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Detection of Herpes Simplex Virus DNA from Genital Lesions by In Situ Hybridization

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Lesion specimens from 118 episodes of recurrent genital herpes were used to compare herpes simplex virus (HSV) isolation with a direct specimen test for in situ DNA hybridization utilizing a biotinylated probe. The frequency of detection of HSV was similar with both tests; HSV was isolated from 81% of vesicular lesions, 76% of pustules, and 67% of ulcers, while HSV DNA was detected in 77, 76, and 55% of lesions in these stages, respectively. Utilizing both methods, HSV was identified in 91, 94, and 79%, respectively. The sensitivity and specificity of the DNA probe in comparison to standard viral isolation in tissue culture were 92 and 63%, respectively. Seven DNA-positive, viral isolation-negative specimens were obtained from patients who had positive culture confirmation at some time subsequent or prior to enrollment, suggesting that these were true positive results. The sensitivity of the DNA probe was dependent on cellular content of the specimen, and 36 (28%) of the 127 submitted specimens had fewer than 20 nonsuperficial cells. The DNA probe was rapid and convenient; its major disadvantage was the lack of type-specific information. The performance of the probe in lower-prevalence populations and in asymptomatic shedding of HSV remains to be evaluated.

The advent of antiviral chemotherapy has made accurate and rapid diagnosis of herpes simplex virus (HSV) infections of increasing importance (3, 4, 15, 19). No described method exceeds the sensitivity of cell culture (2). However, virus isolation from cell culture may take up to 5 days and requires laboratory expertise in cell culture maintenance and recognition of cytopathic effect. Multiple viral antigenic detection techniques, such as enzyme-linked immunosorbent assay, immunoperoxidase, immunofluorescence, and immunohemagglutination, utilizing monoclonal antibodies, have been evaluated in an effort to expedite cell culture processing time and to detect HSV antigen from lesion samples (1, 2, 8–10, 16, 21, 22, 24, 25). DNA hybridization has also been utilized as a rapid means of detecting HSV infection from tissue or lesion samples (5, 6, 11, 14). In this study we evaluated the sensitivity, specificity, and ease of method of a commercially available, nonradioactive, biotinylated (12, 20), viral DNA probe (ENZO Biochem, Inc.) and compared it with standard cell culture with monoclonal antibody confirmation. The probe is a combination of two previously described HSV DNA restriction enzyme fragments, designated Bam F probe (HSV type 1 [HSV-1]) and PDG 305 probe (HSV-2) (7, 13). It hybridizes with both HSV-1 and HSV-2 DNA.

We found that the DNA probe performed well in a well-defined population with recurrent genital herpes, with an overall sensitivity of 92% in comparison to tissue cultures. Laboratory processing time was 2 to 2.5 h.

MATERIALS AND METHODS

Clinical population. Eighty-six patients who had a history of recurrent genital ulcerations and presented with an acute episode of probable recurrent genital herpes to a referral clinic located at the Harborview Medical Center, Seattle, Wash., were enrolled. Genital exam was performed upon enrollment, and external lesions typical of HSV infection were sampled and recorded for stage (vesicles, pustules, ulcers). Nongenital lesions suspected of HSV infection were examined and recorded separately. Specimens for both culture and DNA probe were obtained from a single lesion or a group of lesions. For sampling, the lesion was cleansed with sterile saline, and mucous was removed, if present. Vesicular or pustular lesions were opened with a 25-gauge needle, and fluid was collected and sent to the laboratory in the needle for viral isolation only. A second sample was then obtained from the base of the opened lesion by vigorously rubbing with a Dacron swab moistened with saline. Promptly after sampling, the swab was slowly rolled across the specimen area of a glass slide, allowed to air dry, fixed in acetone, and refrigerated (2 to 8°C). Ulcers were sampled with an initial swab specimen for viral isolation and transported in viral transport media, and a subsequent swab sample was obtained from the base as described for vesicles and pustules. Specimens were transported to the laboratory within 24 h.

Cultures, monoclonal antibody confirmation, and DNA probe testing were performed and evaluated without knowledge of the clinical presentation of the patient.

Cell cultures. Viral isolation methods used have been described previously (14). In summary, 0.2-ml samples of the specimen in viral transport medium (veal infusion broth with penicillin, mycostatin, and gentamicin) were inoculated into duplicate tubes of a diploid fibroblast tissue culture (human embryonic tonsil) within 24 h of collection. Cultures were examined daily for the presence of cytopathic effect.

Cells exhibiting typical HSV cytopathic effect were washed with phosphate-buffered saline, transferred to glass slides, fixed for 10 min in cold acetone, and stained by direct immunofluorescence, using fluorescein-labeled mouse monoclonal antibodies against either HSV-1 or HSV-2 type-specific antigens (Microtrak; Syva Corp., Palo Alto, Calif.). In addition, at 14 days, all negative cultures were tested by monoclonal antibody confirmation. Immunofluorescence

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was detected with an episcopic fluorescence microscope equipped with a tungsten-halogen illumination system and an immunofluorescence 450–490 excitation filter.

In situ hybridization with biotinylated HSV DNA probe. The in situ hybridization procedures were performed per the manufacturer's recommendations. In brief, 50 μl of biotinylated HSV DNA probe (ENZO Biochem, Inc.) was added to each slide (15-mm slide well) and a cover slip was applied. The specimen was heated at 92°C on a heating block for 2 to 3 min and incubated at room temperature for 10 to 20 min; the cover slip was removed, and probe wash solution (50% formamide, 0.1× phosphate-buffered saline, pH 7.0) was applied at room temperature for 5 to 10 min. The slide was then rinsed with wash buffer (1× phosphate-buffered saline-EDTA) for 5 to 10 s and shaken to remove excess buffer, and avidin-biotinylated horseradish peroxidase complex was added for 10 to 20 min. The chromogen-substrate solution (20 mg 3-amino-9-ethylcarbazole in 1 ml of solvent and 1% hydrogen peroxide in water) was mixed immediately before use. After washing, the chromogen-substrate solution was added for 10 to 20 min at room temperature, excess substrate was removed by rinsing, and a fast green counterstain (0.25% in 0.05% acetic acid) was added. Following repeat washing, a cover slip was applied to the 15-mm well, using water as a mounting medium. The specimen was then viewed with an ordinary light microscope with ×100 and ×400 magnification. The dried slides from which cover slips were removed were stored in the dark. For reexamination, the slides were remounted as described above.

Control slides were prepared with each run of specimens, using known diploid fibroblast cells infected with HSV-2 and uninfected diploid fibroblast cells, respectively, for positive and negative controls.

Grading of slides. Systematic scanning of the entire specimen area was performed at a magnification of ×100, with higher magnification used for confirmation of positive cells. HSV DNA was considered to be present when at least one intact cell displayed characteristic red nuclear staining (Fig. 1). A negative slide was considered to be one in which >20 nonsuperficial (17) epithelial cells exhibited none of the characteristic red nuclear staining patterns. Slides were examined for 3 to 5 min by one observer (D.S.). Specimens with fewer than 20 nonsuperficial epithelial cells per slide were reported as "inadequate." Positive or negative reactivity was also recorded on these specimens. Upon completion of the study, all slides were independently reviewed by another reviewer (C.L.B.).

RESULTS

One hundred twenty-seven specimens (69 males and 58 females) were obtained from 118 separate episodes of recurrent genital HSV lesions in these 86 patients. This included 122 genital, 3 oral, 1 nasal, and 1 extremity lesion sample. Eight patients had 2 or more samples taken during the same clinical episode of genital ulceration (17 samples). Sixteen patients had two samples taken from different episodes of genital ulceration, and eight had three or more samples taken from separate episodes. One patient had four samples taken from three different episodes. Overall, HSV was isolated in tissue culture in 94 (74%) of the 127 specimens. Six isolates were typed as HSV-1 and 88 were typed as HSV-2.

Thirty-six samples were graded as inadequate for evaluation by in situ hybridization; of these, 21 (58%) were positive by viral isolation. The 91 samples adequate for both DNA test interpretation and viral isolation were obtained from 82 episodes in 62 patients. HSV was isolated from 73 (80%) of these samples, and HSV DNA was detected by in situ hybridization in 72 (79%) samples. The frequency of isolating HSV in tissue culture of demonstrating HSV DNA in lesions was similar between males and females, 78 versus 69% and 70 versus 64%, respectively.

Relative sensitivities of these tests varied with the stage of the disease at the time of sampling (Table 1). Viral isolation was positive from 81% of vesicular lesions, 76% of pustules, and 67% of ulcers. HSV DNA was detected in 77, 76, and 55% of lesions in these stages, respectively. Seven culture-negative lesions had HSV DNA detected in them (two vesicles, three pustules, and two ulcers). Conversely, HSV was isolated from 16 DNA-negative specimens. Of these 16 specimens, 9 were negative by in situ hybridization because they had inadequate numbers of cells to examine. Seven specimens (two vesicular, one pustular, and four ulcers) had adequate numbers of cells but were DNA negative. Utilizing both methods, HSV was identified in 91, 94, and 79% of vesicles, pustules, and ulcers, respectively.

Twelve samples were viral isolation positive and DNA positive, although they contained <20 nonsuperficial cells. If these samples are included in the results, HSV DNA was identified in 88, 83, and 73% of vesicular, pustular, and ulcerative lesions, respectively. Utilizing both methods, laboratory evidence of HSV was present in 93, 94, and 82% of vesicular, pustular, and ulcerative lesions, respectively.

For samples >20 exfoliated cells on examination, the overall sensitivity of the DNA detection by in situ hybridization compared with viral isolation was 90.4%; specificity, 66.6%; positive predictive value, 92%; and negative predictive value, 63%. When we included the 12 culture-positive DNA-positive samples with <20 nonsuperficial cells, the sensitivity increased to 92%, specificity was 67%, and the predictive values remained the same as given above. Also, the sensitivity of the DNA probe for specimens from which HSV-1 was isolated (4 of 5 with >20 nonsuperficial cells, or 80%) was similar to the sensitivity for specimens from which HSV-2 was isolated (62 of 68, or 92%).

The sensitivity and specificity of the DNA detection were significantly different for specimens with inadequate cell counts compared with adequate specimens; sensitivity was 57%, specificity was 93%, positive predictive value was 92%, and negative predictive value was 61%.

The overall sensitivity of samples taken at multiple times from the same individual was similar for both assays to that seen from single samples obtained from different individuals.

Relationship between duration of lesion and sensitivity of viral isolation and DNA testing. HSV DNA was detected in 65 (71%) of 91 specimens sampled within 48 h of lesion onset, compared with 20 (56%) of 36 samples collected >48 h after lesion onset. Values for viral isolation were 79 and

TABLE 1. Comparison of viral isolation and DNA testing by lesion stage in recurrent genital HSV infection[a]

Lesion stage (no. of samples)	No. (%) of specimens				
	DNA+/ C+	DNA−/ C+	DNA+/ C−	DNA−/ C−	DNA or C+
Vesicle (36)	29 (81)	2 (5.5)	2 (5.5)	3 (8.3)	33 (92)
Pustule (17)	13 (76)	1 (6)	2 (12)	1 (6)	16 (94)
Ulcer (38)	24 (63)	4 (10.5)	2 (5)	8 (21)	30 (79)
All stages (91)	66 (73)	7 (8)	6 (6)	12 (13)	79 (86.8)

[a] Includes only those specimens with >20 exfoliated cells per slide. C, Cell culture; +, positive; −, negative.

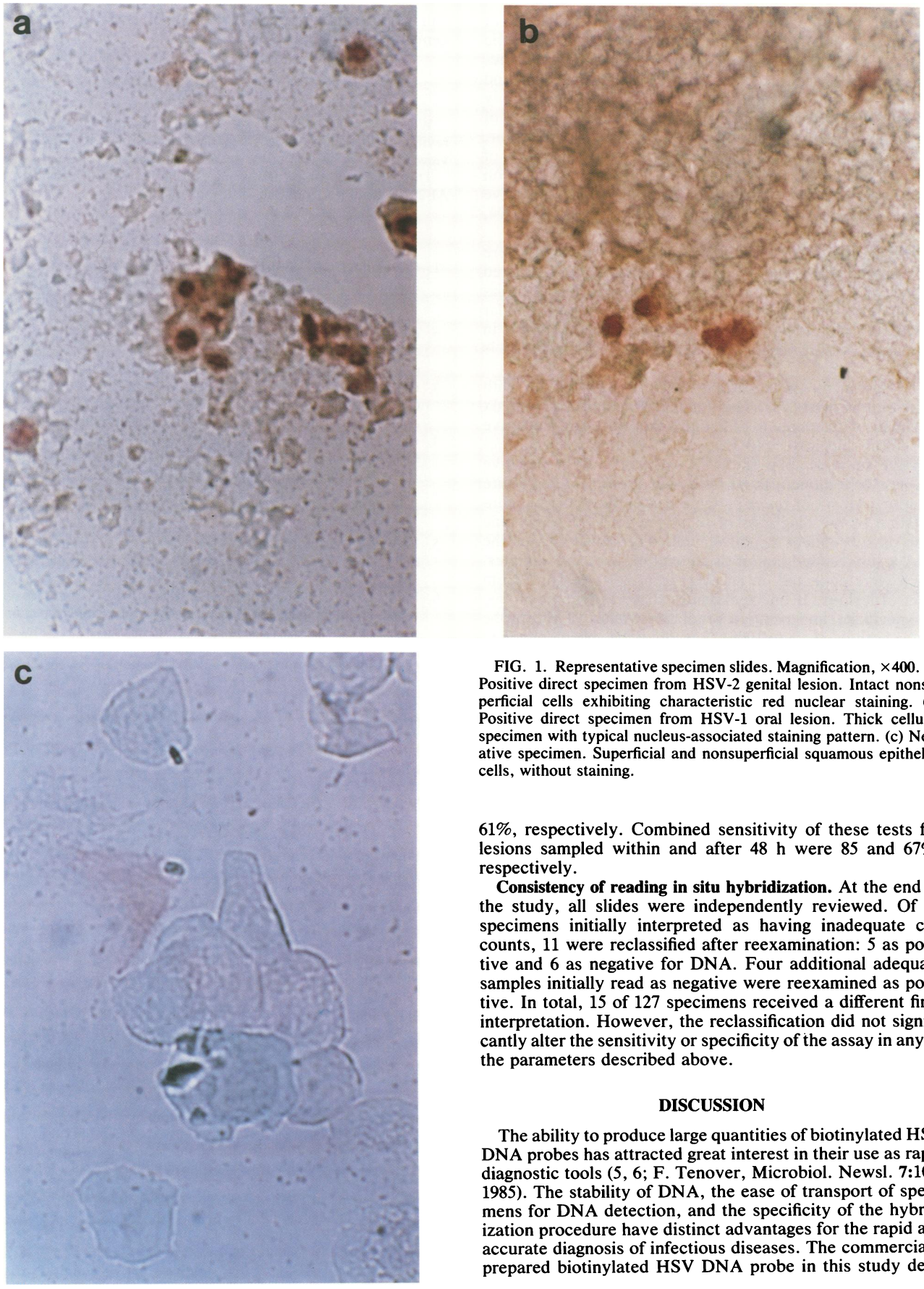

FIG. 1. Representative specimen slides. Magnification, ×400. (a) Positive direct specimen from HSV-2 genital lesion. Intact nonsuperficial cells exhibiting characteristic red nuclear staining. (b) Positive direct specimen from HSV-1 oral lesion. Thick cellular specimen with typical nucleus-associated staining pattern. (c) Negative specimen. Superficial and nonsuperficial squamous epithelial cells, without staining.

61%, respectively. Combined sensitivity of these tests for lesions sampled within and after 48 h were 85 and 67%, respectively.

Consistency of reading in situ hybridization. At the end of the study, all slides were independently reviewed. Of 47 specimens initially interpreted as having inadequate cell counts, 11 were reclassified after reexamination: 5 as positive and 6 as negative for DNA. Four additional adequate samples initially read as negative were reexamined as positive. In total, 15 of 127 specimens received a different final interpretation. However, the reclassification did not significantly alter the sensitivity or specificity of the assay in any of the parameters described above.

DISCUSSION

The ability to produce large quantities of biotinylated HSV DNA probes has attracted great interest in their use as rapid diagnostic tools (5, 6; F. Tenover, Microbiol. Newsl. 7:105, 1985). The stability of DNA, the ease of transport of specimens for DNA detection, and the specificity of the hybridization procedure have distinct advantages for the rapid and accurate diagnosis of infectious diseases. The commercially prepared biotinylated HSV DNA probe in this study dem-

onstrated, in comparison to viral isolation, a sensitivity of 92% and a specificity of 67% in detecting HSV genomic material from clinically suspect HSV lesions. As with previous studies of antigen detection, the stage of the lesion and lesion duration were important determinants of sensitivity for both DNA detection and viral isolation. The sensitivity of viral isolation alone was 81, 76, and 67% for vesicular, pustular, and ulcerative lesions, respectively. At each of these lesion stages, DNA detection by in situ hybridization was 77, 76, and 55% and thus comparable for the respective lesions. The combined sensitivity of the two tests was 92, 94, and 79% of lesions, respectively.

The majority of HSV specimens isolated in this study were from genital sites and hence were HSV-2. In a limited number of specimens from HSV-1 genital and HSV-1 orolabial lesions, the probe appeared to perform with similar sensitivity. However, further evaluation of the probe in populations with a higher prevalence of HSV-1 infection is needed.

The specificity of the probe based on comparison with tissue culture was 67%. This number was derived from six specimens with adequate cell counts that were DNA positive and culture negative and from 12 DNA-negative, culture-negative samples. The six DNA-positive, culture-negative samples were thought to be false negative for tissue culture isolation. All six of these patients had culture-positive confirmation of the diagnosis of recurrent HSV at some time prior or subsequent to enrollment. The sensitivity of the DNA test exceeded that of tissue culture for these samples, which were collected on an average of 35 (6 to 56) h from onset of lesions. If one reclassifies these as true positives, the specificity and positive predictive values of the probe assays both increase to 100%. Specificity has been defined in relation to viral isolation results and may differ with application to a series of nonselected specimens.

The major drawback to the in situ hybridization procedure was the adequacy of the sample. Sensitivity dropped to 57% if samples with <20 cells were evaluated. This problem is, of course, similar for all DNA and HSV antigen detection methods.

Other studies have evaluated the characteristics of similar biotinylated HSV probes in laboratory settings. Forghani et al. reported a sensitivity of 94% and a specificity of 100% for the biotinylated HSV DNA probe in detecting HSV in frozen brain tissue from culture-proven cases of HSV encephalitis (5). Fung et al. evaluated 243 specimens received by the laboratory for HSV culture: 206 genital, 6 rectal, 13 oral, 1 cyst, 5 eye, 9 skin, 1 gastric, 1 lung, and 1 hepatic site. The overall sensitivity of the DNA probe was 71% and specificity was 90% (6).

Direct comparison of this probe with other commercially available HSV antigen detection methods has not been performed. However, in a similar population, using monoclonal antibodies in a direct immunofluorescence assay, viral antigen was identified with sensitivity of 74% and specificity 85% compared with viral isolation (9), suggesting that the probe has equal if not greater sensitivity. Its lack of type specificity is, however, a current disadvantage.

In conclusion, HSV DNA detection appears to be a diagnostic tool with sensitivity equal to that of viral isolation when applied to a high-prevalence population with symptomatic genital lesions. It can be rapidly performed (approximately 2 h 15 min), and equipment costs are projected to be less expensive than for the direct immunofluorescence assay per specimen. Further studies are needed to assess the value of the test in lower-prevalence populations and whether it will detect asymptomatic excretion of HSV in the oral or genital tract.

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