Intent is to provide background information regarding specimen collection and laboratory diagnosis of smallpox for pre-event surveillance and post-event response.

Laboratory support for the diagnosis of smallpox vaccine related adverse events is also important in both a pre-event and post-event vaccination program.

Talk will focus on:
- Characteristics of orthopoxviruses to help us understand the basis for the selection of laboratory tests utilized for the diagnosis of variola and vaccinia infections.
- Specific categories of tests that may be used in laboratory diagnosis of variola or vaccinia virus infections.
- Specimens, collection techniques, and shipping requirements for testing.
In a setting where not smallpox infection has been previously diagnosed (pre-event surveillance setting), the likelihood of a febrile rash illness being caused by smallpox virus is low. Therefore, the use of laboratory testing for this virus should be selective and carefully guided by the clinical presentation in order to prevent false positives.

- Pre-event diagnostic testing should have a high specificity
- This febrile vesicular rash algorithm was developed in the US to help medical and public health personnel work identify the circumstances where smallpox laboratory testing should be done and circumstances where it is not appropriate.
- The algorithm was specifically designed to discriminate infection with variola virus (smallpox) with a typical presentation, from other illnesses (bacterial, viral, immune mediated) that may also manifest with similar vesicular or pustular rash.
- With smallpox vaccine programs being implemented for to enhance responder preparedness, certain vaccinia (smallpox vaccine) related adverse events could fall into this algorithm
- Clinical correlation of differential diagnosis, with appropriate laboratory testing, will be critical for public health understanding and evaluation of adverse events associated with vaccination, as well as differentiation of smallpox from other diseases which may be clinically confused with smallpox.
There are a large number of poxviruses currently recognized to exist in nature, including poxviruses in both vertebrates and arthropods. Those of greatest concern are the ones that belong to the orthopoxviruses, which include variola, the agent of smallpox, and vaccinia, the virus used for smallpox vaccination. Members of the orthopoxviruses are closely enough related such that antibodies developed against one member of the group can also neutralize the other viruses. This is why antibodies developed after vaccination with vaccinia virus, which causes a much less severe infection than variola (smallpox) virus, can protect against infection with variola virus.
Characteristics of Orthopoxviruses

- Brick shaped particles (350 X 270 nm) by cryoelectron microscopy.
- Cytoplasmic replication.
- Double stranded DNA genomes (180 – 200 kbp) encodes:
  - transcription and replication enzymes.
  - multiple proteins aimed at evasion of immune defense molecules.

- Poxviruses are large viruses that replicate within the cytoplasm of cells
- The appearance of the poxvirus particle can provide a basis for non-specific diagnostics
- Their genome is a molecule of double-stranded DNA and the presence of virus-specific DNA sequences provides an important target for more virus specific diagnostic testing.
• Orthopoxviruses have a varied host range.
• Variola (smallpox virus) only infects humans
• Other closely-related orthopoxviruses, such as vaccinia, have a wider host range.
• The reasons for these differences are not completely understood.
• These viruses are genetically and antigenically very similar and provide cross protective immunity from infection. Hence, the basis for vaccination with vaccinia to prevent smallpox.
Among orthopoxviruses, vaccinia and cowpox typically cause localized infections in human hosts with normal immune responses. Variola and monkeypox typically cause systemic diseases. Variola is the only orthopoxvirus for which man is recognized as the only naturally occurring host.
Variety of older and more modern laboratory methods that can be used to confirm an orthopoxvirus diagnosis

List of methods currently used at CDC to confirm and to identify an orthopoxvirus infection, whether it is vaccinia, monkeypox, or variola (smallpox).

Serologic methods may help to evaluate the extent of an immune response, but will not be helpful to determine if replicative vaccinia is the cause of a vaccine associated adverse event.
In regards to smallpox vaccination support, laboratory needs include the confirmation of the various etiologies of adverse events associated with vaccinia (smallpox) vaccination.

Laboratory diagnostic needs also include ability to confirm rashes which are ultimately determined not to be related to the vaccine (such as chickenpox, herpesvirus infections, etc.).

Ability to accurately determine which post-vaccination events are a result of vaccinia virus infection will be important for our public health understanding of adverse events and vaccination risks.

As treatment decisions for the known smallpox vaccine serious adverse events should be made based on the clinical presentation of the patient, laboratory diagnosis to rule in or out vaccinia virus infection will not be an urgent function in most cases, unless potential therapeutic options for other causes are being considered.

Specifically, if a case of encephalitis is associated with vaccination, it will be important to rule out (or in) other treatable causes such as herpes encephalitis. Historically, post-vaccinial encephalitis was a diagnosis of exclusion.

Many newer laboratory assays for vaccinia are considered investigational.

If these newer tests are used guide patient management, results should be always be confirmed with additional tests.
Lab tests should be used, as clinically indicated, to evaluate other non-smallpox virus causes of rash or other non-vaccinia causes of adverse events associated with vaccination.

- General categories for laboratory testing for other etiologic agents are listed in this and the following slide.
- Standard methods can be used to evaluate specimens for the presence of bacteria or enteroviruses.
- Go over slide for diseases and types of diagnostic testing available.
• Go over slide for diseases and types of diagnostic testing available
• Note that in several cases, a biopsy for dermatopathologic examination will be an important diagnostic tool to evaluate the causes of adverse events associated with other etiologies
Now well discuss the types if specimens that can be evaluated for poxvirus DNA. The best specimens for many of the orthopox laboratory tests are the “roofs” or rusts from the lesions which contain large amounts of orthopoxvirus material. Vesicular fluids from the lesions are also sources for diagnostic material. Vesicular fluids are also good starting materials for electron microscopy. Whichever tests are considered for diagnosis, multiple lesions should be sampled for both roof of lesions and vesicular fluids from the lesions since not all lesion specimens are easy to identify. Biopsy material can be used for viral identification with PCR, immuno-histochemical staining, or culture. For electron microscopy, lesion roofs, scab or crust material, or vesicular fluid can be used. For non-dermatologic related adverse event evaluation appropriate material should be taken to evaluate for other potential etiologic causes of the adverse event, such as CSF for herpesvirus testing in suspect post vaccinial encephalitis. In addition, these materials can be tested for the presence of vaccinia.
Slide 12

- This is an example of a negatively stained electron micrographic (EM) preparation of vaccinia virus.
- The EM process may be very quick for a skilled observer and can be used to differentiate generic orthopoxviruses from other groups of viral agents.
- However, EM cannot differentiate between variola and vaccinia viruses.
• This is an example of one of several vaccinia virus lesions associated with a laboratory-acquired case of disseminated vaccinia in a previously unvaccinated laboratory worker.
Specimen Collection

- Vaccinia and variola specimen collection essentially the same.
- CDC website contains guidelines for orthopoxvirus specimen collection:

- Collection procedures for vaccinia virus, in the evaluation of an adverse reaction to vaccination, or variola virus, in the evaluation of a potential smallpox case, are essentially identical.
- Guidelines and updates for specimen collection techniques are maintained on the CDC smallpox website.
Specimen Collection

- Wear appropriate personal protective equipment (PPE), as specified by hospital/clinic infection control.
- Hand hygiene before and after collection.
- Sanitize skin site, with alcohol wipe, prior to specimen collection:
  - ALLOW TO DRY prior to specimen collection.

- Usual practices associated with collection of patient specimens are appropriate for collection of orthopoxvirus lesions, as well.
- These include wearing personal protective equipment, such as gloves, and sanitizing the site prior to collection.
- If alcohol is used to prepare the lesion for collection, it is important to allow the lesion to dry before it is collected.
Slide 16

- These are examples of lab materials useful for collection of orthopox specimens for laboratory testing
- Includes:
  - Plastic specimen container with formalin for the lesion biopsy
  - Punch biopsy collection tool
  - Sterile plastic vials for specimens (without any viral culture medium or fluid)
  - Microscope slides and slide storage container
- If available, electron microscopy (EM) grids are also useful for specimen collection and makes EM examination of materials for the presence of orthopoxvirus particles easier.
Specimen Collection
Vesicles

Use scalpel or 26 gauge needle to unroof vesicle:
- Put roof into collection tube
- Scrape base of vesicle with blunt edge of scalpel or wooden applicator, apply scrapings to microscope slide.
- Lightly apply EM grid, shiny side down, against lesion:
  - Repeat (x2) using more and less pressure.

- For collection of materials from vesicles, use a scalpel or needle to unroof the vesicle.
- The skin or scab that constitutes the roof of the lesion should be put into the plastic vial and sent dry without any viral culture medium or fluid.
- One procedure suggests gently scraping the base of the vesicle with a blunt end of a scalpel or wooden applicator and trying to smear some of this on a microscope slide. An electron microscope grid, with ultra-thin plastic covering, can be gently touched down (shiny side or plastic film-side) against the lesion. This can be repeated perhaps three times per lesion (resulting in three EM grids).
Specimen Collection
Vesicles

- Repetitively touch a microscope slide to the base of the unroofed lesion (touch-prep)
- Allow slide, and grids to air dry for 10 minutes. Store in slide holder, and grid box, respectively

- Touch preparations are made by repetitively touching a glass microscope slide to the base of the unroofed lesion.
  - Touch preps from microscope slides can also be used for EM examination if EM grids are not available
- The slide and/or EM grid are allowed to air dry for ten minutes.
- Store the slide in a plastic slide holder for shipping (do not store slides from different patients together)
- Store EM grid in the appropriate EM grid box
  - Note the number of the slot on the grid box where the EM grid used for the touch prep is stored
- Label all specimens with the date, patient information, and all other appropriate information
Lifting a crust or ‘roof’ from the skin.

- Pictures demonstrating specimen collection techniques from a lesion that has already scabbed
- Demonstration using a vaccinia vaccination site scab
- Lift the roof of the scab using a small-gauge, sterile needle or a scalpel
• Remove the scab and place it into the sterile plastic vial container
• The same process would be used to remove the roof of a vesicle or pustule, though a scalpel may be more useful for removing the roof of a vesicular or pustular lesion and then placing it into the sterile plastic vial container.
After removing the roof of the lesion, the base of the lesion should be scraped with the blunt end of the scalpel or the wood end of a medical wooden, cotton-tipped applicator and the scraped material would be transferred to a microscope slide.

A touch prep (using a separate microscope slide than the one containing the base scrapings) would then be prepared by touching the base of the unroofed lesion 3 times with the slide.
These are electron microscope grids, forceps for handling the grids, and the box for storage of the grids.

- One side of the box contains sterile, unused grids
- The other side of the box can be used to store used grids containing specimens
- All storage slots have numbers that can be used to match the used EM grids containing specimens with the correct patient and specimen site descriptions.
- If available, an electron microscopic grid should then be carefully touched 1 time to the base of the unroofed lesion
- 2-3 different grids should be touched to the base using a different touch pressure for each grid
  - This allows for increasing amounts of material to be collected on the grid for EM examination
- Grids should be touched to the lesion with the shiny side of the grid down, coming into direct contact to the base of the lesion
- Also note how the grid forceps are used to hold the grid towards the edge and not in the middle of the grid.
EM grids and grid box.

- EM grid boxes are divided into 2 sides. One side contains the clean grids and the other side of the box is for storing the grids after specimen collection.
- The grids touched to the base of the lesion should then be placed in the side of the box for used grid storage and the letter and number of the slot noted so that the EM grid can be matched to the appropriate patient and specimen collection site identification.
• Biopsy of a separate intact lesion (with the roof of the lesion still on) should be obtained using a punch biopsy tool as shown here
• Can do a punch biopsy on 2 separate lesions and place one in formalin and one in a sterile, plastic tube (without any viral media or fluid)
• OR
• Can split a single punch biopsy specimen in half and place one half in formalin and the other half in the sterile, plastic tube.
• The formalin fixed specimen would be used for histopathology, while the other, non-fixed specimen would be used for DNA detection or virus isolation.
• Serum, if considered useful diagnostically, can be collected as well.
For specimens for vaccinia virus testing, standard shipping guidelines are appropriate. Standard diagnostic specimen shipping guidelines are available at the website listed on the slide here ([www.bt.cdc.gov/labissues/packaginginfo.pdf](http://www.bt.cdc.gov/labissues/packaginginfo.pdf)). If serum is collected, it is highly advisable to separate the serum from the blood on site. If this is not possible, however, one can send refrigerated whole blood to the LRN laboratory.
- Formalin-fixed tissue must be shipped at room temperature, not frozen.
- The Electron microscopic grids must also be shipped at room temperature.
Specimen Transport
How to Send

• All other virus-containing material:
  – Must be stored and shipped frozen.
  – If overnight delivery possible, specimen may be shipped immediately at room temperature or refrigerated.

• Keep all virus-containing material out of direct sunlight.

- All other virus-containing material should be stored and shipped frozen.
- Virus containing materials include:
  - Roof of lesion
  - Base scrapings of lesion
  - Microscope slide touch preps
  - Non-formalin fixed punch biopsy
  - Serum
- If overnight transportation to a diagnostic lab can be arranged, specimens may be shipped refrigerated instead of frozen
- Keep all virus-containing material out of direct sunlight as the virus and DNA material is UV light sensitive and exposure may decrease the ability to culture virus.
Infectious Substances are defined as United Nations or UN Hazard Class 6, Division 6.2 and must be packaged, marked, and labeled as such.

Packing and shipping of specimens potentially containing vaccinia or variola must be done under the UN guidelines.
• The primary receptacle is the one that contains the specimen or the potentially infectious substance (for example, the microscope slide holder, the plastic specimen tubes, etc.). This recepticle must be water tight and the lids should be sealed with adhesive tape or other appropriate sealing material. The entire content of the primary receptacle is considered infectious.

• Multiple primary receptacles must be separated or individually wrapped to prevent breakage. Use enough absorbent material to absorb the entire contents of the primary receptacles if it were to break.

• The primary receptacles must then be placed in secondary packaging to protect them against damage and leakage so that the specimens arrive at the destination in good condition. This also helps protect the hundreds of people that will be handling your shipment until it arrives at its final destination.
After the primary receptacles are sealed and wrapped with absorbant material, place them in the secondary container.

Add enough filler so that the primary receptacles fit snugly into the secondary container. If the bottom of the primary receptacle isn’t wrapped, put padding in the bottom of the container and add some to the top if necessary to absorb any shock to the outer shipping container.
• Shipment of infectious substances require special packaging known as UN Specification packaging.
• This packaging has been rigorously tested and meets the packaging requirements per the DOT and IATA regulations for infectious substances.
Past and Future of Orthopoxivirus Diagnostics

• The Past (when low tech worked):
  – During smallpox epidemics clinical diagnosis drove immediate medical response
  – Electronmicroscopy more common
  – Gel-diffusion antigen detection
  – Virus isolations done on egg embryos

• How can past experience with smallpox diagnostics help us define our future needs?
• Encouraging to note that smallpox was eradicated as a naturally occurring disease in the absence of high-tech diagnostic tools.
• During the time when smallpox was epidemic, clinical diagnosis drove the immediate medical response, and presumably this would be expected to reoccur if smallpox were to reemerge in the future.
• Diagnostic electron microscopy capability was more common in the past, and relatively low-tech gel diffusion serological assays were also available
As smallpox no longer occurs naturally, the likelihood of a rash illness being smallpox is very low. Today there is a greater need for more highly specific tests in order to minimize the chance of false positives when evaluating a rash illness for its potential to be smallpox.

- Additional sensitive diagnostic tests are currently being developed.
- It is anticipated that in addition to a wide variety of PCR-based tests for detection of DNA, relatively simple tests will be developed that can be used at the point of patient care.
- It is also worth noting that in the event of a validated outbreak of smallpox, expectations for smallpox diagnosis would change considerably. Once smallpox has been confirmed, the likelihood of a compatible rash illness being smallpox increases dramatically and a clinical based diagnosis for the initiation of treatment and control measures (vaccination) would be appropriate, even in the absence of laboratory confirmation.
For More Information

- CDC Smallpox website: www.cdc.gov/smallpox
- National Immunization Program website: www.cdc.gov/nip