JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1983, p. 548–551
0095-1137/83/030548-04$02.00/0

Vol. 17, No. 3

Simplified Method for Typing Herpes Simplex Virus by Restriction Endonuclease Analysis

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Received 23 August 1982/Accepted 22 November 1982

We have developed a simplified method for unambiguously typing herpes simplex virus. The method depends on the production of cell-associated virus at 34°C and subsequently, on the separation of cellular DNA and viral DNA by Dounce homogenization and the removal of nuclei by centrifugation. Viral nucleic acid was prepared from the cytoplasmic fraction and analyzed after restriction endonuclease cleavage. The method obviates the use of radioactive isotopes, and the viral DNA is effectively free of interfering cellular DNA.

The two serotypes of herpes simplex virus (HSV) may be distinguished by a number of techniques. Isolates of HSV type 1 (HSV-1) and HSV type 2 (HSV-2) generally display different sensitivity to certain inhibitors (3, 7, 15) and different abilities to form plaques on chicken embryo cells (4, 17), as well as various other biological differences of varying reliability (9, 13). Methods for examining serological differences also have been widely used to distinguish the two HSV types. These methods include immunofluorescence staining (5, 11, 12), indirect immunoperoxidase staining (1), and an enzyme-linked immunosorbent assay (10). These methods are subject to some error owing to variability among isolates or cross-reactivity of antisera or both.

Methods of typing HSV that rely on direct analysis of the viral DNA by restriction endonuclease digestion are unequivocal, since HSV-1 restriction patterns are distinguished readily from HSV-2 patterns. Current methods employing this technique require ^{32}P-labeling of viral DNA (2, 8). We describe here a simplified method of typing by restriction enzyme analysis that obviates the use of radioisotopes to label the viral DNA. The latter is a procedure that is not desirable for routine use in diagnostic laboratories.

Vero cells were grown in 10-cm tissue culture plates at 37°C in an atmosphere of 5% CO_2. Cells were maintained in Eagle minimal essential medium (Flow Laboratories, Inc., Rockville, Md.) containing penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (25 μg/ml), amphotericin B (2.5 μg/ml), and 10% agamma calf serum (KC Biologicals, Lenexa, Kans.). Monolayers of cells that were 80 to 90% confluent were infected with once-passaged clinical isolates of HSV at a multiplicity of infection of about 0.01 PFU per cell. Adsorption of the virus (diluted in Hanks balanced salt solution plus 0.1% gelatin) was done at 37°C for 90 min, with occasional tilting of the plates. Prewarmed medium (10 ml) was then added, and the infected cells were incubated further at 34°C to optimize the amount of cell-associated virus (6, 14) until 75 to 100% of the cells showed characteristic cytopathic effects.

Cells were harvested by scraping into the medium and collected by centrifugation at 220 × *g* for 10 min at 4°C. The cell pellet was washed with phosphate-buffered saline (0.15 M NaCl–0.01 M sodium phosphate [pH 7.2]) and again centrifuged as described above. Cells were then suspended in 2.5 ml of reticulocyte standard buffer (10 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1.5 mM $MgCl_2$) and allowed to swell at 0°C for 1 h with occasional gentle agitation. Swollen cells were ruptured by homogenization with a tight-fitting glass Dounce homogenizer, and the nuclei were removed by centrifugation at 2,000 × *g* for 5 min at 4°C.

The nuclear pellet was discarded, and the supernatant (crude cytoplasmic extract) was adjusted to 5 mM EDTA, 200 μg of proteinase K per ml, and 0.05% sodium dodecyl sulfate and incubated at room temperature for 30 min. After digestion, the cytoplasmic extract was extracted twice with an equal volume of redistilled phenol which had been saturated with 0.01 M Tris-hydrochloride (pH 8.1)–1 mM EDTA and once with an equal volume of ethyl ether. The nucleic acids were precipitated with 2 volumes of ethanol at −20°C overnight after the addition of NaCl to 0.17 M.

The cytoplasmic nucleic acid precipitate was collected by centrifugation at 12,000 × *g* for 1 h at 4°C, dissolved in 150 μl of 10 mM Tris-hydrochloride (pH 7.0)–1 mM EDTA, and stored at 4°C. The recovery of cytoplasmic nucleic acids was estimated by measuring the absorbance at 260 nm to range from 200 to 400 μg of total nucleic acid taken from two 10-cm plates of infected cells. The nucleic acids at this stage of purification are rather unstable and should be used quickly if good gel patterns are to be obtained.

Purified HSV DNA was prepared essentially as described previously (16). Briefly, cells were infected, incubated, and harvested as described above, and then they were digested with proteinase K in the presence of sodium dodecyl sulfate. This digest was centrifuged to equilibrium in sodium iodide gradients containing ethidium bromide. The band of viral DNA was withdrawn by side puncture with a needle and syringe, extracted three times with isoamyl alcohol, dialyzed to remove sodium iodide, and precipitated with ethanol at −20°C. This procedure generally yielded 25 to 35 μg of pure HSV DNA from four 10-cm plates.

Of the total cytoplasmic nucleic acids, 20 μl, or 1.5 μg of purified HSV DNA, was digested with 6 to 10 units of restriction enzyme (Bethesda Research Laboratories, Gaithersburg, Md.) in the appropriate reaction mixture at 37°C for 2 h in a final reaction volume of 25 μl. The reaction was terminated by the addition of bromophenol blue to 0.025% and EDTA to 10 mM. The entire digest was then subjected to electrophoresis in 0.6% agarose gels containing 1 μg of ethidium bromide per ml in 50 mM Tris-acetate (pH 8.1)–2 mM EDTA–18 mM NaCl. Electrophoresis was for 17 h at 30 V. DNA bands were visualized in the gel by exposure to UV light, and a photograph was taken for permanent record.

The clinical isolates of HSV used in this study were chosen to be representative of isolates routinely obtained in our laboratory. Since we expected to carry out a number of infections with these isolates, we prepared a stock of each isolate by infecting Vero cells on plates with the original isolate grown in tube culture. The infected cells for the stock were harvested, washed, suspended in skim milk, and sonicated. HSV-1 (F) and HSV-2 (G) obtained from B. Roizman (University of Chicago, Chicago, Ill.) were used as prototype strains.

Figures 1 and 2 show the utility of this method and confirm the precision with which clinical isolates of HSV can be typed with restriction enzymes. The major bands of the DNA patterns in Fig. 1A and Fig. 2A were virtually identical to the fragment patterns of the respective purified DNAs in Fig. 1B and Fig. 2B. Isolates SLU-210, 110, 310, 280, and 360 were HSV-1, and isolates SLU-190, 240, 290, and 340 were HSV-2. Some differences in minor bands existed due to the presence of partial digestion products in less purified DNA preparations or to the inability to visualize certain half-molar and quarter-molar

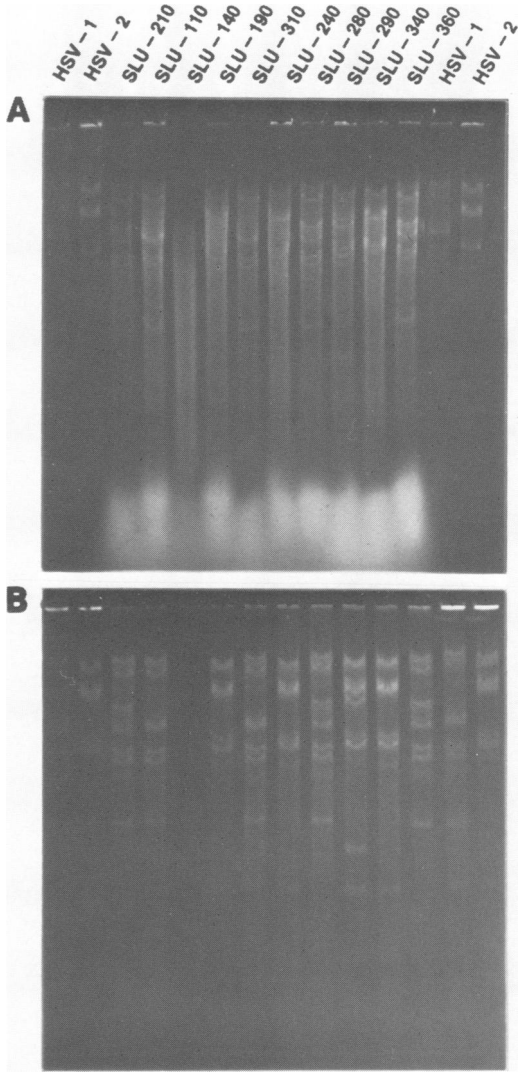

FIG. 1. Agarose gel electrophoresis of DNA digested with restriction endonuclease *Hin*dIII. (A) DNA (20 μl), prepared by the Dounce homogenization method, was digested as described in the text. (B) Viral DNA (1.5 μg), purified in NaI gradients, was digested essentially as previously described (16). Lanes 1 and 13 and 2 and 14 contain prototype strains of HSV-1 and HSV-2, respectively. The digested DNA preparations were subjected to electrophoresis in 0.6% agarose containing 1 μg of ethidium bromide per ml for 17 h, and the gel was then photographed under UV light.

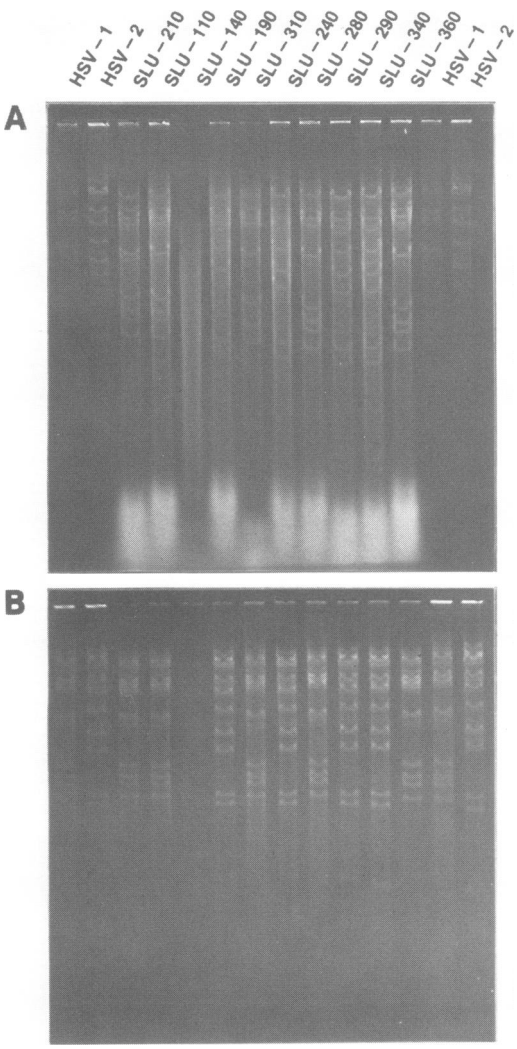

FIG. 2. Agarose gel electrophoresis of DNA digested with restriction endonuclease *Bgl*II. DNA was prepared by the Dounce homogenization method (A) as described in the text or by purification in NaI gradients (B) and subjected to electrophoresis as described in the legend to Fig. 1. Lanes 1 and 13 and 2 and 14 contain prototype strains of HSV-1 and HSV-2, respectively.

fragments. For purposes of HSV type determinations, the visual comparison of molar fragments has been sufficient with most restriction enzymes we have used. At least two independent digestions with different restriction enzymes should be performed on each set of DNA, since we have observed that certain samples are not digested by a particular enzyme. Digested DNAs from HSV-1 and HSV-2 prototype strains were run on each gel.

The major advantage of this method over others previously reported (2, 8) for the preparation and analysis of viral DNA is that it does not require the use of radioisotopes. Radioisotopes generally are expensive and require special storage, handling, and disposal methods, and thus, their routine use in diagnostic laboratories is undesirable. The current method also has the advantage of removing virtually all of the cellular DNA by centrifuging out the nuclei after Dounce homogenization.

In the many cases analyzed, we were unable to identify only one HSV type by this procedure. In that case (isolate SLU-140), although rapid and extensive cytopathic effects were observed in the inoculated plates, the virus apparently produced insufficient DNA to allow positive identification. As stated above, we generally recovered between 200 and 400 μg of total cytoplasmic nucleic acid, as estimated by measuring the absorbance at 260 nm, from two 10-cm tissue culture plates. However, only a small fraction of this (ca. 5%) was DNA, as judged by examining the relative fluorescence of the large RNA spots near the bottom of the gels in Fig. 1A and Fig. 2A and by measuring the total purified DNA obtained by the NaI purification method. Apparently, a small fraction of clinical isolates produce cytopathic effects even without producing large amounts of viral DNA.

If inoculated Vero cells require 2 days to reach 3 to 4+ cytopathic effects, then the final typing results can be read from the agarose gels 2 days after that (i.e., on day 4 if inoculation is on day 0). Thus, the results are available relatively rapidly, and 8 to 10 samples can be processed at the same time. The only specialized equipment necessary is that required for electrophoresis of DNA in agarose gels.

We believe that this technique will be useful in diagnostic laboratories because the results are unambiguous, radioisotopes are not required, and the method is relatively straightforward and uncomplicated, so that it is applicable to clinical laboratories.

This investigation was supported in part by a grant from the Fleur-de-Lis Foundation of Cardinal Glennon Memorial Hospital for Children, St. Louis, Mo. We acknowledge the helpful advice of Anthony Conley.

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