

Detection of Varicella-Zoster Virus (VZV) DNA in Clinical Samples from Patients with VZV by the Polymerase Chain Reaction

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A polymerase chain reaction system for the detection of varicella-zoster virus was established. Of 25 nucleotides, 4 oligonucleotide pairs (regions of thymidine kinase, thymidylate synthetase, glycoprotein I, and immediate early gene) were synthesized. The first three oligonucleotide pairs could be used as primers on the basis of specific DNA amplification. Varicella-zoster virus DNA was amplified by this polymerase chain reaction system in 20 of 20 vesicle samples, 5 of 6 crusts, and 12 of 13 throat swabs collected from patients with clinical varicella.

Varicella-zoster virus (VZV) is a human herpesvirus that causes chicken pox (varicella) and shingles (zoster). VZV is highly infectious and causes significant morbidity in immunosuppressed patients. The cell-associated nature of VZV has hampered studies of it. On primary infection with VZV, viremia has been demonstrated before and after the onset of disease (1, 8). The isolation of VZV from the pharynx is usually difficult and has seldom been achieved (9).

The polymerase chain reaction (PCR) is a novel technique that amplifies a specific sequence of DNA efficiently. This technique has been used to define HLA polymorphism, the T-cell receptor, and immunoglobulin diversity for the detections of pathogens, lymphomas, and leukemias and for the quantification of lymphokines (2, 4, 6). In this study, we established a system of PCR for VZV and used it to detect VZV DNA in clinical specimens.

MATERIALS AND METHODS

Cells and viruses. The Kawaguchi and Oka strains of VZV, the AD 169 strain of human cytomegalovirus, and the Seibert and UW268 strains of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) were used. Human embryonic fibroblast cells, which had been passaged 10 to 15 times, were cultured in a mixture of Eagle minimal essential medium and medium 199 containing 10% fetal calf serum for growth and 3% fetal calf serum for maintenance. Human embryonic fibroblast cells were infected with each virus and cultured for certain periods. When infected cells showed cytopathic effect, they were harvested and their DNA was extracted as described below. A cell line producing Epstein-Barr virus (the B95-8 cell line) was also propagated, and its DNA was extracted in the same fashion. The DNA of human herpesvirus 6 (HHV-6) was extracted from umbilical cord blood mononuclear cells infected with the HST strain, which was isolated from a patient with exanthem subitum.

Collection of specimens from patients and purification of DNA. Vesicles of 18 varicella patients (ages, 4 months to 6 years), throat swabs of 6 varicella patients (ages, 1 to 5

years) and crusts of 6 other varicella patients (ages, 4 months to 8 years) were collected. DNA was prepared from each sample as follows: the sample was centrifuged at $200,000 \times g$ for 2 h, and the pellet was incubated for 6 h at 37°C in 500 μ l of NET buffer (150 mM NaCl, 15 mM Tris hydrochloride, 1 mM EDTA) with 0.1% sodium dodecyl sulfate (SDS) and 1.0 mg of proteinase K (Boehringer Mannheim Biochemicals) per ml. Then the preparation was mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). This procedure was repeated five times. The DNA was precipitated by adding ethanol, washed with 80% ethanol, and suspended in distilled water, and its concentration was determined by measuring the ratio of A_{260} to A_{280} . Samples of about 100 ng of DNA were used for the PCR.

Oligomer synthesis. The sequences used as primers were determined from the results of Davison and Scott (3) and are shown in Table 1. The sequences of the eight oligomers, which were regions of thymidine kinase (TK), thymidylate synthetase (TS), glycoprotein I (gpI) and the immediate early (IE) gene, were synthesized in a DNA synthesizer (Applied Biosystems).

PCR. DNA samples were first heated at 94°C for 10 min to denature the DNA and then subjected to 30 amplification cycles. DNA was amplified in a total volume of 100 μ l of reaction mixture consisting of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μ M each dATP, dGTP, dTTP, and dCTP, and 2.5 U of *Taq* polymerase (Perkin-Elmer/Cetus). Annealing was performed at 60°C for 2 min, extension was performed at 72°C for 5 min, and denaturation was performed at 90°C for 1 min. The primers were used at 1.0 μ M each. The amplification reaction was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus). The sample which contains all reagents except sample DNA was reacted in this system as a negative control in every experiment.

Detection of amplified product. The amplified product was detected by direct gel analysis and by Southern blot hybridization assay with cloned DNA probe (gpI) which was included in the sequences of each DNA region (5). Bacteriophage lambda DNA was digested with *Eco*T14I and used as the DNA molecular markers.

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TABLE 1. Primer pairs for DNA amplification by PCR

Gene and primer pair	Sequence (5'-3')	Length (bp) of amplified product	Location of VZV DNA (nt) ^a
Gene 13			
TS1	ACGATTATTACCGGTACCATGGGAG	959	18422-18446 19358-19382
TS2	CAACACATCTACTGTCTTGACAACA		
Gene 36			
TK1	ATGTCAACGGATAAAACCGATGTA	1,024	64807-64831 65805-65829
TK2	AGGAAGTGTGTCTGCAACGGCATT		
Gene 62			
IE1	GTCTCTTGTGCGAAACCTCGACCTG	496	105447-105471 106018-106042
IE2	GTGTGCCACATACGCTGCAAGGGTG		
Gene 68			
gpl-1	CCGTATATGAGCCTTACTACCATTTC	651	115953-115977 116605-116629
gpl-2	GAGTTCATCAAACAGTGTGCTCGTG		

^a nt, Nucleotides.

For direct gel analysis, 10 μ l of reaction mixture was subjected to electrophoresis on an 0.8% agarose gel and DNA was located as UV fluorescence after staining with ethidium bromide. Molecular weight markers were included in each gel. A band was seen when samples were amplified. For Southern blot analysis, DNA was separated by electrophoresis and transferred to a nylon membrane filter (Hybond N⁺; Amersham) after treatment with 0.4 N NaOH for 4 h (alkali blotting and fixation). Then the filter was neutralized with 2 \times SSPE (0.3 M NaCl, 20 mM NaH₂PO₄ [pH 7.4], 2 mM disodium EDTA) for a few minutes. The DNA samples were then hybridized for 8 h with a homologous ³²P-labeled cloned probe (2.0 \times 10⁶ cpm/ml) in hybridization fluid (6 \times SSPE, 3% skim milk [Difco Laboratories], 1% SDS). The filter was then washed twice with 2 \times SSPE-0.1% SDS for 10 min each time at room temperature, once with 1 \times SSPE-0.1% SDS for 15 min at 65°C, and then twice with 0.1 \times SSPE-0.1% SDS for 20 min each time at 65°C. Bound probe was detected by autoradiography at -70°C for 8 h with intensifying screens.

RESULTS

Amplification of two VZV strains by using four pairs of primers. The abilities of our primers to detect two different clinical isolates were evaluated. Samples of approximately 100 ng of DNA from infected cells were used as templates in the PCR. Both strains gave a positive band on direct gel electrophoresis with three pairs of primers (TK, TS, and gpl) (Fig. 1, lanes 2 to 4 and 6 to 8). The molecular weights of the amplified DNAs were as expected from the DNA sequences. When primers for the IE region were used in this system, two faint bands were detected (lanes 1 and 5), but the sizes of these amplified DNAs did not correspond to the molecular weights of sequences of the DNA. Therefore, only DNAs from these the first three pairs of primers were used in subsequent experiments.

Specificity and sensitivity of the PCR. The specificity of the PCR and the primers for VZV were evaluated by using five other human herpesviruses (HSV-1, HSV-2, HHV-6, human cytomegalovirus, and Epstein-Barr virus). DNAs from cells infected with these viruses were extracted and purified as described above, and samples of 0.01 to 1 μ g of DNA were used as templates in the PCR. No amplification was detected by direct gel electrophoresis or by Southern blot hybridiza-

tion (data not shown). Because samples might be contaminated with inhibitors, DNA samples of approximately 1 μ g containing 10 ng of phage DNA were used as templates and the PCR was performed with a Gene Amp DNA Amplification Kit (Perkin-Elmer/Cetus) according to the manufacturer's instructions. Positive bands were observed on direct gel electrophoresis (data not shown). This finding suggested that this PCR system amplifies VZV DNA specifically.

For determination of the sensitivity of the PCR in this system, a sample of cloned VZV DNA (gpl region) was mixed with human placental DNA and this DNA was amplified with primers of gpl. VZV DNA fragments with the same molecular weight as the expected DNA could be detected in an agarose gel by ethidium bromide staining at a level of 5 \times 10³ copies, and 50 copies of VZV DNA could be detected by Southern blot analysis (Fig. 2).

Detection of VZV DNA in vesicle, crust, and throat swab clinical specimens from patients. Twenty vesicles were collected from 18 patients diagnosed clinically as having chicken pox, and VZV DNA in these samples was examined by PCR. VZV DNA amplified by PCR was detected by ethidium bromide staining on an agarose gel in all vesicle samples (Fig. 3). Furthermore, all samples were amplified by

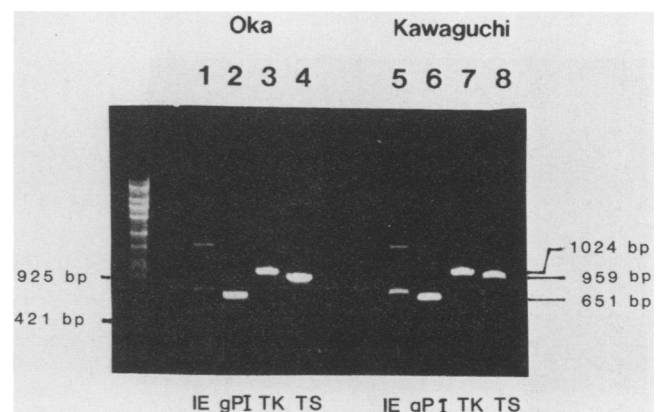


FIG 1. Separation on agarose gel of PCR products of samples containing VZV DNA extracted from cells infected with the Kawaguchi and Oka strains by using four different primer pairs: IE, gpl, TK, and TS.

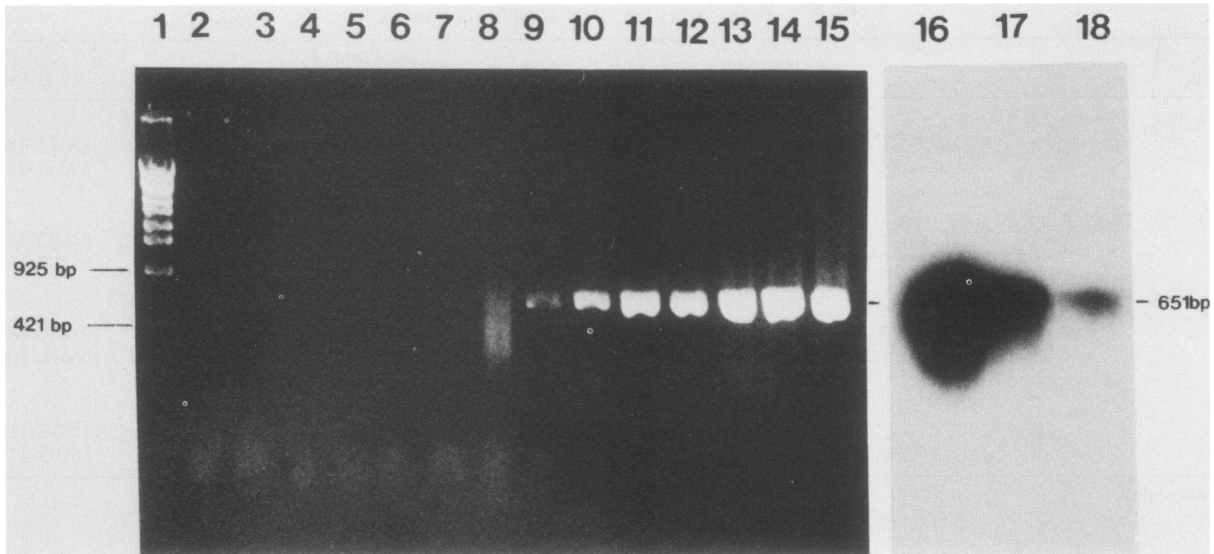


FIG. 2. Sensitivity of the PCR system determined by gpI primer. Lanes: 1, molecular size markers; 2 to 15, 10-fold-diluted cloned DNA (lane 15 contains 30 ng of cloned DNA [one copy is equal to about 10^{-5} pg] and lane 9 contains 3×10^{-2} pg of cloned DNA); 16 to 18, autoradiograph after hybridization with ^{32}P -labeled probe (lanes 16, 17, and 18 correspond to lanes 9, 8, and 7, respectively, on direct gel electrophoresis).

PCR with the three different primer pairs. In addition, six samples of crusts were obtained from the patients during the convalescent phase, and DNAs were extracted from these samples. On direct gel electrophoresis, three specimens gave a positive band but the other samples did not. On Southern blot analysis, VZV DNA was detected in two samples but no VZV DNA was detected in the sample from one patient (data not shown). Finally, we tried to detect VZV DNA in clinical specimens (throat swabs) from varicella patients. VZV DNA was detected in 3 of 13 throat swab samples from six patients with varicella by ethidium bromide staining of an agarose gel, and VZV DNA was detected by Southern blot analysis in 9 of the 10 samples in which it was not detected by direct gel electrophoresis (Fig. 4). DNAs from throat swab specimens of 16 healthy children could not be amplified (data not shown).

DISCUSSION

In this work, we first evaluated the abilities of four paired primers which determined the TK, TS, gpI, and IE genes to amplify VZV DNA. In our PCR system, three of these primers amplified DNA with the exact molecular weight of the DNA probes (Fig. 1). However, when the primers related to the IE gene were used, two faint bands with unexpected molecular weights were observed for some unknown reason. Possibly, these primers amplified cellular DNA that was homologous to the IE region of VZV.

We have found that VZV can usually be isolated from vesicles of patients but that it cannot be often isolated from other samples such as throat swabs (8, 9). As we expected, the VZV DNA in all samples of vesicles could be amplified (Fig. 3). This result suggested that our primers could be

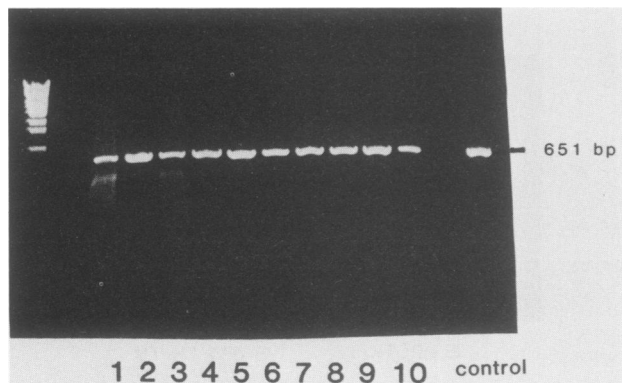


FIG. 3. Separation on agarose gel of products of the PCR with gpI as the primer pair. Results are for 10 vesicle samples containing VZV DNA from 10 patients.

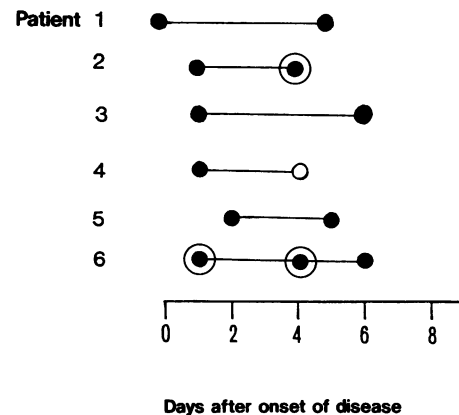


FIG. 4. Detection of VZV DNA in throat swab specimens of varicella patients. ●, VZV DNA detected; ○, VZV DNA detected by direct gel electrophoresis; ○, VZV not detected. The onset of disease means the time of appearance of a skin rash.

useful to specify VZV. Transmission of VZV from one person to another could be by an airborne route from throat swabs or lesions of the skin or by contact with lesions of patients. The isolation of VZV from the pharyngeal area has been reported, but at a very low rate (approximately 4% from throat swabs) (8). However, as viral DNA could be detected even 6 days after the onset of disease in the throat swab specimens by PCR (Fig. 4), it is likely that the airborne route contributes to VZV transmission, although the isolation of the virus was not attempted.

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