Retrospective Evaluation of the Isolation and Identification of Herpes Simplex Virus with Cultureset and Human Fibroblasts

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A total of 442 specimens from various anatomic sites was cultured for herpes simplex virus during the past 2 years. Most specimens were obtained from the respiratory tracts and cutaneous lesions of immunocompromised hosts (232 specimens) or the female genital tract (138 specimens). Two tubes containing human newborn foreskin fibroblasts and two Ortho Cultureset tubes containing Vero cells were inoculated with each specimen. The 384 inoculated specimens were stained with Cultureset peroxidase-antiperoxidase reagents within 48 h and again at 3, 4, or 5 days if initially negative. Fibroblasts were inspected for cytopathic effect for 7 days. Of these 384 specimens, Cultureset detected 87 of 62 positive specimens within 48 h; fibroblasts detected 58 positive specimens by 7 days. The calculated sensitivity and specificity for Cultureset at 48 h were 91.9 and 100%, respectively. However, when all results were considered, including those that became positive in Cultureset after 48 h, the calculated sensitivity and specificity for Cultureset were 98.8 and 100%, respectively. We conclude that Cultureset is a reliable method for detection of herpes simplex virus when two tubes are inoculated and stained as described.

Until recently, the only alternatives available to most clinical laboratory for virus isolation and identification were to institute full-scale virology services or to send specimens to the nearest reference laboratory which offered virology services. Herpes simplex virus (HSV) is the most commonly isolated viral agent. The increasing number of immunocompromised patients has made this agent of clinical importance in other than sexually transmitted diseases. The advent of antiviral therapy has added impetus to the importance of its rapid diagnosis. It is unrealistic, however, to invest the initial capital outlay in space, equipment, and personnel necessary for a clinical virology laboratory for the isolation of a single agent. On the other hand, transport of specimens to an outside reference laboratory often leads to a delay in reporting of results and unsatisfactory recovery rates.

A culture system for HSV isolation and confirmatory staining is available from Ortho Diagnostics, Inc., Carpenteria, Calif., which can be utilized in most hospital microbiology laboratories. Previous reports (2, 5, 12, 13) suggested shortcomings of this system, called Cultureset (CS). In view of these reports, we have retrospectively evaluated our experience with CS. This report is derived from the results obtained from 442 clinical specimens submitted to St. Luke's Pathology Laboratory for isolation of HSV during the period from 1 January 1983 through 7 January 1985.

MATERIALS AND METHODS

Processing and inoculation. Specimens collected on swabs were placed in viral transport medium obtained from Bartels Immunodiagnostics, Bellevue, Wash. This transport medium contains minimal essential medium, fetal bovine serum, carbon and nitrogen sources, and gentamicin, streptomycin, penicillin, and amphotericin B as antibacterial and antifungal agents. Specimens were mixed on a Vortex apparatus for 30 s, and when the swab was present in the transport medium, it was removed after expressing excess liquid on the side of the transport tube. Specimens containing visible debris were centrifuged at 800 × g for 10 min. Portions (0.5 ml) of respiratory specimens were placed in transport medium after receipt in the laboratory and then placed in the refrigerator for 2 to 3 h in an effort to minimize contamination by the action of the antibiotics contained in the transport medium. These specimens were then mixed on a Vortex apparatus and treated as described above. Tissue specimens were homogenized in either saline or viral transport medium by the Stomacher 80 (Fisher Scientific Co., Pittsburgh, Pa.). Body fluids from normally sterile body sites were inoculated directly.

The holding medium was removed from two tubes containing human newborn foreskin fibroblasts (Whittaker M. A. Bioproducts, Walkersville, Md.), and each was inoculated with 0.2 ml of specimen. The tubes were incubated for 1 h at 35°C to allow viral particles to adsorb. Minimal essential medium (2 ml) containing 2% fetal bovine serum and antibiotics was then added to each tube. The tubes were incubated for 7 days and examined for cytopathic effect (CPE) typical of HSV.

Holding medium (8 ml) was removed from two CS tubes containing Vero cells. The CS tubes were Leighton tubes containing a removable paddle, and the Vero cells were grown on the paddle. A 0.6-ml volume of inoculum was added to each tube, and the tubes were incubated at 35°C. For 384 specimens, one tube was fixed and stained either at 24 or 48 h, and the second tube was fixed and stained at 3 to 5 days if the first tube was negative. An additional 58 specimens were not stained until after 3 to 4 days due to weekends which intervened between the time of inoculation and staining. The second tube inoculated with these specimens was stained at 5 to 6 days if initially negative.

Staining procedure. The peroxidase-antiperoxidase reagents supplied by Ortho Diagnostics were used to stain the Vero cells. Eight milliliters of 10% buffered Formalin (pH 6
to 7) was added to each tube, and the tubes were incubated at room temperature for 30 min. The paddles containing the Vero cells were removed and washed by dipping three times in a large beaker containing 400 ml of water. After being washed, the paddles were placed on a staining plate (Ortho) prewarmed to a temperature of 35°C, and 5 drops of the primary antibody (rabbit anti-HSV) was added to cover the surface of the paddles. The paddles were incubated for 10 min and washed three times as described. Five drops of the second reagent (sheep anti-rabbit immunoglobulin) was added, and the paddles were incubated for 5 min and washed. Five drops of the labeling reagent (peroxidase-antiperoxidase) was then added to the paddles, and they were incubated for another 5 min and washed. Five drops of freshly prepared color developer was added, and the paddles were incubated for 10 min and washed again. The counterstain was added, and the paddles were incubated for 3 min and washed. The paddles were flooded with bluing agent (Richard-Allan Medical Industries, Richland, Mo.) and mounted on glass microscope slides with cell-side down. Slides containing the paddles were scanned under low power (×100) to find characteristic red-staining foci of virus-infected cells. When the intensity of reaction was graded for both the CS system and fibroblasts, the following guidelines were used: 1+, less than 25% of the cell layer showed typical CPE or staining reaction; 2+, 25 to 50% of the cell layer demonstrated typical CPE or staining for HSV; 3+, 50 to 75% of the cell layer showed typical CPE or staining; and 4+, greater than 75% of the cell layer demonstrated typical CPE or staining.

The method used for comparing the two systems was that described by Galen. (3) Isolation in either system was used to determine the number of true positive specimens.

RESULTS

The highest number of specimens, 139, came from the respiratory tract and included bronchial washings, throat swabs, mouth lesions, and lung tissue, taken primarily from immunosuppressed patients (heart transplants) and newborn infants. The 93 cutaneous specimens were also primarily from immunocompromised patients. The rates of recovery from these specimens were 14.0% (respiratory) and 17.2% (cutaneous). Of 29 specimens from the central nervous system, 2 (two brain biopsies) were positive, giving a recovery rate of 6.9%. Rates of recovery from specimens taken from the male (24 specimens) and female (138 specimens) genital tract were 58.3 and 23.9%, respectively.

According to the Ortho protocol for CS, the Vero cells should be stained with the peroxidase-antiperoxidase stain within the first 48 h after inoculation. However, this was not always possible since some specimens were received and inoculated on Thursday or Friday, in which case they most often were not observed until the following Monday. Thus we present our data in two ways: one based on results obtained according to the recommendation of the manufacturer and one according to the overall rates of recovery. Of the total 442 specimens, 384 were stained within 48 h. Of these there were 62 positive specimens and 322 negative specimens (Table 1). CS demonstrated a sensitivity and a specificity of 91.9 and 100%, respectively, at 48 h. The predictive value of a positive test was 100%, and that for a negative test was 98.5%. The calculated sensitivity and specificity for CPE in fibroblasts by 7 days were 93.6 and 100%, respectively. The predictive value of a positive test was 100%, and that for a negative test was 98.8%. The agreement between the two methods (97.7%) was statistically significant (Kappa = 0.9096, Z = 17.82, P < 0.0001) by a one-tailed Z test.

Of the five specimens which were negative in CS at 48 h but positive in the fibroblasts by 7 days (Table 1), four became positive in CS at 4 days. In addition, there were 58 specimens which were first stained after 3 to 4 days (after a weekend) and again at 5 to 6 days if initially negative. Table 1 shows the results when the overall rate of recovery of both systems within 7 days was considered. CS detected 84 of 85 positive specimens (98.8% recovery), and fibroblasts detected 81 of 85 positive specimens (95.3% recovery). With these data, the sensitivity of CS was raised from 91.9 to 98.8%. The specificity was 100%, the predictive value of a positive result was 100%, and the predictive value of a negative result was 99.7%. The calculated sensitivity and specificity of CPE in fibroblasts for all specimens were 95.3 and 100%, respectively. The predictive value of a positive result was 100%, and that for a negative result was 98.9%. The overall agreement between the two methods (98.9%) was statistically significant (Kappa = 0.9638, Z = 20.28, P < 0.0001) by a one-tailed Z test.

Of the 85 positive specimens, 41 were graded according to intensity of reaction. The intensity of staining in CS was equal to that of CPE observed in fibroblasts in 19 (46.3%) of these positive specimens. Stronger reactions were observed in CS for 17 (41.5%) of the positive specimens, while the reaction intensity was stronger in fibroblasts for five specimens (12.2%).

DISCUSSION

Of the 442 specimens cultured for HSV, most came from immunocompromised hosts. Our total rate of recovery and the rate of recovery from specimens of the genital tract were similar to those reported by other workers (1, 2, 8). There was overall agreement between the results in fibroblasts and in CS in all but five instances. Four of these were considered to be false-negative reactions in the fibroblasts, two of which were verified by concomitant culturing in another laboratory. The other two results were considered to be falsely negative on the basis of a clinical presentation which was highly suggestive of HSV infection. The fifth disagreement was a false-negative reaction in CS verified by subculturing from the positive fibroblast tube into a CS tube and subsequent staining at 48 h. Hence, of the verified positive specimens, CS detected 98.8%, and fibroblasts detected 95.3%.

When we considered the 384 specimens stained within 48 h and compared the data in terms of what is claimed by the manufacturer, the results were somewhat different. There has also been a discrepancy between the claim of the manufacturer and the results of other investigations (2, 5, 12,
VOL. 22, with 66 positive specimens, there was 94% correlation between CS and standard tissue culture techniques at 24 h and 98% correlation at 48 h. However, the standard tissue culture technique used as the reference method is not described.

The techniques which have been used by investigators as the reference method to which CS was compared have varied in terms of the cell lines used, the number of replicate tubes set up, the amount of inoculum used, and the time period during which the tissue culture tubes were incubated. Fayram et al. (2) inoculated three MRC-5 human embryonic lung fibroblast tubes and three cynomologous monkey kidney cell tubes with 0.25 ml of inoculum, incubated the tubes for up to 10 days, and confirmed the presence of HSV by immunofluorescence. The results observed in those tubes were compared with either a single CS tube inoculated with 0.3 ml of specimen which was stained at 48 h or with two tubes inoculated with 0.6 ml of inoculum and stained at 48 and 72 h. Of 54 positive specimens detected by tissue culture, 19 were interpreted as false-negative cultures in CS at 48 h. Hayden et al. (5) used two MRC-5 human embryonic lung fibroblast monolayers inoculated with 0.2 ml of the specimen and incubated for 1 week as the standard method. They compared results obtained in those tubes to a single CS tube stained at 24 h. Of 63 culture-positive specimens in MRC-5, 17 were interpreted as false-negative reactions in CS. No confirmatory stain was used for the MRC-5 culture tubes. Sewell et al. (13) used one tube of human fibroblasts, type unspecified, inoculated with 0.5 ml of specimen and incubated for 1 week as the standard reference method. The presence of HSV in the fibroblasts was confirmed by passing to a CS tube and staining with the CS reagents. They compared the results of this method with the results in one CS tube inoculated with 0.5 ml and stained at 48 h. Of 72 positive specimens, 15 were interpreted as false negatives in CS at 48 h. Rubin ad Rogers (12) used two primary rabbit kidney cell tubes inoculated with 0.3 ml of specimen and incubated for 1 week as the reference method. Two CS tubes were inoculated with 0.6 ml of specimen and stained at 24 and 48 h. CS performed better than rabbit kidney cells at 24 h, but there were 12 specimens interpreted as false negatives in CS at 48 h compared with the 1-week culture results. All of these investigators reported 100% recovery of HSV in standard cell cultures and suggested that CS is not an adequate substitute for standard virus isolation or recommended that CS not be used as the only method for recovery of HSV.

Results of the present study correlate more closely with the sensitivity at 48 h stated in the package insert than those previously published. It is conceivable that differences in source of specimens and minor alterations in specimen handling and procedures could account for this result.

Discrepancies between the claims of the manufacturer and observations made by other investigators should not discount the contribution CS can make toward HSV detection in clinical specimens. When the overall results of the specimens we examined were considered (all CS results between 24 h and 6 days), we found that CS performed as well as or better than the fibroblasts with regard to the number of positive specimens detected, the time of detection, and the intensity of positive reactions. The positive specimens that were missed completely by fibroblasts were all detected in CS by 48 h (Table 1). One of these was a specimen from the female genital tract, two were brain biopsies, and one was from a neck wound of an oncology patient. The need for accurate and rapid diagnosis in these kinds of patients is paramount since the management of pregnant females can be influenced by HSV detection in the birth canal (7), and the decision to use potentially toxic but lifesaving antiviral drugs in seriously ill patients will also be influenced by HSV detection (10). The fact that HSV was not recovered from these specimens in the fibroblasts can probably be explained by the differences in inoculum size, since the CS tubes received 0.6 ml of inoculum as compared with the 0.2 ml used for fibroblasts. The presence of a low number of viral particles can probably explain the one false-negative result observed in CS. In addition, it should be pointed out that one CS tube inoculated with that specimen (ear swab from a newborn) had been stained at 24 h and the other at 48 h. Thus, there were not any CS tubes to be stained after 48 h.

The goal in detecting HSV is to have a rapid, reliable, simple, and inexpensive test which will provide results within a time period that will be clinically useful. Direct examination of clinical specimens is a reasonable alternative to culture if one is dealing with specimens likely to harbor many viral particles (4, 9, 11). However, the number of viral particles in asymptomatic females and immunosuppressed patients may not always be great enough to allow virus detection by a direct test, and so cultivation is still the most sensitive method. A second approach is to use the most sensitive cell line available which allows the quickest detection time of the virus. Some investigators (12) have suggested that primary rabbit kidney cells are more sensitive than Vero cells, while other workers (6) have presented data demonstrating a higher sensitivity of primary rabbit kidney cells over that of various fibroblast cell lines. Other reports have shown no significant differences in rates of HSV recovery in fibroblasts and rabbit kidney cells (1, 8).

The inoculation procedure for the CS system is very simple and requires only centrifugation and pipetting. We consider the staining procedure to be technically simple, but it requires about 35 min of concentrated attention. The interpretation of the stained monolayer of cells is also not very difficult. One needs only to be able to differentiate precipitated stain which is not cell associated from the usually well-formed foci of cells which stain red due to HSV antigen within the cells.

The major disadvantage of the CS system is its cost. At the time of our studies, the cost of two culture tubes and staining reagents was $17.80. The cost of two fibroblast tubes without confirmatory staining was $3.80. However, the fact that the CS system includes a simple confirmatory staining method is

| Table 2. Summary of reported sensitivity of HSV detection by conventional cell culture and CS methods |
|--------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Investigator | 24 h with: | 48 h with: | 1 week with |
|              | Cell culture | CS | Cell culture | CS | cell culture |
| Ortho insert | ND* | 94 | ND | 98 | 100 |
| Fayram et al. | ND | ND | ND | 64.8 | 100 |
| Hayden et al. | 49.2 | 73 | 87.3 | ND | 100 |
| Sewell et al. | ND | ND | ND | 79.2 | 100 |
| Ruben and Rogers | 45.5 | 56.4 | 83.6 | 78.2 | 100 |
| Phillips et al. | ND | ND | ND | 91.9 | 93.6 |

* ND, Not done.
one reason why we chose the CS system to help us initiate virology services for HSV detection. CPE alone is not adequate for identification of HSV isolates.

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LITERATURE CITED