

Note

Diagnosis of Herpetic Keratoconjunctivitis by Nested Polymerase Chain Reaction in Human Tear Film

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Abstract A study was performed to evaluate nested PCR (nPCR) versus viral cultures as method and tear film versus corneal scrapings as specimen in the diagnosis of viral keratoconjunctivitis. Tear film specimens were taken from both eyes and corneal scrapings from the affected eye only in 17 patients with suspected viral keratoconjunctivitis. In 15 of the 17 patients the viral agent of the infection could be detected: 11 patients had herpes simplex virus type 1, two varicella-zoster virus, one both herpes simplex virus type 1 and varicella-zoster virus, and one adenovirus. Overall there was no significant difference between the detection rate for corneal scrapings (85%) and tear film (75%). In both types of specimens nPCR showed a higher detection rate than viral cultures (corneal scrapings: 87.5% vs 31.25%; tear film: 75% vs 12.5%; $P < 0.05$). For the diagnosis of keratoconjunctivitis nPCR is superior to viral culture and tear film is an adequate sample that is easier to collect, causing the patient less discomfort.

Key words Keratoconjunctivitis · Polymerase chain reaction · Viral culture · Human tear film · Corneal scrapings

Introduction

Herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) are aetiological agents of herpetic keratoconjunctivitis and a cause of corneal blindness in all parts of the world. For correct therapy rapid diagnosis is very important. Herpetic keratoconjunctivitis infections are usually diagnosed on the basis of their typical presentation, but in the case of an atypical ocular presentation a laboratory diagnosis is usually necessary and corneal scrapings (CS) are required as clinical specimens. The methods commonly used for diagnosis (di-

rect immunofluorescence and viral culture) show a low detection rate [1, 2]. In this study we evaluated the nested polymerase chain reaction (nPCR) as method and human tear film (TF) as clinical specimen in the diagnosis of viral keratoconjunctivitis.

Patients and Methods

Tear film from both eyes and corneal scrapings from the affected eye in each of 17 patients with clinical findings suggestive of herpetic keratoconjunctivitis (14 with dendritic keratitis, 2 with superficial punctate keratitis and 1 with marginal keratitis) were investigated by means of viral culture and nPCR techniques to detect HSV-1 and VZV. To obtain a TF specimen, a swab was placed on the lower temporal eyelid margin of each eye for one second or less. Each swab was then placed in a vial containing 2 ml of viral transport medium (Difco, USA). Corneal scrapings were also collected in 2 ml of viral transport medium. The samples were immediately sent to the laboratory, stored at 4°C and processed within the next 24 h. All swabs were mixed by vortexing in the viral transport medium; half of each sample obtained was stored at 4°C until processing for viral culture and the other half was kept frozen at -40°C until the nPCRs were performed.

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To perform the viral culture the specimens were first decontaminated (penicillin 200 U/ml, streptomycin 200 µg/ml, amphotericin B 200 µg/ml); 200 µl were then inoculated into a tube with the human embryonic fibroblast cell line MRC-5 (Vircell-Ingelheim Diagnostic, Spain) and allowed to adsorb for 1 h at 37°C in 5% CO₂. The sample suspension was then replaced by inoculation medium (Minimum Essential Medium with Earle's balanced salt solution; Biowhittaker, Belgium). The cultures were incubated for 15 days at 37°C in a 5% CO₂ atmosphere and observed periodically (twice a week) during this time. Positive cultures were identified using monoclonal antibody.

The samples for nPCR were thawed, homogenized and pelleted by centrifugation at 1800 × g for 5 min. Then 100 µl of lysis solution (10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.5% Nonidet P-40, 0.5% Tween 20 and 1 µg of proteinase K) was added to 25 µl of pellet, mixed and incubated for 45 min at 56°C, followed by 10 min at 96°C. The amount of DNA in the samples was determined using a spectrophotometer (Biotech LKB-Ultrospec III, Pharmacia, Sweden) and adjusted to a final concentration of 0.1 µg/µl. Nested PCR assay was performed as previously described by Aurelius et al. [3] and Duglosch et al. [4] with slight modifications in the annealing temperature and the preparation of reaction mixture (these modifications are described below). The oligonucleotides used in the HSV-1 nPCR assay amplify a 138 bp fragment belonging to the glycoprotein D encoding-gene (Table 1). The oligonucleotides used in the VZV nPCR assay amplify a 326 bp fragment belonging to the immediate-early gene 63 (Table 1). The reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 1 U recombinant *Taq* DNA polymerase (Gibco BRL, UK), 125 µM of each deoxynucleoside triphosphate, and 0.25 µM of each of the outer oligonucleotides for HSV-1 or VZV, was both freshly prepared and batch-prepared. Forty microlitres of the batch-prepared mixture were distributed into 500 µl reaction-tubes, overlaid with 30 µl of mineral oil, stored at -20°C and used up to 90 days after preparation. Aliquot tubes were thawed immediately before the reaction was to be carried out and 1 µg DNA was added. All samples were amplified in parallel with both the batch-prepared mixture and the freshly prepared mixture. The first round of amplification for both HSV-1 and VZV comprised twenty-two cycles; for HSV-1 each cycle consisted of denaturation at 95°C for 30 sec, anneal-

ing at 58°C for 30 sec and elongation at 72°C for 1 min, followed by an additional final extension step of 10 min at 72°C; for VZV each cycle consisted of denaturation at 94°C for 60 sec, annealing at 44°C for 72 sec and elongation at 70°C for 150 sec, followed by an additional final extension step of 10 min at 70°C. After the first round of amplification, 1U of recombinant *Taq* DNA polymerase (Gibco BRL) and 25 µM of each of the inner oligonucleotides for HSV-1 or VZV, were added to the same tube and a second round of amplification was performed. For both nPCRs, this second round comprised 35 cycles; in the case of HSV-1 each cycle consisted of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec and elongation at 72°C for 1 min, followed by a final extension step at 72°C for an additional 10 min; in the case of VZV a two-step procedure each cycle consisting of denaturation at 94°C for 60 sec annealing and extension at 72°C for 96 sec, followed by a final extension step at 72°C for 10 min. Ten microlitres of the second round nPCR products were subjected to electrophoresis on 3% agarose (NuSieve GTG; FMC Bioproducts, USA), containing ethidium bromide and were then examined under ultraviolet (UV) light. In order to avoid contamination, the usual measures were adopted [5]. One positive control and two negative controls (DNA from HSV-1 or VZV-infected and uninfected MRC-5 cells) were used for each nPCR assay. All nPCR products were stored at 4°C until hybridization. For hybridization, five microlitres of the nPCR products were denatured by treatment with 200 µl denaturation buffer (NaOH 0.4 N, EDTA 10 mM) at room temperature for 15 min. Each sample was then applied to nylon membrane (Bio-Rad, Zeta-Probe, USA). The filter was subsequently washed in 2 × SSC and fixed with UV light. On the day of the hybridization assay the filter was incubated in prehybridization buffer [2% sodium dodecyl sulfate (SDS), 2 × PBS, 5 × Denhardt's solution (DS), 1 mM EDTA, 40 µg salmon sperm DNA per ml] at 65°C for 30 min and was hybridized for 60 min at 59°C for HSV-1 or 57°C for VZV in hybridization buffer (2% SDS, 2 × PBS, 5 × DS, 1 mM EDTA, probe 4 µM) with the HSV-1 probe [3] or VZV probe [4] (Table 1), 5'-labelled with [γ -³²P]ATP and T4 polynucleotide kinase. The filter was subsequently washed in 2 × PBS, 1 mM EDTA at room temperature for 10 min, then twice in 2 × PBS, 1 mM EDTA, 1% SDS at hybridization temperature for 10 min and once more in PBS at room temperature for 5 min. Finally, the filter was autoradiographed (Kodak XRP) for 24 h at -70°C.

Table 1 Oligonucleotides sequences of herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV)

HSV-1
Outer primers:
BJHSV1.1.-5' ATCACGGTAGCCCGGCCGTGTGACA 3' (19-43)
BJHSV1.2.-5' CATACCGGAACGCACCACACAA 3' (239-218)
Inner primers:
BJHSV1.3.-5' CCAACCGACCACACCGACGA 3' (51-71)
BJHSV1.4.-5' GGTAGTTGGTTCGTCGCTGAA 3' (188-166)
Probe:
BJHSV1.-5' TACGAGGAGGAGGGGTATAA-CAAAGTCTGT 3' (96-125)
VZV
Outer primers:
VZV.1.-5' GTTTTGTACTCCGGGTTG 3' (721-738)
VZV.2.-5' TTACATCCGATGGCGTAG 3' (106-123)
Inner primers:
VZV.3.-5' GCTCGTTGAGGACATCAACCGTGTT 3' (757-781)
VZV.4.-5' CATCGTCGCTATCGTCTTCACCAC 3' (82-105)
Probe:
VZV.-5' AGACGCAGTGCTTACGCGCTACTT 3' (930-953)

Results and Discussion

An aetiological diagnosis could be established in 15 (88.23%) of the 17 patients studied. Eleven patients with a clinical diagnosis of dendritic keratitis were diagnosed as having HSV-1 infections (5 by culture and 11 by nPCR). Of three patients with a clinical dendritic keratitis two were diagnosed as having a VZV infection (diagnosis by PCR only) and one was diagnosed as having a mixed infection with HSV-1 and VZV (diagnosis by PCR only) (Table 2). One patient with a clinical diagnosis of superficial punctate keratitis was diagnosed by culture as having an adenovirus infection. In two patients, a laboratory diagnosis could not be made; one had a clinical diagnosis of marginal keratitis and the other a clinical diagnosis of superficial punctate keratitis.

Two methods for detecting HSV-1 and VZV were compared in this study, namely viral culture and nPCR. Using CS, viral culture to detect HSV-1 and/or VZV was positive in five cases, which represents a detection rate of 31.25%, whereas the detection rate rose to 87.5%

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