



EVALUATION OF VIROLOGICAL LABORATORY METHODS  
FOR SMALLPOX DIAGNOSIS

by

James H. Nakano, Ph.D.  
Center for Disease Control  
Health Services and Mental Health Administration  
United States Department of Health, Education, and Welfare  
Atlanta, Georgia 30333

SUMMARY

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Between July 1966 and May 1972, the Vesicular Disease Laboratory of the Center for Disease Control tested specimens from 849 suspected smallpox cases by at least two methods, electron microscopy (EM) and chick embryo chorioallantoic membrane (CAM) cultures. Lesser numbers of specimens were tested by a battery of four methods currently used in the laboratory: EM, CAM, agar gel-precipitation, and tissue culture.

For field-handled specimens, the CAM culture method, traditionally preferred for smallpox diagnosis, was found to be less sensitive than the EM procedure, because the adverse field conditions frequently inactivated the virus. CAM cultures were valuable for identifying members of the poxvirus subgroups, however, particularly when supplemented by tissue culture methods. The simply performed agar gel-precipitation test was the least sensitive but was of value in confirming EM results.

EM was highly effective for diagnosis of varicella, but dependably identified only about half of the vaccinia infections; for vaccinia, the CAM technique was essential.

The realization that human monkeypox cases occurred in West Africa emphasized that the usual smallpox diagnostic methods were inadequate. More sophisticated tests, such as the rabbit dermal sensitivity test, were necessary for accurate diagnosis of these cases as monkeypox.

INTRODUCTION

A number of laboratory methods have been used in the diagnosis of smallpox infections; these are summarized by Dumbell<sup>1</sup> and Downie & Kempe.<sup>2</sup> Since 1966, the Vesicular Disease Laboratory of the Center for Disease Control (CDC), Atlanta, Georgia, has made use of most of these methods both in its capacity as the reference laboratory for CDC's Smallpox Eradication Program conducted in 20 West African countries and as a WHO Regional Reference Laboratory for Smallpox. The diagnostic methods presently used in this laboratory were chosen over the years because of their proven reliability and short time required for performance.

This report presents results that demonstrate the sensitivity of our selected methods. The results also show that the selected methods were not always adequate when unusual events occurred, and emphasize the need for development of even more precise and effective techniques.

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## MATERIALS AND METHODS

### Diagnostic specimens

Between July 1966 and May 1972, specimens of lesion crusts and vesicular fluid were received from 849 persons suspected of smallpox in the United States of America, Africa, South-East Asia, and the Near East. These specimens do not include those from 165 persons with Brazilian variola minor already reported from this laboratory by Noble and co-workers.<sup>3</sup>

### Diagnostic methods

Table 1, summarized from Downie & Kempe,<sup>2</sup> lists the seven methods which can be used for the virological diagnosis of smallpox. Of these, the CDC laboratory currently uses the first four methods: EM, the gel-precipitation test, CAM culture, and tissue culture. The procedure used for EM is described by Long et al.,<sup>4</sup> and the procedures used for CAM and gel-precipitation are those of Downie & Dumbell<sup>5</sup> and Dumbell & Nizamuddin,<sup>6</sup> respectively, as applied by Nobel et al.<sup>3</sup> The tissue cultures used are primary Rhesus monkey kidney and embryonic human fibroblast grown in test-tubes, and for poxvirus strain characterization, the VERO line of green monkey kidney cells is used. These are inoculated with 0.2 ml of specimens ground or homogenized with 0.004M McIlvaine's buffer containing penicillin and streptomycin. The last three methods listed in Table 1 (stained smear examination, complement fixation, and fluorescent antibody tests) are not used because they do not provide additional advantages.

The methods used for poxvirus strain characterization are described by Lourie et al.;<sup>7</sup> they are summarized below:

1. Pock morphology on CAM.
2. Tissue culture plaque morphology in VERO line of green monkey kidney cells.
3. Dermal reaction in rabbits.
4. Ceiling temperature for poxvirus growth on CAM.<sup>8</sup>
5. Chick embryo lethality.<sup>8,9</sup>
6. Suckling mice virulence test by intracerebral and footpad inoculation.<sup>10</sup>

## RESULTS

Table 2 shows the results of specimens that were tested from persons with suspected cases by three combinations of test methods. In the early phase of our work, specimens were tested by EM and CAM only; then a period followed when most specimens were tested by EM, CAM, and gel-precipitation. Since July 1971, however, specimens have been tested by EM, CAM, gel-precipitation, and tissue culture. The data in this table show the number of cases tested, the number and percentage of cases for which no virus was found by any method, the number and percentage for which a virus (of one type or another) was found by one or more methods, the number and percentage diagnosed as variola, and the number and percentage diagnosed as herpes-varicella group infection.

Table 3 shows the relative efficacy of each method. Results on specimens from 801 cases studied during the period when EM, CAM, and gel-precipitation tests were being used, indicate that the percentage of positives (30.0%) observed with the gel-precipitation method was significantly (statistically) less than that observed with either the EM or CAM method. None of the other differences observed proved to be statistically significant, although the observed proportion of positives by EM was consistently greater than for the other two methods.

Also shown in Table 3 (last test grouping) is the advantage of adding "tissue culture" to the group of routine methods. Of the 48 positive cases of variola, only 41 were positive by CAM, but 45 were positive by tissue culture. This situation can occur when for some reason the sensitivity of the CAM decreases. We did notice that during the time tests were performed, the plaque forming unit (PFU) titre of the "house standard smallpox vaccine" was about  $0.5-1.0 \log_{10}$  lower than it was usually. When decreased sensitivity of CAM is suspected, each specimen with a negative CAM should certainly be cross-checked in tissue culture. In this series of tests (but not shown in Table 3) 30 specimens were found which were EM positive for poxvirus but CAM negative, and 17 were found which were EM negative but CAM positive.

As shown in Table 4, all the positive diagnoses of varicella-herpes cases were made by EM. Thus, for the positive differentiation of varicella from smallpox, EM is essential. Table 4 also shows that the number of varicella isolations is very small. Specimens submitted from these cases generally consisted of dried lesion scabs and/or vesicular fluid shipped without refrigeration; such conditions are very detrimental to the viability of varicella viruses.

The effectiveness of the various test methods in the diagnosis of cases of vaccinia complications is shown in Table 5. Of the 25 vaccinia virus positive cases, CAM detected 100% and EM only 52%. The gel-precipitation detected only 12%; it detected none that was not also detected by both of the other methods.

#### Credibility of the "negative" results for variola

Table 2 in column 3 shows the number of cases and percentages for which no virus was detected by the three combined test methods. The percentages of these cases, which were 37.8, 38.3, and 47.1 respectively, were sizable, and should be given more than a passing acknowledgement because these cases in the final laboratory diagnosis were declared negative for smallpox. But could some of these cases have been smallpox?

This question can be partially answered by Table 6. Of the 83 cases of suspected smallpox tested in the United States of America between July 1966 and May 1972, 44 were negative for virus, none were positive for variola, and 39 were positive for varicella-herpes. The West African countries had a "twilight" period the last few months of 1970 in which no case of bona fide smallpox was found. Between January 1971 and May 1972, 93 cases of suspected smallpox were tested and of these 54 were negative for virus, none were positive for variola, and 39 were positive for varicella-herpes. Because no secondary case of smallpox erupted in the unprotected contacts of the 98 "negative" cases which were under close surveillance, we can assume that they were correctly diagnosed as negative for smallpox.

With a sufficient quantity of specimen from a bona fide smallpox case, it is very likely (45/45 or 100%) that variola can be detected by at least two of the four methods. This confidence failed for three of the 48 cases in Table 3 that were diagnosed positive for variola by the four-test-method system. For these three cases only one of the four methods detected the virus.

Insufficient amounts of specimen material contributed to the deviation of the test results for the three cases. From two of the cases, only a single small smear of vesicular fluid was available, and from the other case, only a single swab of vesicular fluid. In these cases, specimen suspensions are diluted excessively to assure an adequate volume for testing by a variety of tests. However, it is imperative that the portion of a specimen tested by EM be the least diluted, which probably accounts for EM yielding the only positive results.

In view of the requirement of all four tests giving negative results before a specimen is accepted as negative, we believe that when adequate specimens are available, a case with "negative" diagnosis is most likely not a case of smallpox.

Laboratory diagnosis of human monkeypox

As a result of the mass smallpox vaccination programme carried out by CDC in 20 West African countries over the past five years, no case of bona fide smallpox has been found in these countries since May 1970.<sup>11</sup> However, between September 1970 and October 1971, seven cases of pustular disease, clinically indistinguishable from smallpox, were found in West Africa (Table 7). In addition, one similar case from Zaire with an onset date of 20 August 1970 was reported by Dr S. S. Marennikova of Moscow, USSR. These cases differed from the usual smallpox cases in that no secondary cases resulted from human to human contact of unprotected individuals.

The data presented in Table 8 show that the four African isolants, V70-I-187, V70-I-199, V70-I-266, and V71-I-82, are monkeypox. Their characteristics in respect to the various identifying criteria are described in more detail below.

Pock morphology on CAM. The two Liberian isolants (V70-I-199) and the Sierra Leone isolant (V70-I-266) produced small white pocks with central necrosis and haemorrhage on CAM. The central necrosis appeared as a "punched-out" area, the size of a pinpoint. These pocks resembled those produced by the monkeypox (Utrecht) and differed from those produced by the variola (Harvey), which showed no central necrosis or haemorrhage. The pocks produced by the Nigerian isolant (V71-I-82), however, were not clearly distinguishable from those of variola. The pocks produced by the four isolants were much smaller than those produced by vaccinia and were easily differentiated.

Plaque morphology in VERO cell line. In VERO cell monolayers, the four isolants (including the Nigerian strain) and the monkeypox (Utrecht) produced large plaques with relatively clear centres, surrounded by cells piled up along the edges, but the variola (Harvey) produced hyperplastic clumping of cells followed by formation of small plaques. Although the pocks of the Nigerian isolant on CAM were not noticeably different from those of variola, its plaques on VERO cells were different from those of variola. The plaque morphology of the four isolants and the monkeypox did not differ noticeably from that of vaccinia.

Dermal reaction in rabbits. The four isolants each produced large necrotic, haemorrhagic local lesions at the site of the intradermal inoculation. The inoculated rabbits developed generalized illness and exhibited secondary "satellite" exanthems. A 0.1 ml volume of viral inoculum of each isolant with a titre of  $10^{1.5}$  pock forming unit (PFU)/0.1 ml produced a local haemorrhagic lesion about 15 mm in diameter; in contrast, an inoculum of variola (Harvey) with a titre of  $10^{5.5}$  PFU/0.1 ml produced an almost invisible reaction, and vaccinia produced an only slightly visible reaction. The monkeypox (Utrecht) was inoculated intradermally and produced reactions similar to those produced by the four isolants. As a single screening method, the test for dermal reaction in rabbits probably is most appropriate for differentiating human monkeypox from variola.

Ceiling temperature for poxvirus growth on CAM. The four isolants and the monkeypox (Utrecht) were easily differentiated from variola (Harvey) and vaccinia (Wyeth) by their ability to grow at 39.0°C, but not at 39.5°C. Thus, the characteristics by the ceiling temperature test for the four isolants and the monkeypox (Utrecht) were identical.

Chick embryo lethality. The average  $\log_{10}$  PFU of virus per 0.1 ml that gave a mean survival time of four days ( $D_4$  value) for chick embryos for the four isolants was 2.1; for the monkeypox (Utrecht), 2.2; and for vaccinia, 2.2. For the variola this value was 5.0. The four isolants, the monkeypox (Utrecht), and the vaccinia (Wyeth) were about 1000 times as lethal for chick embryo as the variola (Harvey).

### Suckling mice virulence test by intracerebral and footpad inoculations

The average  $\log_{10}$  PFU of virus per 0.1 ml which gave a mean survival time of four days ( $D_4$  value) when inoculated intracerebrally into one-day-old suckling mice were 3.0 for the four isolants and 3.1 for the monkeypox (Utrecht). The variola's value was 6.2, which indicates how much more lethal the four isolants and the monkeypox (Utrecht) were than the variola.

Inoculation of 0.02 ml of either of the four isolants or the monkeypox (Utrecht) virus in a titre of  $10^{2.0}$  PFU/0.1 ml into footpads of one-day-old suckling mice produced generalized infection and 100% mortality by day 7; the same volume of inoculum of variola or vaccinia with a titre of  $10^{5.0}$  PFU/0.1 ml produced only local infection of the limb and occasional runting.

### DISCUSSION AND CONCLUSION

An important factor in the laboratory diagnosis of smallpox is collecting adequate amounts of specimens. The minimum should be three large scabs (2-3 mm in diameter) or two capillary tubes of vesicular fluid, each with 5-10 mm of a column of fluid. By testing either one of these specimens or both by the four methods (EM, gel-precipitation, CAM culture, and tissue culture), we feel that smallpox cases will rarely be misdiagnosed. This confidence decreases when an inadequate amount of specimen is collected.

Working with adequate amounts of specimens increases the likelihood of obtaining accurate positive diagnoses, and even more significantly, gives us confidence in our negative results.

Traditionally, culturing on the CAM has been thought to be the most sensitive and accurate single test for laboratory confirmation of smallpox. In our laboratory, however, we have found that EM examination gives us the highest percentage of positive poxvirus identifications. Most of the specimens received in our laboratory have been shipped from Africa or other distant areas and may have been repeatedly subjected to adverse conditions en route. Virus inactivation as a result of such handling can explain our failure to isolate the virus in many instances.

Although the agar gel-precipitation method is less sensitive than the other methods, it is simple to perform and serves well as a confirmatory test for EM results. It is always comforting to support a negative EM result by a negative gel-precipitation result.

EM is essential for a positive varicella diagnosis, but is not always dependable in detecting a vaccinia infection. With vaccinia infections, the virus is apparently much less abundant in the test specimens than is the case with variola or with varicella, thereby considerably reducing the likelihood of EM visualization.

The discussion until now has been focused on specimens and situations involved in the laboratory diagnosis, but what can be said about the tester's experiences and reactions?

Upon receiving a specimen, a reliably experienced tester must correctly judge whether the specimen is sufficient for the detection of variola. This judgement becomes very important when all tests produce negative results. Because then it must be decided whether the tests produced negative results because the specimen was insufficient or because the specimen actually did not contain variola.

When testing by EM, a tester must examine a specimen of vesicular fluid or a homogenized scab and make appropriate dilutions to avoid overloading the grids, obscuring good visualization of viral particles, or underloading the grids, which would result in a false negative. An experienced electron microscopist will question a negative result obtained by grids prepared with insufficient materials.

For testing by agar gel-precipitation, a tester certainly should be careful to place the reagents in the correct wells and to fill the wells adequately with the reagents to avoid results that could be false negative. The tester must also recognize a non-specific precipitation line and be equally careful not to overlook a faint specific line.

For CAM culture, a tester must not only be experienced, but also careful and alert and constantly expect unusual developments. He must recognize the non-specific pocks and differentiate variola pocks from those of Herpes simplex. He must also watch for pocks such as those of human monkeypox which may differ only subtly from those of variola.

For tissue culture, a tester must not only have a general knowledge of the normal tissue cultures in use, but also must be experienced and alert enough to recognize development of cytopathogenic effect which differs slightly from that caused by the usual strains of viruses.

As many of the global areas approach the goal of complete eradication of smallpox, I wish to emphasize the increasing importance of complete and careful laboratory investigation of every suspected smallpox illness. With the discovery of the human monkeypox cases, we realized that our dependable routine methods of smallpox diagnosis were not sufficient to completely define these unusual cases. We must employ more sophisticated tests which give more specific results.

It is likely that other outbreaks of smallpox-like illness due to viruses other than variola have occurred and will continue to occur. Only through thorough laboratory testing will the true etiology of these outbreaks be defined.

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TABLE 1. METHODS AVAILABLE FOR VIROLOGICAL  
LABORATORY DIAGNOSIS OF SMALLPOX

Methods	Identification of
Electron microscopy	Poxvirus
Gel-precipitation test	Poxvirus
Chick CAM culture	Smallpox
Tissue cultures (Primary monkey kidney or embryonic human fibroblast)	Isolation and partial identification of smallpox
Stained smear	Poxvirus
Complement fixation	Poxvirus
Fluorescent antibody	Poxvirus

TABLE 2. SPECIMENS SUSPECTED OF SMALLPOX TESTED BY THREE COMBINATIONS OF METHODS

Methods	Number of smallpox suspects	No virus detected	Virus detected	Variola identified	Herpes- varicella identified
EM <sup>a</sup> + CAM <sup>b</sup>	849	321 (37.8%)	528 (62.2%)	337 (39.7%)	191 (22.5%)
EM + CAM + Gel-precipit. <sup>c</sup>	801	307 (38.3%)	494 (61.7%)	303 (37.8%)	191 (23.8%)
EM + CAM + Gel-precipit. + T.C. <sup>d</sup>	153	72 (47.1%)	81 (52.9%)	48 (31.4%)	33 (21.6%)

<sup>a</sup> EM = Electron microscopy.

<sup>b</sup> CAM = Chick embryo chorioallantoic membrane.

<sup>c</sup> Gel-precipit. = Agar gel-precipitation.

<sup>d</sup> T.C. = Tissue culture.

TABLE 3. DETECTION OF POXVIRUS INFECTION BY VARIOUS TEST METHODS

Methods	No. of smallpox suspects	Positives for variola by any one method or more	Positives for pox-virus by EM	Positives for pox-virus by CAM	Positives for poxvirus by Gel-precipit.	Positives for poxvirus by T.C.
EM + CAM	849	337 (39.7%)	320 (37.7%) <sup>a</sup>	307 (36.2%) <sup>a</sup>	N.D.	N.D.
EM + CAM + Gel-precipit.	801	303 (37.8%)	286 (35.7%) <sup>b</sup>	279 (34.8%) <sup>b</sup>	240 (30.0%) <sup>b</sup>	N.D.
EM + CAM + Gel-precipit. + T.C.	153	48 (31.4%)	47 (30.7%) <sup>c</sup>	41 (26.8%) <sup>c</sup>	39 (25.5%) <sup>c</sup>	45 (29.4%) <sup>c</sup>

<sup>a</sup> - No significant difference (by Chi-square test, P = 0.50, degrees of freedom = 1).

<sup>b</sup> - Significant differences among methods (by Chi-square test, P = 0.03, degrees of freedom = 2).

<sup>c</sup> - No significant difference (by Chi-square test, P = 0.73, degrees of freedom = 3).

TABLE 4. DETECTION OF HERPES-VARICELLA GROUP INFECTIONS  
BY ELECTRON MICROSCOPY AND VIRUS ISOLATION

Methods	Total positive cases	EM	CAM	Gel-precipit.	T.C.
EM + CAM	191	191 (100%)	0	N.D.	N.D.
EM + CAM + Gel-precipit.	191	191 (100%)	0	0	N.D.
EM + CAM + Gel-precipit. + T.C.	33	33 (100%)	0	0	4 (12.1%) <sup>a</sup>

<sup>a</sup> - Three Herpes simplex and one varicella.

TABLE 5. DETECTION OF VACCINIA INFECTIONS BY VARIOUS TEST METHODS

Method	Total positive cases	Positive with all three tests (EM, Gel-precipit. and CAM)	With EM only	Gel-precipit. only	With CAM only
EM + CAM	25	3 (12%)	13 (52%)	3 (12%)	25 (100%)

TABLE 6. DETECTION OF POXVIRUS INFECTION BY VARIOUS TEST METHODS

Location	Time interval	Total suspects	No virus	Variola	Herpes- varicella
United States of America	July 1966 through May 1972	83	44	0	39
West African countries	Jan. 1971 through May 1972	93	54	0	39

TABLE 7. HUMAN MONKEYPOX CASES THROUGH MAY 1972<sup>a</sup>

Case No.	Country	Age	Sex	Vaccination	Onset date	Basis for judgement
1	Liberia	4	F	No	13.9.70	Strain V70-I-187 isolated
2	Liberia	4	M	No	12.9.70	Serological and epidemiological evidence
3	Liberia	6	F	No	13.9.70	Serological and epidemiological evidence
4	Liberia	9	M	No	2.10.70	Strain V70-I-199 isolated
5	Sierra Leone	24	M	No	1.12.70	Strain V70-I-266 isolated
6	Nigeria	4	F	No	9.4.71	Strain V71-I-82 isolated
7 (possible)	Ivory Coast	5	?	No	8.10.71	Serological and epidemiological evidence

<sup>a</sup> One additional case from Zaire with onset date of 24 August 1970 was tested by Dr S. S. Marrenikova of the Institute for Research on Virus Preparations.

TABLE 8. LABORATORY RESULTS OBTAINED ON TESTING VIRUS STRAINS V70-I-187, V70-I-199, V70-I-266, V71-I-82, MONKEYPOX (UTRECHT), VARIOLA (HARVEY), AND VACCINIA (WYETH)<sup>a</sup>

	Four West African isolants	Monkeypox (Utrecht)	Variola (Harvey)	Vaccinia (Wyeth)
Small pocks with haemorrhagic centre on CAM	<u>b</u>	+	- <u>c</u>	- <u>d</u>
Tissue culture plaque (VERO)	large	large	small	large
Haemorrhagic reaction on rabbit dermis	strong	strong	absent	weak
Growth on CAM at 39°C	+	+	-	+
Growth on CAM at 39.5°C	-	-	-	+
Chick embryo lethality (D <sub>4</sub> value) <sup>e</sup>	10 <sup>2.1</sup> (average)	10 <sup>2.2</sup>	10 <sup>5.0</sup>	10 <sup>2.2</sup>
Suckling mice lethality (D <sub>4</sub> value) <sup>e,f</sup>	10 <sup>3.0</sup> (average)	10 <sup>3.1</sup>	10 <sup>6.2</sup>	10 <sup>5.6</sup>

<sup>a</sup> - Includes some results from Lourie et al.<sup>7</sup>

<sup>b</sup> - Except V71-I-82 which produced pocks not clearly distinguishable from those of Variola (Harvey).

<sup>c</sup> - Small pocks without haemorrhagic centre.

<sup>d</sup> - Pocks very large.

<sup>e</sup> - D<sub>4</sub> value = log dose of virus giving harmonic mean survival time of four days.

<sup>f</sup> - Results of mice inoculated intracerebrally only.