JOURNAL OF CLINICAL MICROBIOLOGY, July 1989, p. 1623–1625
0095-1137/89/071623-03$02.00/0

Vol. 27, No. 7

Double-Label Immunofluorescence Method for Simultaneous Detection of Adenovirus and Herpes Simplex Virus from the Eye

P. WALPITA†* AND S. DAROUGAR
Institute of Ophthalmology, Judd Street, London W1, England

Received 24 October 1988/Accepted 13 March 1989

The development and application of a double-label immunofluorescence method which has the potential to screen for single or dual infections from any site, in single shell vial cultures, is described. In this study, a total of 1,141 ocular specimens were inoculated in shell vials, centrifuged at 15,000 × *g* for 1 h, incubated at 37°C for 48 h, and fixed in methanol at room temperature for 15 min. The virus inclusions were detected by staining with a double-label indirect immunofluorescence procedure using mixtures of appropriate first antibodies, followed by fluorescein- and rhodamine-conjugated second antibodies. Each specimen was also inoculated in parallel by the conventional virus isolation method. The sensitivity and specificity of the double-label shell vial procedure were comparable to those with the conventional method, and the former test took only 48 h to complete. The test offers a rapid and simple single-vial procedure which allows for individual or simultaneous detection of multiple pathogens. It results in savings in time and cost over the conventional virus isolation method and other shell vial procedures.

The traditional methods of laboratory diagnosis of viral infections, either by virus isolation in cell culture or by demonstration of rising antibody titer in paired serum specimens, may take days or weeks to complete. Direct demonstration of virus in exfoliated cells (3, 12) is generally less sensitive than virus isolation and requires high-quality smears and strict criteria of identification of viral inclusions.

Shell vial systems have been described for the rapid detection of several viruses (6, 11, 13) and chlamydia (4) from several specimen types, but these have required separate vial cultures and separate staining procedures for the detection of each of the pathogens. We describe the development and application of a double-label shell vial test (DLST). This system allows for the simultaneous screening of two pathogens from a single shell vial culture, resulting in savings in time and cost. It has the additional advantage in that it has the potential to detect dual infections from a site. The specimen is inoculated in a shell vial for 48 h, and virus inclusions are detected by a double-label indirect immunofluorescence antibody (IFA) method. In this study, the sensitivity of the DLST is compared with that of the reference conventional cell culture test (CCT) for the simultaneous detection of adenovirus (ADV) and herpes simplex virus (HSV) from ocular specimens.

MATERIALS AND METHODS

Patients and specimens. Conjunctival specimens were collected from patients who presented to the External Eye Disease Clinic at Moorfields Eye Hospital at London. The specimens were collected in plastic vials containing 2SP transport medium (7) with antibiotics. They were transported to the laboratory in a liquid-nitrogen refrigerator and stored at −70°C until cultured.

Cell culture and inoculation. Methods for cell culture and inoculation have been described previously (6, 13). Briefly, HEp-2 cell monolayers were prepared by seeding at a concentration of 25,000 to 30,000 cells per ml of growth medium in cell culture test tubes and shell vials. Growth medium consisted of Eagle minimum essential medium supplemented with vitamins, glutamine, antibiotics (vancomycin, 100 μg/ml, and streptomycin, 50 μg/ml), and 10% fetal bovine serum. At the end of 48 h of incubation, when the monolayers were approximately 80% confluent, growth medium was replaced with maintenance medium (same as growth medium but with 3% fetal bovine serum).

DLST. Each specimen was vortexed, and equal volumes (approximately 300 μl) were inoculated in parallel by the two methods. DLST involved inoculation of each specimen into a single shell vial containing a 13-mm glass cover slip with an HEp-2 cell monolayer. The inoculated vials were centrifuged at 15,000 × *g* for 1 h, incubated at 35°C for 48 h, and fixed in methanol at room temperature for 15 min. Virus inclusions were detected by a double-label IFA procedure (see below).

CCT. For CCT, each specimen was inoculated into a test tube containing an HEp-2 cell monolayer. The tubes were rolled in drums and examined at least every other day for cytopathic effect (CPE). The negative tubes were passaged at weekly intervals and discarded at the end of 3 weeks. Virus in tubes showing CPE was identified by individual IFA procedures for HSV and ADV.

Double-label IFA procedure. Group-specific anti-HSV serum was raised in our laboratory by inoculation of rabbits by the conjunctival route using an HSV type 1 strain isolated from a corneal ulcer of a patient. Anti-ADV type 5 hexon serum raised in horses was obtained from Burroughs Wellcome Laboratories, Ltd., England. Both the sera were titrated and tested for sensitivity and specificity against a panel of related and unrelated viruses to obtain a working dilution (1/10) of each. Equal volumes of the two antisera at double strength (1/5) were mixed and applied to cover each cover slip.

Fluorescein isothiocyanate (FITC)-conjugated sheep anti-horse immunoglobulin G (IgG) (Burroughs Wellcome Laboratories, Ltd.) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Cappel Laboratories) were titrated, and the working dilutions of each were obtained similarly. Volumes of each conjugate at double

* Corresponding author.
† Present address: Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093.

TABLE 1. Sensitivity and correlation of positivity for ocular ADV and HSV detection by the DLST and the conventional CCT[a]

Result of paired tests	No. (%) of specimens positive for:	
	ADV	HSV
DLST +	157 (95.7)	65 (95.5)
CCT +	149 (90.8)	57 (83.8)
DLST +, CCT −	15 (9.1)	11 (16.2)
DLST −, CCT +	7 (4.3)	3 (4.4)
DLST +, CCT +	142 (86.6)	54 (79.4)
Total + by either or both tests	164 (100)	68 (100)

[a] McNemar's test (DLST versus CCT): ADV, $U = 1.6$, $P > 0.05$; HSV, $U = 3.5$, $P > 0.05$.

strength (2× working dilution) were mixed and applied to each cover slip.

The fixed cover slips were mounted on a staining frame, and each was covered with a drop of the mixed anti-HSV and anti-ADV antisera. They were incubated in a moist chamber at 35°C for 30 min. They were then thoroughly washed for 15 min in phosphate-buffered saline (pH 7.3), using a magnetic stirrer, and then air dried. The cover slips were covered with a drop of the mixed conjugates. The incubation and washing procedures were carried out as before except for an extra final wash in distilled water for 5 min. The cover slips were dried and mounted in buffered glycerol at pH 8.6 (9).

The cover slips were examined using a standard 18UV Zeiss microscope fitted with an HBO 50W high-pressure mercury lamp and an epifluorescence condenser, which facilitates the selection of both the FITC and TRITC filter sets (Zeiss). The FITC filter sets comprised a blue exciter filter, an FT 510 beam splitter, and a 520-560 band pass barrier filter. For TRITC fluorescence, a BP 546/7 exciter filter, an FT 580 beam splitter, and an LP 590 red barrier filter were used. An ×16 Zeiss neofluor objective and a ×10 eyepiece were used throughout. A ×40 objective was also used as and when required to check the quality of inclusions. Each cover slip was examined twice, once for the FITC-stained ADV and once for the TRITC-stained HSV inclusions. A positive control (a shell vial infected simultaneously with ADV and HSV) and a negative control were carried with each batch of tests to control the quality of tissue culture and the staining procedure.

The criterion of positivity was the finding of one or more brightly fluorescing, granular, intracellular inclusions.

RESULTS

A total of 1,141 ocular specimens were inoculated in parallel by the DLST and the reference CCT. A total of 164 (14.4%) of the specimens were positive for ADV and 68 (6%) were positive for HSV by either or both of the methods. Sensitivity and correlation of positivity between the two tests is shown (Table 1). There was no significant difference between the two methods in terms of rate of detection of either of the two pathogens.

The DLST took only 48 h to complete. By comparison, the mean time taken to develop CPE by the CCT for ADV isolation was 8.3 days (range, 2 to 21 days). Only 10.6% of these specimens were positive at 48 h, and more than half (55%) took 8 days or more. For HSV isolation by the CCT, the mean time taken to develop CPE was 5.1 days. Although 54% of these specimens were positive at 48 h, 17% took 8 days or more for detection.

DISCUSSION

Several recent studies have described double-label fluorescent-antibody (FA) procedures for a variety of applications (1, 2, 10). We have developed a similar method for the simultaneous rapid screening of multiple viral pathogens from clinical material. A single shell vial culture followed by a double-label IFA procedure allows for the possibility of detection of either or both pathogens from a single specimen within 48 h. The sensitivity of the test for the detection of ADV was equal to that of the CCT and confirms our previous findings made on the basis of use of single shell vials and a single-label IFA staining procedure (6). In this study, DLST appeared to be more sensitive than the CCT for the detection of HSV, although the difference did not reach statistical significance. In a previous report employing a much larger sample size, we have shown that the shell vial test which involves a centrifugation step is indeed significantly more sensitive than the cell culture method for the detection of HSV from the eye (13).

The screening of two pathogens from the same specimen in a single shell vial culture, as we tested in this study for the two most common viral pathogens of the eye, resulted in considerable savings in time and effort and in the reduction of cost per specimen (data not presented). An additional advantage of the DLST is its potential to detect dual infections as evident from our double-infected positive controls. In the present study, dual infections with ADV and HSV from the eye were not detectable either by the DLST or the CCT, and we assume that in the population we studied such infections were not present. However, the DLST described here could be adapted for the detection of multiple pathogens simultaneously infecting other sites. Such multiple infections of the genital tract have been described previously.

Quality reagents and attention to technical details are essential for all FA procedures. For the double-label FA procedure, the two lots of antisera must be raised in two different animal species to avoid cross-reactivity when the appropriate conjugates are added. We used a horse anti-ADV plus sheep anti-horse IgG FITC conjugate system for the detection of ADV and a rabbit anti-HSV plus goat anti-rabbit IgG TRITC conjugate for the detection of HSV inclusions. Each batch of sera was titrated before use and checked for sensitivity and specificity. Mixing the two antisera and the two conjugates did not result in any loss of clarity in comparison to a staining procedure that involved application of each serum and conjugate separately.

The double-label staining procedure is economical in reagents, and the commercially available conjugated sera were of good quality. The background staining was minimal, and the distinction between positive and negative was clear cut. The virus inclusions were brightly fluorescing, granular, and intracellular. Such inclusions were never found in the negative control cover slips. When the strict criteria laid down for culture, staining, and recognition of inclusions were used, the finding of even one such inclusion was adequate for a positive diagnosis and the chances of false-positive DLST were remote. The increased sensitivity of DLST for HSV may have resulted from the centrifugation step involved in this method. Centrifugation has been shown to increase the HSV isolation rate in shell vial cultures as compared to the CCT (5). Slow CPE-producing viruses such

as ADV type 8 are often difficult to cultivate by the CCT. Whether such strains were easier to detect by the DLST and were a contributing factor in some of the discrepant results needs to be evaluated in further studies. Individual discrepant results might also occur by chance in the processing of low-titered specimens (unpublished observations).

In a pilot study, we compared the sensitivity of DLST at 24-, 48-, and 72-h incubation periods, using the polyclonal antibodies described in Materials and Methods. Sensitivity was similar at 48 and 72 h but marginally reduced at 24 h. We therefore chose 48 h as the optimum incubation period. The DLST could be further simplified, resulting in further savings in labor and cost, by doing a direct instead of an indirect FA staining using appropriate high-affinity, high-titer panels of monoclonal antibodies. The fact that the shell vial test results in considerable savings in time for the detection of ADV and HSV has been reported previously (6, 13) and is confirmed in this study.

Both HSV and ADV replicate well in different cell lines. Human embryo fibroblasts and HEK or A549 cells are the frequently used cell lines for the isolation of HSV and ADV, respectively. We used HEp-2 cells, which appear to have sensitivity equal to that of the more frequently used cell lines (8). The DLST could be adapted to use any cell line or combination of cell lines of choice, depending on the viruses of interest.

In this study, we centrifuged shell vials containing their full complement of maintenance medium (1 ml) and inoculum (approximately 300 μl) at 15,000 × *g* for 1 h (13). Our subsequent work suggests that centrifuging at speeds as low as 1,500 × *g* would suffice with no loss of sensitivity. In these experiments, we added the media to the vials after the centrifugation step.

Finally, the double-label shell vial test offered a rapid, sensitive, and simple means of detecting from a single sample the two most frequent ocular viral pathogens.

LITERATURE CITED

1. **Ayliffe, M. J.** 1985. Double staining immunofluorescence procedure for enumeration of cells containing cytoplasmic immunoglobulin in human bone marrow: 40 selected cases. J. Clin. Pathol. **38**:989–994.
2. **Bologna, M., R. Allen, and R. Dulbecco.** 1986. A method for double immunofluorescent staining by the indirect procedure with antibodies of the same isotype. J. Immunol. Methods **86**:151–153.
3. **Chien, L., and R. Llaues-Rodas.** 1972. Application of immunofluorescent staining technique to the study of pathogenesis and rapid diagnosis of viral infections. Am. J. Clin. Pathol. **57**: 829–834.
4. **Darougar, S.** 1983. Chlamydial disease. Br. Med. Bull. **39**: 181–186.
5. **Darougar, S., J. A. Gibson, and V. Thaker.** 1981. Effects of centrifugation on herpes simplex virus isolation. J. Med. Virol. **8**:231–235.
6. **Darougar, S., P. Walpita, U. Thaker, and N. Vishwalingam.** 1983. Rapid culture test for adenovirus isolation. Br. J. Ophthalmol. **67**:111–114.
7. **Gorden, F. B., J. A. Harper, L. A. Quan, J. D. Treharne, R. St. C. Dwayer, and J. A. Garland.** 1969. Detection of Chlamydia (Bedsonia) in certain infections in man. 1. Laboratory procedures: comparison of yolk sac and cell culture techniques for detection and isolation. J. Infect. Dis. **120**:451–462.
8. **Grist, N. R., E. J. Bell, E. A. C. Follett, and G. E. D. Urquhart.** 1979. Diagnostic methods in clinical virology, 3rd ed. Blackwell Scientific Publications, Edinburgh.
9. **Johnson, G. D., R. S. Davidson, K. C. McNamee, G. Russell, D. Goodwin, and E. J. Halborow.** 1982. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J. Immunol. Methods **55**:231–242.
10. **Ozturk, G. E., A. R. Hayward, R. Weil III, and P. F. Kohler.** 1985. Double fluorescence technique for measurement of complement-fixing antibody to lymphocyte subsets. J. Immunol. Methods **85**:163–168.
11. **Paya, C. V., A. D. Wold, and T. F. Smith.** 1987. Detection of cytomegalovirus infections in specimens other than urine by the shell viral assay and the conventional tube cell cultures. J. Clin. Microbiol. **25**:755–757.
12. **Schwartz, H. S., D. W. Vashine, H. Yamashioya, and C. E. West.** 1976. Immunofluorescent detection of adenovirus antigen in epidermic keritoconjunctivitis. Invest. Ophthalmol. Vis. Sci. **15**:199–207.
13. **Walpita, P., S. Darougar, and U. Thaker.** 1985. A rapid and sensitive culture test for detecting herpes simplex virus from the eye. Br. J. Ophthalmol. **69**:637–639.