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Experience with Electron Microscopy in the Differential Diagnosis of Smallpox

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The usefulness of negative-contrast electron microscopy in the rapid differential diagnosis of poxvirus and herpesvirus exanthems is described in this study of 301 specimens from patients with vesicular exanthematous diseases. Specimens from patients with smallpox, various forms of vaccination complications, varicella, zoster (shingles), and herpes simplex are included in this evaluation. Electron microscopy, when applied to the study of lesion material, was found to be more sensitive than the classical techniques of virus isolation in the diagnosis of both poxvirus and herpes/varicella virus infections. However, since specific identification of a virus within a group cannot be made morphologically by electron microscopy, it is recommended that both electron microscopy and virus isolation methods be employed for the routine differential diagnosis of vesicular exanthematous diseases in the reference diagnostic laboratory.

Electron microscopy of lesion material in the differential diagnosis of poxvirus and herpesvirus exanthems was first employed in 1947 during an outbreak of smallpox in New York (7). However, not until the present decade, with the advent of negative-contrast techniques involving the use of phosphotungstate salts (1), has electron microscopy become generally accepted as a practical diagnostic method. Its use has increased as the necessary facilities have become available. The rapidity, sensitivity, and limitations of negative-contrast electron microscopy have been well described by several investigators (2, 5, 6, 8). However, each of the reported studies has dealt with specimens from fewer than 75 patients.

The problems encountered by a regional or international reference diagnostic laboratory related to quantity and quality of specimens and adequacy of accompanying clinical information are quite different from those of a research laboratory. The Vesicular Disease Laboratory of the National Communicable Disease Center is the smallpox diagnostic reference laboratory for the United States and provides reference diagnostic support to the World Health Organization. Negative-contrast electron microscopy has been one of the routine diagnostic techniques employed by the Vesicular Disease Laboratory since 1966. Our report presents the results obtained by this laboratory on specimens submitted since 1 January 1967. All smallpox and some nonsmallpox specimens were from patients in West Africa and Southeast Asia. Other nonsmallpox specimens were from patients in the United States.

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MATERIALS AND METHODS

Electron microscopic observations were made between 1 January 1967 and 30 April 1969 on 301 specimens from 287 patients. Specimens were submitted to this laboratory because either a poxvirus or a herpesvirus was presumed to be responsible for the patient's illness. The poxvirus specimens for this study were from patients with smallpox (variola), vaccinia, or paravaccinia; the herpesvirus specimens were from patients with chickenpox (varicella), zoster, or herpes simplex. The clinical information which accompanied the specimens was frequently inadequate and raised serious doubts regarding the validity of the primary clinical diagnosis. A specimen was included in the study only if at least one laboratory test confirmed the presence of a virus, either by isolation in an appropriate host system or by electron microscopic identification. Accordingly, the 301 specimens reported here were selected from a much larger group.

Usually the specimens submitted were dried smears or swabs of lesion exudate, lesion fluid in capillary tubes, or crusts from desiccated lesions. However, viral isolates or tissues obtained from autopsy were also submitted. Most specimens were shipped unrefrigerated. Upon arrival, they were either examined immediately or frozen.

For virus identification by electron microscopy, the specimen was suspended in McIlvaine's phosphate buffer, *p*H 7.2. The volume of buffer used was dependent upon the quantity of the specimen.

Smears. Smears were suspended in 2 to 3 drops of buffer for grid preparation; the specimen suspension was then further diluted to facilitate inoculation into the appropriate host systems.

Swabs. Swabs were soaked and then vigorously agitated in 0.5 to 1.0 ml of buffer.

Fluid specimens. Fluid specimens were diluted with an equal volume of buffer. High-quality grids could not be prepared from undiluted lesion fluid because it contained excessive protein and cellular debris.

Crusts. Crusts were ground in thick-walled glass tissue grinders with sufficient buffer to produce an opalescent suspension.

Virus isolates from tissue cultures. Chorioallantoic membranes were homogenized by vigorous agitation with glass beads. No additional preparatory procedures were performed.

Autopsy tissues. Autopsy tissues were ground without an abrasive in mortars with an equal volume of buffer. The suspension was clarified by low-speed centrifugation.

A drop of the specimen suspension was placed on a paraffin-coated microscope slide alongside a drop of 2% sodium phosphotungstate, *p*H 7.0. Two Formvar-coated grids were prepared from each specimen by touching them successively to the drop of specimen suspension and phosphotungstate. Excess liquid was removed by touching the edge of each grid to filter paper. The grids were then disinfected by exposure to ultraviolet light for 30 min before they were removed from the containment facilities of the laboratory to the electron microscope area. The grids were examined until a viral particle with diagnostic morphology was observed. The grids were routinely scanned at a magnification of 18,500, and a higher magnification was used to confirm morphological detail. Examination for 1 min or less would frequently reveal diagnostic poxvirus particles in a positive specimen; finding herpesvirus particles of diagnostic quality was usually more difficult and required longer periods of examination. Each of the two grids was examined for at least 5 min before the specimen was considered negative.

Virus isolation was attempted in embryonated eggs, in tissue cultures, or in both. The chorioallantoic membranes (CAM) of 12-day-old embryonated hens' eggs (3) were inoculated if variola, vaccinia, cowpox, or herpes simplex was suspected. Tissue cultures (human diploid lung fibroblast cells) were inoculated only if the specimen was frozen when received and if the clinical history suggested a varicella zoster infection.

RESULTS

The 301 specimens were separated into three groups according to primary clinical diagnoses. Thirty-three (34.0%) were submitted with the diagnosis of a herpesvirus infection (Table 1). Electron microscopy failed to make an identification in only one specimen when isolation studies were successful. No poxvirus particles were found in this group of specimens.

Table 2 summarizes the diagnostic experience with 61 specimens in which herpesvirus was isolated or identified (sum of herpesvirus specimens from Tables 1 and 3). Herpesvirus was isolated in 16 (26.2%) and identified by electron microscopy in 60 (98.4%) of the specimens. Only one false-negative diagnosis was made by electron microscopy, as compared with 45 false-negative diagnoses by isolation procedures.

The second group was composed of 268 specimens submitted with the primary clinical diagnosis of a poxvirus infection (Table 3). Twenty-eight (10.4%) were found to contain herpesvirus particles despite the fact that many were obtained from patients in endemic areas for smallpox. Both CAM cultures and electron microscopy were positive for poxvirus with 201 (83.8%) of the remaining 240 poxvirus specimens. Eighteen false-negative diagnoses were obtained by electron microscopy and 19 false-negative diagnoses by CAM culture (Table 4). Two specimens of paravaccinia were excluded from these figures comparing the accuracy of electron microscopy and culture techniques because these viruses cannot be cultured on the CAM or in human fibroblast cells. These two paravaccinia cases illustrate that the laboratory diagnosis of some infrequently en-

TABLE 1. *Virus isolations[a] and identifications[b] for 33 specimens submitted with a primary clinical diagnosis of a herpesvirus infection*

Result	No. of specimens
Herpesvirus isolated	15
Herpesvirus identified	32
Herpesvirus isolated and identified	14
Herpesvirus isolated or identified	33
Poxvirus isolated or identified	0

[a] Isolations by culture on the chorioallantoic membrane of embryonated hens' eggs, in human diploid lung fibroblast tissue culture, or in both.

[b] Identification accomplished by electron microscopy.

TABLE 2. *Summary of laboratory diagnoses for 61 herpesvirus specimens*

Result	No. of specimens
Herpesvirus isolated	16
Herpesvirus identified	60
Herpesvirus isolated and identified	15
Herpesvirus isolated or identified	61

TABLE 3. *Virus isolations[a] and identifications[b] for 268 specimens submitted with a primary clinical diagnosis of a poxvirus infection*

Result	No. of specimens
Poxvirus isolated	219
Poxvirus identified	222
Poxvirus isolated and identified	201
Poxvirus isolated or identified	240
Herpesvirus isolated	1
Herpesvirus identified	28
Herpesvirus isolated and identified	1
Herpesvirus isolated or identified	28

[a] Isolations by culture on the chorioallantoic membrane of embryonated hens' eggs, in human diploid lung fibroblast tissue culture, or in both.

[b] Identification accomplished by electron microscopy.

TABLE 4. *Electron microscopic (EM) and chorioallantoic membrane (CAM) culture results obtained for 240 poxvirus specimens*

Virus	No. of specimens		
	CAM positive, EM positive	CAM positive, EM negative	CAM negative, EM positive
Poxvirus[a]	201	18	10
(Variola)	(179)	(10)	—
(Vaccinia)	(22)	(8)	—
(Cowpox)	(0)	(0)	—
Paravaccinia[b]	—	—	(2)[c]
Totals	201	18	21

[a] Refers to the genus *Poxvirus* which comprises the variola-vaccinia-cowpox group.

[b] Synonyms are milkers' nodes or pseudocowpox.

[c] Members of this group of poxviruses do not produce pocks on the CAM; therefore, a negative result does not represent a failure of the CAM culture technique.

countered poxviruses (molloscum contagiosum, Milker's nodules, orf, and Yaba monkey virus) may depend on electron microscopy when appropriate tissue culture systems are not available.

The distinct morphological differences between particles of paravaccinia and those of the variola-vaccinia-cowpox group can be seen by comparing Fig. 1 and 2. The particles in both preparations are superficially penetrated by phosphotungstate, demonstrating the character and pattern of surface filaments. The vaccinia particle (Fig. 1) was obtained from a highly purified, washed suspension, and phosphotungstate did not aggregate around it. The virion appears rectangular or brick-shaped when viewed lengthwise and circular when viewed on end (Fig. 1 inset). The surface filaments are short and a round central body was frequently seen. The paravaccinia particle (Fig. 2) was obtained directly from unpurified specimen material. Phosphotungstate may be seen aggregated around this particle. It appears cylindrical with a spiral arrangement of the surface filaments; a central body is not visible.

Poxvirus particles deeply penetrated by phosphotungstate were frequently encountered in diagnostic specimens (Fig. 3). The surface filaments are not visible in such preparations. Instead, the virion consists of a sharply defined, relatively dense core surrounded by several concentrically laminated zones of differing densities. The innermost zone, immediately adjacent to the core, and the outermost zone are of low density and appear lighter. Between them lies a denser, darker zone. Frequently, the middle and outermost zones have a crinkled appearance. Those poxvirus virions which are superficially penetrated with phosphotungstate are slightly smaller than their deeply penetrated counterparts (Fig. 4). Poxvirus virions are much smaller than bacilli and significantly larger than herpesvirus virions (Fig. 5; a composite micrograph).

The spectrum of viral particle degeneration was broad and extended from nearly typical particles (Fig. 1 and 3–6) through degenerated, yet identifiable, virus particles (Fig. 7 and 8) to entities which were nondiagnostic (Fig. 9). Some partially degenerated particles (Fig. 7) were considered diagnostic because sufficient morphological detail was evident. Particles such as the one in the lower right of Fig. 4 and the one in the uper left of Fig. 8 were too degenerated to be considered diagnostic in themselves, despite the fact that their size, shape, and immediate proximity to a nearly typical particle would suggest that they were a part of poxvirus aggregate. Such particles, had they been found individually, would have presented much the same diagnostic problem as the one illustrated in Fig. 9. This last particle, although nondiagnostic as previously stated, was sufficiently suggestive that additional time was spent searching the grids for particles of diagnostic quality. Frequently, suspicious but nondiagnostic objects were found in crust specimens at low, scanning magnifications (far left of Fig. 4). Further examination of such particles at higher magnification failed to reveal diagnostic characteristics of the poxvirus group.

Although herpesvirus particles were significantly smaller than those of poxviruses (Fig. 5)

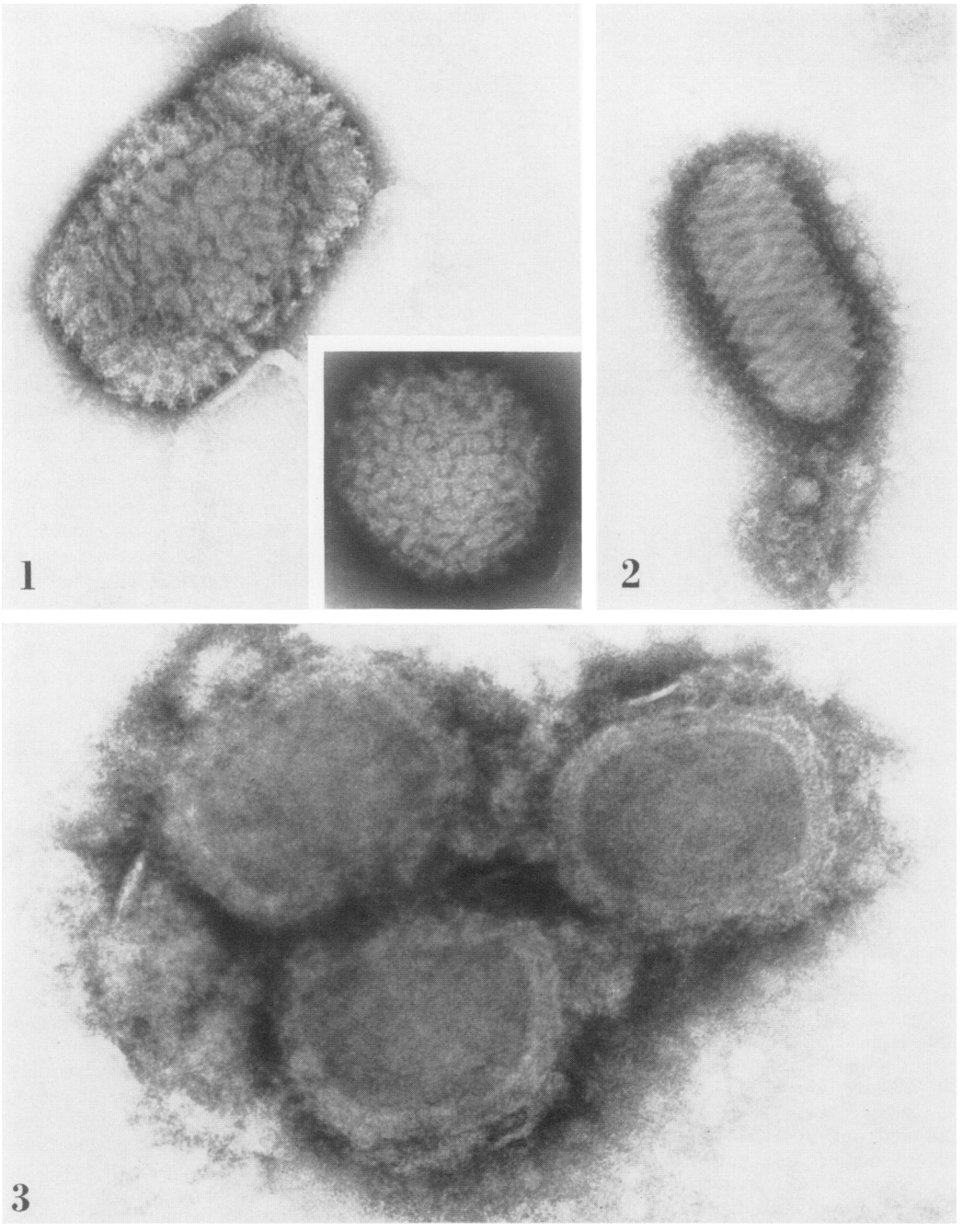

FIG. 1. *Purified poxvirus (variola-vaccinia-cowpox group), phosphotungstate stain (superficial penetration). Note the short surface filaments and the round central body within the rectangular virion. Inset provides an on-end view. × 150,000.*

FIG. 2. *Paravaccinia virus from specimen material, phosphotungstate stain (superficial penetration). Note the smaller size, cylindrical shape, spiral arrangement of surface filaments and absence of a central body. × 150,000.*

FIG. 3. *Poxvirus (variola-vaccinia-cowpox group) from specimen material, phosphotungstate stain. The deep penetration of the stain permits observation of three concentrically laminated zones around the core. × 150,000.*

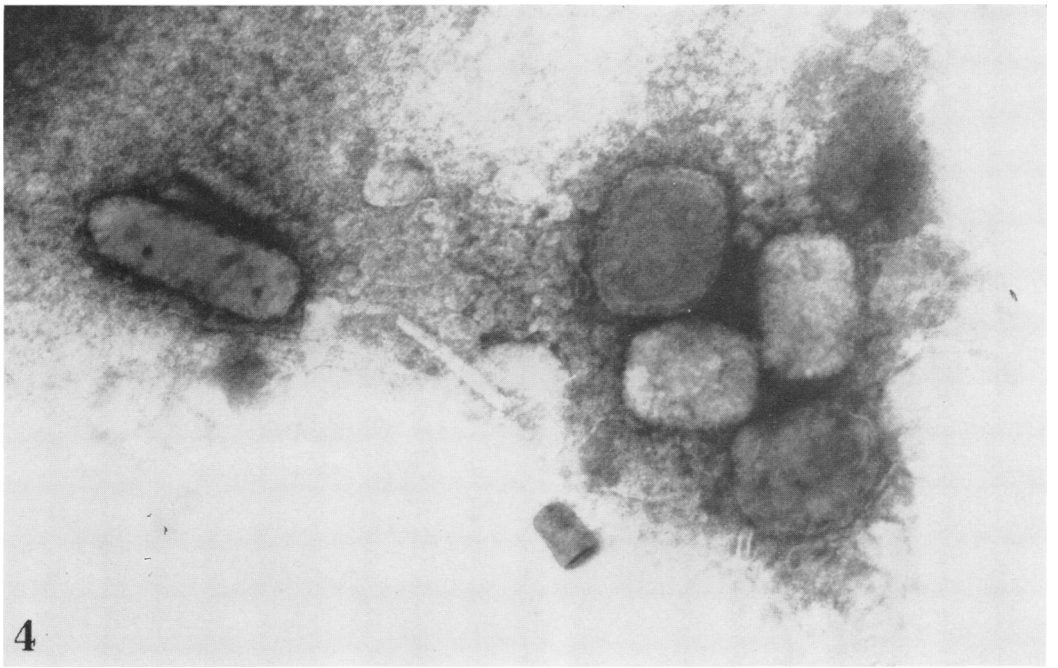

FIG. 4. *Variola virus, phosphotungstate stain. Superficially penetrated variola virus particles appear slightly smaller than the deeply penetrated one. Object at the left is a frequently encountered nonvirus artifact. Phosphotungstate stain of specimen material.* × *65,000.*

they could be detected with relative ease at the low magnifications used for scanning specimens for poxviruses. Indeed, the 28 herpesvirus diagnoses established by electron microscopy (Table 3) were made under such conditions. Fully enveloped herpesvirus particles (Fig. 10 and 12) were most frequently found in specimens of vesicular fluid, whereas naked capsids (Fig. 11 and 14) were usually found in crust specimens. However, numerous enveloped particles were occasionally found in crust specimens.

The morphological detail of herpesvirus particles was usually not as well preserved as that of the poxviruses. Enveloped particles as illustrated in Fig. 5 and 10 and naked capsids as in Fig. 14 were fairly representative of the virus quality encountered in specimens submitted to a reference diagnostic laboratory. Although less than ideal, the quality of such a single particle was sufficient to permit a positive diagnosis. Occasionally, specimens contained only more severely degenerated particles (Fig. 15 and 16). Such a specimen was considered positive only if three or more particles were found.

DISCUSSION

Negative-contrast electron microscopy is well established as a rapid means of detecting both poxvirus and herpesvirus particles in diagnostic specimen materials. The technique is rapid and sensitive, and can be used to detect inactivated virus. However, the technique has several shortcomings, the major one being a lack of specificity. Differentiation is possible between the virus groups (e.g., poxvirus versus herpesvirus) but not between the viruses within a group (e.g., variola versus vaccinia). For this reason, specific identification of the virus by isolation techniques should be attempted. The hazard of smallpox to a susceptible population is of such magnitude that specific identification of any poxvirus is essential.

Another shortcoming of electron microscopy is the relatively high concentration of virus particles necessary in a specimen. Macrae (4) estimated that 1 million to 10 million virus particles per milliliter must be present for electron microscopy to give successful results. It is possible that even higher concentrations of herpesvirus particles may be necessary, because they appear to degenerate more quickly under adverse conditions of transit and storage than do poxviruses.

Although virus isolation is more specific, it is slower in yielding an answer and it does not appear to be any more sensitive than electron microscopy in the diagnosis of poxviruses. Even the hardy poxvirus may be inactivated in a small but

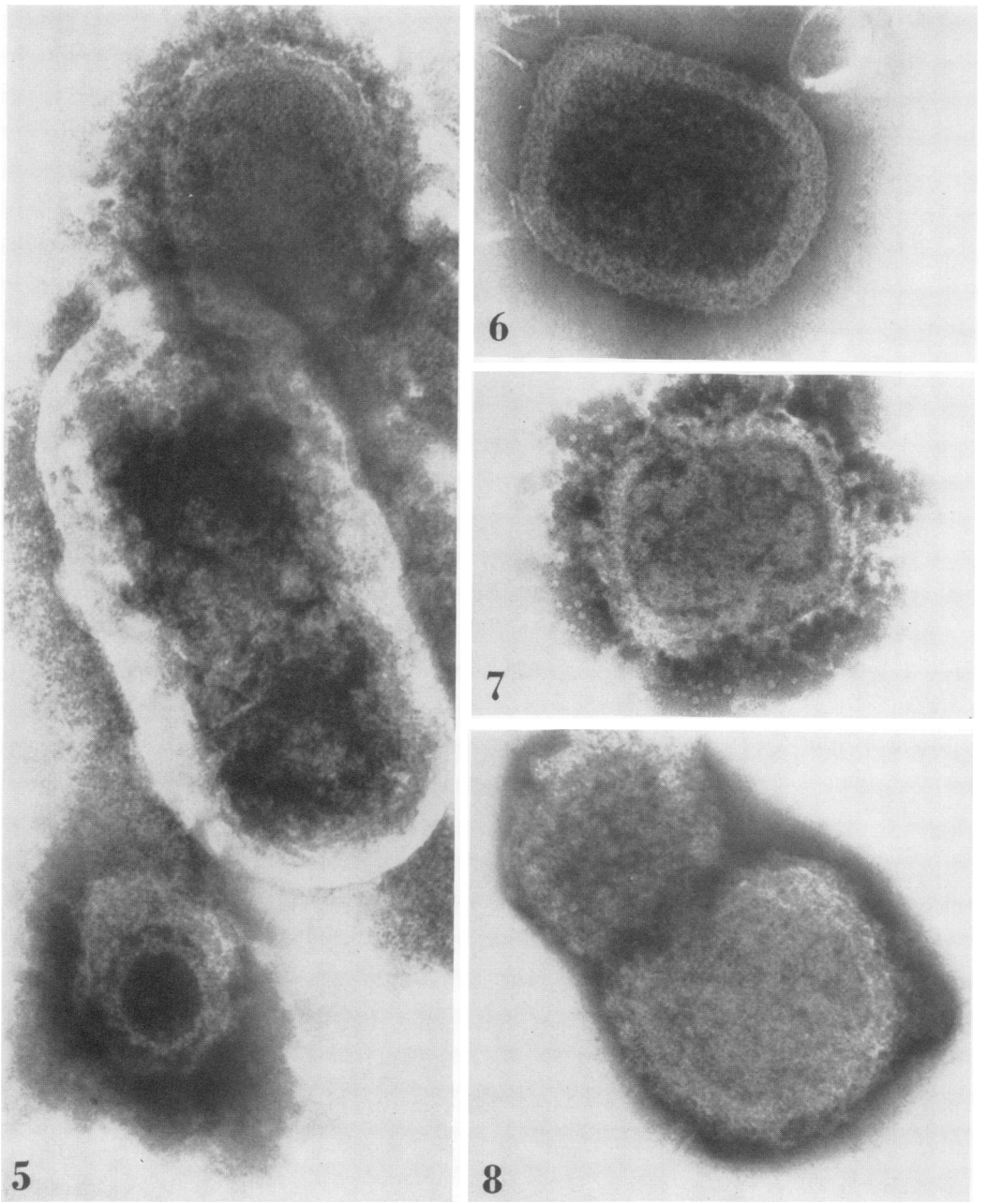

FIG. 5. *Composite micrograph for size comparison of poxvirus (top), bacillus (center), and herpesvirus (bottom). Phosphotungstate stain of specimen materials.* × *150,000.*

FIG. 6–8. *Variola virus particles in different degrees of degeneration. All particler (except for the one in the upper left of Fig. 8) retain sufficient morphological detail to permit diagnostic identification. Phosphotungstate stain of specimen material.* × *150,000.*

significant portion of specimens. The marked lability of the herpesviruses is a definite factor in accounting for the superiority of electron microscopy over isolation techniques.

This study is rather unique in that it was concerned with specimens which in large part were obtained, stored, and shipped under adverse conditions to a geographically distant reference

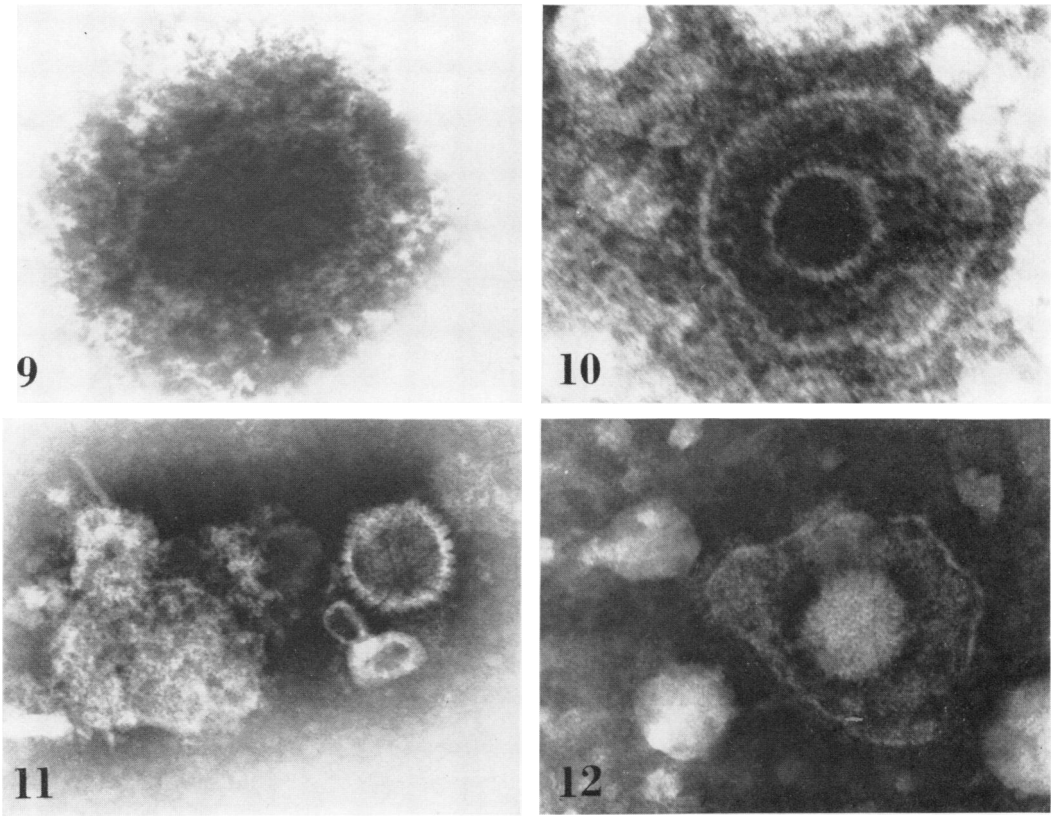

FIG. 9. *Particles with a degree of degeneration making diagnostic identification impossible. Phosphotungstate stain of specimen material.* × *150,000.*

FIG. 10. *Enveloped herpesvirus particles are common in specimens of vesicular fluid, but rare in crusts. Phosphotungstate stain.* × *150,000.*

FIG. 11 and 12. *Naked and enveloped herpesvirus particles of exceptional preservation for specimen material. Phosphotungstate stain.* × *150,000.*

laboratory. The specimens of lesion material submitted were, in order of increasing frequency, smears, fluids, and crusts.

Several generalizations may be made concerning the adequacy of the various types of specimens for diagnostic procedures. For poxviruses, specimens of lesion fluid and crusts were found to be satisfactory for both virus isolation and identification, but poor results were obtained with smear specimens. *Herpesvirus hominus* or *Herpesvirus varicellae* was successfully isolated and identified from most lesion fluid specimens. Crust specimens were usually satisfactory for electron microscopic identification but not for isolation of herpesviruses. As Cruickshank (2) has observed, herpesvirus particles in crust specimens are usually not numerous and frequently are present as naked capsids with poorly preserved fine structure. As a result of these limitations of crusts, electron microscopy presents the only practical means of obtaining diagnostic information from such specimens.

The importation of contagious diseases into nonendemic countries is a current problem resulting from the use of jet aircraft for international travel. Outbreaks of imported contagious disease have already occurred in several countries, emphasizing the need for adequate surveillance and diagnostic facilities. Smallpox has been one of the diseases involved in several such outbreaks of imported disease. Negative-contrast electron microscopy is of great value in the rapid differential diagnosis of vesiculating viral exanthems. The techniques of electron microscopic identification and virus isolation are complementary, as each corrects the major diagnostic deficiencies of the other.

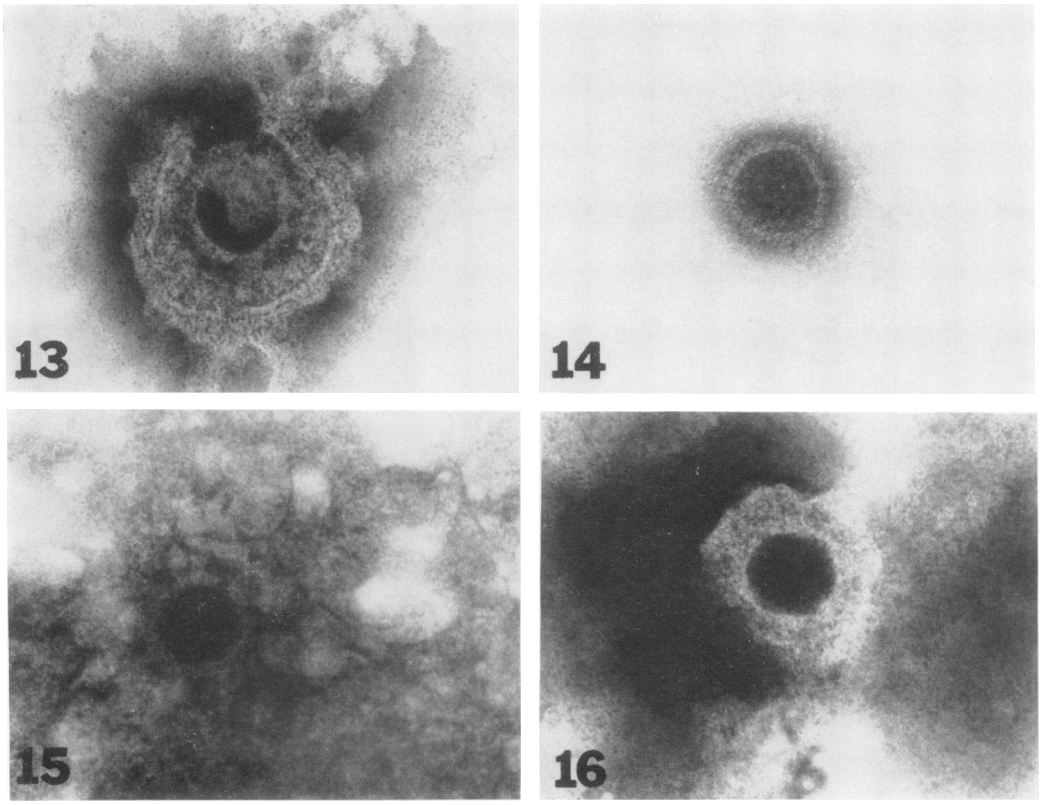

FIG. 13 and 14. *Herpesvirus particles in various states of degeneration. Naked capsids are most frequently encountered in crust specimens. Phosphotungstate stain of specimen material.* × *150,000.*

FIG. 15 and 16. *Severely degenerated herpesvirus particles which are not adequate for diagnostic identification. Phosphotungstate stain of specimen material.* × *150,000.*

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