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 a poxvirus by itself would not constitute proof of a smallpox infection because different poxviruses, such as variola, vaccinia, monkeypox, and molluscum viruses, are morphologically indistinguishable.

While EM laboratories will be integral members of the biopreparedness team, several issues should be considered before an EM laboratory agrees to process specimens from patients with suspect poxvirus infection. First to consider is the diagnostic capabilities of the lab personnel, who should have experience with preparing negative stain EM grids. More importantly, the microscopists must have experience with analyzing negative stain EM preparations to identify virus morphology, and to differentiate these from look-alikes and artifacts. Second, EM personnel who will handle specimens need to have been recently vaccinated, or have no contra-indications to post-exposure vaccination. Lastly, the EM laboratory will need to have access to a BSL-2 containment facility that uses BSL-3 precautions.

EM laboratories involved in negative stain viral diagnostics are encouraged to participate in the External Quality Assessment program administered by the Robert Koch Institut in Berlin. Email Dr. Norbert Bannert at the Robert Koch at Bannertn@rki.de
Details are available at:
http://www.rki.de/cln_006/nn_231536/EN/Content/Institute/DepartmentsUnits/NRC/CONSULAB/consulab__node.html__nnn=true

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 http://www.bt.cdc.gov/agent/smallpox/lab-testing/index.asp

MATERIALS

Acceptable specimens, from lesions:

Vesicular fluid on EM grid obtained by the direct-touch method
Vesicular fluid as smears on glass slides
Crusts and tissue biopsies
Swabs
Vesicular fluid in plastic capillary tube, 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)
EM grade paraformaldehyde or EM grade glutaraldehyde (Electron Microscopy Sciences; catalog #15710 and #16010, respectively)
Sodium hypochlorite
Optional, for alternate EM grid processing method (see step II. A. 2.): 1% Alcian Blue (Electron Microscopy Sciences; catalog #26385-01)
Optional, for alternate chemical fixation method (see step III. A. 2.): 37% Formaldehyde

Supplies:

Formvar/carbon-coated 400-mesh copper grids (Electron Microscopy Sciences; catalog #FCF400-CU)
Grid storage box or plastic vials (Electron Microscopy Sciences; catalog #71150 and #72621-10, respectively)
Parafilm
Sterile gauze pad, soaked in 70% alcohol
Syringe filters (for step II. B. 4.)
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)
Optional, adapter for EM grid in Airfuge (Beranek; catalog #11093)

Equipment:

Transmission electron microscope
Tweezers, EM grade (examples include: Ted Pella, Inc., catalog #507-NM, #5630-4, 5724, #5326; Electron Microscopy Sciences, catalog #72803-01, #78322-7E, 78320-5B, and #72750-F)
Glow Discharge Unit and Vacuum pump (Electron Microscopy Sciences; catalog numbers 94000 and 91005, respectively). Alternately, a glow discharge unit may be constructed in the laboratory, following the design described by Aebi and Pollard (see references).
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
Tabletop centrifuge
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
Beckman Instruments; Tel: (800) 742-2345; http://www.beckman.com
Beranek Laborgerate; Tel: +40 (0) 6201/590031; http://www.Laborgeraete-Beranek.de; email: kberanek@t-online.de

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 unfixed 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. B. 4.

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 1ml 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., capillary tube, syringe, 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).

1. Clear the diagnostic suspension by low speed centrifugation at 4000 rpm 10min
   a. 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.
   b. Alternately, use adapters for EM grids to concentrate specimen directly onto a plastic-coated grid. Run the airfuge for 10-15 min at 20 PSI (85,000 rpm; 100,000xg). Place the airfuge rotor back into the BSC to remove grid. Proceed to step II. B. 4.

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

Notes: To avoid cross-contamination, either clean tweezers with gauze pad soaked in 70% alcohol between each specimen preparation, or use different tweezers for each specimen.
Make at least 2 specimen grids whenever possible.

A. IN PREPARATION:

Enhance hydrophilicity of EM grids:

1. If available, use glow discharge treatment on plastic/carbon-coated grid just prior to applying grid to specimen drop.
2. Alternatively, grids may be placed on a drop of 1% Alcian Blue for 10 min, then rinsed on 3 drops of dH_2O.

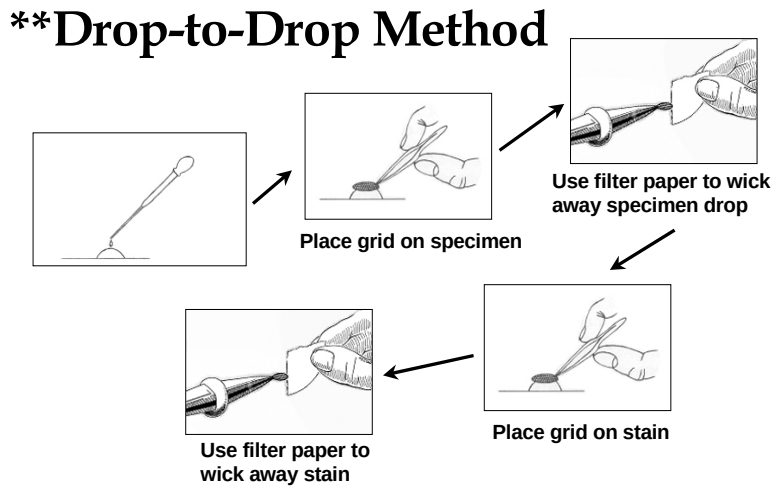
B. DROP-TO-DROP METHOD (see Fig. 1):

1. Place 5 µl of liquid specimen onto a sheet of Parafilm.
2. Place plastic/carbon-coated 400-mesh copper grid (plastic-side down) on drop and let absorb for approximately 10 min.
3. Wick away excess fluid with filter paper.
4. Stain.
   a. Place grid (plastic-side down) on drop of filtered 2% PTA, pH 7.0, and let stain for 30-60 sec.
   b. If 2nd specimen grid is available, stain with filtered 0.5% UA for 10-30 sec.
5. Wick away excess fluid with filter paper, and place grids, specimen-side up, in specimen petri dish (described above in step II. A. 1.).
6. 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°C.

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



Adapted from FW Doane and N Anderson, "Electron Microscopy in Diagnostic Virology"

Figure 1.

III. Inactivation

Note: Inactivate grids while still within the Containment Area.

A. Chemical fixation

1. While under the BSC, grids with specimens should be inactivated by placing the grid **within** a large drop of fresh 2% paraformaldehyde (or methanol-free formaldehyde) or 2% glutaraldehyde for 15 min. replace the cover of the specimen petri dish containing the grids and formaldehyde.
2. Alternatively, exposing the grids to formaldehyde fumes, only, may decrease the adverse affects of direct fixation and help reduce the possibilities of washing away viral particles.

a. 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.
b. Place petri dish inside BSC and place grids with specimens on the filter paper. Cover with the top of the petri dish.
c. Expose grids to formaldehyde fumes for 30 min.

3. Proceed to step B.

B. UV irradiation and bleach inactivation (see Fig. 2B):

Note: These steps, using UV irradiation and bleach, are only necessary for specimens that have been designated as a High Risk for variola virus. They are used to ensure the inactivation of any virus particles, including those on the filter paper or on the outside of the petri dish.

1. 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).
2. From the specimen petri dish, remove the cover and the cap containing formaldehyde.
3. Place bottom of specimen petri dish (with grids) within the larger dish.
4. Place under UV light 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.

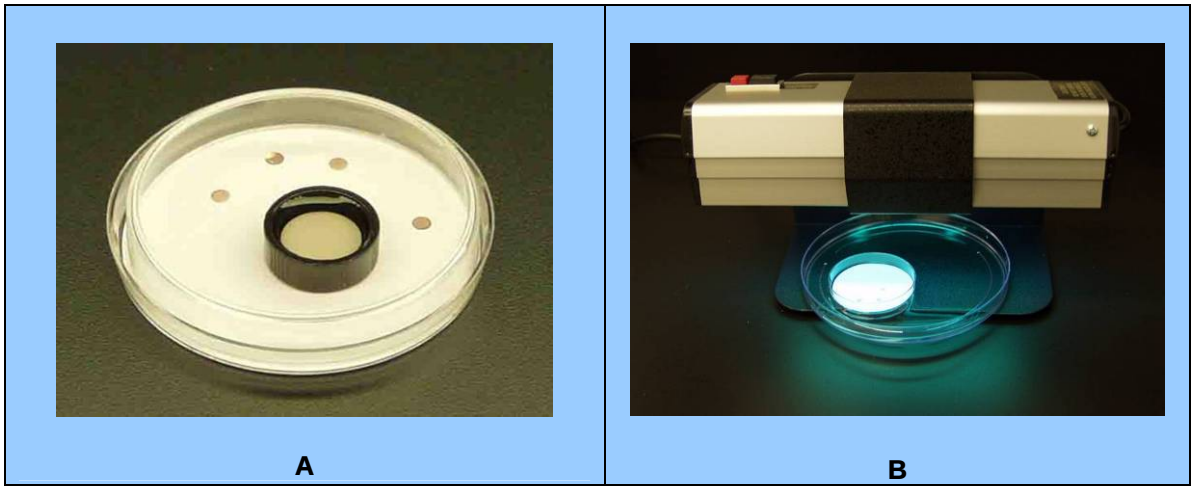


Figure 2. Inactivation Steps.
A – Chemical fixation.
B – UV irradiation and bleach inactivation.

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.

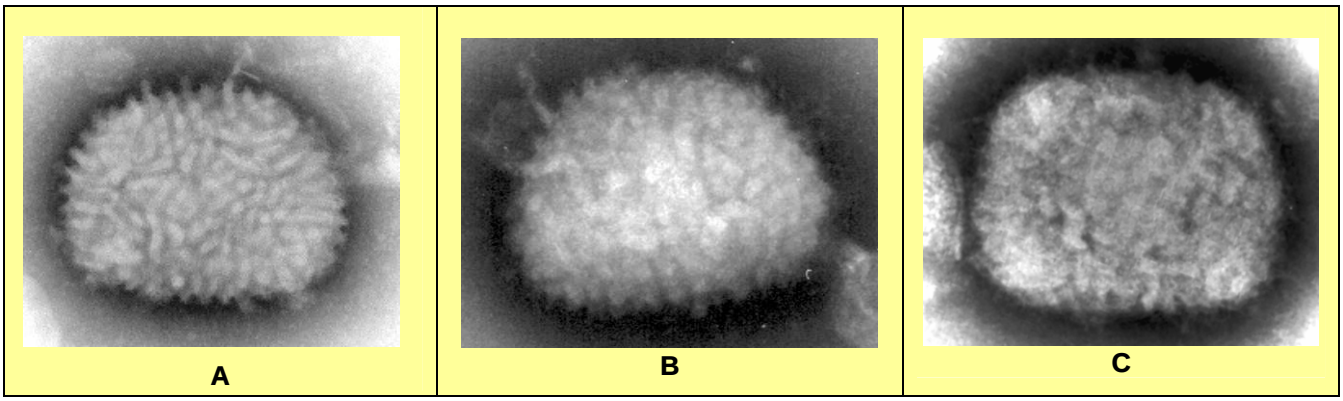


Figure 3. **A –** Vaccinia virus from tissue culture, "M" form.
B and **C –** Vaccinia and monkeypox viruses, respectively, from clinical specimens. Note that in clinical specimens the morphology may be less distinct than in tissue culture specimens.

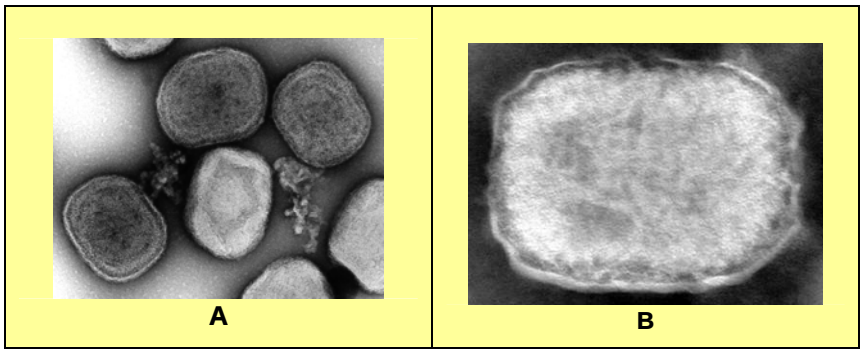


Figure 4. **A –** Fowlpox virus, showing 3 virions with "C" form.
B – Tanapox virus, clinical specimen (enveloped virion).

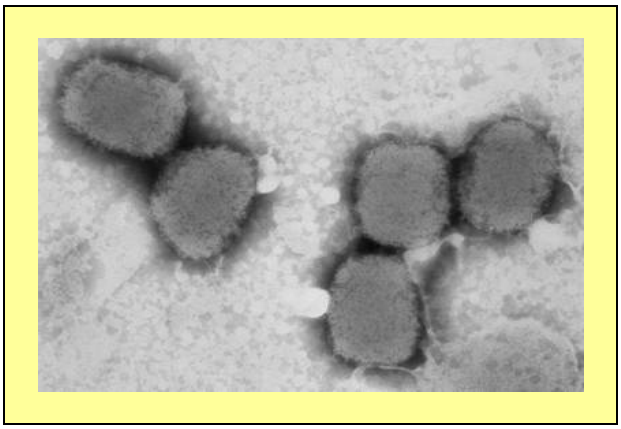


Figure 5. Variola virus, clinical specimen from experimentally infected monkey obtained by directly touching an EM grid to the open lesion.

Parapoxviruses (e.g., Orf): Parapoxvirus particles appear more ovoid than other poxviruses, and the surface filaments have a spiral arrangement. Particles measure approximately 150 X 200 nm.

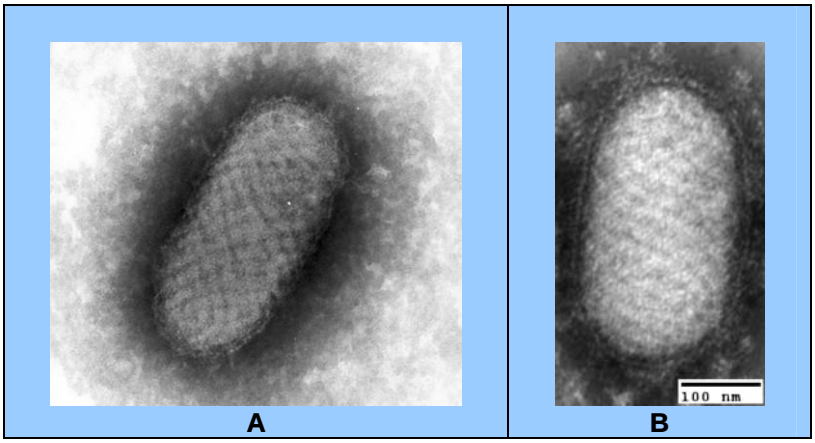


Figure 6. Parapox virus (Orf virus) from tissue culture (A) and clinical specimen (B).

Herpesviruses (e.g., varicella zoster virus, herpes simplex viruses type 1 and type 2): Enveloped virions may be identified when the stain penetrates the viral envelope and outlines the nucleocapsid. 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.

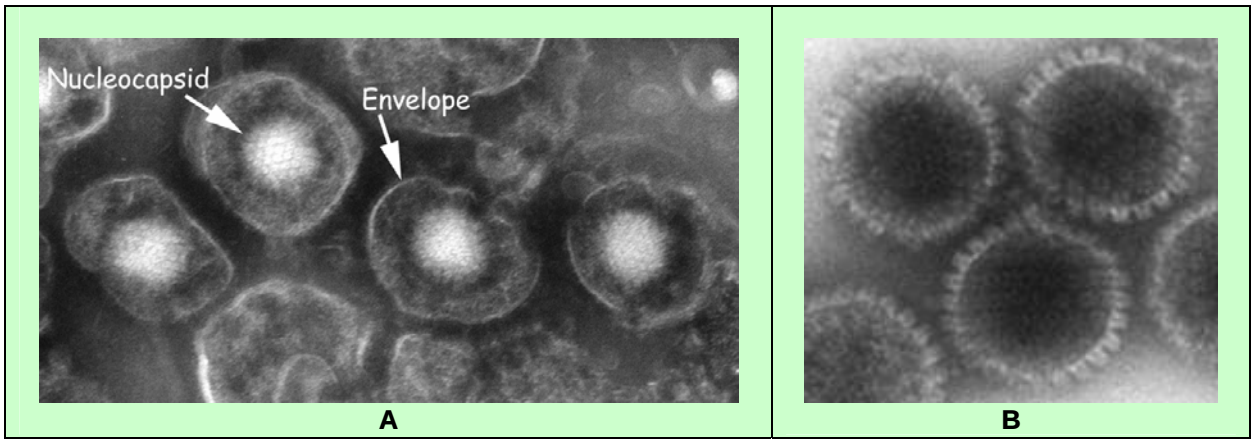


Figure 7. Herpesvirus particles from tissue culture.
A - Enveloped virions. **B** - Naked nucleocapsids, rimmed by hollow capsomers.

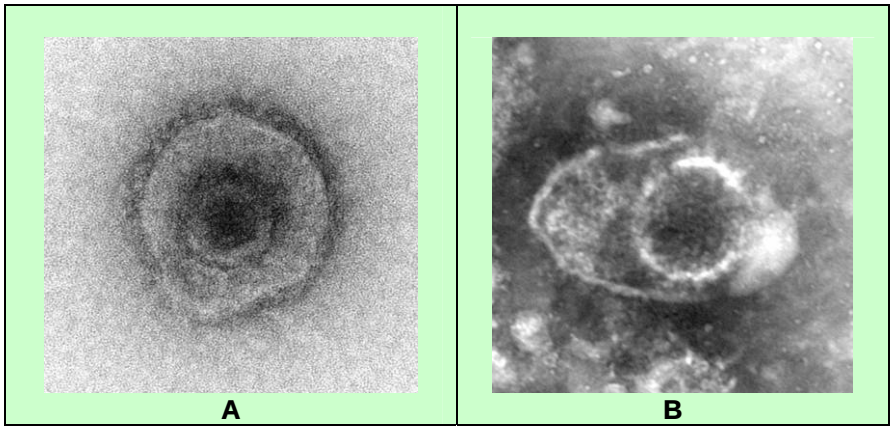


Figure 8. Herpesvirus, clinical specimens. Note that in clinical specimens the morphology may be less distinct than in tissue culture specimens.

Melanosomes: Care must be taken to distinguish between poxvirus particles and this look-alike structure found in normal skin. Melanosomes are found in skin epidermis and hair bulbs and measure approximately 370 nm in diameter and 0.7-1.15 µm in length.

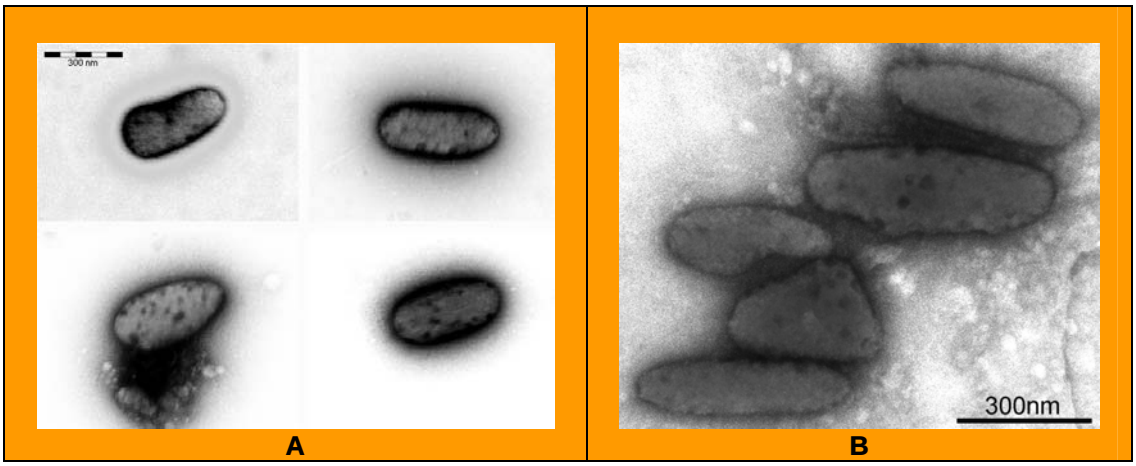


Figure 9. Melanosomes.

References

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