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Comparison of Viral Isolation, Direct Immunofluorescence, and Indirect Immunoperoxidase Techniques for Detection of Genital Herpes Simplex Virus Infection

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Seventy-six consecutive patients presenting to a genital herpes simplex virus (HSV) clinic were enrolled in a study comparing viral isolation (VI), indirect immunoperoxidase (indirect IP), and direct immunofluorescence (direct FA) techniques for the detection of HSV antigen. Of the 76 patients, 61 (80%) demonstrated HSV by VI, compared with 66% by indirect IP and 55% by direct FA ($P < 0.05$). Genital lesions from nine patients demonstrated HSV antigen by direct FA or indirect IP but were VI negative; eight of nine patients had subsequent episodes of genital HSV confirmed by VI. During the vesicular-pustular stage of the disease, VI was positive in 90%, indirect IP was positive in 76%, and direct FA was positive in 71% of the lesions, whereas with ulcerative lesions, VI was positive in 72%, indirect IP was positive in 55%, and direct FA was positive in 38%. These commercially available rapid viral diagnostic techniques are specific and useful, if adequate specimens are obtained from early genital lesions.

Genital herpes simplex virus (HSV) infection is a disease of increasing incidence and growing importance. At the Harborview Medical Center Sexually Transmitted Disease (STD) Clinic during 1976-77, of 24,758 patient visits, *Neisseria gonorrhoeae* infection was diagnosed in 11.4%, and genital HSV infection was diagnosed in 5.0%, 1 case of HSV to every 2.2 cases of *N. gonorrhoeae*. The morbidity of the illness, the occasional complications such as aseptic meningitis (9) and sacral radiculopathy (6), the high rate of recurrence (8), the devastating effects on the neonate (13, 16), and the epidemiological association with cervical carcinoma (15, 18) make genital HSV a disease of major public health importance.

Although no effective therapy for genital HSV presently exists, a simple, rapid, and reliable diagnostic test would be a valuable clinical tool for several reasons: (i) the patient should be informed that he or she has the disease and be instructed as to how to minimize the chances for its spread; (ii) in view of the association of HSV infection and cervical carcinoma (15, 18), female patients should be advised to obtain regular Papanicolaou smears (19); and (iii) a rapid diagnostic test to detect the presence of cervical HSV infection at the onset of labor would be useful in determining whether vaginal delivery or caesarean section should be performed in a woman having a history of recurrent genital disease (12).

Although viral isolation (VI) is sensitive and specific, it is not widely available. Three available methods for the rapid diagnosis of HSV infection are (i) cytology (Papanicolaou or Tzanck preparations) (17), (ii) direct immunofluorescence (direct FA) (5, 11), and (iii) indirect immunoperoxidase staining (indirect IP) (3). A commercially available indirect IP test for detecting HSV antigen was developed in 1976 (G. Dolana, E. Kern, Z. Brown, J. C. Overall, manuscript in preparation). As part of a multicenter evaluation of this product, we compared the results of indirect IP testing, VI, and cytological examination in 103 patients who presented with suspected genital herpes. HSV was isolated from 80.6% of these patients and was detected by indirect IP in 57.3%, and Papanicolaou smears from genital lesions revealed giant cells or cells with intranuclear inclusions in 37.6% of specimens (L. Corey, unpublished data). These results were similar to those obtained in four other centers enrolling an additional 352 patients with

oral-labial or genital HSV infections—75.4, 54.2, and 47.9% for VI, indirect IP, and cytological examination, respectively (M. Caputo, personal communication). As an extension of these observations, we desired to compare in a different patient population the sensitivity and specificity of the indirect IP test with the direct FA test for the rapid detection of genital HSV infection and to determine whether the stage of the lesion influenced the sensitivity of these tests.

MATERIALS AND METHODS

Clinical population. Seventy-six patients with suspected genital HSV infection who were referred to the University of Washington Genital HSV Clinic were enrolled in the study. At the initial visit, a standardized interview and genital examination were performed. If vesicular or pustular lesions were present, the lesions were opened with a 27-gauge needle, and the fluid was collected for VI. The bases of all suspected lesions were then vigorously rubbed with a calcium alginate swab (Inolex Co., Glenwood, Ill.). The exfoliated cells obtained were smeared onto two glass slides which were then fixed in cold (4°C) acetone. Thirty-two women who presented with initial genital infection also had duplicate slides made from exfoliated cervical epithelial cells, as well as a swab of the cervix submitted for VI. As a control group, cervical specimens were collected from 40 women attending the Genital HSV Clinic with a past history of laboratory-confirmed HSV but with no evidence of current active lesions (intercurrent HSV). Duplicate slides were made, and VI specimens were submitted in the same way as outlined for the experimental group.

Processing of slides. After fixing, the slides were transported to the laboratory and stored at −20°C until staining was performed. One slide from each pair was tested by direct FA, and the other was tested by indirect IP. For the direct FA method, fluorescein isothiocyanate-conjugated HSV type 1 (HSV-1) and type 2 (HSV-2) antisera (rabbit) were purchased from Flow Laboraotries, Inc., Rockville, Md. One lot of each (no. 45607107 for HSV-1 and no. 45677208 for HSV-2) was used throughout the study. Staining procedures followed the manufacturer's recommendations, and 1:10 working dilutions were used for both conjugates. All runs included a positive and negative control slide made from suspensions of HSV-infected and uninfected diploid fibroblasts, respectively. Slides were read the day of staining with a Zeiss fluorescence microscope using epiillumination at 400×.

The indirect IP reagents were obtained from Hyland Laboratories and used according to the manufacturer's instructions. Slides were rehydrated in phosphate-buffered saline, incubated with a bivalent, i.e., HSV-1 and HSV-2 rabbit antiserum (undiluted), and, after a rinse, incubated with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G. After rinsing in phosphate-buffered saline, an immunoperoxidase substrate-hydrogen peroxide mixture was added. After a final rinse in phosphate-buffered saline, slides were mounted and scanned at 200×, with individual cells inspected at 400× by standard light microscopy. All slides were stained and read without knowledge of their source or the results of the duplicate slide or virus culture.

Grading of slides. All slides were read by one person and graded by the following criteria: (i) the presence or absence of greater than 25 exfoliated epithelial cells per smear, and (ii) the quality of the staining. Staining quality was judged as follows: 4+ cells showed nuclear and surface staining of high intensity (apple green with direct FA and dark red with indirect IP); 3+ cells displayed strong cytoplasmic rimming, but only faint nuclear staining; 2+ cells showed distinct rimming only; 1+ cells showed faint rimming. Only slides containing cells graded 2+ or greater were considered positive for HSV antigen.

VI. Samples (0.2 ml) of the specimen in transport medium (sucrose-phosphate buffer with mycostatin and gentamicin) were inoculated into duplicate tubes of diploid fibroblast tissue culture within 4 h of collection. The tubes were read at 2-day intervals, and all specimens exhibiting characteristic cytopathic effect were passed into HeLa M or Vero cells to confirm the presence of HSV (22). Positive isolates were harvested, and 64 were typed by an indirect IP technique (4), using a type-specific rabbit antiserum (4, 8) made in the laboratory of E. R. Alexander. A total of 55 specimens were typed as HSV-2, and 9 were typed as HSV-1.

Statistical analysis. Statistical evaluation was performed using a chi-square test.

RESULTS

Genital lesions. Of the 76 specimens obtained from genital lesions from patients with suspected genital HSV infection, HSV was isolated in 61 (80%). Indirect IP slides were positive in 50 (66%), and exfoliated cells demonstrated HSV antigen by direct FA in 42 (55%) ($P < 0.05$, VI versus indirect IP or direct FA or both).

The relative sensitivities of these immunological techniques to VI for specimens obtained from genital lesions, as well as cervical scrapings, are summarized in Table 1. Adequate smears, that is, those containing more than 25 exfoliated cells per preparation, were available on 95% of the slides from VI-positive genital lesions. HSV-infected exfoliated epithelial cells were detected by the indirect IP and direct FA tests in 46 (79%) and 41 (71%), respectively, of 58 cases.

The most important determinant of the ability to detect HSV or antigen was the stage of the lesions at the time of sampling. Clinical description of the stage of the lesion at sampling was available for 71 (93%) of 76 patients (Table 2). In the vesicular or pustular stages of the disease, HSV was isolated from 90% of the lesions, whereas only 21 (72%) of 29 ulcerative lesions had HSV isolated ($P < 0.05$). The indirect IP test detected HSV antigen in 76%, and the direct FA test detected it in 71% of vesicular-pustular lesions. In contrast, the indirect IP test detected

TABLE 1. *Comparison of indirect IP and direct FA with VI in genital HSV infection*

Type of lesion	Assay with all specimens		Assay with adequate specimens[a]	
	IP(+)/isolate(+)	FA(+)/isolate(+)	IP(+)/isolate(+)	FA(+)/isolate(+)
Male genital	22/27 (81%)	17/27 (63%)	22/26 (85%)	17/24 (71%)
Female genital	24/34 (71%)	24/34 (71%)	24/32 (75%)	24/34 (71%)
Female cervical	11/21 (52%)	10/21 (48%)	11/21 (52%)	10/21 (48%)

[a] Slides containing greater than 25 exfoliated cells.

TABLE 2. *Sensitivity of VI, indirect IP, and direct FA according to clinical stage of HSV lesions*

Source of HSV lesion in:	VI [no. positive (%)]	IP [no. positive (%)]	FA [no. positive (%)]
Initial disease patients			
Vesicle (n = 10)	10 (100)	9 (90)	8 (80)
Pustule (n = 9)	8 (89)	6 (67)	6 (67)
Ulcer (n = 22)	18 (82)	14 (64)	9 (41)
Recurrent disease patients			
Vesicle (n = 20)	18 (90)	14 (70)	15 (75)
Pustule (n = 3)	2 (67)	3 (100)	1 (33)
Ulcer (n = 7)	3 (43)	2 (29)	2 (29)
Total			
Vesicle (n = 30)	28 (93)	23 (77)	23 (77)
Pustule (n = 12)	10 (83)	9 (75)	7 (58)
Ulcer (n = 29)	21 (72)	16 (55)	11 (38)

HSV antigen in 55%, and the direct FA test detected it in 38% of specimens obtained from herpetic lesions in the ulcerative stage of disease.

Specimens obtained from patients with initial genital herpes demonstrated HSV by either VI, direct FA, indirect IP, or all three more frequently than did specimens collected from patients with a history of previous genital lesions. HSV was isolated from genital ulcers from 18 (82%) of 22 persons having their first episode of HSV infection compared, with 3 of 7 specimens from genital ulcers collected from patients with recurrent genital disease ($P = 0.05$). Indirect IP and direct FA demonstrated HSV antigen in 64 and 41%, respectively, of ulcers from initial-disease patients compared with a 29% positivity rate with both immunological tests for specimens obtained from ulcers from patients with recurrent genital herpes.

Cervical specimens. Thirty-two women with initial genital herpes had specimens obtained from both vulvar and cervical sources. HSV was isolated from vulvar lesions in 26 (81%) and from cervical cultures in 21 (66%). Virus was isolated only from the cervix in 2, only from the vulva in 7, from both sites in 19, and from neither site in 4 (three of these four patients subsequently demonstrated HSV in specimens obtained 1 to 2 days later; the fourth seroconverted to HSV by both complement fixation (21) and plaque reduction neutralization assays (14). HSV antigen was detected in the cervical specimens by indirect IP in 14 (44%) and by direct FA in 12 (38%). In the 21 VI-positive cervical specimens, HSV antigen was demonstrated by indirect IP in 11 (52%) and by direct FA in 10 (48%) (Table 1). Among the 11 women from whom HSV was not isolated from the cervix, HSV antigen was detected in exfoliated cervical cells by indirect IP in 3 and by direct FA in 2. Both methods were negative in six patients. Of the five patients whose cervical specimens were VI negative but whose exfoliated epithelial cervical cells demonstrated HSV by direct FA or indirect IP, four had the virus isolated from cervical specimens at another time during the same clinical episode (range, 1 to 10 days).

Cervical specimens in intercurrent herpes. The 40 patients in the control group were seen when neither signs nor symptoms of genital herpes were apparent to the patient or the examiner. The cervix appeared normal on inspection in all patients, and HSV was not isolated from the vulva or cervix in any of these 40 cases. One individual demonstrated HSV-infected cells by both direct FA and indirect IP techniques, despite a negative VI; three women each had a cervical specimen that was positive by direct FA but negative by indirect IP, and one had a positive indirect IP but negative direct FA slide. These six slides were considered false-positives.

Concordance between indirect IP, direct FA, and VI. The overall concordance (genital and cervical results grouped together) between VI and direct FA was 74% (110 of 148), and between VI and indirect IP it was 77% (114 of 148) (Table 3). Of 26 persons with suspected genital HSV infection in which HSV was not detected by VI, 9 (35%) demonstrated HSV antigen in exfoliated epithelial cells. Eight of these nine persons has HSV isolated from subsequent episodes of genital lesions.

Predictive value. The overall predictive value of HSV being isolated from a genital or cervical lesion that was either indirect IP or direct FA positive was 86 and 88%, respectively (Table 4). The predictive value of a positive

TABLE 3. *Concordance between VI, direct FA, and indirect IP*

Assay	No. (%) of specimens in which HSV was isolated	No. (%) of specimens in which HSV was not isolated
IP+ FA+	43 (52)	2 (3)
IP+ FA−	14 (17)	7 (11)
IP− FA+	8 (10)	5 (8)
IP− FA−	17 (21)	52 (79)

TABLE 4. *Predictive value of indirect IP and direct FA in relation to viral isolation*

Patient population	Predictive value (%) of positive test with:		Predictive value (%) of negative test with:	
	IP	FA	IP	FA
Patients with genital lesions	92	94	42	41
Cervical specimens in women with initial genital disease	76	83	44	43
Cervical specimens in women with initial and intercurrent HSV	69	67	80	79
Cervical specimens in patients attending an STD clinic with no evidence of HSV	100	ND[a]	100	ND
All genital and cervical specimens in patients with suspected genital HSV	86	88	70	66

[a] ND, Not determined.

direct FA or indirect IP test from a genital lesion was 92 and 94%, respectively. In our patients with genital lesions, however, the predictive value of a negative test was low: 42% for indirect IP and 41% for direct FA, presumably because patients were selected for a high likelihood of having genital HSV infection.

Relationship between virus type and detection of HSV antigen. In the 54 direct FA-positive slides from genital or cervical lesions or both, 2+ to 4+ fluorescence was seen in 37 (69%) specimens with HSV-1 conjugate and in 46 (85%) specimens with HSV-2 conjugate. In 19 (35%) of the smears, 2+ to 4+ fluorescence for HSV antigen was seen with only 1 of the 2 direct FA antisera. In 28 slides obtained from patients with documented HSV-2 isolates, the direct FA conjugate was positive only with the HSV-1 conjugate in 3 (11%), with the HSV-2 conjugate alone in 10 (36%), and with both conjugates in 15 (54%). Fluorescence was more pronounced in the HSV-2 conjugate in 10 of these latter 15. Positive direct FA slides were available on only two of the nine HSV-1 patients; one demonstrated reactivity to both types (HSV-1 more than HSV-2), and one was reactive with the HSV-2 conjugate only. Generally, the background staining was more prominent in the direct FA test than in the indirect IP test. This was especially so with the HSV-1 direct FA antiserum, although in no case was the background fluorescence felt to interfere with the proper reading of the slide.

DISCUSSION

This study indicates that both commercially available immunological detection methods for HSV, although not as sensitive as VI, are useful for laboratory confirmation of genital HSV infection. The absolute sensitivity in patients with suspected genital lesions was 80% for VI, 66% by the indirect IP technique, and 55% by the direct FA technique. The relative sensitivities could be increased to 79% for indirect IP and 71% for direct FA when adequate specimens (i.e., slides containing greater than 25 exfoliated cells) were obtained from genital lesions.

The clinical stage of the lesions and whether the patient had initial or recurrent genital herpes were important determinants of the sensitivity of all three tests. HSV was isolated from 90% of vesicular-pustular lesions and 82% of ulcerative lesions from patients with initial genital herpes, compared with a 43% isolation rate from specimens obtained from ulcerative lesions of patients with recurrent genital disease. A similar trend with the indirect IP and direct FA procedures was apparent: HSV was detected by direct FA in 71% and by indirect IP in 76% of specimens obtained in the vesicular-pustular stage of disease compared with only 38 and 55%, respectively, of specimens obtained from genital ulcers.

Previous studies have indicated that higher titers of virus are present in vesicular than in ulcerative herpetic lesions (20). In addition, the mean duration of viral excretion in initial genital herpes is significantly longer (12 days) than in recurrent genital disease (4.5 days) (2, 7). Thus, the ability to detect HSV antigen by these immunological assays appears to be related to both the quantity of virus present and the duration of viral excretion. An alternative explanation for the difference between initial and recurrent genital HSV may be that local or humoral antibody or both may coat HSV-infected cells early in recurrent disease and, hence, decrease the ability to detect HSV antigens by these techniques, a mechanism analogous to that seen in respiratory tract infection with respiratory syncytial virus (10).

In an earlier study, we evaluated the specificity of the indirect IP test by comparing it with VI in 96 women attending the Harborview Medical Center STD Clinic with illnesses other than HSV infection. Of these 96 women, 40% had signs and symptoms of vaginitis, 35% had *N. gonorrhoeae* infection, and 25% were sexual con-

tacts of patients with nongonococcal urethritis. In 95 of these women, both VI and indirect IP did not reveal HSV. In one woman, both tests detected asymptomatic cervical HSV infection. Thus, the specificity of the indirect IP in this clinical setting was 99% (Table 4). In this investigation, we evaluated the specificity of the direct FA and indirect IP procedures by obtaining specimens from women with documented genital HSV who were seen when no clinical signs or symptoms of HSV were apparent (intercurrent genital herpes). In this population HSV antigen was detected by either direct FA or indirect IP but not by VI in six women, indicating that the specificity of the indirect IP method for the detection of cervical HSV infection was 95% and that of the direct FA method was 90%. It is of interest that Adam et al. also reported instances in which HSV antigen was detected by immunofluorescence at time periods in which the virus was not isolated in tissue culture (1). Whether these discrepancies between VI and these immunological assays represent vagaries in sampling techniques, inactivation of infectious virus during transport, periods of defective or "noninfectious" viral antigen production, or truly methodological false-positives is uncertain. Studies evaluating the potential of experimentally transmitting HSV infection from cervical secretions taken during these time periods are needed to clarify these observations.

The concordance between direct FA and VI was 74%, and that between indirect IP and VI was 77%, with most of the difference due to the greater sensitivity of VI. In nine patients, however, HSV antigen was detected by indirect IP or direct FA or both but not by VI. Eight of these nine patients had HSV subsequently documented by VI, indicating that these specimens were most likely not false-positives but instances in which VI was not the most sensitive method for the detection of genital HSV infection.

Although the differences in overall sensitivity between indirect IP and direct FA were not statistically significant throughout the study, the indirect IP method detected HSV antigen more frequently than did the direct FA method, in 8% more genital lesions and 4% more cervical scrapings. These results suggest that the indirect antigen detection technique is slightly more sensitive than the direct technique. Although the indirect IP technique is not type specific, we found that characterizing a herpetic infection as HSV-1 or HSV-2 by direct FA staining of exfoliated cells from lesions was unreliable. In fact, the HSV-1 conjugate was either the only positive indicator of HSV infection or had more intense immunofluorescence in 8 (29%) of 28 isolates from patients with documented HSV-2 disease. Another advantage of the indirect IP technique was that background cellular debris was easier to identify with light than with fluorescence microscopy. This problem can be minimized, however, if an equipment setup is available that allows one to switch from dark-field to light-field microscopy. Although this type of specialized fluorescence microscopy equipment is available in many large hospital laboratories, the easier readability of the indirect IP procedure and the use of light microscopy make the HSV indirect IP procedure more suitable for STD clinics and many laboratories.

These data indicate that whereas VI is the most sensitive method for confirming the diagnosis of genital HSV in patients presenting with genital ulcerations, both the indirect IP and direct FA tests can be useful adjuncts to VI in situations where a rapid laboratory diagnosis is needed.

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