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# Herpes Simplex Virus Detection by Macroscopic Reading after Overnight Incubation and Immunoperoxidase Staining

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**Human diploid foreskin fibroblast cells grown in 24-well plates were inoculated with clinical specimens by centrifugation at 1,000 × *g* for 45 min. Cultures were incubated at 37°C overnight, fixed, and stained with peroxidase-labeled monoclonal antibodies against herpes simplex virus types 1 and 2. Stained plaques of infected cells were large enough to be detected with the naked eye, and microscopic examination did not reveal any further positive specimens. The method was compared with standard isolation in human fibroblasts grown in shell vials and inoculated by centrifugation at 4,000 × *g*, observed microscopically for the occurrence of typical cytopathogenic effect three times a week for 10 days, and then typed by enzyme immunoassay. Of the 289 specimens tested, 105 were positive and 174 were negative by both methods. Six specimens were positive by standard isolation only, two of them containing varicella-zoster virus, and two specimens were stored frozen before being tested by immunoperoxidase staining. Two specimens found negative by standard isolation were positive by immunoperoxidase staining. For two specimens negative by immunoperoxidase staining, the standard isolation cultures were lost due to microbial contamination. Forty-two specimens found positive by standard isolation were clearly positive when stained only 8 h after inoculation. By standard isolation, positive results were reported on the average 3 to 4 days after inoculation, whereas by immunoperoxidase staining the result was available within less than 24 h. Immunoperoxidase staining of infected cells is a sensitive method for rapid laboratory diagnosis of herpes simplex virus infections, and 24-well plates are convenient for the handling of a large number of specimens.**

Rapid diagnosis of infections caused by herpes simplex viruses (HSVs) is still a task for the diagnostic laboratory. For vesicular rashes in immunosuppressed patients, infections of newborns during the first weeks of life, and genital infections during pregnancy, laboratory diagnosis has an influence on the management of the patient. If laboratory findings are available within a reasonable time after collection of the specimen, they can facilitate the decision of whether to commence antiviral therapy and may also help to determine the route of delivery.

Virus isolation, especially in combination with centrifuged inoculation of the specimens, is a highly sensitive technique (3, 10) and should therefore serve as a reference method for the evaluation of each new test. To be suitable for routine use, a laboratory test for the rapid diagnosis of HSV infections should fulfill several requirements. First, in order to yield reliable results, the sensitivity of the new method should be comparable to that of virus isolation methods. Second, to have any influence on clinical decisions, the test result should be available within a few hours or at most 1 day after collection of the specimen. Furthermore, to make the test practicable in many diagnostic laboratories, its performance must not involve the use of unusual and expensive equipment, and to make possible the handling of a substantial number of specimens, the work caused by the test should be minimal. Finally, it ought to be possible for the staff of the clinical virology laboratory to read and interpret the test without any extensive additional training.

The test described here uses centrifuged inoculation of cell cultures and immunoperoxidase staining (IPS) with HSV type-specific monoclonal antibodies after overnight incubation. Stained plaques of infected cells are sufficiently large to be easily detectable macroscopically.

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## MATERIALS AND METHODS

**Cells and reference viruses.** All experiments were done with human diploid foreskin fibroblasts (HDF). HSV type 1 (HSV-1; strain F) and type 2 (HSV-2; strain G), both obtained from the American Type Culture Collection (Rockville, Md.), served as positive controls.

**Clinical specimens.** Twenty specimens that were previously found to be HSV positive by standard isolation and had been stored at −70°C for up to 2 months and 269 fresh consecutive specimens from patients with a clinical diagnosis of either genital herpes infection, vesicular rash, stomatitis, or ocular infection were studied (Table 1). The lesions were swabbed with a cotton stick, and the stick was placed in a vial containing 2 ml of tryptose-phosphate broth supplemented with 0.5% bovine serum albumin and antibiotics. Without cooling, the specimens were transported to the laboratory, where they were vortexed and then clarified in an Eppendorf centrifuge at 9,000 × *g* for 30 s, and the supernatant was used for inoculation. Most specimens were inoculated the same day they arrived at the laboratory, but a few were stored at 4°C overnight before inoculation the following day.

**Monoclonal antibodies.** Mouse monoclonal antibodies with a specificity for either HSV-1 or HSV-2 have been described earlier (18). For IPS, the antibodies were labeled with horseradish peroxidase (HRPO, type 4; Sigma Chemical Co., St. Louis, Mo.) as described elsewhere (13).

**Standard isolation and typing by enzyme immunoassay.** Approximately 100,000 HDF cells in 1 ml of Eagle minimum essential medium containing 10% fetal bovine serum were seeded into shell vials containing a 13-mm round cover slip (Trac bottle; Sterilin Ltd., Feltham, Middlesex, United Kingdom) and incubated at 37°C until subconfluency was reached. The growth medium was replaced by 1 ml of medium containing 2% fetal bovine serum and antibiotics.

TABLE 1. Origin of clinical specimens

| Source of specimen | No. of specimens | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Females | | | Males | | | Total | | |
| | Negative | HSV-1 | HSV-2 | Negative | HSV-1 | HSV-2 | Negative | HSV-1 | HSV-2 |
| Vesicle | 33[a] | 8 | 3 | 15[a] | 7 | 1 | 48 | 15 | 4 |
| Genitals | 91 | 6 | 50 | 11 | 0 | 25 | 102 | 6 | 75 |
| Eye | 5 | 0 | 0 | 4 | 0 | 0 | 9 | 0 | 0 |
| Throat | 4 | 5 | 0 | 6 | 0 | 0 | 10 | 5 | 0 |
| Unknown | 3 | 0 | 5 | 6 | 0 | 1 | 9 | 0 | 6 |

[a] One specimen VZV positive by standard isolation.

Duplicate cultures were inoculated with clinical specimens (200 μl per tube) and centrifuged at 35°C in a Sorvall GSA rotor at 4,000 × *g* for 45 min. Thereafter, the tubes were incubated at 37°C in a 5% $CO_2$ atmosphere for 1 h before the inoculate was aspirated and replaced by 1 ml of fresh maintenance medium. The tubes were further incubated and observed for cytopathic effect (CPE) at 40× magnification under an inverted microscope three times a week for 10 days or until pronounced typical CPE was visible.

The typing of isolates was carried out as described previously (18). In brief, when CPE affected at least 25% of the cells, the supernatant was aspirated, the cells were rinsed twice with phosphate-buffered saline (PBS), and 1 ml of carbonate buffer (pH 9.6) was added. Virus antigens were then extracted by freezing and thawing and short sonication. The resulting suspension was finally clarified by low-speed centrifugation. The supernatant (100 μl) was added to each of four flat-bottomed wells on a polystyrene microstrip (Eflab, Helsinki, Finland). The strips were sealed with cellophane tape and incubated at room temperature overnight. Unadsorbed material was then aspirated, and the wells were washed twice with PBS containing 0.1% Tween 20 (PBS-T). HRPO-labeled MAbs against HSV-1 and HSV-2, appropriately diluted in PBS supplemented with 1% bovine serum albumin and 0.5% Tween 20 (PBS-BT), were added to duplicate wells, and the strips were kept at 37°C for 1 h. After washing, 0.03% hydrogen peroxide in a citrate-phosphate buffer (pH 5.0), containing 1,2-phenylenediamine dihydrochloride (0.7 mg/ml), was added, and the strips were kept in the dark at room temperature for 15 min. Color reaction was stopped by adding 1 N HCl, and the $A_{492}$ was measured by a Titertek Multiskan photometer (Eflab, Helsinki, Finland). The $A_{492}$ value obtained with the HSV-1-specific MAb was divided by the $A_{492}$ value obtained with the HSV-2-specific antibody. If a ratio of >2 was obtained, the isolate was classified as HSV-1, and if the ratio was <0.5, it was classified as HSV-2.

**IPS.** HDF cells were grown in 24-well cell cluster dishes (well diameter, 16 mm; Costar, Cambridge, Mass.). Approximately 100,000 cells in 1 ml of growth medium were seeded to each well. At confluency, the growth medium was replaced by 1 ml of maintenance medium containing antibiotics. HSV-1 and HSV-2 reference viruses as positive controls in an appropriate dilution and the clinical specimens were added to duplicate wells (200 μl per well), and the plates were centrifuged at 35°C in a Damon CRU-5000 centrifuge (International Equipment Company, Needham Heights, Mass.) at 1,000 × *g* for 45 min. The plates were then immediately placed in a 5% $CO_2$ atmosphere at 37°C and incubated overnight, usually for 16 to 18 h. Next, the medium was aspirated off, and the cells were rinsed twice with PBS, air dried, and fixed with methanol for 10 min. Methanol was removed, and the cells were washed twice with PBS. HRPO-labeled monoclonal antibodies against HSV-1 and HSV-2, diluted 1:1,000 in PBS-BT, were added to the wells, and the plates were incubated at 37°C for 1 h before the cells were washed three times for 1 min each with PBS-T and once with PBS. As a chromogenic substance, 20 mg of 3-amino-9-ethylcarbazole (Sigma Chemical Co.) was dissolved in 5 ml of dimethylformamide, and sodium-acetate buffer (pH 5.0) was added to a final volume of 1 liter. The solution was filtered through a 0.22-μm bottle top filter (Becton Dickinson, Oxnard, Calif.) and stored in aliquots of 10 ml at −20°C. Prior to use, 10 μl of 3% $H_2O_2$ was added, and 300 μl of the solution was distributed to each well. The plates were kept at room temperature for approximately 30 min, until clearly stained plaques could be distinguished with the naked eye in the wells inoculated with the reference viruses.

**Reduction of incubation time after centrifuged inoculation.** The reference strains of HSV-1 and HSV-2 in 10-fold dilutions were incubated in duplicate wells on each of four different plates. The plates were centrifuged as above and further incubated for 3, 7, 11, or 17 h before fixing and staining. The plates were then read under an inverted microscope at 40× magnification. At 4 h after inoculation, faint staining of single cells was visible microscopically in wells inoculated with a low dilution of either serotype. At 8 h, small plaques were seen in all virus dilutions. In a separate experiment, duplicate plates were inoculated with 42 clinical specimens previously found to be positive. After centrifugation, one plate was incubated for 7 h and the other for 17 h before fixation and staining.

## RESULTS

**Comparison of IPS with standard isolation.** In preliminary experiments with HSV-1 and HSV-2 reference viruses as well as with known positive clinical specimens, it was found that viral plaques grew large enough during overnight incubation to be distinguished with the naked eye. Plaques usually consisted of approximately 5 to 30 cells, depending on the cell density in the wells (Fig. 1). Microscopy was used only to verify the finding.

A total of 289 specimens from 282 patients were available for evaluation. Results obtained with the two techniques were in agreement for 279 specimens, 174 being negative and 105 positive. Of the positive specimens, 24 were HSV-1 and 81 were HSV-2, as determined by both techniques (Table 2). The results were discrepant for 10 specimens, 4 being HSV positive only by standard isolation and 2 only by IPS (Table 3). In standard isolation, cultures of three specimens were contaminated with bacteria or yeasts. Only from one specimen was the result of IPS lost on account of microbial

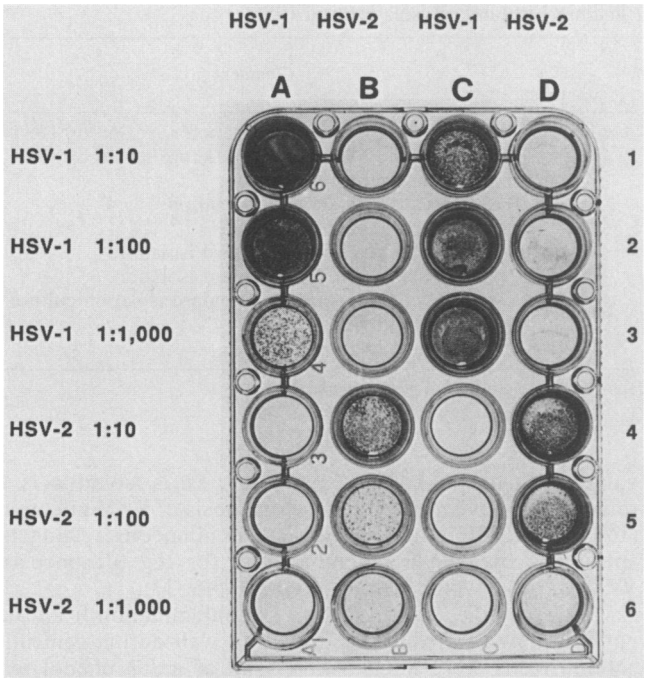


FIG. 1. Representative plate fixed and stained 18 h after inoculation with HSV-1 and HSV-2 reference viruses in three 10-fold dilutions and with six positive clinical specimens. Dilutions of reference viruses (columns A and B) and clinical specimens (columns C and D) were inoculated to duplicate wells and stained with an HRPO-labeled monoclonal antibody against HSV-1 (columns A and C) or HSV-2 (columns B and D). Specimens 1, 2, and 3 are strongly positive isolates of HSV-1, and specimens 4 and 5 are strongly positive and specimen 6 is a weakly positive isolate of HSV-2. Faint background staining in column D of specimens 1 to 3 is due to nonspecific precipitation of chromogen during prolonged storage of the plate before the photograph was taken.

contamination. If the evaluation is restricted to those 269 specimens that were studied by both methods without prior freezing, 87 of the 89 specimens found positive by standard isolation were revealed by IPS, showing a test sensitivity of 97.8%.

Standard isolation cultures were microscopically observed for the occurrence of typical CPE three times a week. For 107 specimens found to be positive by standard isolation, the average time elapsing between inoculation and reporting as HSV positive was 3 to 4 days (range, 1 to 8 days; Fig. 2).

**IPS after a reduced period of incubation.** Cultures inoculated with 42 specimens known to be HSV positive were incubated for 7 or 17 h before fixation and staining. Strongly positive specimens could be detected macroscopically, whereas weakly positive specimens were only detected by microscopic examination. It was possible to identify all specimens as positive without microscopic examination after overnight incubation.

TABLE 2. Comparison of standard isolation with IPS for 289 clinical specimens

| IPS result | Standard isolation result (no. of specimens) | | | |
|---|---|---|---|---|
| | Negative | HSV-1 positive | HSV-2 positive | NA[a] |
| Negative | 176[b] | 0 | 3 | 2 |
| HSV-1 positive | 0 | 24 | 0 | 1 |
| HSV-2 positive | 1 | 0 | 81 | 0 |
| NA | 0 | 1 | 0 | 0 |

[a] NA, Result not available due to microbial contamination of the cultures.
[b] VZV was found in two specimens by standard isolation.

## DISCUSSION

The findings of the present study indicate that IPS of infected cell cultures is a rapid, sensitive, and simple method for the laboratory diagnosis of HSV infections. Centrifuged inoculation of clinical specimens enhances the sensitivity of HSV isolation (3, 10). During a previous study of 441 consecutive specimens sent to this laboratory for isolation of HSV, 118 specimens were found to be positive without centrifugation, and with centrifuged inoculation 18 additional specimens were found to be positive, an increase of 15.3% (T. Ziegler, unpublished data). When the cultures were checked microscopically three times a week, on an average 3 to 4 days elapsed between inoculation and a positive result, the range being 1 to 8 days. The purpose of the present study was to find a method for the diagnosis of HSV infections which would have the sensitivity of virus isolation with centrifuged inoculation but give a definite result within 24 h of the time when the specimen was received at the laboratory.

Direct staining of infected cells on smears can be performed within 1 or 2 h. Reading and interpretation of such smears require skill and experience, and the technique is less sensitive than virus isolation (6, 11). With clinical specimens, immunological staining of cultured HSV-infected cells 1 to 2 days after inoculation has been found in recent studies (5, 10, 15) to be a convenient method for the diagnosis of HSV infections. Depending on the cell line and on the immunoreagents used, IPS had a sensitivity of between 70 and 100% compared with standard virus isolation (15). This is on a par with the 98% sensitivity we found for specimens studied by either method without prior freezing. In the present study, three specimens found to be positive by standard isolation proved to be negative when tested by IPS. Two of these had been stored frozen at −70°C for several weeks before testing by IPS, whereas the third was a fresh specimen. All three specimens were found to be positive in only one of the two standard isolation tubes inoculated, and CPE was not identified until 6 days after inoculation, indicating that all three specimens originally contained minimal amounts of infectious virus. Inactivation of virus by freezing and thawing and also the lower centrifugal forces used during inoculation for IPS (12) may explain these false-negative results. One specimen was found to be positive by IPS, showing a few distinct plaques in the well stained with the HSV-2-specific monoclonal antibody, but it was found to be negative by standard isolation. None of the original material was left for reinoculation, but as the patient had a clinical diagnosis of genital herpes infection, the result obtained with IPS could be regarded as a true-positive.

Contamination of cultures with yeasts or bacteria is a common problem in virus isolation. In the present study, inconclusive results due to microbial contamination were obtained from four specimens, two of which were found to be HSV positive by either standard isolation or IPS. Contamination was seen three times in standard isolation cultures but only once in IPS, indicating that contaminants probably derive from laboratory manipulations rather than from the specimen itself, since in IPS the inoculum was not removed after centrifugation. Reducing the incubation period from a maximal 10 days to 18 h also minimizes the risk of contamination.

TABLE 3. Specimens producing discrepant findings by standard isolation and IPS

| Specimen no. | Result | | Comment |
|---|---|---|---|
| | Standard isolation | IPS | |
| 1 | HSV-2 | Negative | False-negative by IPS; frozen before tested by IPS[a] |
| 2 | HSV-2 | Negative | False-negative by IPS; frozen before tested by IPS[a] |
| 3 | HSV-2 | Negative | False-negative by IPS[a] |
| 4 | Negative | HSV-2 | False-negative by standard isolation |
| 5 | HSV-1 | Cont.[b] | Bacterial contamination in IPS |
| 6 | Cont. | HSV-1 | Yeast contamination in standard isolation |
| 7 | Cont. | Negative | Yeast contamination in standard isolation |
| 8 | Cont. | Negative | Bacterial contamination in standard isolation culture |
| 9 | VZV | Negative | Not detected by IPS |
| 10 | VZV | Negative | Not detected by IPS |

[a] Only one of the two tubes inoculated was positive; all three specimens were reported positive on day 6 after inoculation.
[b] Cont., Contaminated.

Plaques of infected cells became large enough during overnight incubation to be detected macroscopically after staining with HRPO-labeled monoclonal antibodies (Fig. 1). When using illumination from a light box, plaques consisting of as few as three to five cells were clearly visible. Microscopic observation of the wells did not reveal any further positive specimens. Brief centrifugation of the clinical specimen prior to inoculation removed cellular and other debris that might cause unspecific staining. Background staining of uninfected cells was minimal and usually only occurred several hours after substrate and chromogen had been added to the cultures. As we have shown earlier (18), the two monoclonal antibodies used in the present study are highly specific for either HSV-1 or HSV-2. IPS makes possible the unambiguous identification and differentiation of positive cultures, and the test can easily be read without special training. Even after the incubation period was reduced to 8 h, all of the 42 known positive specimens studied were found to be positive. Specimens arriving at the laboratory late in the afternoon can still be inoculated the same day, and the results are available early the following day.

From two specimens, varicella-zoster virus (VZV) was isolated by the standard technique. It still remains to be studied whether the introduction of a monoclonal antibody specific for VZV would significantly improve the diagnostic value of this test. In our experience, virus isolation is a rather insensitive method for the diagnosis of VZV infection (16). For a differential diagnosis, simultaneous testing of specimens by enzyme immunoassay for the presence of VZV antigens would be more reliable (16, 17).

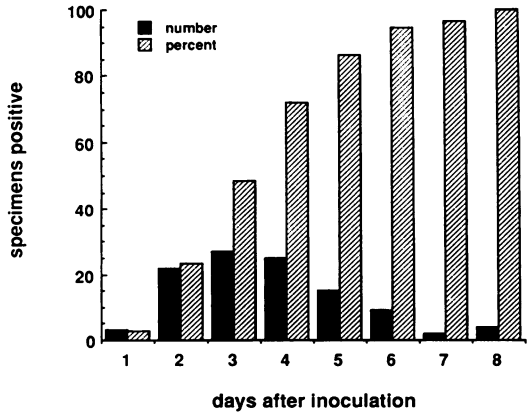


FIG. 2. Number and cumulative percentage of specimens reported positive by standard isolation by day after inoculation. For 107 specimens found to be HSV positive by standard isolation, the time between inoculation and recognition of CPE typical for HSV was recorded. Cultures were observed microscopically three times a week.

We also studied the risk of cross-contamination from the possible flow of medium from well to well during centrifugation. Wells were filled with 1.5 ml of a 1% phenol red solution, and a sheet of filter paper was placed over the wells before the cover was closed. No traces of stain could be detected on the filter paper after centrifugation of the plate as described in Materials and Methods (results not shown). Plates with 24 wells are convenient for routine use. Especially in a laboratory receiving a large number of specimens daily, such plates are time-saving and cost effective compared with shell vials (14) and also allow the economical use of reagents.

The test described here could be further simplified by using a type-common monoclonal antibody or by pooling the two monoclonal antibodies used in the present study. The clinical course of infection and the prognosis are, however, often influenced by the serotype (2, 4, 9), so that typing of isolates is desirable. Cutting down the time required for getting a definite result from HSV isolation from up to 10 days to less than 24 h meets the need for rapid diagnosis of HSV infections in immunocompromised patients, pregnant women, and newborns (1, 2, 7, 8).

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