'HSV au moyen de la technique ELISA. Ces tests ont montré des valeurs de sensibilité globales de 22,4 %, 39,8 %, 30,4 % et 20,3 % respectivement.

**Résultats :** Dans la kératite épithéliale, tous les spécimens (35) à partir desquels le virus a été cultivé se sont révélés positifs par la PCR. Celle-ci a donné des résultats positifs dans 23 (82,1 %) des 28 spécimens où l'antigène anti-HSV a été décelé et dans 4 (57,1 %) des 7 spécimens qui présentaient un IgG spécifique à l'HSV. De plus, elle a permis de déceler l'ADN de l'HSV dans 5 des 30 cas où ces trois tests ont donné des résultats négatifs. La PCR de deux échantillons de larmes mis en commun (prélevés à une semaine d'intervalle chez le même patient) provenant de 40 patients avec une kératite stromale a donné des résultats positifs dans 12 cas (30,0 %). Dans la kératite stromale, la sensibilité de la PCR pour déceler l'ADN de l'HSV dans les échantillons de larmes a été de 85,7 % avec la culture, l'immunofluorescence indirecte et la détection de l'IgG anti-HSV comme étalon-or et de 80,0 % avec la détection du sIgA comme étalon-or.

**Interprétation : Les résultats confirment que l'analyse au moyen de la PCR permet d'obtenir une bonne corrélation avec le tableau clinique. Ils font aussi ressortir l'utilité diagnostique de la PCR pour déceler l'ADN de l'HSV dans les échantillons de larmes. C'est particulièrement important dans la kératite stromale herpétique, où la collecte de prélèvements cornéens n'est pas conseillée et où les techniques traditionnelles comme l'isolation du virus et la détection de l'antigène deviennent par conséquent difficiles.**

Herpetic ocular disease is a major cause of blindness worldwide.[1] Since a wide range of effective antherpetic drugs are available for the treatment of herpetic keratitis, rapid and accurate diagnosis is essential for proper and prompt therapy. The diagnosis of herpetic keratitis is generally based on the clinical presentation; however, clinical presentation may be an inadequate indicator of the presence of herpes simplex virus (HSV).[1,2] Although virus isolation is considered the "gold standard," its relative insensitivity and technical complexity as well as the delay in diagnosis prevent its routine use.[3] The indirect immunofluorescence assay for the detection of antigen is rapid but requires skill in the interpretation of specific fluorescence.[4,5] The enzyme-linked immunosorbent assay (ELISA) for the detection of HSV-specific secretory IgA (sIgA) in tears, although specific, is less sensitive than antigen detection. We assessed the diagnostic utility of polymerase chain reaction (PCR) analysis in detecting HSV DNA in both epithelial and stromal herpetic keratitis.

## Methods

In a cross-sectional study to analyse the prevalence of herpetic keratitis among patients attending the cornea clinics in south India, 234 patients were enrolled.

The patients attended the cornea clinic at the Regional Ophthalmic Institute, Chennai, India, between March 1995 and September 1997. The patients were examined at the slit-lamp, and the clinical lesions were recorded. Inclusion in the study was based on clinical diagnosis of herpetic keratitis. Characteristic punctate epithelial keratitis, a dendrite or a geographic ulcer indicated corneal involvement. Stromal keratitis included disciform and necrotizing stromal keratitis. In compliance with the Declaration of Helsinki, written consent was obtained from all patients enrolled in the study after the nature of the study and possible outcome were described.

### Specimens

Corneal scrapings and serum and tear samples were collected according to the lesion. Corneal scrapings were collected only from patients who had any epithelial manifestation (161 cases), as we did not want to disturb the intact epithelium. Scrapings were collected with a sterile surgical blade and were transported in viral transport medium (Hanks Balanced Salt Solution, HiMedia, Mumbai, India) with antibiotics and 3% fetal bovine serum (Sigma Chemical Co., St. Louis).

We were able to collect paired serum samples obtained 3 to 4 weeks apart from 56 patients.

Tear samples (two samples obtained 1 week apart and pooled) were collected in a capillary tube after tear production was stimulated with onion vapour or aromatic ammonia.[6] Care was taken not to touch or irritate the eye directly. The tear samples were divided in two. One fraction was mixed with an equal volume of sterile glycerol (Sigma Chemical Co.), and the other was added to 50 μL of buffer containing 20*mM* tris-hydrochloride, 0.5% sodium dodecyl sulfate (Sigma Chemical Co.), 20*mM* ethylenediamine tetraacetic acid (EDTA) (Stratagene GmbH, Heidelberg, Germany) and 100 μg/mL proteinase K (Boehringer Mannheim, GmbH, Mannheim, Germany). Tear samples sufficient for analysis were collected from 138 patients.

All the specimens were transported on ice and processed immediately. When there was a delay in processing, corneal scrapings were stored at −70°C, and serum and tear samples at −20°C.

### Laboratory analyses

Corneal scrapings were cultured for HSV using a vero cell line and were tested for HSV-specific antigen by means of an indirect immunofluorescence assay. We detected anti-HSV IgG in serum samples and sIgA in tear samples using ELISA. The details of these techniques have previously been reported.[7–9] We confirmed the identity of the HSV isolates using a monoclonal-antibody-based direct immunofluorescence kit (MicroTrak HSV-1/HSV-2 culture identification/typing test, Syva Company, San Jose, Calif.).

#### *Polymerase chain reaction*

DNA was isolated from the transport medium containing corneal scrapings by proteinase K digestion (100 μg/mL at 56°C for 2 hours) in proteinase digestion buffer (20*mM* tris-hydrochloride, 0.5% sodium dodecyl sulfate and 20*mM* EDTA).[10] The samples were extracted twice with phenol–chloroform, and DNA was precipitated with ethanol and 3*M* sodium acetate. The PCR assay was carried out as described by Laycock and colleagues[11] using a primer pair, which brackets 110 base pair segment of HSV-1 thymidine kinase gene. The primer sequences were p1: 5′-ATA CCG ACG ATA TGC GAC CT-3′, and p2: 5′-TTA TTG CCG TCA TAG CGC GG-3′. Amplification was done in a reaction mixture containing 10× Taq polymerase buffer, deoxyribonucleotide triphosphates (both 200μ*M*), 1μ*M* p1 and 1μ*M* p2, in a total volume of 50 μL. We used 2.5 U of Taq DNA polymerase for each reaction (reagents obtained from Amersham Life Sciences, Amersham, Buckinghamshire, UK). The thermal profile was 94°C for 2.5 minutes, 56°C for 2 minutes and 72°C for 3 minutes. The cycle was repeated 40 times in an automated thermal cycler (PTC-100, MJ Research Inc., Watertown, Mass.), and the amplification products were analysed by electrophoresis in 4% agarose gel.

The detection limit of the PCR assay, as determined by serial dilutions of titrated HSV-1 strain KOS, was 1 plaque-forming unit/mL. We determined the specificity by including DNA samples isolated from HSV-2 standard strain (strain 753167, National Institute of Virology, Pune, India) and varicella–zoster clinical strain (gift from Dr. Vasanthapuram Ravi, National Institute for Mental Health and Neurosciences, Bangalore, India) as a control group.

We compared the results with those of the conventional diagnostic tests and computed their sensitivity, specificity, and positive and negative predictive values.

## Results

Of the 234 patients 153 had epithelial keratitis and

**Table 1—Number of specimens with a positive result of polymerase chain reaction (PCR) testing for herpes simplex virus (HSV) DNA obtained from 234 patients with herpetic keratitis**

| | Laboratory test; no. of specimens | | | | | |
|---|---|---|---|---|---|---|
| | | | | | Polymerase chain reaction | |
| Description of keratitis | Cell culture (n = 161) | Indirect immuno-fluorescence (n = 161) | HSV-specific IgG (n = 56) | HSV-specific secretory IgA (n = 138) | Corneal scrapings (n = 93) | Tears (n = 40) |
| Dendritic (n = 104) | 30/104 | 51/104 | 8/26 | 10/56 | 52/56 | – |
| Geographic (n = 26) | 4/26 | 3/26 | 3/10 | 5/21 | 6/13 | – |
| Epithelial punctate (n = 23) | 1/23 | 7/23 | 2/5 | 4/13 | 2/17 | – |
| Disciform (n = 66) | 0/1 | 0/1 | 2/9 | 4/36 | – | 6/30 |
| Necrotizing stromal (n = 15) | 1/7 | 3/7 | 2/6 | 5/12 | 2/7 | 6/10 |
| Positivity, % | 22.4 | 39.8 | 30.4 | 20.3 | 66.7 | 30.0 |

81 had stromal infiltration. Of the 153 patients with epithelial keratitis 104 had a dendritic ulcer, 26 had a geographic ulcer, and 23 had punctate epithelial keratitis. Disciform and necrotizing stromal keratitis were seen in 66 and 15 patients respectively. Eight (9.9%) of the patients with stromal keratitis had epithelial manifestations, such as ruptured bullae.

### *HSV isolation and antigen detection*

HSV was isolated by cell culture from 36 (22.4%) of the 161 corneal scraping specimens. HSV-specific antigen was detected by indirect immunofluorescence in 28 additional cases with a negative result of culture. Antigen was not detected in one case of geographic keratitis in which HSV was isolated. Of the 64 cases in which HSV was isolated or HSV-specific antigen was detected, or both, 61 (95.3%) were from patients with epithelial keratitis. Serotyping of the HSV isolates showed 35 to be HSV-1 and 1 to be HSV-2.

### *Serum anti-HSV IgG*

A rise in the titre of HSV-specific IgG was seen in 17 (30.4%) of the 56 paired serum samples. Of the 17, 10 had a positive result of cell culture or indirect immunofluorescence, or both, and 7 had a negative result of these tests.

### *HSV-specific secretory IgA*

HSV-specific sIgA was detected in 28 (20.3%) of the 138 tear samples. Of the 28 patients 19 had epithelial keratitis (dendritic, geographic or epithelial punctate), and 9 had stromal keratitis. In 16 of the 19 cases of epithelial keratitis, HSV was isolated or HSV-specific antigen was detected, or both. Corneal scrapings were not obtained from the patients with stromal keratitis; however, a paired serum sample was available in four cases, and testing for anti-HSV IgG showed a rise in titre in all four cases. HSV-specific sIgA was detected in eight cases (five stromal and three epithelial) in which there was no other evidence of infection.

### *Polymerase chain reaction*

PCR analysis was done on corneal scraping specimens with positive results of cell culture for HSV-1 (35 cases), indirect immunofluorescence (28 cases) and testing for anti-HSV IgG (7 cases). PCR was also done on 30 corneal scrapings with negative results of these tests to determine the utility of the assay in epithelial keratitis. PCR gave a positive result for all 35 corneal scrapings from which HSV was isolated, for 23 of the 28 specimens in which anti-HSV antigen was detected, and for 4 of the 7 specimens with a rise in the titre of anti-HSV IgG. Of the 30 cases in which a negative result was obtained with these three tests, 5 had a positive result of PCR. The specificity of PCR in detecting only HSV-1 DNA was shown by the negative result obtained for the control group. The number of specimens analysed in the various clinical categories and the positivity obtained in each are shown in Table 1.

**Table 2—Correlation analysis of PCR testing for HSV DNA on corneal scrapings against conventional techniques (alone and in combination)**

| | Measure (and 95% confidence interval), % | | | |
|---|---|---|---|---|
| "Gold standard" | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
| Culture alone (n = 35) | 100 (87.7–100) | 50.8 (38.2–63.3) | 52.2 (39.8–64.4) | 100 (87.0–100) |
| Indirect immunofluorescence alone (n = 28) | 82.1 (62.4–93.2) | 38.9 (27.8–51.1) | 34.3 (23.4–47.0) | 84.8 (67.3–94.3) |
| Culture and indirect immunofluorescence (n = 63) | 92.1 (81.7–97.0) | 75.7 (58.4–87.6) | 86.6 (75.5–93.3) | 84.8 (67.3–94.3) |
| Culture, indirect immunofluorescence and HSV-specific IgG (n = 70) | 88.6 (78.2–94.6) | 83.3 (64.5–93.7) | 92.5 (82.7–97.2) | 75.8 (57.4–88.3) |

The correlation of PCR positivity in epithelial keratitis against the other markers is shown in Table 2.

Of the 40 tear samples from patients with stromal keratitis (30 disciform and 10 necrotizing), 4 were positive for HSV-specific IgG and HSV-specific sIgA, 1 was positive for HSV-specific sIgA alone, 3 had a positive result of culture and indirect immunofluorescence, and 32 were negative for all these markers. PCR analysis gave a positive result in 3, 1, 2 and 6 of these cases respectively. The correlation of PCR positivity for tear samples from patients with stromal keratitis against the other markers is shown in Table 3. The additional sensitivity of PCR testing of tear samples compared with culture, indirect immunofluorescence and testing for HSV-specific IgG and with testing for HSV-specific sIgA was statistically significant ($p < 0.05$, McNemar's test).

## Interpretation

Clinically, dendritic or geographic ulcers are generally sufficient to make a diagnosis of herpetic keratitis. However, the atypical corneal presentations of herpetic keratitis may be confused with other diseases. Recently, Koizumi and associates[12] showed that 8 of 20 atypical corneal presentations that could not be identified by a panel of ophthalmologists were shown by PCR analysis to be due to HSV. A clear-cut diagnosis is needed to avoid unnecessary treatment with antibiotics or the hazards of steroid therapy in epithe-

**Table 3—Correlation analysis of PCR testing for HSV DNA on tear samples against cell culture, indirect immunofluorescence and detection of HSV-specific IgG, and detection of HSV-specific secretory IgA**

| | Measure (and 95% confidence interval), % | | | |
|---|---|---|---|---|
| Gold standard | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
| Culture, indirect immunofluorescence and HSV-specific IgG (n = 7) | 85.7 (42.0–99.2) | 78.8 (60.6–90.4) | 46.2 (20.4–73.9) | 96.3 (79.1–99.8) |
| HSV-specific secretory IgA (n = 5) | 80.0 (29.9–98.9) | 74.3 (56.4–86.9) | 30.8 (10.4–61.1) | 96.3 (79.1–99.8) |

lial keratitis.[13,14] Correlation of the clinical presentation with virologic confirmation is thus essential for proper patient management. In an earlier study we found that with conventional techniques such as culture, indirect immunofluorescence and testing for sIgA, only 44.4% of cases with epithelial manifestations and 14.8% of cases with only stromal disease could be linked to HSV.[15] Hence, we adopted PCR analysis to increase the power of diagnosis.

The initial results regarding the utility of PCR in ocular HSV infections were contradictory. Wu and coworkers[16] reported that the technique gave a positive result in five of seven cases of suspected herpetic keratitis and a negative result in nine of ten healthy corneas. In contrast, Kowalski and colleagues[17] concluded that PCR has lower sensitivity and specificity than culture. These reports were followed by a series of publications, most of which showed the value of PCR with various ocular specimens in ocular HSV infections.[18–20]

The diagnosis of herpetic stromal keratitis, particularly in patients with no previous history of epithelial manifestations, is important.[21] The differential diagnosis of HSV stromal disease is extensive as it includes other forms of microbial keratitis, such as varicella–zoster, Epstein–Barr virus, *Acanthamoeba* and syphilitic keratitis, as well as nonmicrobial conditions, such as Cogan's syndrome and keratitis secondary to connective tissue disorders.[22] As collection of corneal scrapings is not advised in some cases (to avoid disturbing the intact epithelium), virus isolation or antigen detection becomes difficult, and newer diagnostic markers must be considered. In a South American study of the utility of PCR in herpetic keratitis, the technique detected 3 of 15 cases of stromal keratitis due to HSV (all 15 culture-negative), and culture and PCR were found to be equally sensitive (77%) in cases of epithelial keratitis.[23] Kudo and associates[24] reported that PCR gave a positive result in 33.3% of cases of stromal keratitis with tear samples collected at the active and the quiescent phase. They suggested that repeated tear samples should be collected from the same patient regardless of the phase of activity of the condition. They also found that the average number of tear samples needed to detect the HSV genome was 3.3. In our analysis we pooled two samples collected 1 week apart from the same patient. In a developing country like India, specimen collection more than twice from the same patient is difficult. The possibility that PCR can provide a satisfactory result with a pooled sample is a great advantage under these conditions.

In our study the correlation analysis of PCR against culture as the gold standard showed 100% sensitivity but only 50.8% specificity owing to the additional PCR positivity in culture-negative cases. For statistical purposes these were considered false-positive results. Animal models have clearly shown that HSV DNA persists longer in the corneal tissues after infection than does virus or antigen.[25,26] Thus, culture recovery of virus and antigen detection have a narrower diagnostic window than detection of DNA. In view of the limited value of other laboratory markers and the value of PCR in the diagnosis of herpetic stromal keratitis, PCR should be considered an emerging gold standard for the laboratory diagnosis of keratitis due to HSV.

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