Last Revised: Jan. 17, 2003

NEGATIVE STAINING ELECTRON MICROSCOPIC PROTOCOL FOR RASH ILLNESS

This protocol may be subject to minor changes. Before proceeding, please ensure an updated version is being used.

Introduction:

Electron microscopic (EM) visualization of negatively stained poxvirus virions was a valuable technique for confirming poxvirus infections during the smallpox eradication campaign. Historically, negative-stain EM successfully detected orthopoxvirus particles in approximately 95% of clinical specimens from patients with variola/monkeypox infections, and approximately 65% from patients with vaccinia infections. In the event of a deliberate release of smallpox virus and subsequent human disease, or in generalized vaccinia infections resulting from vaccination, negatively stained preparations derived from lesions or scab material would again provide a valuable method for assisting in poxvirus diagnosis and/or ruling out other causes of rash illness. However, EM visualization of virions compatible with orthopoxvirus by itself would not constitute proof of a smallpox infection because variola, vaccinia, monkeypox, and molluscum viruses, for example, are morphologically indistinguishable.

Reporting and appropriate action:

1. Pre-event, clinical specimens with high levels of suspicion for presence of variola virus, as described by the Febrile Vesicular Rash Illness Algorithm, should be immediately forwarded to CDC for specialized diagnostic evaluation. Several tests to confirm or rule-out smallpox infection will be performed at CDC, given that a positive result for smallpox would precipitate an immediate and extensive public health response.
2. Details are available at www.bt.cdc.gov/agent/smallpox/lab-testing/index.asp

MATERIALS

Acceptable specimens, from lesions:

Vesicular fluid on EM grid
Vesicular fluid as smears on glass slides
Crusts and tissue biopsies
Swabs
Vesicular fluid in tuberculin syringe or other collection device

These collection devices should be shipped inside a sealed plastic container to avoid spillage. Safety precautions are required when using needle and syringe. Consult your local safety officer.

Reagents:

Phosphotungstic acid (see section II. for recipe)
Uranyl acetate (see section II. for recipe)
Sterile distilled water (dH_2O)
37% Formaldehyde
Sodium hypochlorite
Optional, for alternate EM grid processing method (see step II. A. 2.): 0.01% Poly-L-lysine
Optional, for alternate chemical fixation method (see step III. D.): Paraformaldehyde, methanol-free (Polysciences, Inc; catalog #18814), or EM grade glutaraldehyde.

Supplies:

Formvar/carbon-coated 400-mesh copper grids (Electron Microscopy Sciences; catalog #FCF400-CU)
Grid storage box
Parafilm (or wafer of dental wax)
Sterile gauze pad, soaked in 70% alcohol
Petri dishes, plastic, 60 x 15 mm and 100 x 15 mm
Filter paper, round, 55 mm
Small cap (e.g., from 8 dram viral, 15 ml centrifuge tube, etc.)
Conical centrifuge tubes, plastic, 15 ml
Syringes, plastic, 3 ml
Microcentrifuge tubes
Pestle and Grinder Tube set (Fisher Scientific; catalog #1371215)

Equipment:

Transmission electron microscope
Tweezers, EM grade
Glow Discharge Unit and Vacuum pump (Electron Microscopy Sciences; catalog numbers 94000 and 91005, respectively).
E-Series Germicidal Ultraviolet Lamps, lamp stand, UV meter, UV goggles (Spectroline; catalog numbers EF-160, SE-140, DM-254XA, and UVF-50, respectively)
Microcentrifuge
Optional, for virus concentration (see step I. F.): Airfuge (Beckman Instruments)

Materials Sources:

Electron Microscopy Sciences; Tel: (800) 523-5874; http://www.emsdiasum.com/ems
Ted Pella, Inc.; Tel: (800) 237-3526; http://www.tedpella.com
Fisher Scientific; Tel: (800) 766-7000; http://www.fishersci.com
Spectroline; Tel: (800) 274-8888; http://www.spectroline.com
Polysciences; Tel: (800)523-2575; http://www.polysciences.com
Beckman Instruments; Tel: (800) 742-2345; http://www.beckman.com

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources; inclusion does not imply endorsement by the Centers for Disease Control and Prevention or the Department of Health and Human Services.

Negative Staining Procedure:

I. Specimen Preparation

All manipulations of infixed material must be carried out in a Class II Biological Safety Cabinet while using BSL-3 practices and safety equipment.

Note: When possible, prepare at least 2 grids per specimen.

A. Vesicular fluid on EM grids previously prepared by the direct-touch method:
Proceed to step II. E.

B. Vesicular fluid as smears on glass slides:
1. Add 1-2 drops sterile dH_2O.
2. Scratch dry material to resuspend.
3. Make EM grids directly off this material (see step II.).
4. Transfer remaining liquid to microcentrifuge tube for storage/further testing.

C. Crusts and tissue biopsies (use pestle and grinder tube):
1. Place crust or tissue in grinder tube and add 1-2 ml sterile dH_2O.
2. Grind to produce an opalescent suspension.
3. Centrifuge at 1,000g for 5 min.
4. Use supernatant as the specimen for step II.

D. Swabs:
1. Place swab in 15 ml conical centrifuge tube containing approximately 0.3 ml sterile dH_2O.
2. Soak for 10-15 min.
3. With a wooden applicator stick, scrape any remaining specimen off the cotton swab directly into the dH_2O.
4. Temporarily remove swab from centrifuge tube. Place the barrel only of a 3 cc syringe into the 15 ml conical tube, then place swab in syringe barrel. Break off stick, if necessary. Screw on cap to prevent aerosolization.
5. Place conical tube into a centrifuge canister in an aerosol-barrier rotor.
6. Centrifuge at 2,000g for 20 min.
7. Place entire centrifuge canister back into the BSC. Remove conical tube from canister.
8. Remove and discard swab and syringe barrel into a discard bin containing 1:10 dilution of commercial hypochlorite bleach solution.
9. Resuspend any precipitate. Use the resulting liquid as the specimen for step II.

E. Vesicular fluid in collective devices (e.g., syringe, capillary tube, etc.):
1. Expel fluid into microfuge tube.
2. Place two 2-5 µl drops of specimen onto sheet of Parafilm.
2. Dilute the 2nd drop by adding an equal amount of dH_2O and mixing.
4. Proceed to step II.
5. Keep remaining specimen in microcentrifuge tube for storage/further testing.

F. Virus concentration (optional): If available, an airfuge may be used to concentrate the virus in specimens from step I. B. 4. (vesicular fluid as smears on glass slides), step I. C. 4. (crusts and tissue biopsies), and step I. D .9. (swabs). Spin specimens at 30 lb/in^2 for 30 min, decant supernatant into a discard pan containing bleach solution, resuspend pellet in 10-20 µl of dH_2O, and use liquid as the specimen for step II.

II. EM Grid Processing by the Drop-To-Drop Method

Notes: Make at least 2 specimen grids whenever possible.

To avoid cross-contamination, tweezers **must** be cleaned with gauze pad soaked in 70% alcohol between each specimen preparation.

IN PREPARATION:

1. Prepare specimen petri dish (see Fig. 2A):
   - Place round filter paper into the bottom of an inverted 60 x 15 mm plastic petri dish.
   - Place a small cap on the filter paper and fill with 37% formaldehyde, using at least 10 drops. Cover with the top of the petri dish.
   - Place petri dish inside BSC.
2. Enhance hydrophilicity of EM grids:
   - If available, use glow discharge treatment on plastic/carbon-coated grid just prior to applying grid to specimen drop.
   - Alternatively, grids may be coated with a 0.01% poly-L-lysine solution in place of the glow discharge treatment.

DROP-TO-DROP METHOD (see Fig. 1):

A. Place 5 µl of liquid specimen onto a sheet of Parafilm.
B. Place plastic/carbon-coated 400-mesh copper grid (plastic-side down) on drop and let absorb for approximately 10 min
C. Wick away excess fluid with filter paper.
D. Stain
   1. Place grid (plastic-side down) on drop of 2% PTA, pH 7.0, and let stain for 10-30 sec.
   2. If 2nd specimen grid is available, stain with 0.5% UA for 10-30 sec.
F. Wick away excess fluid with filter paper, and place grids, specimen-side up, in specimen petri dish (described above).
G. Proceed to inactivation steps.

Negative Stain Reagents:

2% Phosphotungstic acid (PTA):
2 g phosphotungstic acid in 100 ml dH_2O
pH to 7.0 with KOH.
Store at 2-8^oC.

0.5% Uranyl Acetate (UA):
0.5 g uranyl acetate in 100 ml dH_2O
Let stand overnight.
Store at 2-8^oC, in the dark.

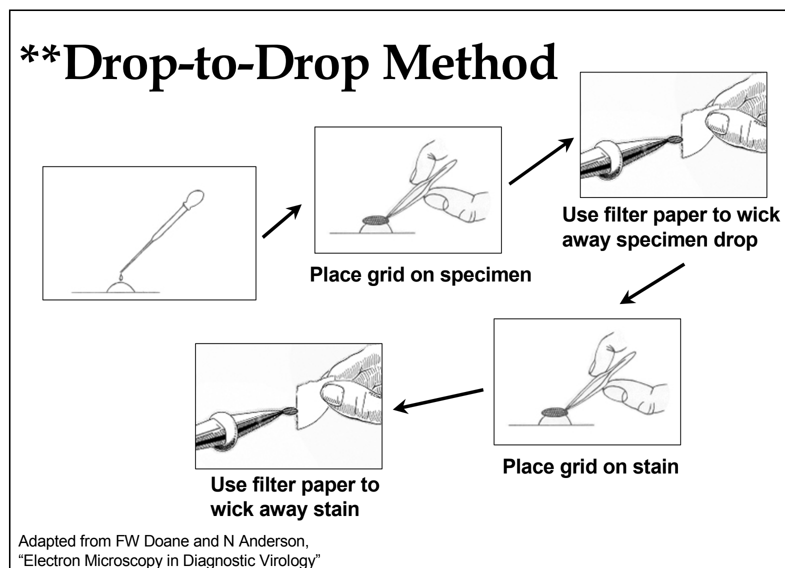

Figure 1.

III. Inactivation

Note: Inactivate grids while still within the Containment Area.

A. Chemical fixation (see Fig. 2A)

1. While under the BSC, replace the cover of the specimen petri dish containing the grids and formaldehyde.
2. Expose grids to formaldehyde fumes for 30 min.
3. Proceed to step B.

   *** Alternate chemical fixation method: Grids with specimens may be inactivated by placing the grid within a large drop of fresh 2% paraformaldehyde (or methanol-free formaldehyde) or 2% glutaraldehyde for 30 min. Proceed with UV irradiation. Note: This method of fixation may adversely affect the morphology of the virions.

B. UV irradiation (see Fig. 2B):

1. From the specimen petri dish, remove the cover and the cap containing formaldehyde.
2. Add 10% solution of commercial hypochlorite bleach to the 100 x 15 mm dish, just enough to cover bottom of the dish (approximately 50 ml).
3. Place bottom of specimen petri dish (with grids) within the larger dish.
4. Place under UV light, at a distance of 2.5 cm, and irradiate for 10 min.
5. Turn grids over, and irradiate an additional 10 min.

C. Using clean tweezers, place grids into grid storage boxes after inactivation steps. Carefully record which slot is used for each patient specimen.

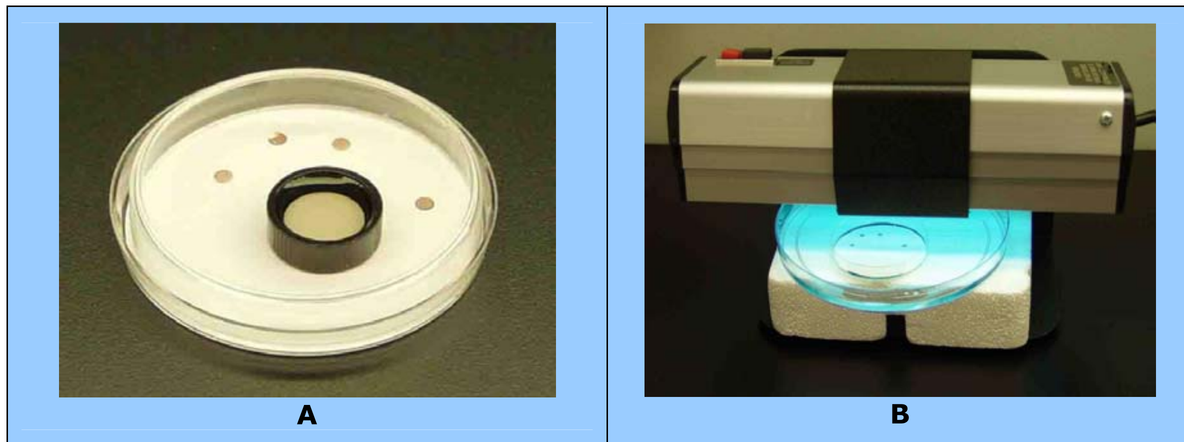

Figure 2. Inactivation Steps
A – Chemical fixation
B – UV Irradiation

Interpretation of results:

Poxviruses, excluding parapoxviruses: The virions measure approximately 225 X 300 nm, and appear rectangular or brick-shaped when viewed lengthwise and circular or ovoid when viewed on end. Depending on penetration of the stain, two forms may be seen. In the "M" (or "mulberry") form, the surface is covered with short, whorled filaments, and a circular depression is sometimes seen in the center of the virion. In particles penetrated by stain, the "C" (or "capsular") form, surface filaments are not visible; instead, the virion consists of a sharply defined, dense core surrounded by several laminated zones of differing densities. In addition, enveloped particles are sometimes found in clinical specimens.

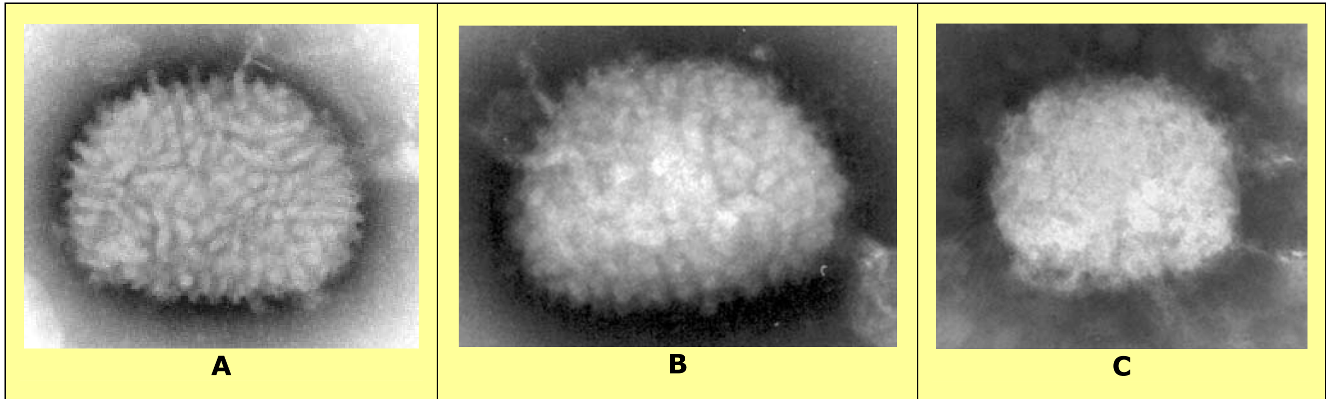

Figure 3. **A** – Vaccinia virus from tissue culture
B and **C** – Vaccinia virus from clinical specimens

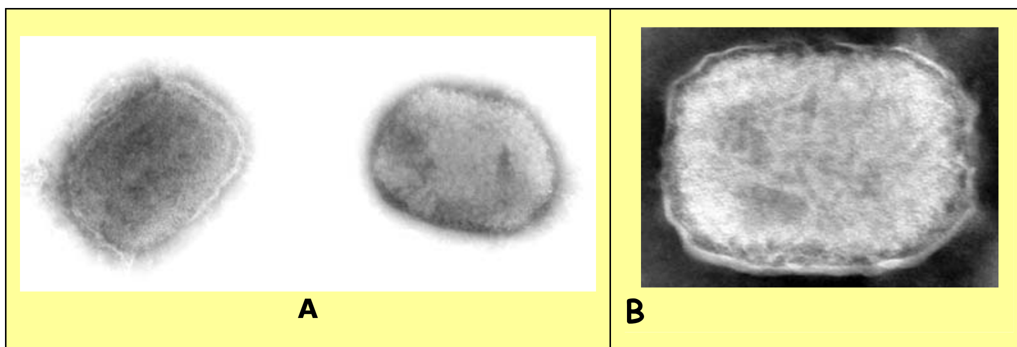

Figure 4. **A** – Monkeypox virus from tissue culture – "C "form on left, "M" form on right
B – Tanapox virus, clinical specimen (enveloped virion)

Figure 5. Variola virus, clinical specimen from experimental monkey

Parapoxviruses (e.g., Orf) virus: Parapoxvirus particles appear more ovoid than other poxviruses,

and the surface filaments have a spiral arrangement. Particles measure approximately 150 X 200 nm.

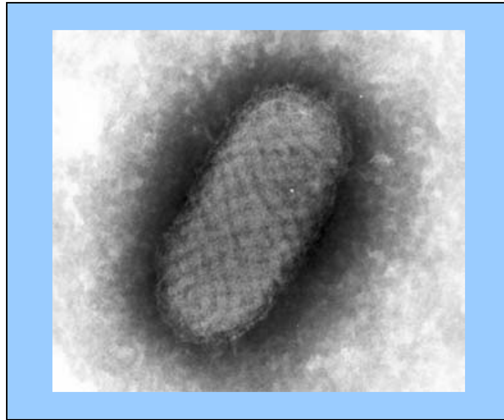

Figure 6. Parapox virus (Orf virus) from tissue culture

Herpesviruses (e.g., varicella zoster virus, herpes simplex viruses type 1 and type 2): The naked nucleocapsid, measuring approximately 100 nm in diameter, is composed of an iscosahedron formed by hollow capsomers. Stain-penetrated nucleocapsids may have the appearance of a hexagon rimmed by the hollow capsomers. Enveloped virions may be identified when the stain penetrates the viral envelope and outlines the nucleocapsid.

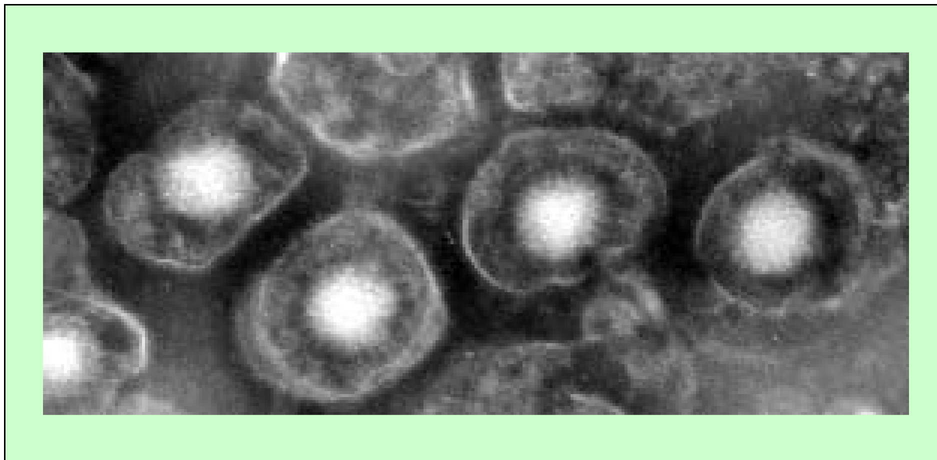

Figure 7. Herpesvirus particles from tissue culture (enveloped virions)

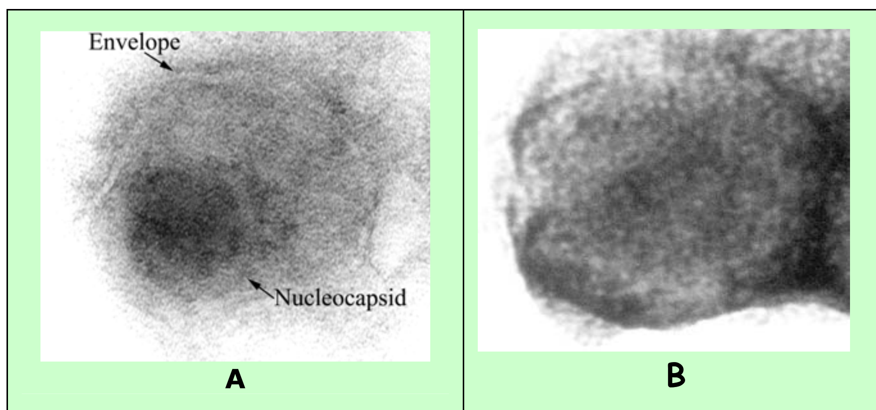

Figure 8. Herpesvirus, clinical specimens: **A** – Enveloped virion
B – Naked nucleocapsid

References

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Nakano JH (1979) Poxviruses. In: Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, 5th edition. Eds. Lennette, EH & Schmidt, NJ, American Public Health Association, pp. 257-308.