

Accuracy of Smallpox Diagnosis by Immunofluorescence with a Purified Conjugate

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A direct fluorescent-antibody test for smallpox is described which utilizes a conjugated antivaccinia serum that was purified by diethylaminoethyl fractionation. The purity of the conjugate was analyzed by density gradient centrifugation, and specific staining of smallpox and nonsmallpox specimens was measured quantitatively by a photovoltmeter. Variola elementary bodies were identified in all of the specimens of vesicular and pustular fluid collected from 50 smallpox patients, and no false-positive diagnoses were made on specimens from 27 patients with varicella or other nonpox viral exanthems. However, 63.8% of the specimens of vesicular and pustular fluid smeared on slides in the field were unusable because of bright nonspecific fluorescence. For this reason, the fluorescent-antibody test does not compare favorably with other routine laboratory tests for smallpox.

The rapid laboratory diagnosis of viral and bacterial illness by fluorescent-antibody (FA) techniques is well established and has been applied to specimens from patients suspected of having smallpox. Avakian (1) and Murray (13) first demonstrated specific staining of variola elementary bodies and antigen in smears of lesion material from smallpox patients by direct and indirect FA testing. Kirsh and Kissling (9) utilized a direct FA test in the diagnosis of a smallpox patient and documented the specificity of the fluorescent staining by pretreating one smear with an unconjugated hyperimmune serum in an inhibition test. Although the FA technique permits rapid testing of small amounts of specimens, its diagnostic accuracy has been debated. False-negative results and one false-positive test for smallpox were reported by El-Ganzoury (5), and difficulties were encountered in the interpretation of an FA test on a varicella specimen at the National Communicable Disease Center in 1965.

The accuracy of an FA test for smallpox made by the identification of poxvirus antigen in specimens from 91 smallpox patients is analyzed in this study.

METHODS AND MATERIALS

Immune serum production. The Lister strain of vaccinia was adapted to Dutch rabbits by three serial intradermal passages to produce a dermal virus preparation titering 3×10^9 pock-forming units per

ml. Two New Zealand rabbits were vaccinated with this antigen and observed for 4 weeks or until the vaccination site was completely healed. They were then inoculated intravenously with three doses of 2 ml of dermal virus suspension at 5- to 6-day intervals and exsanguinated 7 days after the final booster dose. The sera had vaccinia-neutralizing antibody titers of 1:1,100 and 1:1,600, respectively, and were pooled before conjugation.

Preparation of conjugate. The gamma globulin was extracted from the hyperimmune serum on a diethylaminoethyl (DEAE) column by the method of Levy and Sober (11). However, a degree of overloading the column was indicated by the detection of a small amount of albumin by cellulose acetate strip electrophoresis. The globulin preparation was conjugated with fluorescein isothiocyanate (FITC) by the method of Marshall et al. (12). After removal of unconjugated fluorescein by filtration through Sephadex G-25 (Pharmacia, Upsala, Sweden), the conjugated globulins were fractionated on a DEAE cellulose column, and fluorescein to protein (F/P) ratios were determined as described by Riggs et al. (16). The conjugate was not concentrated back to the original protein content but was used at a 1:2 dilution.

Density gradient centrifugation. The fluorescent conjugates, before and after purification with DEAE cellulose, were analyzed by density gradient centrifugation. By using nitrocellulose centrifuge tubes [1 by 3.5 inches (2.54 by 8.9 cm)] 1.0-ml samples were layered into the top of 24 ml of buffered sucrose gradients [10 to 33% (w/w) sucrose containing 0.02 M phosphate buffer (pH 7.2) and 0.15 M NaCl]. These gradients had been formed previously by permitting

18 hr of diffusion between four layers of buffered sucrose of the following composition: 6 ml of 35%, 6 ml of 26.7%, 6 ml of 18%, and 6 ml of 10%. The sample was overlaid with 15 ml of buffer solution without sucrose and centrifuged at 27,000 rev/min for 22 hr at 4 C in the Beckman SW 27 rotor. Upon completion of the run, the centrifuge tubes were unloaded from the bottom through tubing (1 mm inner diameter) with a tubing pump at 1.3 ml/min, through an ultraviolet (280 nm) recording spectrophotometer. Fractions (1 ml) were collected for further analysis.

Specimen material. Patient specimens of vesicular and pustular fluid were collected from 91 smallpox patients hospitalized at the Emilio Ribas Infectious Disease Hospital, Sao Paulo, Brazil. Smears were made at the bedside (field smears) from vesicular or pustular fluid and also in the laboratory (laboratory smears) from fluid collected in capillary tubes and frozen until the time of laboratory testing. The clinical diagnosis of smallpox was confirmed for all cases by the isolation of variola on the chorioallantoic membrane (CAM) from lesion materials. Specimens of varicella and other exanthems were collected from 27 patients in Atlanta and used as control preparations in the FA test (varicella smears).

Smears of vaccinia-infected BHK cells were made from clone 13S of BHK-21 cells, supplied by Kenneth Herrmann, and maintained in suspension culture by the technique reported by Halonen et al. (8). Cell suspensions were infected with 1 TCID₅₀ per cell of vaccinia, Lister strain. At 24 hr, a 15-ml sample of cell suspension was removed and centrifuged, and the pellet was smeared on glass slides (BHK smears).

Fixation and staining. Slides with smears were fixed in cold acetone for two 10-min periods. The slides were dried and comparative areas for diagnostic and inhibition tests on each were marked with a Tek pen. A 1:1 mixture of normal rabbit serum and conjugate was placed in the diagnostic area, and a 1:1 mixture of unconjugated hyperimmune rabbit serum and conjugate was placed in the inhibition area on each slide. The slides were incubated at 37 C for 1 hr, rinsed twice in 0.02 M phosphate-buffered saline (pH 7.2) and four times in distilled water, and air-dried. The stained smears were covered with a Corning no. 1 cover slip (22 by 40 mm) after one drop

of mounting medium (nine parts glycerol to one part 0.02 M phosphate-buffered saline, pH 7.2) was added to the center of each ringed area.

Microscopy and photography. A Carl Zeiss microscope (standard WL model) with a cardioid dark field condenser and an HBO 200 L-2 lamp was used in this study. The combination of a UG-1 excitor filter with Zeiss number 27 and 44 barrier filters gave optimal fluorescence with a dark background. For photography, the UG-1 excitor and the 44 barrier filter were used with a 10× periplan ocular and 40× panapochromatic oil immersion objective lens. The magnification was 400×; "ansochrome 200D," 35 mm film was used and processed in the usual manner. Cargille (type A) nonfluorescent immersion oil and Corning no. 1 cover slips (22 by 40 mm) were used.

A photovoltmeter (model 520-M, Photovolt Corp, New York, N.Y.) was attached to the phototube of the microscope by a tight aluminum sleeve as described by Pittman et al. (15). Light from the specimen was attenuated by a neutral density filter to prevent "fatiguing" of the photomultiplier tube. Measurements were made of the most brightly fluorescing areas on the test and inhibition sides of each smear.

RESULTS AND DISCUSSION

Stain specificity. The sera that were conjugated with FITC had a vaccinia neutralizing titer of > 1:1,000. The F/P ratio and protein concentration of conjugate no. 1 were 1.65 and 0.534 mg/ml. Conjugate no. 2 had an F/P ratio of 2.4 and a protein concentration of 0.117 mg/ml.

The definition of specific staining of Goldstein et al. (6) was used; therefore, fluorescent-staining material was considered positive for vaccinia poxvirus group antigen only if the inhibition test performed on a different area of the same slide showed definite inhibition of the staining when pretreated with an unconjugated vaccinia antiserum. Nonspecific staining in this study was defined as all fluorescent staining which was not significantly reduced by the inhibition test. The conjugates were tested for specific staining on smears prepared from vaccinia-infected and un-

Table 1. *Stain specificity of anti-vaccinia conjugates*

Conjugate	Method of purification	Specimen	Fluorescence		
			Test slide	Inhibition slide	Controls
1	G-25 Sephadex and DEAE cellulose fractionation	LLC monolayer	4+	2+	0
		BHK smears	4+	0	0
		Smallpox smears	4+	0	
		Smallpox crust suspension	4+	0	
		Varicella smears	0	0	
2	G-25 Sephadex and DEAE cellulose fractionation	BHK smears	4+	0	0
		Smallpox smears	4+	0	

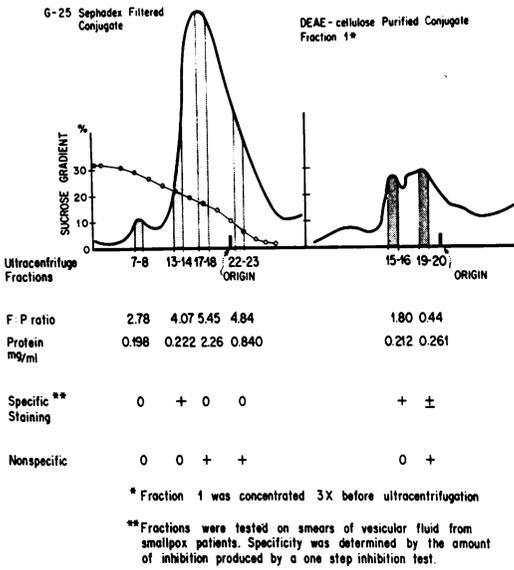


FIG. 1. Staining characteristics of filtered and purified anti-vaccinia conjugated globulin.

infected tissue cultures and from lesion material from smallpox and varicella patients (Table 1). The conjugates showed marked nonspecific staining before being subjected to fractionation on DEAE cellulose. Absorption of the conjugates with calf liver powder and with wet cell packs of LLC-MK₂ cells failed to remove the nonspecific staining. Counter staining with rhodamine-conjugated bovine serum albumin merely reduced the overall fluorescence and did not improve stain specificity.

The sedimentation profiles of conjugate no. 2 on sucrose gradients before and after refractionation are shown in Fig. 1. In both cases, specific staining was optimal in fractions comprising the faster portion of the major protein peak. These fractions gave brilliant specific staining that could be completely inhibited (Table 1). They were tested on vaccinia-infected BHK-21 cells. Specific staining of intracytoplasmic vaccinia virus (Fig. 2) was reduced to the level of autofluorescence by the inhibition test (Fig. 3). Fluorescence or extracellular virus particles was specifically inhibited.

The conjugates were tested on specimens from 91 smallpox patients and 27 patients with varicella or other illnesses. Table 2 shows that a positive diagnosis of poxvirus antigen was made on all smears of smallpox materials, when these smears were made in the laboratory from specimens of vesicular or pustular fluid which had been kept frozen since collection. No false-positive diagnoses of smallpox were obtained on smears from

patients with varicella or other exanthems. Specifically fluorescing viral particles were not found in these specimens.

Specifically stained viral particles (Fig. 4) in the smears of smallpox vesicular and pustular fluids corresponded to the size of elementary bodies stained by the Gispens technique (11). Extracellular virus particles were usually abundant, and specific fluorescence of these particles was completely inhibited by treatment with unconjugated hyperimmune serum (Fig. 5). Smallpox crust suspensions had virus particles and larger fragments of antigen-containing tissue. These all stained specifically; however, inhibition of antigen within the tissue fragments was not complete. It was noted both in varicella and smallpox specimens that leukocytes often fluoresced brightly in both the test and inhibition preparations. Thick smallpox smears often had moderate to bright autofluorescence. Although specific-staining viral particles were present on the test side in these smears and were less bright on the inhibition side, it was difficult to make a diagnosis of smallpox because of the autofluorescence. Only 46% of the field smears from known smallpox patients were positive for poxvirus antigen in our tests. The remaining slides were unusable because of bright fluorescence of the entire protein film and dense accumulations of leukocytes. Subsequent study (J. Noble, unpublished data) has revealed that smears of smallpox lesions made in the field must be frozen promptly and maintained frozen until

TABLE 2. Fluorescent-antibody test results

Type of specimen	No. of patients tested	No. of positive tests for smallpox	Per cent positive smallpox tests
Smallpox vesicular laboratory smears ^a	50	50	100.0
Smallpox field smears ^b	26	12	46.2
Smallpox crust suspensions	15	15	100.0
Varicella ^c and other vesicular laboratory smears	10	0	0.0
Varicella ^c and other field smears	17	0	0.0

^a Smears prepared from continuously frozen vesicular or pustular fluids.

^b Smears prepared at bedside of patients and not continuously frozen.

^c Smallpox specimens were not included in these categories.

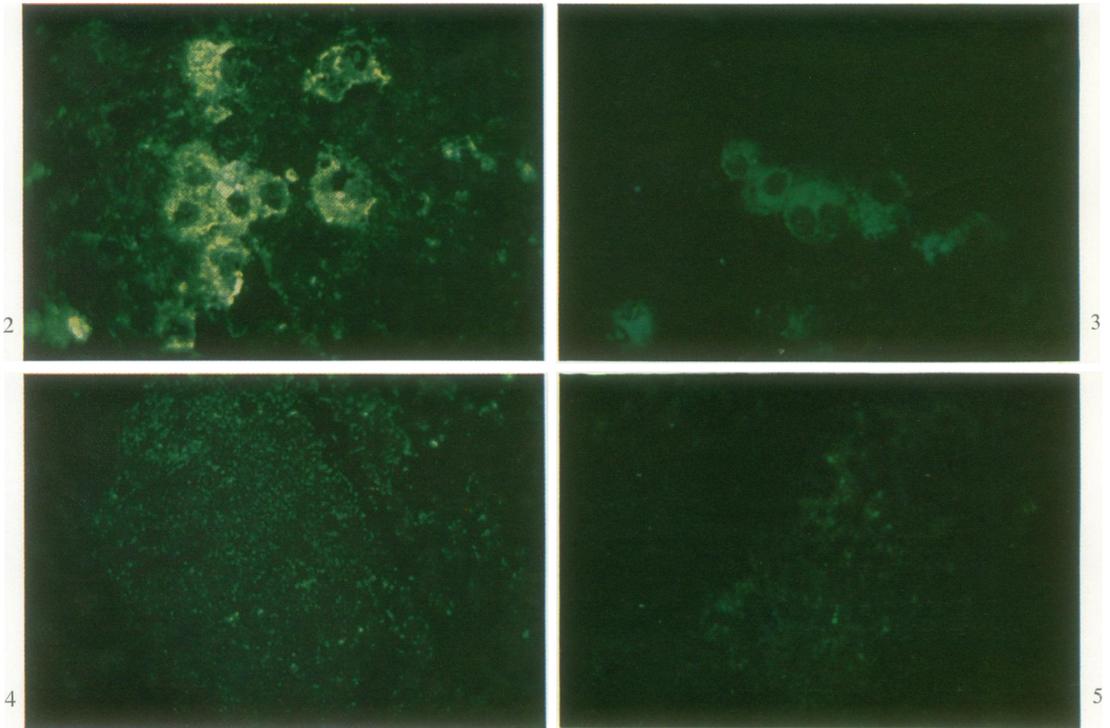


FIG. 2. Intracellular and extracellular vaccinia virus in a smear of BHK-21 cell culture stained with hyperimmune rabbit anti-vaccinia serum conjugated to fluorescein isothiocyanate.

FIG. 3. Inhibition of specific viral fluorescence in the direct fluorescent-antibody test by treatment of a different area of the same smear illustrated in Fig. 2 with unconjugated rabbit vaccinia antiserum in a one-step inhibition test.

FIG. 4. Fluorescing virus particles in a smear of vesicular fluid obtained from a smallpox patient.

FIG. 5. Inhibition of specific viral fluorescence in inhibition test performed on a different area of the smear illustrated in Fig. 4.

tested to obtain the high diagnostic accuracy that was obtained with laboratory smears.

Quantitation of specific staining. Fluorescent light emitted by test and inhibition areas of smears from 24 smallpox patients and 15 patients with other exanthems was quantitatively measured. The test and inhibition readings for each smear are charted in Fig. 6. The median difference between test and inhibition readings for the smallpox smears was 698.2 (arbitrary) photometric units. The range of differences of test minus inhibition readings for these smears was 95.0 to 3,450.0 photometric units. The median difference of values obtained on nonsmallpox smears was minus (−) 40.0 photometric units, and values ranged from −950.0 to 192.0 photometric units. The one nonsmallpox value of 821.7 photometric units (point plotted at test, 1,600; inhibition at 778.3 in Fig. 6) was obtained on a varicella smear which had most of the specimen smeared in the test area and very little in the inhibition area. Only one of the smallpox specimens overlapped the range of the other nonsmallpox readings.

When small amounts of specimen were present on smallpox smears, there was little difference between test and inhibition readings (i.e., point plotted at test 350, inhibition 290; Fig. 6). However, specifically staining granules could be visually identified on the test side of this smear. All photometric readings obtained from smallpox specimens fell to the right of the line of equal illumination (Fig. 6), whereas nonsmallpox values clustered around the line or to the left of it. The negative median difference for nonsmallpox specimens reflects an observation that there was often brighter background illumination on the inhibition than on the test sides of nonsmallpox smears. Variation in the amount of specimen in test and inhibition areas and in the background illumination prevented us from developing photometric criteria for smallpox diagnosis.

A total of 60 smears of vesicular fluid were made from one patient to determine the internal consistency of FA diagnosis and the stability of the smears. Twenty smears examined on the day of preparation were positive with equal amounts

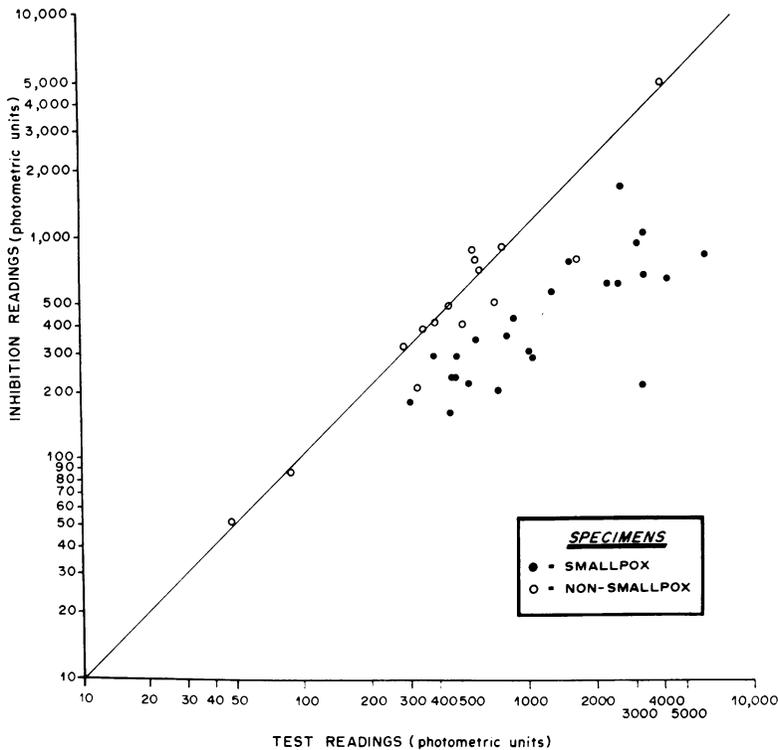


FIG. 6. Average amount of fluorescence in the test and inhibition areas of smallpox and nonsmallpox vesicular fluid smears.

TABLE 3. Internal consistency and stability of smears made from smallpox vesicular fluid^a

Interval	No. of slides	No. positive	No. negative	Nonspecific staining
Immediate	20	20	0	0
24 hr	12	12	0	0
7 days	14	14	0	0-1 ^b
14 days	12	12	0	0-1
Total	58	58		

^a Sixty slides were made from vesicular fluid of three smallpox patients.

^b There was a slight rise in nonspecific staining.

of specific staining. After the smears had been stored for 1 and 2 weeks at -70 C, a slight increase was noticed in the autofluorescence of the background and nonspecific staining (Table 3). This did not affect the interpretation of the test. Slides stored in a fixed condition appeared to have a little more nonspecific fluorescence than slides fixed at the time of testing. Fluorescence of stained smears was brightest on the day of preparation; however, diagnostic interpretation could still be made after 5 days of storage at 4 C.

Accuracy of smallpox diagnosis by immuno-

fluorescence. FA methods used to diagnose smallpox include the direct technique (1, 9), the indirect technique (13), and FA staining of tissue culture inoculated with smallpox specimens (3, 7, 10). We chose a direct FA technique and a one-step inhibition test to document specific viral staining. We found that the appearance of conjugated antibody bound to viral particles or elementary bodies on smallpox smears was similar to the "extracellular fluorescing particles" described by Kirsh and Kissling (9) and the "numerous points of bright fluorescence" described by El-Ganzoury (6). With the exception of some special problems with field smears, staining of smallpox viral particles was completely inhibited by the one-step inhibition test (Fig. 4 and 5). Thus, the criterion of Goldstein et al. (6) for specific fluorescence was fulfilled. Like Benda et al. (2), we made a positive identification of poxvirus antigen only when extracellular fluorescent particles were identified on the test side and were absent in the inhibition side of a smear. Smallpox was correctly diagnosed in 100% of the vesicular and pustular fluid specimens from 50 patients when these fluid specimens were stored at -20 C from the time of collection until smears were made just before test-

ing. Smallpox field smears from 14 patients gave inconclusive results. Although smears of crust suspension are reported to give nonspecific results (9, 5), DEAE cellulose-purified conjugate produced unequivocally positive results on all of the 15 crust specimens. All of the specimens from varicella and other exanthematous rashes had equal amounts of fluorescence on test and inhibition sides and were negative for smallpox by the FA test.

The plot of mean test and inhibition area readings clearly separated the smallpox and nonsmallpox specimens (Fig. 6) and confirmed the ability of our conjugate to stain smallpox preparations specifically. Thus, the FA technique used in this study gave an accurate diagnosis of smallpox on all specimens maintained in a frozen condition after collection and tested within 4 months.

The problem of nonspecific staining was overcome by purification of conjugated globulin and careful preparation of the test specimens. Nonspecific staining could not be removed by adsorption of the filtered conjugate with calf liver powder or wet cell packs. Counter-staining with Lissamine-rhodamine may be of use when testing Formalin-fixed tissues (17); however, it did not lessen nonspecific staining in our acetone-fixed specimens of BHK cells or smallpox smears.

Specific and nonspecific staining fractions of conjugates were separable by the sucrose density gradient sedimentation process. This method of conjugate purification may be applicable to other FA tests that require minimal amounts of background or nonspecific staining.

In most nations of the world, smallpox does not exist, and surveillance is maintained to prevent importation of the disease. Specimens from smallpox suspects in these areas are submitted in small numbers for laboratory testing (14). Thus, standard laboratory tests for smallpox must not only be accurate but must also be easy to perform and interpret; they must require stable reagents. The agar-diffusion test and electron microscopy are utilized to provide a rapid preliminary diagnosis of smallpox. The final and definitive laboratory diagnosis of smallpox is obtained by isolation of variola on the CAM after 72 hr of incubation. CAM culture, electron microscopy, and agar-gel testing have been recommended as rapid, reliable, and simple laboratory tests for smallpox (4, 18). Although the FA test described in this report is accurate, conjugate preparation is a complicated procedure and the accuracy of the test is not reliable unless specimens are kept frozen after collection. Thus, our FA test does not compare favorably with these other well-established methods for

diagnosing smallpox. If a diagnostic laboratory routinely uses other viral or bacterial FA tests, it may be justified in using an FA test for smallpox. However, sufficient reference smallpox specimens must be available for use as positive controls. In other laboratories, purified conjugate and immunofluorescence would better be used as a research tool than for the diagnosis of smallpox.

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