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CHAPTER

SMALLPOX, VACCINIA, AND HUMAN MONKEYPOX VIRUSES

by

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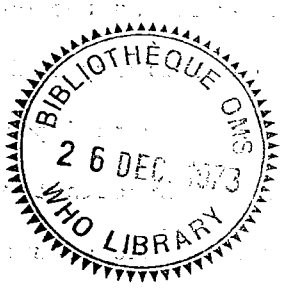

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CLINICAL BACKGROUND

" 'Could this be smallpox?' If this question occurs to the first physician seeing a case of a severe prostration or acute skin eruption in a person who within two weeks has been exposed to travelers or to hospital personnel, half of the danger from smallpox could be averted." So wrote Louis Jacobs in the foreword to the 1966 reproduction of the book by Ricketts and Byles, entitled The Diagnosis of Smallpox, originally published in 1908 (25). Jacobs' statement is even more important today. Physicians in the nonendemic areas, such as the United States, have less and less chance to see smallpox and develop clinical diagnostic acuity as the endemic areas for smallpox decrease. Furthermore, since compulsory smallpox vaccination has been abolished in many countries, it is more important than ever to prevent the spread of smallpox in an unvaccinated population. To help prevent the spread of smallpox and to help eradicate the disease, rapid and accurate laboratory diagnosis is needed.

Variola, human monkeypox and vaccinia are immunologically closely related but distinct poxviruses, each causing an exanthematous infection in humans.

Variola causessmallpox, the most severe exanthematous communicable disease of humans. By 1971, smallpox was eradicated in South America, Indonesia and all the West African countries, but infections still occur in some parts of Africa and southeast Asia.

Smallpox is grouped into three types of illness based on differences in the degree of severity and the proportion of fatal cases occurring during an outbreak. Variola major, the severe type, with case fatality ratios of 15% to 40% prevails in the Asiatic subcontinent; variola minor [alastrim, amass, Kaffir pox, Marsden, 1948 (16)], the mild form with fatalities of less than 1%, was most recently prevalent in South America and southern Africa; variola intermediate, the intermediate type with case fatality ratios 5% to 15%, reported by Bedson et al, 1963 (1), formerly prevailed in East Africa and Indonesia. The viruses causing these types of illnesses can be distinguished in the laboratory by their growth characteristics at certain supraoptimal temperatures.

According to Downie's 1951 description of the pathogenesis of samllpox (3), the virus enters through the mucosa of the upper respiratory tract and quickly passes to the lymphatic glands and is carried by the bloodstream to the internal organs. During an incubation period of 7 to 17 days, the virus multiplies in the internal organs and overflows into the bloodstream, causing viremia. Widespread infection of the skin and mucous membranes follows, and skin lesions become apparent after an additional 2 or 3 days. As the lesions break down, virus is liberated, and the patient becomes highly infective. The immune response which follows determines to some extent the severity of the disease. The cause of death in persons in the late pustular stage, despite a high antibody titer, may be the late effects of earlier virus activity, some complicating disability, or a secondary infection.

The clinical picture of human disease caused by monkeypox, first recognized in Africa in 1970 (19), is similar to that of smallpox except that secondary infections in unimmunized household contacts are all but unknown to occur. The mode of transmission is not clearly understood, but intimate contact with monkeys has been apparent in some cases. Through 1973, only 17 cases had been identified, all in western and central Africa. Although outbreaks have occurred in monkeys and other mammals in captivity, the natural reservoir of the virus has not been elucidated.

Several different strains of vaccinia virus are used in vaccination. Following vaccination, vaccinia infection may occur elsewhere on the skin and mucous membranes as a result of manual transfer of the virus from the vaccination site or following viremia. Rare but possible serious complications following vaccination include post vaccinal encephalitis, eczema vaccinatum and progressive vaccinia (vaccinia gangrenosa).

DESCRIPTION OF AGENTS

The viruses of variola major, variola minor, variola intermediate, vaccinia and human monkeypox, all of which are classified under the genus group of poxvirus, also belong in the Subgenus A, of which vaccinia is its representative type (28). They are double-stranded deoxyribonucleic acid viruses, are "brick-shaped" by electron microscopy, range in size from 250-300 nm in length X 200-250 nm in width, and are morphologically indistinguishable from each other. They are stable in dried condition; variola viruses, especially in crusts, may survive at room temperature for many years. They are serologically closely related and can be identified as poxviruses belonging to one group by using the agar gel precipitation test with antivaccinia serum. The viruses produce soluble hemagglutinin. They grow on chick allantoic membrane and in tissue cultures.

COLLECTION AND STORAGE OF SPECIMENS

In the U.S.A. and its territories, all suspected cases of smallpox are, upon discovery, immediately reportable by telephone to the respective state or territorial health department.

All persons routinely handling specimens from patients with suspected smallpox must be vaccinated every 12 months. All laboratories located in geographic zones nonendemic for smallpox which handle speciments of suspected smallpox cases should be isolated from other work areas, and all personnel in these laboratories must practice strict isolation techniques.

It is important to collect an amount of specimen sufficient to permit effective testing; use of too little specimen decreases the dependability of laboratory tests in the diagnosis of smallpox.

During the pre-eruptive phase of illness, blood collected with sterile anticoagulant can be used as a virus isolation source.

During the macular phase, at least four smears should be collected. These smears are made by lesion scrapings from the lower epithelial layers, with a No. 11 or No. 12 scalpel blade. Each smear made on a slide should be as small and as thick as possible. The slides should be shipped separately in a plastic slide holder with a tight-fitting cap, or several slides may be bundled together with a rubber band, with the individual slides separated by broken matches or tooth picks, and placed in a screw-capped metal container.

During the vesicular phase, a volume of vesicular fluid equivalent to at least two capillary tubes filled to a height of 10 mm should be collected. The tubes are sealed with clay or plastic cement. When the fluid is too viscous to permit collection in capillary tubes, as during the pustular phase, smears may be made on glass slides instead. Capillary tubes are placed in a tightly stoppered or screw-capped vial, then in a larger protective container, as described above.

During the crusting phase no fewer than three crusts, each measuring about 5 mm in diameter, should be collected; for thorough testing, at least 12 crusts should be collected. All crusts should be shipped in a screw-capped vial.

The packaging and shipment of smallpox diagnostic specimens warrants special attention to avoid damage and leakage of the specimen in transit and possible accidental exposure of anyone enroute or at the destination. The Interstate Quarantine Regulations (42 CFR, Part 72.25, "Etiologic Agents" revised July 30, 1972) specify minimum packaging, labeling and notification requirements to assure safe arrival and delivery.

Figure 1a illustrates proper packaging of a smallpox specimen. Clinical materials are placed in a primary container (test tube or screw-capped vial), which is securely closed, and sealed with waterproof tape and then enclosed in a second durable, watertight container (secondary container). The space at the top, bottom, and sides between the primary and secondary containers should contain sufficient nonparticulate

absorbent material (paper towel) to absorb the entire contents of the primary container if it breaks or leaks. Each set of primary and secondary containers is placed in an outer shipping container constructed of corrugated fiberboard, cardboard, or other material of equivalent strength. A copy of the laboratory record or other identifying information should be enclosed between the secondary container and the outer shipping container. The "Etiologic Agents" label (Fig. 1b) is affixed to the outer shipping container. Smallpox specimens should be transported by special air transportation system, registered mail or an equivalent system which provides for the shipper to be notified immediately upon delivery.

In addition to the packaging, labeling and transportation requirements of the Interstate Quarantine Regulations, the importation of smallpox speciments into the United States from abroad requires an authorizing permit issued by the Center for Disease Control (CDC). For more detailed information on importation permits and other requirements applicable to the shipment and receipt of smallpox specimens, write the Center for Disease Control, Attention: Biohazards Control Officer, 1600 Clifton Road, Atlanta, Georgia 30333, or phone the Center at 404-633-3311.

DIAGNOSTIC TESTS FOR SMALLPOX

General statements

Laboratory diagnostic tests for smallpox should produce accurate, easily interpreted results. They should be relatively quickly performed, simple and direct. Seven methods that may be used for the laboratory diagnosis of smallpox are listed in Table 1. The first four methods, namely, electron microscopy (EM), agar gel precipitation (AGP), chick chorioallantoic membrane (CAM) culture, and tissue cultures (TC), have been shown over the past 7 years (1966-1973) at CDC to be the combination of choice, giving the most dependable results with the least confusion. Each of these methods will be discussed in detail. The other three methods, stained smears, fluorescent antibody (FA), and complement fixation (CF), do not provide additional advantages to the laboratory diagnosis. They are not used routinely, but, for completeness, they will be referenced.

Serological tests are not generally employed in the definitive diagnosis of smallpox, but they may be useful when specimens for direct detection of viruses are not available.

Electron microscopy

The electron microscopy (EM) method described here is essentially that described by Long et al (13) and requires the following equipment and reagents: 400-mesh grids coated with formvar, platinum-tipped forceps, plastic Petri dishes (60 mm in diameter) lined with filter

paper, 2.0% sodium phosphotungstate with pH 7.0, wax-coated glass microscope slides prepared by dipping the slides into melted paraffin wax, and glass capillary tubes with individual micro rubber bulbs.

Preparation of grids with specimens. Vesicular fluid collected in capillary tubes, lesion fluid collected as smears, and ground scab suspensions are all excellent sources of virus for visualization by EM.

Capillary tubes containing vesicular fluid are nicked with a file, and the sealed ends are removed. A microbulb is attached to one end of the tubes, and two drops of the fluid are squeezed out to form a single larger drop on a wax-coated slide. If a fibrin plug is present in the fluid, it is included in the drop on the slide. An equal-size drop of phosphate buffer, pH 7.2, 0.01 M, is delivered in close proximity to the vesicular fluid, and the two drops are mixed with a clean capillary tube and microbulb to give an approximate 1:2 dilution. A drop of 2.0% sodium phosphotungstate is placed on another section of the same wax-coated slide. Two EM grids coated with formvar are placed first on the drop of the diluted vesicular fluid, coated side down, and allowed to float for about 30 seconds. The grids are then transferred to the drop of 2.0% sodium phosphotungstate and allowed to float there for 30 to 60 seconds. The grids are then placed, coated side up, on filter paper lining a small Petri dish and labeled as the 1:2-diluted specimen. Subsequent dilutions of 1:4 and 1:8 are made in a similar manner, and two grids are prepared for each dilution. Undiluted

vesicular fluid is not usually used because it would make the grids too dense for examination.

When lesion fluid collected as smears is used to prepare grids, the smears are softened with two drops of the buffer, and a suspension is made by the sucking-expelling action of a capillary tube equipped with a microbulb. Two or three smears are used to provide sufficient material. The suspension thus prepared is used, without further dilution, to prepare grids.

Scabs are ground with the buffer in a thick-walled glass tissue grinder. The volume of buffer added depends on the size and the number of scabs. About 0.3 to 0.5 ml of the buffer is used for each scab approximately 5 mm in diameter; smaller scabs are diluted with less volume and larger scabs with somewhat more. The suspension prepared in this manner is considered undiluted, and subsequent twofold dilutions are made from it on the wax-coated slide. Two grids are prepared for each dilution, as described for the vesicular fluid.

The grids and contaminated containers must be rendered noninfectious before they are taken out of the isolation laboratory for EM examination. To accomplish this, the small Petri dish containing the prepared grids is placed open (base dish, right side up, and the cover dish, upside down) in a larger dish containing a shallow volume of 5% sodium hypochlorite and exposed for 30 minutes to ultraviolet light at

a distance of 2.5 cm from the light source* in a pass-through box. The effectiveness of the ultraviolet light source must be checked at weekly intervals.

Electron microscopic examination. Initially, the grids are examined for density at a magnification of about 2,000 X to 3,000 X. A grid too dense indicates that too much material has been put onto the grid, and a grid not dense enough indicates that too little material has been used. Either condition greatly diminishes the reliability of the test. Those grids which are found to have a satisfactory density are then scanned at a magnification of 10,000 X to 20,000 X. Scanning at 10,000 X rather than at 20,000 X gives more thorough coverage, but requires that the operator be thoroughly experienced in detecting the virions of pox and herpes group viruses. Once a virion is found, the detailed structure of the particle is examined at a higher magnification of 50,000 X to 184,000 X.

Figure 2(3) illustrates the "C" form of poxvirus described by Harris and Westwood (10) [the type 1b form of Nagington and Horne (20)] found

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*General Electric "Germacidal Lamp" (G15T8), 15 watt, with specification of 2.95 watts/sq. ft. at the exposure distance of 5 cm. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

in most scabs collected from patients with smallpox. Figure 2(1) illustrates the "M" form of Harris and Westwood (type 1a of Nagington and Horne) most prevalent in vesicular fluid from patients with smallpox. Figure 2(7) shows the naked capsids of herpes-type viruses, the most abundant form in scabs collected from cases of chickenpox. The typical enveloped virions of herpes-type viruses [Figure 2(5)] also occur, but they are more abundant in vesicular fluid.

In examining grids, one must learn to differentiate nonviral particles that resemble poxvirus or herpes virus from the real viral particles. Nonviral particles that resemble the "M" and "C" forms of poxviruses are illustrated in Figure 2(2) and Figure 2(4).

What is the reliability of EM for the detection of viruses in specimens collected from patients with suspected smallpox? Of the specimens from 1,066 patients examined by our laboratory since 1966, a total of 367 were identified as variola or human monkeypox by the combination of EM, CAM and AGP methods. Of the 367 positives, 351, or 95.6%, were detected by EM alone. It was apparent that an even higher percentage positive could have been detected by EM alone if the quantity of vesicular, pustular or crust material in all specimens had been adequate.

Of the specimens from the 1,066 patients of suspected smallpox, 282 were identified as having varicella-herpes viruses by EM. This constituted the entire number identified as such. Varicella-herpes viruses are relatively labile as compared with poxviruses; and since

many specimens received were scabs or vesicular fluids which were not fresh, virus isolation was generally impossible. EM remained the only dependable method for the detection of these viruses.

Stained smears

Stained smears can be examined by light microscope laboratories where no EM is available, but this type of examination certainly does not equal EM in reliability. For details consult Downie and Kempe (4) who describe and evaluate Gutstein's method (8) and Gispen's modification of Morosow's silver method (7).

Fluorescent antibody

Although several reports describe the use of FA for the diagnosis of smallpox, the technique is not considered reliable enough for routine diagnosis and is not recommended (23).

Chick embryo chorioallantoic membrane culture

Preparation of eggs. Fertile hen's eggs must be incubated at 38 to 39 C for 11 to 13 days to be useful for isolating and identifying the viruses of variola, vaccinia, and most known strains of human monkeypox. Lower incubation temperatures render the CAM less sensitive or totally insusceptible to poxviruses at the recommended time of 12 days.

Excellent photographic illustrations of methods for preparing an embryonated egg for inoculation may be found in the WHO Guide to the Diagnosis of Smallpox (9).

With the embryonated eggs in a rack, blunt ends up, a small hole is made in the shell and shell membrane of each egg over the air space

by rotating the pointed end of a hand punch. [An electric-powered punching device has also been discussed (17).] Each egg is then candled to determine the embryo's viability, and while the egg is being candled, a small hole is made with the hand punch (with rotary motion) through the shell at a spot over the CAM in an area without large blood vessels. Care is taken at this time not to puncture the shell membrane. All eggs are then placed with the CAM "hole" side up on a rack. The shell dust is blown away, and a drop of sterile phosphate buffered saline (PBS) is placed over the CAM hole. A sterile tip of a bent disposable blood lancet or a curved pen nib is inserted through the drop of PBS at an angle of about 20° to the horizontal and passed directly under the shell as far as it will go. The lancet handle is then raised towards the vertical; this motion tears the shell membrane and the drop of PBS is drawn inside as the CAM falls. The eggs are candled to confirm the creation of a new air space above the dropped CAM. If the new air space is not found, slight suction is applied with a rubber bulb over the hole at the original air space on the blunt end of the egg. If the CAM still does not drop to create a new air space, the egg should be discarded. The eggs are returned to the incubator and inoculated within 2 hours.

Preparation and inoculation of specimens. Blood which has been collected with sterile anticoagulant is centrifuged at 1,500 RPM. The plasma is drawn off by pipette and bulb first, and then the buffy coat

(layer of white blood cells) is drawn off separately with the same pipette. Each is mixed 1:2 with McIlvaine's buffer, pH 7.8, 0.004 M, containing 500 units of penicillin, 500 µg of streptomycin and 250 units of neomycin sulfate per ml. The plasma and the white cell suspension are inoculated separately, each into a set of four eggs. Each egg is inoculated with 0.1 ml directly onto the CAM by a disposable tuberculin syringe equipped with a 25-gauge 5/8-inch needle.

The scab suspension, vesicular fluid suspension or smear suspension made for the preparation of EM grids is also used to inoculate eggs. Each suspension is diluted 1:10, 1:1,000, and 1:10,000 with McIlvaine's buffer containing the antibiotics. Four eggs are inoculated with each dilution, as described above, and rocked back and forth for better distribution of the inoculum over the CAM. The holes in the shell are then sealed with a drop of plastic cement. The three dilutions of each specimen may be inoculated with one syringe, provided the highest dilution is inoculated first.

As a routine procedure, the inoculated eggs are incubated at 35 C instead of 36 to 37 C to avoid the complicating effects which result from supraoptimal temperatures. Some eggs are opened for examination at 48 hours, if necessary (see "Harvesting" below), and the remainder are incubated for 72 hours. Negative CAM's are passed again in eggs (blind passage).

To monitor the susceptibility of CAM's to the poxviruses, some of each lot of embryonated eggs should be inoculated with variola and

vaccinia virus as controls. (Controls should always be inoculated after test specimens are inoculated to avoid cross-contamination.) The controls are necessary because it has been observed since 1966 that at times CAM's do not support the growth of variola, vaccinia, human monkeypox or herpes simplex. Possible reasons are: (1) eggs from a physiologically different flock of hens, (2) use of unusual antibiotics in the flock, (3) viral infection in the flock, causing infection of the embryo and interference, (4) incubation of the eggs at a temperature lower than 38 C, resulting in physiologically less developed embryos than the normal 12-day-old embryos, (5) insufficient humidity during incubation, (6) use of a too concentrated buffer solution to dilute the test specimens, and (7) incubation of inoculated eggs at a supraoptimal temperature.

Although the exact cause of the variable susceptibility is uncertain, the effects are real, and they are manifested in several ways: (1) CAM's support no growth of control strains of variola, vaccinia, human monkeypox and herpes simplex; (2) inoculation of a control strain of vaccinia virus promotes growth of atypically small pocks; or (3) when a "house standard" vaccinia is inoculated, the pock titer may be 0.5 to 1.0 $\log_{10}$ lower than usual, although the pock morphology is characteristic of vaccinia.

Harvesting. Inoculated eggs should be harvested in an isolation cabinet, and the cabinet should be disinfected after harvesting each specimen lot. A separate set of sterile forceps and scissors is used for each specimen.

When an early result is required, some of the eggs are opened and the CAM's are examined at 48 hours instead of 72 hours. To harvest, hold the egg with the inoculated CAM uppermost, insert sharp-pointed scissors into the hole in the blunt end, and cut the shell along the horizontal axis. Thus, the egg is cut into half. The lower half, containing the yolk and embryo, is allowed to drop into a discard pan; only the inoculated CAM should adhere to the top half of the egg shell. The CAM is peeled from the shell with forceps and placed in a large Petri dish containing sterile saline. All eggs are harvested in a similar manner, with the CAM's from each inoculum dilution deposited in separate Petri dishes. The CAM's are examined for pocks by using a magnifying glass under a strong light. Since pocks stand out better against a black background, the Petri dishes containing the CAM's are placed on a wet, black counter top for examination.

Differentiation of virus on the basis of pock morphology. The viruses of variola and vaccinia, most strains of human monkeypox, and types 1 and 2 herpes simplex are differentiated primarily by the morphology of the pocks they form on the CAM. Varicella-zoster virus does not grow on the chick CAM.

Variola pocks at 72 hours of incubation are about 1 mm in diameter, grayish white to white, opaque, convex or dome-shaped, raised above the the CAM, round, regular, smooth on the surface, not hemorrhagic, and of about the same size.

Vaccinia pocks at 72 hours are 3 to 4 mm in diameter, flattened with central necrosis and ulceration, and sometimes slightly hemorrhagic.

Human monkeypox pocks at 72 hours are about the same size as those of variola, but they are not as raised; and most of the pocks have a pinpoint hole in the center and are sometimes hemorrhagic. Marrenikova reported that hemorrhage appears when the inoculated eggs are incubated at 34 C (15).

Herpes simplex type 1 pocks at 72 hours are pinpoint size, not raised, not opaque, not regular shaped and, when many are present, are in a lattice-work arrangement.

Herpes simplex type 2 pocks at 72 hours are about 1 mm in diameter, white, flat and irregular in shape and size.

If there is any doubt in the identification of pocks formed by herpes simplex, the virus can be directly identified by the EM, CF, or agar gel test.

Pocks of various poxviruses and herpes simplex are clearly illustrated in excellent colored photographs by Dumbell (5).

The CAM culture method is highly effective for detecting variola and human monkeypox, as shown by the CDC comparative tests. Of 1,066 specimens from suspect cases of smallpox, 367 (34.4%) were found to be positive by EM, CAM and AGP combined. Of these 367 (89.1%) were positive on the CAM, despite the fact that many of the specimens had been in transit for up to 2-4 weeks at relatively high ambient temperatures

before testing. Furthermore, the occasional unpredictable variation in CAM susceptibility already described may have allowed some positive specimens to go undetected. Special care to avoid these two problems will increase the efficiency of the CAM method.

A number of pitfalls can, with foresight, be avoided. For example, when the eggs are candled, examine the pointed end of the eggs to determine whether the CAM is adequately developed into that area; eggs with an underdeveloped CAM should not be used because they may be less sensitive to poxviruses. In this same vein, Westwood et al (27) reported that eggs with albumin sac encroachment on the dropped area of the CAM gave only about 1/10 of the vaccinia pock count of the control.

In examining the pocks on the CAM, one must be careful not to mistake nonspecific lesions for true pocks. Among the several causes for the appearance of nonspecific lesions, the most common is mechanical trauma. In addition, Westwood et al (27) reported that isotonic sodium phosphate buffer 0.122 M used for inoculation induced a high incidence of nonspecific lesions.

Dumbell (5) reported that large doses of variola virus inactivated by heat or ultraviolet irradiation can cause a general thickening of the CAM and obscure the effect of a small amount of viable virus which may be present. We have observed similar effects when the CAM is inoculated with diagnostic specimens containing a very high virus titer; they could be avoided by diluting the specimens 10^{-3} or 10^{-4} before inoculation. CAM's showing the thickening effect should always be put through a

a second passage at several tenfold dilutions in attempts to obtain definitive evidence of pock growth.

Tissue cultures

The use of tissue cultures is a necessary alternate virus isolation method for the CAM, in view of the periodic unpredictable insensitivity of the CAM. The tissue cultures used are primary rhesus monkey kidney cells (MKC), infant foreskin fibroblast cells (FS-32), and VERO cells. These cells are grown in round screw-capped tissue culture tubes. The MKC are grown in lactalbumin hydrolysate (LAH) with 5% fetal calf serum and maintained in LAH without calf serum. FS-32 cells are grown in Kissling's modification of Eagle's minimum essential medium (MEM) (12) (appendix) with 10% fetal calf serum and maintained in Eagle's MEM with 2% fetal calf serum. VERO cells are grown in Eagle's MEM with 10% fetal calf serum and maintained in Eagle's MEM without calf serum.

The MKC and the FS-32 cells are used routinely for isolation of variola, vaccinia and monkeypox, and the FS-32 cells for isolation of varicella and herpes simplex viruses. The VERO cells are used to differentiate variola and human monkeypox on the basis of plaque characteristics, since these two viruses are sometimes difficult to differentiate by pocks produced on CAM's. The plaques formed by variola and vaccinia in VERO cells also differ from each other, but those of human monkeypox and vaccinia are not clearly different.

Specimen suspensions previously made for EM examination are prepared for tissue culture inoculation by diluting 1:2 with McIlvaine's buffer containing 4,000 units of penicillin, 2,000 µg of streptomycin and 2,500 units of neomycin sulfate. Usually, 0.1 ml of this dilution is inoculated into each of three tubes of the tissue culture and incubated at 35.5 C. The inoculated cultures are examined daily for signs of cytopathogenic effect (CPE). Those which fail to show CPE are observed for 10-12 days and then passaged in additional cultures. A test is not declared negative until the second passage cultures are also observed for 10-12 days without evidence of CPE.

Monkey kidney cells. Variola virus in clinical specimens may evoke signs of CPE within 1 day in MKC, with a rounding-up of the cells and the presence of hyperplastic foci. The CPE spreads rapidly when high-titered inoculum is used, and the cells will eventually slough off. Vaccinia and human monkeypox virus may also cause CPE in 1 day, evidenced by the rounding up of cells and the formation of foci. However, they will also form plaques in 2 to 3 days which measure 2 to 3 mm in diameter and usually show cytoplasmic bridging. Again, when a high titered inoculum is used, the entire cell sheet becomes involved, and the cells will eventually slough off.

FS-32 cells. When inoculated with variola, cells fuse and form multinucleated foci in 1 to 3 days, with little cytoplasmic bridging. With vaccinia and human monkeypox, cells fuse and fall apart, forming

large plaques and a meshwork of cytoplasmic bridging in 1 to 3 days. After inoculation with varicella-zoster virus, foci of small groups of swollen, rounded, refractile cells appear in 4 to 5 days. With herpes simplex virus, on the other hand, some degenerating cells may be rounded and others are fattened while elongated. Some cells become fused in 24 hours, and CPE spreads rapidly.

Vero cells. Variola causes hyperplastic clumping of cells within 48 hours, followed by the formation of small plaques. These plaques are easily differentiated from those of vaccinia and human monkeypox. Vaccinia produces large plaques within 48 hours, with a noticeable amount of cytoplasmic bridging. Human monkeypox produces large plaques within 48 hours with relatively clear centers surrounded by cells piled up along the edges.

As a general rule, tissue cultures inoculated with high-titered viral materials show CPE on the entire cell sheets so that no individual plaques can be discerned. Therefore, when tissue cultures are used to produce viral plaques that can be characterized morphologically, one must inoculate several dilutions of the stock viruses (or specimens) so that the plaques formed are properly separated on the cell sheets.

Animals

Rabbits and suckling mice are not generally used for isolating the exanthematous viruses, but they are useful in differentiating variola and human monkeypox (14).

Dermal reaction in rabbits. A 0.1 ml volume of human monkeypox virus with a titer of $10^{2.5}$ pock-forming units (PoFU)/ml, when inoculated intradermally on the shaved skin of a rabbit, produces a local hemorrhagic lesion about 15 mm in diameter; a similar inoculum of variola with a titer of $10^{6.5}$ PoFU/ ml produces only a slightly visible reaction. In contrast, a 0.1-ml inoculum of human monkeypox virus with a titer of $10^{6.0}$ PoFU/ml causes a generalized illness in the rabbit, with secondary "satellite" exanthems in about 3 to 4 days.

Suckling mouse virulence test by footpad inoculation. Inoculation of 0.02 ml of human monkeypox virus with a titer of $10^{3.0}$ PoFU/ml into a hind footpad of 1-day-old suckling mice produces a generalized infection and 100% mortality by day 7, but the same volume of variola virus inoculated at a titer of $10^{6.0}$ PoFU/ml produces only local infection of the limb and occasional runting.

Agar gel precipitation test

Methods for the preparation of vaccinia antigen in CAM and hyperimmune antivaccinia serum in rabbits are described in the appendix. An end-frosted 75- X 25-mm glass slide, precoated with 0.2% purified agar (Difco) in distilled water, is prepared in the following manner for use as an agar gel slide. A line is drawn between the frosted and the clear area with a marking pen containing a fast-drying oil-base paint.

The slightly raised line produced prevents the melted agar from running into the frosted area. One-and-a-half milliliter of melted 1.0% purified agar in distilled water containing a 1:10,000 dilution of thimerosal is carefully delivered and spread onto the entire clear area of the slide. This forms a layer of agar about 2 mm thick. After the agar hardens, a plastic template with a pattern, as shown in Figure 3, is used to punch out wells in the agar. Agar cores are removed by suction produced with a Pasteur pipette attached to a vacuum line. (The dimension of the template is such that the wells arranged in the pattern shown in Figure 3 are each 4 mm in diameter and are 1 mm apart from each other.)

The original suspensions of specimens prepared for the EM test are used for the AGP test without diluting. [According to Dumbell and Nizamuddin (6), the limit of dilution of crusts is about 1:60 by W/V.] The specimen suspension to be tested is placed in wells Nos. 1 and 4, as shown in Figure 3. The positive control vaccinia antigen is placed in well No. 2, and the normal rabbit serum in well No. 3. The antivaccinia rabbit serum is placed in the central well, "C." The slide is then placed in a humid chamber and incubated at 35 C. Lines of precipitation (positive reaction) will occur within 2 to 4 hours between the wells containing the specimen and the antivaccinia serum, if the specimen is from lesions of smallpox, vaccinia or human monkeypox. The line(s) of precipitation formed between the wells containing the

specimen and the antivaccinia serum must fuse or join (form a line of identity), with at least one of the lines between the wells of the positive control vaccinia antigen and the antivaccinia rabbit serum. Specimens are not considered negative unless diagnostic lines fail to appear by 24 hours of incubation.

A specimen negative on first testing should be retested with antivaccinia rabbit serum diluted 1:2, 1:4, and 1:8, provided adequate specimen remains. The use of the diluted reagent antiserum in the test may result in an optimal antigen-antibody proportion and consequently give a positive result.

In evaluating the reliability of the AGP test for detecting poxviruses, we found 288 (78.8%) of 1,066 specimens from patients with suspected smallpox positive by AGP versus 367 for EM, CAM and AGP combined. The major reason that 21.2% were not detected was inadequate amount of specimen for testing. The next was probably degeneration of the soluble precipitating antigens brought about by the prolonged exposure of some of the specimens to high ambient temperatures during shipment. Dumbell and Nizamuddin (6) have shown that heating crust extracts at 60 C for 15 minutes greatly weakens AGP reactions.

Human convalescent smallpox serum should not be substituted for hyperimmune antivaccinia rabbit serum as a testing reagent. In human serum, antibodies other than those specific for vaccinia, variola or

human monkeypox may be present, in which case a precipitation line other than that against vaccinia may be observed and may confuse the diagnosis.

SEROLOGIC DIAGNOSIS

Serologic methods are almost useless for the diagnosis of smallpox because conditions under which the disease occur demand more speed and accuracy than such procedures provide. The various serologic procedures will not be described in detail, but the relative merits of each will be discussed briefly.

Four serologic tests are applicable to assay antibodies evoked by smallpox, human monkeypox and vaccinia infections: AGP (in which a patient's undiluted serum is tested instead of lesion material--Fig. 3), CF, hemagglutination inhibition (HI), and neutralization, using tissue cultures such as LLC-MK_2 (11) or eggs. Vaccinia virus is used as the test antigen in all four methods.

For the CF, HI and neutralization tests, a fourfold rise in titer between acute and convalescent serum specimens is considered diagnostic. However, only a single serum specimen, taken late in the illness or during convalescence, may be all that is available. It is important to understand the limitations of the various tests when attempting to interpret the serological results obtained with such sera. For a single serum collected 8 or more days after the onset of symptoms of smallpox, the following range of results may be expected;

Agar gel precipitation test. After actual smallpox infection, the test is generally clearly positive. On the other hand, sera from individuals recently vaccinated or revaccinated without a history of smallpox are rarely positive.

Complement fixation test. Following smallpox infection, the CF antibody titer is usually greater than 1:20; the titer in specimens from individuals vaccinated or revaccinated without a history of smallpox is usually less than 1:20 or not detectable.

Hemagglutination inhibition test. The HI titer is generally greater than 1:1000. However, in some individuals without a history of smallpox the titers resulting from vaccination or revaccination may be as high as 1:320, making diagnosis uncertain at best.

Neutralization test. The neutralization titers after smallpox infections are usually higher than in vaccinated individuals, but since some vaccinated individuals do show high titers, results of tests of a single serum are not definitive and cannot be considered very helpful in diagnosis.

These shortcomings greatly limit the usefulness of serologic tests in the diagnosis of the diseases except when a fourfold rise in titer is demonstrated. These tests are useful, however, for general serologic surveys and special serologic surveys to establish the fact of and date of past epidemic experience and in special studies of vaccination responses, and to establish the existence of sub-clinical infection in individuals.

EVALUATION OF SMALLPOX DIAGNOSTIC METHODS

Smallpox can be confidently diagnosed in the laboratory by the "four-test-combination" method, namely, the combined use of EM, AGP, CAM culture, and TC methods for each specimen. The occasional shortcomings of one method are compensated for by the strong points of another. It must be emphasized, however, that an adequate amount of specimen is needed. Strongly positive specimens pose no problem; the problem is in making a negative diagnosis with confidence, and this cannot be done if the specimen received is inadequate.

In the past 2 years at CDC, specimens from 345 patients with suspected smallpox from endemic and nonendemic areas were tested by the "four-test combination" method; 221 (64.1% were found to be negative. All of these negative cases, under close surveillance, were confirmed beyond a reasonable doubt to indeed be negative for smallpox.

Traditionally, culturing on CAM has been thought to be the most sensitive and accurate single test for the laboratory confirmation of smallpox; however, the accumulated findings of many workers over the past 25 years have shown that EM methodology equals or surpasses the CAM method. The proficiency of EM for smallpox diagnosis was first demonstrated by Van Rooyen and Scott (26) and Nagler and Rake (22) in 1948; their findings were reinforced by more extensive investigations reported by Peters et al in 1962 (24), Nagington and Macrae in 1965 (21), Cruickshank et al in 1966 (2), Macrae et al in 1969 (18) and, most recently, by CDC.

On the other hand, EM has not been very reliable in detecting poxviruses in specimens from patients whose diseases were clinically diagnosed as vaccinia infection. In tests at CDC, 30 of 77 specimens from patients with clinical vaccinia were found positive by combined CAM, TC and EM and only 12 (40.0%) were found positive by EM alone, whereas 29 (96.7%) were found positive by culturing on CAM or in TC. The AGP test was not used for these specimens, since the amount of specimen received was usually small.

Although the AGP test is not as sensitive as the EM, CAM and TC test, the procedure is so simple and direct, and the results from positive specimens are obtained so rapidly, that it is a convenient companion for the EM test and a comfortable support for a positive EM result.

CAM culture is very sensitive, provided that the several pitfalls already discussed are avoided or dealt with appropriately. In addition to its sensitivity, the CAM culture method has a broad spectrum, permitting positive identification of variola, vaccinia, most known strains of monkeypox and herpes simplex viruses. When test specimens are received in the laboratory in a fresh condition, viral inactivation should be minimal and the method should be highly reliable.

Tissue culture is an essential backup test for the CAM culture method. Its use in combination with the CAM culture method has amply confirmed its sensitivity.

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Table 1

Accepted methods for the laboratory diagnosis of smallpox, vaccinia infection and human monkeypox.

Preferred Methods	Accomplishment
Electron microscopy (EM)	Direct visualization of virus
Agar gel precipitation (AGP)	Antigenic identification
Chick chorioallantoic membrane culture (CAM)	Growth of variola, vaccinia, human monkeypox and herpes simplex viruses, with definitive pock characteristics
Tissue culture (TC)	Growth of variola, vaccinia, human monkeypox and herpes simplex viruses, with definitive CPE characteristics
Other Methods	
Stained smear	Visualization of elementary bodies
Fluorescent antibody (FA)	Visualization of virus-antibody complexes
Complement fixation (CF)	Visualization of virus-antibody complexes. Antigenic identification

APPENDICES

1. Growth Medium for Monkey Kidney Cells (MKC)

10 X Hank's BSS	95 ml
Fetal bovine serum	50 ml
Glucose	1.0 g
Lactalbumin Hydrolysate	5.0 g

Double distilled water added to 1,000 ml. Dissolve the lactalbumin hydrolysate in 800 ml of water with the aid of mild heat and stirring. Cool. Dissolve glucose in 100 ml of water and add to the lactalbumin hydrolysate solution while stirring. Add the serum and qs to 1,000 ml. Sterilize by filtration. Before use, add sterile antibiotics and sufficient volume of sterile 8.8% $NaHCo_3$ to give a pH of 7.2-7.4. Hank's BSS contains phenol red.

2. Maintenance Medium for Monkey Kidney Cells (MKC)

50% Lactalbumin Hydrolysate	100 ml
1 X Earle's BSS	900 ml
22.6% Sodium Acetate	5 ml
20% Glucose	5 ml
20% Yeastolate	5 ml
With necessary phenol red.	

Components sterilized by autoclaving before mixing. Add antibiotics and enough 8.8% sterile $NaHCo_3$ to give the desired pH.

3. Kissling's modification of Eagle's MEM (12)

For each liter of Eagle's MEM add glycine 75 mg, thymidine 0.8 mg, adenine 6.8 mg and galactose 800 mg. The growth medium contains 10% fetal calf serum, and the maintenance medium contains 2% fetal calf serum.

4. Eagle's Minimum Essentail Medium

(See chapter on general reagents)

5. McIlvaine's Buffer 0.004 M, pH 7.8

Solution A = Citric acid 0.1 M ($H_3C_6O_7 \cdot H_2O$)

Solution B = Na_2HPO_4 0.2 M (28.393 g/1000 ml H_2O)

Combine 63.5 ml Solution A and 936.5 ml of Solution B; this gives a solution with pH 7.6.

Sterilize by millipore filter.

To prepare 0.004 M buffer, pH 7.8, dilute 1.0 ml of the above pH 7.6 stock to 48.5 ml with sterile distilled H_2O.

6. Phosphate Buffered Saline (PBS) 0.01 M, pH 7.2

Na_2HPO_4(anhydrous)	1.096 g
$NaH_2PO_4 \cdot H_2O$	0.315 g
NaCl	8.5 g

Distilled water, add to 1,000 ml.

7. Vaccinia Antigen Produced on CAM, Positive Control for Agar Gel Precipitation

a. Obtain 20 CAM's, confluently infected with the Wyeth* smallpox vaccine strain of vaccinia.

b. Place the 20 infected CAM's in 250 ml Sorvall's Omni-Mixer* cup and homogenize for 3 minutes at full speed with the cup immersed in the ice-water bath.

c. Add 20 ml of sterile PBS and homogenize the mixture 2 minutes with the cup immersed in the ice-water bath.

d. Add 1 part of Genetron* 113 to 3 parts of the homogenate. Homogenize for 3 minutes with the cup immersed in the ice-water bath.

e. Centrifuge the mixture at 2,000 RPM for 10 minutes. Draw off and save the supernatant fluid. Add 1 part of 0.01% trypsin to 9 parts of the supernatant fluid, bringing the trypsin concentration to 0.001%. Mix and place in the incubator at 36 C for 1 hour.

f. Dialyze the mixture against polyethylene glycol-20 M to a final volume of 20 ml. Distribute in 1 ml aliquots and store at -20 C.

For normal CAM control, 20 uninfected CAM's are treated in the same manner.

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*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of Health, Education, and Welfare.

8. Preparation of Vaccinia Antigen Grown on Rabbit Skin (used to inject rabbit to produce antivaccinia rabbit serum)

a. Clip and shave the entire back of a 6-month-old or older New Zealand white rabbit.

b. Wash the shaved area, first with soap and water, then with 70% alcohol, to remove dirt and oil.

c. Scarify the animal's back with a 20-gauge needle with the point bent to form a burr. The scratchings should be about 5 mm apart, red but not bleeding.

3. With a gloved hand, rub the glycerinated vaccinia stock into the skin. (Vaccinia virus stock is the Lister strain which has been adapted to grow on rabbit skin.)

e. A confluent growth of the virus on the skin is obtained in about 3-5 days.

f. To harvest the growth: 1) anesthetize the rabbit with ether, 2) exsanguinate by cardiac puncture, 3) excise the portion of the skin with the confluent growth, and 4) spread and tack down the hide on a wooden board.

g. Flood the hide with ether and allow to dry.

h. Scrape the crust and upper epithelial layers with a scalpel blade and save the "pulp." Keep the hide moist with McIlvaine's buffer 0.004 M, pH 7.8, while it is being scraped.

i. Homogenize the pulp with about 20 ml of McIlvaine's buffer in a 50 ml Sorvall's Omni-mixer cup for 2-3 minutes. During this time the cup should be immersed in the ice-water bath.

j. Centrifuge the mixture in a refrigerated centrifuge at 1,500 RPM for 10 minutes.

k. Discard hair and light debris and save the supernatant fluid.

l. Repeat steps i, j, and k three more times, collecting the sediment at the end of each cycle.

m. Discard the sediment after the fourth extraction.

n. Pool the four samples of supernatant fluid and centrifuge at 2,000 RPM for 10 minutes; discard the sediment.

o. Centrifuge the supernatant fluid through a 40% wt/wt sucrose cushion.

p. Suspend the pellet of vaccinia virus in 10 ml of McIlvaine's buffer 0.004 M, pH 7.2, and add 10 ml of sterile glycerine (final glycerine concentrate of 50%). Store at -20 C.

9. Production of Antivaccinia Rabbit Serum for Agar Gel Precipitation

a. The antigen used is the Lister rabbit dermal vaccinia antigen with a titer of about 10^7 pock-forming unit/ml.

b. First inoculation = 1.5 ml subcutaneously.

c. After 2 weeks, give second inoculation of 1.0 ml intravenously.

d. After 2 weeks, give third inoculation of 1.0 ml intravenously.

e. After 2 weeks, give fourth inoculation of 1.0 ml intravenously.

f. After 1 week, give fifth inoculation of 1.0 ml intravenously.

Bleed out after 1 week.

A total of 8 weeks is required to prepare the serum.

Figure 1a. Illustration of proper packaging of etiological agents for-shipment

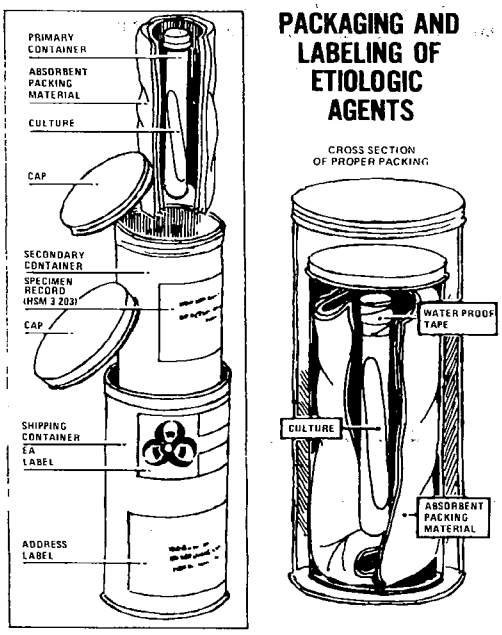

Figure 1b. The "Etiologic Agents" label which should be affixed to the outer shipping container

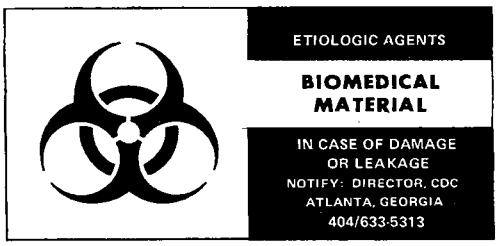

Fig. 2. Electron micrographs of particles at the original magnification of about 109,000 X.

Fig. 2(1) "M" form of variola.

Fig. 2(2) Nonviral particles resembling the "M" form of variola.

Fig. 2(3) "C" form of variola.

Fig. 2(4) Nonviral particles resembling the "C" forms of variola.

Fig. 2(5) Typical varicella-herpes group virus with envelope, from vesicular fluid.

Fig. 2(6) Particles found in a scab, may be varicella-herpes virus capsids, each surrounded by a degenerated contracted envelope; further search revealed typical enveloped virions on the same grid.

Fig. 2(7) Varicella-herpes group virus capsids without the envelope.

Fig. 2(8) Particles perhaps similar to those in Fig. 2(6).

FIGURE 2

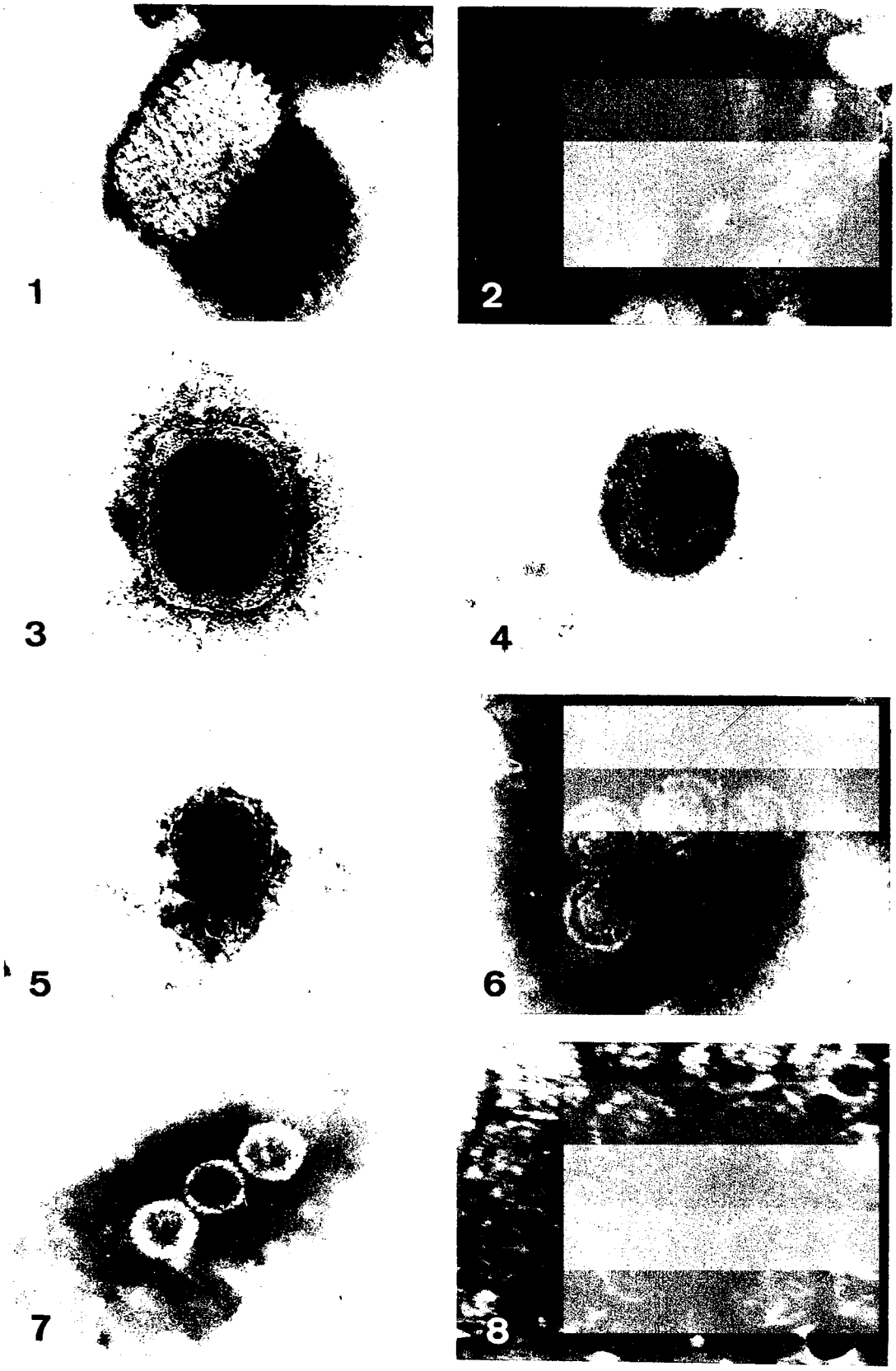

Fig. 3. Agar gel precipitation tests. Left pattern represents positive results with an unknown specimen and left pattern represents negative results with an unknown specimen. Nos. 1 and 4 = positive unknown specimens, Nos. 5 and 8 = negative unknown specimens, Nos. 2 and 6 = positive control vaccinia antigen, Nos. 3 and 7 = normal rabbit serum, and C = positive anti-vaccinia rabbit serum.

Note the fusion of lines between wells 1 and C and wells 2 and C.

FIGURE 3