

Comparison of Direct Immunofluorescence and Direct Immunoperoxidase Procedures for Detection of Herpes Simplex Virus Antigen in Lesion Specimens

NATHALIE J. SCHMIDT,^{1*} JUANITA DENNIS,¹ VERONICA DEVLIN,¹ DANA GALLO,¹ AND JOHN MILLS²

Viral and Rickettsial Disease Laboratory, California Department of Health Services, Berkeley, California 94704,¹ and Division of Infectious Diseases, San Francisco General Hospital, San Francisco, California 94110²

Received 17 February 1983/Accepted 11 April 1983

Direct immunofluorescence and direct immunoperoxidase staining were equally sensitive and specific for detection of herpes simplex virus antigen in lesion specimens, and each method showed 82% agreement with virus isolation results.

The value of immunofluorescence (IF) staining methods for rapid identification of herpes simplex virus (HSV) directly in lesion specimens is well documented (5, 6, 9, 12, 13, 16). In our accumulated experience and in reports of various other workers, IF methods have shown well over 80% agreement with cell culture isolation for detection of HSV in clinical materials (9, 12, 13, 16), although some studies (2) have shown a lower sensitivity for IF. Immunoperoxidase (IP) staining procedures also have been advanced in recent years for detection of HSV in lesion materials (1, 3, 8, 9, 10). These are considered to have advantages over IF methods in that results can be read with an ordinary light microscope and permanent preparations can be made. Furthermore, IP staining has the potential for greater sensitivity than IF, since the enzyme label on the antibodies can have a continuous action on the substrate which results in a buildup of reaction product at the site where virus is present in the specimen, thus amplifying the virus content. In contrast, activity seen in IF staining is directly dependent upon the amount of antigen available in the specimen for binding of the labeled antibodies. IP staining has been used to a limited extent for identification of HSV in clinical specimens, and although generally less sensitive than virus isolation, it has identified a greater number of positive specimens than were detected with cytological methods (3, 9, 10). In one comparative study with indirect IP and direct IF (DIF) staining, IP was shown to be equally or slightly more sensitive than IF for detection of HSV in lesion specimens (9).

There is a need for more data on the comparative sensitivity, specificity, and simplicity of IF and IP staining on clinical materials to determine the actual advantages which one method may have over the other. Because of this, and be-

cause of our interest in evaluating commercially available reagents for the detection of HSV by IP staining, we undertook a study comparing DIF and direct IP (DIP) procedures, together with cell culture isolation, for the detection of HSV in specimens collected at a venereal disease clinic.

Specimens were from patients seen at the San Francisco City Clinic who were suspected of having genital or orolabial HSV infection. There were 123 male and 11 female patients. The sites of specimen collection were as follows: penile lesions, 59; rectal or perianal lesions, 42; female genital tract, 10; other sites, including mouth, finger, neck, sacrum, and leg, 17; and unspecified, 6. Vesicle fluid or swabs of material from ulcers or papules were inoculated directly into cultures of Flow 2000 fibroblasts maintained on minimal essential medium with 2% fetal bovine serum. These were transported daily to the Viral Diagnostic Laboratory at San Francisco General Hospital, incubated at 37°C, and examined daily for a viral cytopathic effect. Isolates were identified and typed by DIF staining (13) at the Viral and Rickettsial Disease Laboratory (VRDL), California Department of Health Services, Berkeley. Cellular material from the lesions was collected onto swabs, and two to three smears were prepared on each of two microscope slides; these were air dried and held at -70°C until DIF and DIP staining were done at the VRDL. Lesion smears from typical varicella-zoster infections were included for control purposes. Smears were fixed in acetone for 10 min at room temperature before DIF and DIP procedures were performed.

DIF staining was done as previously described (5, 13), using HSV immune hamster globulins produced and conjugated with fluorescein isothiocyanate in the VRDL. Specimens

from sites below the waist were stained with a conjugate to HSV type 2 (HSV-2), and those from sites above the waist were stained with a conjugate to HSV-1, although the conjugates were used at predetermined working dilutions which gave strong staining with both HSV types. A conjugate to varicella-zoster virus (14) was used as a specificity control. Positive findings were based upon the demonstration of typical 3- to 4-plus nuclear and cytoplasmic staining with the HSV conjugate, but little or no reaction with the varicella-zoster virus conjugate. Occasionally, very weak fluorescence was seen only in the cytoplasm of cells stained with the conjugates, and this was attributed to Fc receptors binding the conjugates non-specifically.

DIP staining was done with HSV-2 immune rabbit globulins conjugated with horseradish peroxidase (Dakopatts Antibodies, Accurate Chemical and Scientific Corp., Westburn, N.Y.). This conjugate was used at a working dilution (1:25) which gave strong staining with both HSV-1- and HSV-2-infected cells. In preliminary studies it was noted that smears occasionally contained cells with endogenous peroxidase activity which could give false-positive staining. Accordingly, it was decided to employ routinely an initial step for differential staining (11) of any endogenous peroxidase which might be present in the specimen so that it could be distinguished from the specific IP staining of the immune reaction. This consisted of pretreating the smears with Hanker-Yates reagent (*p*-phenylenediamine-pyrocatechol) (7) and H₂O₂ to stain endogenous peroxidase a brown color. At the time of use, a stock solution of Hanker-Yates reagent was added to 0.1 M Tris buffer, pH 7.6, to give a concentration of 50 mg of *p*-phenylenediamine and 100 mg of pyrocatechol per 100 ml, and to each 100 ml of this was added 0.08 ml of a 3% solution of H₂O₂. Slides were treated with this solution for 15 min at room temperature, followed by a 10-min rinse in phosphate-buffered saline on a shaker and two 1-min rinses in distilled water. After drying, the HSV conjugate diluted in a 20% suspension of normal beef brain in phosphate-buffered saline, pH 7.5, was applied, and slides were incubated for 40 min at 37°C in a humidified atmosphere. Slides then received two 10-min rinses in phosphate-buffered saline, followed by two 1-min rinses in distilled water. Substrate solution was freshly prepared by adding 40 mg of 4-chloro-1-naphthol (4) dissolved in 0.25 ml of absolute ethanol to 100 ml of 0.05 M Tris buffer, pH 7.5, filtering through no. 40 Whatman paper, and then adding 0.05 ml of 3% H₂O₂. While still wet, slides in a large petri dish were flooded with the substrate and held for 15 min at room temperature. After a

TABLE 1. Agreement between virus isolation and immunoassays for detection of HSV

Im-muno-assay	Result (no. of specimens)	Isolation results		% Agreement on satisfactory specimens ^a
		Positive (10 HSV-1, 79 HSV-2)	Negative (45)	
IF	Positive (62)	60	2	82
	Negative (47)	18	29	
	Unsatisfactory (25)	11	14	
IP	Positive (61)	59	2	82
	Negative (50)	18	32	
	Unsatisfactory (23)	12	11	

^a There were 109 specimens satisfactory for DIF and 111 satisfactory for DIP.

brief rinse in distilled water, slides were mounted in Gelvatol 20-30 (Monsanto, Indian Orchard, Maine), a pH 7.8 polyvinyl alcohol medium (15), and examined with an ordinary light microscope. Specific staining of HSV antigen appeared as intracellular blue-black staining, whereas any endogenous peroxidase showed brown staining with the Hanker-Yates reagent.

DIF and DIP staining were done in separate laboratory units by individuals with no knowledge of results obtained with the other test.

The results are summarized in Table 1. HSV was recovered from 89 of the 134 specimens, giving a positive rate of 66%. Ten of the isolates were HSV-1 and 79 were HSV-2. Sixty-two specimens were positive for HSV by DIF, giving a positive rate of 46% for total specimens or 57% for those specimens on which a satisfactory DIF examination could be performed. Twenty-five of the specimens contained insufficient cells to permit a valid interpretation of DIF results, and thus they were classified as unsatisfactory. For a smear to be satisfactory for examination by the immunoassays, it had to contain 5 to 10 basal epithelial cells; 23 specimens were unsatisfactory for DIP examination. Only nine specimens were unsatisfactory for both DIF and DIP. Sixty-one specimens were positive by DIP, giving a positive rate of 45.5% for total specimens or 55% for the 111 specimens which were satisfactory for DIP examination. For specimens which were satisfactory for the immunoassays, DIF and DIP each showed 82% agreement with virus isolation. Of the total specimens positive for HSV (93), isolation identified 96%, whereas IF and IP each identified 66%. Each of the immunoassays showed positive results on two specimens which were isolation negative. Based upon the characteristic staining patterns, the lack of reactivity with controls, and the past experience of this

TABLE 2. Comparison of IF and IP results on specimens which were satisfactory for both tests^a

IF result	No.	IP results	
		Positive	Negative
Positive	54	44	10
Negative	41	9	32

^a There were 35 unsatisfactory specimens, but only 9 were unsatisfactory in both systems. There was 80% agreement between the two assays.

laboratory (5, 13, 14) and others (6), these results were considered to represent true-positive reactions. One of the two specimens positive only by DIF was unsatisfactory for DIP, and one was negative by DIP. Similarly, one of the two specimens positive by DIP only was unsatisfactory for DIF and one was negative by DIF.

In comparing the results of the DIF and DIP procedures on specimens which were satisfactory for both procedures, there was 80% agreement between the two immunoassays (Table 2).

Although DIF and DIP showed good agreement with virus isolation, the sensitivity of these methods for the detection of HSV-positive specimens was somewhat lower than that seen in certain other studies (1, 3, 9, 10, 13, 16). This may be related to two factors. First, it has clearly been shown that IP and IF procedures are more satisfactory on vesicular HSV lesions than on older, ulcerated lesions (2, 9), and specimens used in the present study were from cases selected without regard to the stage of the lesions. Secondly, adequate specimen collection and slide preparation (5, 6) are essential for satisfactory IF and IP results. It would appear that using the lesion specimens to prepare slides for examination by two immunoassays resulted in a higher number of unsatisfactory specimens than would have resulted if available lesion material had been used to prepare fewer smears. This is suggested by the fact that the 19% unsatisfactory smears encountered in this study was higher than the 9% found in a previous study of DIF from the VRDL (13), and also by the fact that most specimens were unsatisfactory for only one of the immunoassays, indicating uneven distribution of the cellular materials rather than failure to sample cells from the lesions.

In the present study, DIP showed no advantage over DIF in terms of sensitivity for HSV detection, and the additional steps involved might be considered a disadvantage. However, for laboratories lacking IF equipment for HSV examinations, DIP with commercially available reagents would be a highly satisfactory alternative. Although endogenous peroxidase activity

in lesion specimens is a potential source of nonspecific activity, differential staining proved to be a very effective means for distinguishing this from specific HSV staining. This is a more satisfactory approach than methods for inactivation of endogenous peroxidase activity (11), since it avoids the possibility of destroying viral antigens.

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LITERATURE CITED

1. Benjamin, D. R. 1977. Use of immunoperoxidase for rapid diagnosis of mucocutaneous herpes simplex virus infection. *J. Clin. Microbiol.* 6:571-573.
2. Brown, S. T., H. W. Jaffe, A. Zaidi, R. Filker, K. L. Herrmann, H. C. Lyeria, D. F. Jove, and J. W. Budell. 1979. Sensitivity and specificity of diagnostic tests for genital infection with *Herpesvirus hominis*. *Sex. Transm. Dis.* 6:10-13.
3. Corey, L., J. Dragavon, and D. Benjamin. 1982. Immunoperoxidase staining in the clinical virology laboratory, p. 246-253. In R. C. Tilton (ed.), *Rapid methods and automation in microbiology*. American Society for Microbiology, Washington, D.C.
4. Elias, J. M. 1980. A rapid, sensitive myeloperoxidase stain using 4-chloro-1-naphthol. *Am. J. Clin. Pathol.* 73:797-799.
5. Emmons, R. W., and J. L. Riggs. 1977. Application of immunofluorescence to diagnosis of viral infections. *Methods Virol.* 6:1-28.
6. Gardner, P. S., and J. McQuillin. 1980. *Rapid virus diagnosis: applications of immunofluorescence*, 2nd ed. Butterworth & Co., Ltd., Publishers, London.
7. Hanker, J. S., P. E. Yates, C. B. Metz, and A. Rostioni. 1977. A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. *Histochem. J.* 9:789-792.
8. Morisset, R., C. Kurstak, and E. Kurstak. 1974. Diagnosis of herpes simplex virus infections with immunoperoxidase, p. 31-39. In E. Kurstak and R. Morisset (ed.), *Viral immunodiagnosis*. Academic Press, Inc., New York.
9. Moseley, R. C., L. Corey, D. Benjamin, C. Winter, and M. L. Remington. 1981. Comparison of viral isolation, direct immunofluorescence, and indirect immunoperoxidase techniques for detection of genital herpes simplex virus infection. *J. Clin. Microbiol.* 13:913-918.
10. Pearson, N. S., G. Fleagle, and J. J. Docherty. 1979. Detection of herpes simplex infection of female genitalia by the peroxidase-antiperoxidase method alone or in conjunction with the Papanicolaou stain. *J. Clin. Microbiol.* 10:737-746.
11. Robinson, G., and I. Dawson. 1975. Immunochemical studies of the endocrine cells of the gastrointestinal tract. I. The use and value of peroxidase-conjugated antibody techniques for the localization of gastrin-containing cells in the human pyloric antrum. *Histochem. J.* 7:321-333.
12. Rubin, S. J., R. D. Wende, and W. E. Rawls. 1973. Direct immunofluorescence test for the diagnosis of genital herpesvirus infections. *Appl. Microbiol.* 26:373-375.
13. Schmidt, N. J., D. Gallo, V. Devlin, J. D. Woodie, and R. W. Emmons. 1980. Direct immunofluorescence staining for detection of herpes simplex and varicella-zoster

- virus antigens in vesicular lesions and certain tissue specimens. *J. Clin. Microbiol.* 12:651-655.
14. Schmidt, N. J., E. H. Lennette, J. D. Woodie, and H. H. Ho. 1965. Immunofluorescent staining in the laboratory diagnosis of varicella-zoster virus infections. *J. Lab. Clin. Med.* 66:403-412.
 15. Thomason, B. M., and G. S. Cowart. 1967. Evaluation of polyvinyl alcohols as semipermanent mountants for fluorescent-antibody studies. *J. Bacteriol.* 93:768-769.
 16. Vestergaard, B. F., and H. Thybo. 1974. Rapid diagnosis and typing of herpesvirus hominis by the fluorescent antibody method. *Scand. J. Infect. Dis.* 6:113-116.