Clear Detection and Typing of Herpes Simplex Virus Types 1 and 2 by an Indirect ELISA Assay: Comparison With Three Different Combined Methods—Capture ELISA, Restriction Enzymes, and Polymerase Chain Reaction

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The severity and recurrences of Herpes Simplex Virus (HSV) infection depend on the type of the infectious agent (HSV-1 or HSV-2), which induces the necessity of a nonambiguous detecting typing. The commonly used capture ELISA technique has to be often supported by DNA analysis to confirm the detection and the typing of HSV viruses in exposed patients. In this report, we describe a rapid and cheap indirect ELISA method using anti-HSV monospecific polyclonal antibodies prepared in the laboratory.

The typing of the studied samples was clear, did not need series of dilution, and allowed the immediate classification of viruses without further control examination. We tested 51 specimens, which were typed 25 HSV-1 and 26 HSV-2 strains. The comparison with capture ELISA, restriction enzyme and polymerase chain reaction analysis definitely allowed our method to be assessed as a useful tool for a routine diagnostic. *J. Clin. Lab. Anal.* 11:146–153, 1997. © 1997 Wiley-Liss, Inc.

Key words: herpes simplex virus; typing; indirect ELISA; capture ELISA; restriction enzymes; polymerase chain reaction

INTRODUCTION

Herpes simplex virus (HSV) is a widespread infectious agent in the human population and one of the most commonly isolated viral agents from clinical specimens. The severity of HSV infections varies greatly in frequency and severity of recurrence, ranging from asymptomatic infection to often fatal encephalitis. Differences between HSV types 1 and 2 are especially apparent in their susceptibility to antiviral drugs (1,2), the site and the rate of recurrence (3), HSV-2 primary genital lesions being more likely to recur than HSV-1 primary lesions (4). This has been accompanied by an interest in the types of HSV responsible for individual infection and has made the typing of HSV a valuable tool for a better understanding of the disease.

Numerous authors reported typing systems to distinguish between HSV types. Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) may be distinguished by various techniques following initial isolation, including sensitivity to (ε)-5-(2-bromovinyl)-2'-deoxythymidine (BVDU) and differential replication in chick embryo cells versus quinea pig embryo cells (5), restriction endonuclease analysis (5–7), recognition by polyclonal rabbit antisera (6) or monoclonal antibodies (5, 6,

8), DNA/DNA hybridization (9), and finally polymerase chain reaction (10, 11).

Analysis of viral DNA with restriction enzymes is a specific method and the restriction patterns of HSV-1 are readily distinguished from those of HSV-2 (7). Moreover, the individual variation within the type 1 and 2 groups allows, after analysis of the genome, to determine whether a second site of infection results from the spread of the same virus or the transmission of a different virus from another individual. However, typing of HSV isolates by restriction enzyme-DNA analysis is time-consuming, involves the use of radioisotopes, and is too cumbersome for most clinical laboratories to be performed on a large scale. Dot-blot DNA-DNA hybridization and PCR have now been currently introduced for laboratory diagnosis of viral infections. These techniques can supplement the currently used immunological assays for de-

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testing and typing HSV (10–13), although they are expensive, delicate in their procedure, and not yet available for clinical routine analysis laboratories.

For the present study, we designed in our laboratory an indirect ELISA using monospecific polyclonal antibodies prepared from primary rabbit kidney (RK) cells infected by HSV-1 or HSV-2. The preparation of the specific antigens for each HSV type was screened by immunoelectrophoresis through an intermediate gel containing polyclonal antibodies directed against the opposite type. The selected immunoprecipitates when pooled and injected into rabbits induced the production of highly reactive and specific antibodies. When testing for the presence of the HSV-1 and HSV-2 viruses 51 clinical isolates after cell culture, this method gave clear and rapid results that were immediately interpretable. It was compared for its sensitivity and specificity with three other methods: one extra immunological method and two analyses at the DNA level. The immunological method consisted of a commercially available capture enzyme-linked immunosorbent assay kit, which allowed a comparison between two immunological methods. The DNA analysis involved restriction enzyme and PCR techniques. The results obtained with these methods were in good concordance and confirmed our indirect ELISA assay as a currently available tool for a rapid detection and typing of HSV in the course of viral infection.

MATERIALS AND METHODS

Specimen Collection and Inoculation

Clinical specimens were collected from 51 adult patients (32 females and 19 males) using sterile cotton swabs from oral, genital, and skin sites. The swabs were transferred into 3 ml of Hanks balanced salt solution containing 100 µg/ml streptomycin, 100 units/ml penicillin, and 1 µg/ml mycostatin and were frozen at -70°C if not immediately studied. Specimens were inoculated into triplicate tubes (0.3 ml/tube) of MRC-5 cells (American Type Culture Collection, Rockville, MD) and grown in MEM-D medium supplemented with 2% fetal calf serum and examined daily until observation of typical HSV cytopathic effect (CPE). When 80–90% CPE was observed, the tubes were frozen at -70°C until their use in ELISA assays. As positive controls, the F and MacIntyre HSV-1 strains and the G and MS HSV-2 strains (American Type Culture Collection) were propagated in MRC-5 cells. Uninfected MRC-5 cells were used as negative controls.

Preparation of Monospecific Polyclonal Antibodies for Indirect ELISA Typing

Primary RK cells were prepared from 21-day-old rabbits by standard methods. RK cells were grown in Dulbecco's MEM medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, and 0.2% sodium bicarbonate. HSV1-F and HSV2-G strains were grown in RK

cells at a multiplicity of infection of 0.01 infectious viral particle per cell. RK cell monolayers that showed 80–90% CPE 24 hours after infection were washed twice in cold PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) containing the following protease inhibitors: tosyl-lysyl-chloromethyl ketone (TLCK), p-hydroxy-mercuribenzoate and phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma Chemicals, St. Louis, MO) at 1 mM final concentration each. Cells were then scrapped into cold 20 mM glycine, 7.6 mM Tris buffer (pH 8.6) containing 3% (vol/vol) Triton X-100, and the protease inhibitors as indicated above. After 30 min incubation at $+4^{\circ}\text{C}$, the lysed cells were sonicated at 18kc/sec two times for 15 sec each on ice and then centrifuged at 1,000 g for 10 min, and the supernatants were centrifuged at 100,000 g for 1 hr. The supernatant used for the antigen preparations for HSV1-F and HSV2-G strains and mock-infected cells were aliquoted and stored at -70°C . The protein concentration in the soluble fraction was determined by the Lowry method modified for measurement in Triton X-100 containing solutions (14). The protein concentration of the solubilized antigen preparation was adjusted to 10 mg/ml. The soluble fraction was dialysed against TNE buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 0.001 M EDTA) overnight at $+4^{\circ}\text{C}$. An aliquot of 0.3 ml (corresponding to 3 mg) per tube from each HSV-1 and HSV-2 antigen preparation or mock infected cells was layered on the top of 12.3 ml linear sucrose gradient prepared in TNE buffer. The HSV-1 and HSV-2 type specific antigens were prepared on a 10–27% (wt/wt) and a 6–19% (wt/wt) linear sucrose gradient, respectively. The gradients were centrifuged for 24 hr in a SW 41 Ti Beckman rotor at 40,000 rpm, at $+5^{\circ}\text{C}$. They were collected in 29 fractions of 0.4 ml, which were tested by fused rocket immuno-electrophoresis with an intermediate gel as described in our European patent (15).

The upper part of the immunoprecipitates from the reference gel containing the type specific antigens were cut out from the unstained wet gel. The isolated immunoprecipitates were disrupted by a double sonication on ice at 20 kc/sec for 30 sec each, mixed with equal volume of incomplete Freund's adjuvant. Each immunoprecipitate was inoculated into two rabbits, four times with intervals of 2 weeks. The immunoglobulins were purified and their concentration adjusted to 30 mg/ml. This precipitation constituted the monospecific polyclonal anti-HSV-1 or anti-HSV-2 sera.

HSV Typing by Indirect ELISA

The indirect ELISA using monospecific polyclonal anti-HSV-1 or anti-HSV-2 sera was performed when the HSV isolates grown on monolayer tube cultures with a CPE of 80–90% were harvested. One tube (out of three) for each sample was centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and 0.2 ml of a cold Tri-glycine buffer at pH 8.6 (7 mM Tris, 20 mM glycine) containing 3% (vol/vol) Triton X-100 was added to the pellet. The samples were incubated for

30 min at 4°C; then, 1 ml of 0.05 M carbonate-bicarbonate buffer at pH 9.6 was added per tube. The tubes were vortexed and 100 µl/well were distributed in triplicates and tested against anti-HSV-1 as well as anti-HSV-2 monospecific antisera on polystyrene microtiter plates (Nunc Immunoplates 1-96F, certified, Denmark) and incubated overnight at +4°C. On each plate, a positive HSV-1 (F, MacIntyre strains) and HSV-2 (G, MS strains) as well as a negative control (mock infected cells) were run simultaneously against anti-HSV-1 and anti-HSV-2 monospecific sera. The plates were incubated at 37°C for 1 hr and then washed twice in PBS-0.05% Tween 20 named buffer A. A 100 µl sample of the 1/1,000th diluted (30 mg/ml) anti-HSV-1 or anti-HSV-2 monospecific sera in buffer A supplemented with 1% bovine serum albumin (BSA) was added in triplicates and the plates were incubated for 1 hr at 37°C. They were then washed four times in buffer A as above and 100 µl per well of a swine antirabbit horseradish peroxidase (HRP) conjugate (Dako, Glostrup, Denmark) 1/1,000th diluted in dilution buffer was added. The plates were incubated for 30 min at 37°C. After washing the plates four times with buffer A, 200 µl of substrate solution (4 mg of o-phenylene diaminehydrochloride, 0.03% H₂O₂ added immediately before use in 10 ml of 0.1 M citric acid phosphate buffer, pH 5) was added to each well. The reaction was terminated after 20 min by the addition of 50 µl per well of 3 N HCl. The absorbance was measured at 492 nm with a Dynatech ELISA microplate reader MR 5000®. In this assay, positive-negative cutoff values were established as follows. Samples with Optical Density (O.D.) units ranging between the absorbance of the negative control and addition of 0.100 O.D. units were considered as negative (negative cutoff). Samples with O.D. units greater than the absorbance of the negative control plus 0.250 O.D. units (positive cutoff) were considered as positive and HSV types were identified.

HSV Typing by Capture ELISA

The capture ELISA (Herpes Simplex Virus ELISA kit®, code K233 DAKO, Denmark) was performed according to manufacturer's instructions, procedure A being used for the simultaneous detection and typing of HSV. The HSV isolates grown in MRC-5 cells with a CPE of 80–90% were used either undiluted or 1/100th diluted with MEM-D medium supplemented with 2% fetal calf serum, and the ELISA was run simultaneously against anti HSV-1, anti HSV-2 and a negative HSV control serum with both dilutions. The absorbance was measured at 492 nm with a Dynatech ELISA microplate reader MR 5000®. This defined the A₄₉₂ value. The ratio of A₄₉₂ using polyclonal anti HSV-1 and HSV-2 reagents was determined for each undiluted and diluted test sample and represented the type 1/type 2 index. HSV-1/HSV-2 index (or typing index) was calculated by dividing the absorbance of the well from which the sample reacted with antibodies to HSV-1 by the absorbance of the well from which the same

sample reacted with antibodies to HSV-2. The HSV1/HSV-2 index was >1 when the isolate was of HSV-1 type, and the typing index was <1 when the isolate was of HSV-2 type. Samples with a typing index neighboring 1 were considered as ambiguous and tested again with two DNA methods.

HSV DNA Restriction Endonuclease Analysis

MRC-5 cells were grown in Nunc plastic flasks of 25cm² to near confluency. They were then maintained for 24 hr in E-MEM medium without phosphate (Flow Laboratories, Irvine, Scotland) containing 2% fetal calf serum. The medium was removed and 1 ml of each viral isolate was added to each flask. The inoculum was adsorbed for 1 hr at 37°C and washed with E-MEM phosphate free medium. A 3 ml sample of the same medium was added to each flask and the incubation at 37°C was continued for 3 hr. Then, 15 µCi of α-³²P-ATP (Amersham, Amersham, England) was added to each flask, and the incubation was continued at 37°C for additional 20–32 hr until infected cells showed an adequate CPE. Infected cells were treated to isolate ³²P labelled HSV DNA as previously described (7).

Partial digestion of DNA with the Bam HI and BglIII restriction endonucleases (Amersham) was carried out according to manufacturer's recommendations. The DNA digests were analysed by overnight electrophoresis (30 mA) on 0.8% agarose gels. The gels were dried in a vacuum gel slab drier (Biorad Laboratories) and exposed to Kodak-X Omat S films at –70°C for 20 hr. Autoradiographs were analyzed by reference to strain F (HSV-1) and strain G (HSV-2).

HSV DNA PCR Amplification and Product Analysis

Two 20-base oligonucleotides P1 and P2 deduced from a highly conserved region of the DNA polymerase gene of the Herpes virus group (6) were used in PCR assays. A 50 µl PCR reaction mix was prepared containing 1x PCR buffer (Stratagene, Recombinant 10x cloned Pfu buffer®), 50 pM of each oligonucleotide primer, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP; Pharmacia Biotech), and 2 µl of cellular culture supernatant in microcentrifuge tubes (MicroAmp®, Perkin-Elmer).

The mixture contained in tubes was heated at 94°C for 10 min and then immediately chilled on ice. Two units of Taq DNA polymerase (Stratagene, Recombinant Pfu DNA polymerase®) was added to each tube, and the tubes were placed on a thermal cycler (Perkin-Elmer, GeneAmp PCR System 9600®). Each cycle consisted of a 30-sec denaturation at 94°C, a 1-min annealing at 60°C, and a 1-min elongation at 72°C. It was repeated 40 times and followed by a final extension for 10 min at 72°C. Fifteen µl of PCR product was loaded onto a 2% agarose gel (Sigma Chemical, wide range/standard 3:1) containing 1 µg/ml of ethidium bromide in TAE buffer, subjected to electrophoresis, and visualized under UV light.

The specificity and typing of PCR products were further

confirmed by restriction enzyme digestion of 30 µl of the PCR product by 10 units of Bam HI (Amersham) incubated for 2 hr at 37°C in the 10x restriction enzyme buffer. The PCR amplification was predicted to give a 518 base pair (bp) product, which should be digested into two fragments of 225 bp and 293 bp, respectively for HSV type 2, whereas the HSV type 1 PCR product should be intact. The digests were resolved by electrophoresis on 3% agarose gels.

RESULTS

HSV Typing by Indirect ELISA with Monospecific Polyclonal Antibodies

The 51 clinical isolates after cell culture were grown on MRC-5 fibroblasts and harvested when the CPE was 80–90%. They were submitted in triplicates to the indirect ELISA using the monospecific polyclonal antibodies prepared in our laboratory as described in Materials and Methods. All the samples were tested in the presence of positive HSV-1 and HSV-2 as well as negative controls. They were run simultaneously against anti-HSV-1 and HSV-2 monospecific sera, and the typing revelation used a swine antirabbit horseradish peroxidase conjugate. The colorimetric detection was performed at 492 nm and the cutoff value was determined at 0.45 and 0.40 for HSV-1 and HSV-2, respectively.

This method allowed a rapid typing and a clear distinction between HSV-1 and HSV-2 isolates. The average O.D. value was 1.12 for both HSV-1 and HSV-2 and the range of the O.D. values was narrow as varying from 0.77 to 1.62 for HSV-1 and from 0.70 to 1.50 for HSV-2. From the 51 isolates, 25 were typed as HSV-1 and 26 as HSV-2 (Tables 1 and 2). No isolate required to be further typed by another method. However, our data were reinforced by DNA method analyses (restriction enzymes analysis and PCR amplification) which confirmed the typing obtained by indirect ELISA.

HSV Typing by the Capture ELISA Herpes Simplex Virus Kit

The typing index for each isolate as well as for HSV-1 and HSV-2 reference strains (strains F and MacIntyre for HSV-1, G and Ms for HSV-2) was calculated for the undiluted and diluted samples. The average and range values for the typing index of HSV-1 isolates were 1.77 and 1.20 to 2.84 for the undiluted samples and 2.03 and 1.00 to 3.40 for the diluted samples, respectively. The average and range values for the typing index of the HSV-2 isolates were 0.76 and 0.43 to 0.95 for the undiluted samples and 0.75 and 0.50 to 0.94 for the diluted samples, respectively. Based on these data, the 51 HSV could be typed as 25 isolates HSV-1 or as 26 isolates HSV-2. However, this method did not allow an immediate and clear typing for all the studied samples as noted recently. (At the end of the series of experiments, we noticed the replacement of the kit K 223[®] referenced as a “detection and typing kit of

TABLE 1. Typing of 51 HSV Isolates by the Indirect ELISA and the Capture Herpes Simplex Virus ELISA Kit: Detection of HSV-1 Isolates^a

N° of Specimens	Indirect ELISA	Capture ELISA	
		Undiluted	Diluted
1	0.99	1.76	1.44
2	0.92	1.53	2.33
3	1.25	1.73	1.20 (*)
4	1.13	1.87	2.42
5	1.10	1.76	1.60
6	1.20	1.85	1.88
7	0.85	2.13	1.43
8	0.77	1.93	3.04
9	0.91	1.75	2.84
10	1.10	1.83	2.40
11	1.20	1.50	2.80
12	1.25	1.60	2.20
13	1.45	1.50	2.20
14	1.50	1.20	1.00 (A)
15	1.60	1.58	1.40
16	1.10	1.80	3.40
17	1.05	1.60	2.30
18	1.02	1.80	3.40
19	0.85	1.76	1.28 (*)
20	0.92	1.72	1.22 (*)
21	0.90	1.50	1.20 (*)
22	0.88	1.70	2.60
23	0.92	1.80	2.50
24	1.60	2.84	1.50
25	1.55	2.23	1.20 (*)
F-strain	1.42	1.90	2.95
McInt. strain	1.62	2.00	3.00

^aNumbers in the first column represent the O.D. values for the indirect ELISA. For the capture ELISA, the numbers represent the corresponding typing index for undiluted and 1/100th diluted samples. These indices were calculated as reported in Materials and Methods using a positive cutoff value of 0.45.

(A) = ambiguous detection.

* Index ranging between 1.20 and 1.28.

HSV” by a new kit K 6000[®] for which the assay does not differentiate between HSV type 1 and type 2”). Fifteen specimens presented ambiguous typing indices distributed as follows. As shown in Tables 1 and 2, the typing index was 1.00 for one HSV-1 diluted sample (specimen n° 14) and ranged from 0.90 to 0.99 for 14 HSV type 2 samples. These 14 HSV-2 specimens corresponded to the indices observed for six undiluted samples (n° 27, 33, 46, 49–51), seven diluted samples (n° 31, 32, 34, 36, 40, 47, 48) and for both diluted and undiluted samples for one out of 14 specimens (n° 37). To compare the efficiency of capture ELISA to ours, all the 15 samples together with five more HSV-1 isolates with a typing index ranging from 1.20 to 1.28 for diluted samples (specimens n° 3, 19–21, 25) were studied by DNA restriction endonuclease analysis and PCR amplification.

Typing of DNA Restriction Endonuclease Analysis

Twenty HSV isolates were analyzed by restriction endonuclease digestion using the Bam HI and Bgl II enzymes.

TABLE 2. Typing of 51 HSV Isolates by the Indirect ELISA and the Capture Herpes Simplex Virus ELISA Kit: Detection of HSV-2 Isolates^a

N° of Specimens	Indirect ELISA	Capture ELISA	
		Undiluted	Diluted
26	1.30	0.66	0.68
27	1.42	0.93(A)	0.85
28	1.25	0.88	0.75
29	1.65	0.48	0.50
30	1.50	0.50	0.55
31	1.25	0.76	0.90(A)
32	1.05	0.75	0.90(A)
33	0.95	0.90(A)	0.87
34	0.90	0.77	0.92(A)
35	0.85	0.70	0.50
36	0.75	0.65	0.95(A)
37	0.70	0.90(A)	0.95(A)
38	0.70	0.68	0.53
39	0.95	0.85	0.80
40	0.90	0.80	0.90(A)
41	1.10	0.84	0.80
42	1.25	0.75	0.52
43	1.42	0.86	0.83
44	1.25	0.46	0.57
45	1.05	0.78	0.53
46	1.02	0.95(A)	0.53
47	1.20	0.82	0.99(A)
48	1.10	0.43	0.90(A)
49	0.95	0.95(A)	0.86
50	0.82	0.95(A)	0.63
51	0.75	0.92(A)	0.78
G-strain	1.20	0.80	0.64
MS-strain	1.10	0.75	0.60

^aNumbers in the first column represent the O.D. values for the indirect ELISA. For the capture ELISA, the numbers represent the corresponding typing index for undiluted and 1/100th diluted samples. These indices were calculated as reported in Materials and Methods using a positive cutoff value of 0.40. (A) = ambiguous detection.

Fourteen isolates were types as HSV-2 and six were typed as HSV-1. In Figure 1, we present the six HSV-1 we selected after capture ELISA (samples 14 and 3, 19–21, 25) and samples of HSV-2 isolates. We have chosen to show the isolate n° 37 ambiguous for both the diluted and undiluted sample detections, two isolates with a narrow discrepancy between the two detections (samples n° 27 and 33), and four isolates with an important discrepancy between the two detections (samples n° 36, 46, 48, 50). The results were thus in complete agreement with those achieved by indirect ELISA and definitively confirmed the typing of 14 HSV-2 isolates from which the typing index ranged from 0.90 and 0.99 and one HSV-1 isolate whose typing index was 1.00 when obtained by the capture Herpes Simplex Virus ELISA kit.

HSV Typing by PCR Amplification

Using the same set of primers (P1 and P2) located within a highly conserved region of the DNA polymerase gene, it was

Fig. 1. Typing by restriction endonuclease analysis. As an example, 13 isolates typed as HSV-1 and HSV-2 by the indirect ELISA method are presented for restriction enzyme analysis. The strains were cleaved with Bam HI (A) and Bgl II (B) and were subjected to overnight electrophoresis on 0.8% agarose gels at 30 mA. Their restriction profiles were autoradiographed for 20 h at -70°C . The lanes are as follows: lane F: HSV-1 reference strain F, lane G: HSV-2 reference strain G, lanes numbered as 14, 3, 19–21, 25: correspond to HSV-1 isolates typed by the indirect ELISA, lanes numbered as 37, 27, 33, 36, 46, 48, 50: correspond to HSV-2 isolates typed by the indirect ELISA.

possible directly to amplify from infected cell culture HSV-1, HSV-2, CMV, and EBV as previously described (10). The expected size of the PCR product for HSV-1 and HSV-2 of 518 bp was found for the 15 samples already analyzed in Figure 1 and Figure 2. These products were further subtyped between HSV-1 and HSV-2 isolates by restriction enzyme digestion. The 518 bp HSV-2 amplified product was cleaved by Bam HI into two 225 bp and 293 bp fragments, whereas HSV-1 amplified product remained undigested. Twenty HSV isolates were amplified and subjected to restriction enzyme analysis by Bam HI endonuclease. Fourteen out of 20 isolates were typed as HSV-2, and six were typed as HSV-1. The typing of the 15 selected detections is presented as examples

Fig. 2. Detection of HSV strains by PCR. In these experiments, the primers P1 (5' - CGA CTT TGC CAG CCT GTA CC - 3') and P2 (5' - AGT CCG TGT CCC CGT AGA TG - 3') were used. Amplified products were subjected to agarose gel electrophoresis. The lanes are as follows: Upper part: M: molecular weight marker corresponding to ϕ X174 DNA/Hae III restriction fragments, lane F: HSV-1 reference strain F, lanes numbered as 14, 3, 19–21, 25: correspond to HSV-1 strains typed by indirect ELISA, lane nc: negative control. Lower part: M, molecular weight marker corresponding to ϕ X174 DNA/Hae III restriction fragments, lane G: HSV-2 reference strain G, lanes numbered as 37, 27, 33, 36, 46, 48, 50: correspond to HSV-2 strains typed by indirect ELISA. Sizes in base pairs are shown on the right. The amplified product observed at 518 bp represent the predicted fragment for both HSV-1 and HSV-2 types.

on Figure 3. It corresponded to seven HSV-2 (n° 37, 36, 46, 48, 50, 27, 37) and six HSV-1 (n° 14, 3, 19–21, 25) as detailed above. The discrimination between HSV-1 and HSV-2 isolates directly obtained with our method was confirmed by restriction enzyme digestion and emphasized its specificity.

Correlation Among the Four Studied Methods

The typing by indirect ELISA presented high sensitivity and specificity and gave clear results that were confirmed by the DNA methods. The capture ELISA showed 15 ambiguous detections, which revealed a typing index neighbouring 1.00 and five extra HSV-1 samples which had an index comprised between 1.20 and 1.28. The ambiguities were solved after typing by restriction enzyme analysis and PCR amplification. The comparison reported in Table 3 confirmed the rapid and direct type determination by our method based on an indirect ELISA using monospecific polyclonal antibodies.

Fig. 3. Typing of HSV strains. The PCR products visualized in Figure 2 were subjected to enzyme digestion by Bam HI. The resulting restriction fragments were analyzed on a 3% agarose gel stained with ethidium bromide. The lanes are as follows: Upper part: M: molecular weight marker corresponding to ϕ X174 DNA/Hae III restriction fragments, lane F: HSV-1 reference strain F, lanes numbered as 14, 3, 19–21, 25: correspond to HSV-1 strains typed by indirect ELISA. Lower part: M, molecular weight marker corresponding to ϕ X174 DNA/Hae III restriction fragments, lane G: HSV-2 reference strain G, lanes numbered as 37, 27, 33, 36, 46, 48, 50: correspond to HSV-2 strains typed by indirect ELISA. The alignment and numbering of strains was as in Figure 2. The expected size of uncleaved HSV-1 strains (518 bp) or cleaved HSV-2 strains (293 and 225 bp) are shown on the right.

DISCUSSION

This report describes a sensitive method that allowed a rapid diagnostic for an HSV infection and, more, a clear subtyping of the HSV-1 and HSV-2. This was based on an indirect ELISA technique using monospecific polyclonal antibodies that we carefully selected. It permitted us to analyze 51 infected isolates that could be directly classified as 25 HSV-1 and 26 HSV-2, unambiguously.

Some other techniques previously described had used polyclonal antibodies and included double antibody capture ELISA (16), double antibody capture ELISA, and comparison with

TABLE 3. Comparison of Results Obtained in the Different Analyses

Type	Indirect	Capture	Restriction	PCR
HSV-1	25 (25)	25 (25)	6 (6)	6 (6)
HSV-2	26 (26)	26 (26)	14 (14)	14 (14)

This table summarizes the results of the typing of the 51 HSV isolates by indirect and capture ELISA. The confirmation of the typing of the 15 ambiguous detections and of the 5 extra HSV-1 was made by restriction enzyme digestion and PCR. The number of isolates tested in the considered method are indicated in parentheses.

indirect ELISA (17), monoclonal antibodies and indirect ELISA (18–20), or a double-antibody capture ELISA using polyclonal antibodies for capture and monoclonal antibodies for detection (21–23). The results of HSV typing with these various ELISA techniques induced some ambiguous interpretation and need further examination, as was for methods using immunofluorescence and immunoperoxidase with type-specific monoclonal antibodies (6, 8, 23, 24) or other methods that included differential viral replication in chicken embryo cells versus guinea pig embryo cells and typing by the BVDU method (5). When submitting the 51 samples to such a commercial kit using the capture ELISA technique, we found that 15 specimens presented ambiguous typing indices, one for HSV-1 and 15 for HSV-2. To clarify the typing and to assume the validity of our method, further analysis of these samples was made at the DNA level together with five extra HSV-1 specimens presenting indices neighboring the limit of detection.

Methods using synthetic DNA probes (25) gave satisfying results, and now the restriction endonuclease digestion of the viral DNA is considered as the most specific method for HSV typing, as it can give information related to the viral molecular epidemiology, the mode of transmission, intratyping variations, and mixed infections with HSV-1 and HSV-2 (26–28). However, many clinical laboratories are constrained to perform restriction analysis because of the complexity of other assay procedures and the need to use radioactive materials. In these techniques, the design of a single pair of primers selected in a highly conserved region of herpes viruses DNA polymerase gene permitted the detection and the typing of HSV genomes by means of PCR, which is a sensitive and specific procedure (10). Such use of a single pair of primer is of particular importance in order to simplify the delicate current procedure for detection and typing of HSV genome.

In all samples, the PCR product was detected at the predicted size of 518 bp for HSV type I and type II. Further analysis by restriction enzyme Bam HI digestion confirmed the detection and the typing directly realized with our method. This restriction method allowed a differential identification of the HSV types: the amplified products from HSV-1 genomes remained undigested, whereas that of HSV-2 were cleaved into 225 bp and 293 bp fragments, respectively.

In conclusion, it is of importance to note that HSV typing is essential for epidemiological as well as for clinical reasons, helping a better evaluation of the prognosis associated with the type of HSV infection (14, 28–30). The present study showed that it is possible to detect and determine in perfect concordance the type of herpes simplex viruses by three different methods: indirect ELISA, restriction enzyme digestion, and PCR, the reported sensitive and specific “in-house prepared” indirect ELISA assay allowing a clear and direct detection and typing of HSV. The performance of this method is a consequence of the two-step preparation of the specific antiviral antibodies that involved a gel selection of the im-

munoprecipitates followed by the injection of the very specific antigens. The clinical application of this assay should provide a rapid and useful addition to the currently available modalities for the type-specific detection of HSV infection and may facilitate the diagnosis and the management of exposed patients with suspected infections.

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