

Smallpox Vaccination Laboratory Support

Department of Health and Human Services
Centers for Disease Control and Prevention
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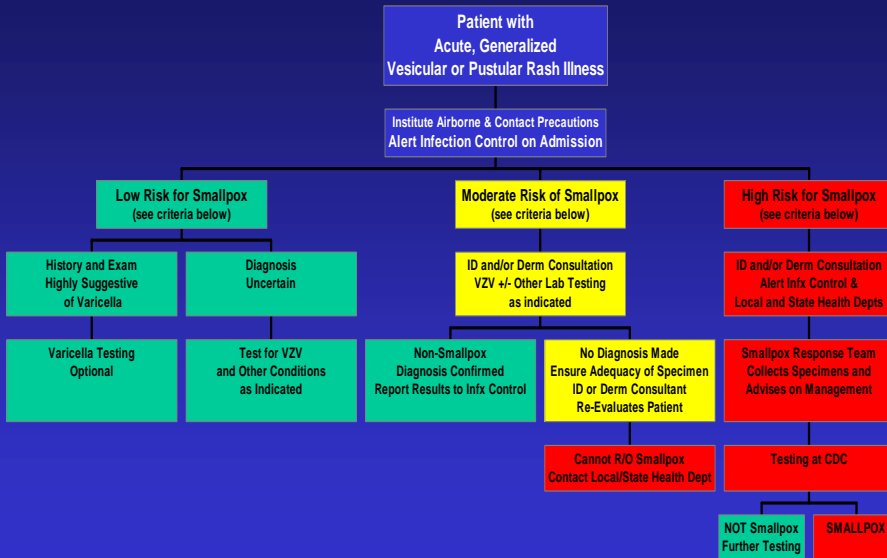
The intent of this Laboratory Support presentation is to provide background information for people who might be involved with smallpox preparedness and response. Of course, thorough clinical evaluation and patient history may be all that is required to conclude that a person has had or is having an adverse reaction to smallpox vaccination. However, persons involved with the vaccination program may be expected to have some degree of familiarity with the basis for laboratory testing, in the event of an adverse reaction, even though they themselves may not be responsible for making sure the testing is facilitated. This talk will try to help: provide some background on the specific tests currently in place within the state's Laboratory Response Network; what sort of specimens would be needed for testing; and how to prepare those specimens for transport to the LRN sites.

Laboratory Support

- **Learning Objectives:**
 - Indicate an awareness of the laboratory testing that exists for the confirmation of an adverse reaction to smallpox vaccine
 - Identify the state's Laboratory Response Network laboratory
 - Describe the types of specimens that would be taken to send to the laboratory
 - Describe specimen collection and transport guidelines



Febrile, Vesicular Rash Illness Algorithm for Evaluating Patients for Smallpox



You've already heard about the febrile vesicular rash algorithm and its application for enhancing rapid diagnosis of smallpox look-alike diseases. Many of the same principles apply to diagnosis of vaccine adverse reactions caused by vaccinia.

Key Concepts of Algorithm

- **Uses existing resources for dx and exclusion of smallpox look-alikes (especially 1.0 million cases of chickenpox)**
- **Provides significant diagnostic benefits, even in absence of smallpox**
- **Encourages careful diagnosis of other rash illnesses (including vaccinia)**



The algorithm provides significant diagnostic benefits even in the absence of smallpox and encourages careful diagnosis of other rash illnesses such as vaccinia.

Key Concepts of Algorithm

- **Minimizes number of cases that require intensive investigation: focuses attention where it is justified; important for both clinician and lab**
- **Provides rapid, thorough response to highly suspect cases**
- **Vaccination response if/when smallpox diagnosis confirmed**



The use of such an algorithm minimizes the number of cases that require intensive investigation and hopefully focuses attention where it is most needed and justified. This is an important consideration for laboratories, just as it is in the clinical setting. It allows for a more rapid, thorough response to highly suspect cases. And in the case of smallpox, of course, the diagnosis of smallpox would result in initiation of vaccination.

Vaccinia Diagnostic Goals

- **Provide methods for laboratory differential diagnosis of vaccinia associated adverse events**
- **Develop rapid dx methods (e.g., real-time PCR)**
- **Encourage development of point-of-care diagnosis (e.g., generic orthopox antigen capture assays)**



The essential features of the febrile vesicular rash disease algorithm can be applied to the clinical diagnosis of possible vaccinia-associated adverse events especially since such event would be associated with histories of vaccination or contact with vaccinees. Many of the laboratory testing requirements for vaccinia infection are also very similar to those expected for variola or smallpox testing. Much of what I say today applies to both vaccinia and variola testing. Rapid implementation of methods for laboratory confirmation of vaccinia-associated adverse events in such cases where the clinical picture may not be sufficient to provide a positive diagnosis has become a high priority for the anticipated vaccination program. Such testing methods have been developed and validated and include realtime PCR analysis of poxvirus DNA. It is anticipated that as vaccination proceeds, additional methods of point-of-care diagnosis will become available as they are developed and approved for clinical use.

Poxviruses

- **Two Subfamilies:**
 - ***Chordopoxvirinae*** (vertebrate poxviruses)
 - **Orthopoxvirus** (variola, vaccinia, cowpox, monkeypox, raccoonpox, camelpox, skunkpox, volepox, ectromelia, taterapox)
 - **Others**
 - ***Entomopoxvirinae*** (insect poxviruses)



As additional background there are a large number of poxviruses currently recognized to exist in nature including poxviruses in both vertebrates and arthropods. Today we're only concerned with those that belong to the closely-related orthopoxviruses, especially variola, the agent of smallpox and vaccinia, the agent that's used for smallpox vaccination.

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 and their genome is a molecule of double-stranded DNA. The presence of virus-specific DNA sequences provides an important target for diagnostic testing.

Characteristics of Orthopoxviruses

- **Infectious forms: IMV, CEV, EEV**
- **No known unique viral receptor protein**
- **Host ranges vary**
 - **Variola vs vaccinia**
- **Antigenically similar; serologic cross reactivity**



There are several closely related forms of infectious orthopoxvirus particles, which are distinguished by how many membranes surround each virion. It is interesting that some orthopoxviruses have limited host ranges, such as variola, which only infects humans, whereas other closely related viruses may have wider ranges, such as vaccinia. The reasons for these host-range differences are not completely understood. Importantly these viruses are genetically and antigenically very similar and provide cross protection immunity from infection; hence the basis for vaccination with vaccinia to prevent variola infections (i.e., smallpox).

Spectrum of Human Disease in Normal Host

- **Vaccinia, cowpox: localized infection**
- **Variola, monkeypox: systemic illness**



Among orthopoxviruses vaccinia and cowpox cause 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.

Lab Methods for Confirmation of Orthopoxvirus Diagnosis

- PCR related methods for DNA identification, (e.g., real-time PCR)
- Electron microscopy
- Histopathology
- Culture
- Serology
 - Antigen detection (IFA, EIA ag capture)
 - IgM capture
 - Neutralization antibodies
 - IgG ELISA



There are a variety of laboratory methods that can be used to confirm an orthopoxvirus diagnosis. Many of these are currently limited to a few reference laboratories including several of the serologic assays that are listed at the bottom of this slide. However, highly sensitive methods for vaccinia DNA identification in the form of real-time PCR assays have been deployed to every state through the Laboratory Response Network. In addition, a number of states have the capacity to do electron microscopic analysis of orthopox specimens as well as histopathologic evaluation.

Real-Time PCR Assay: Detection of the E9L Gene for Vaccinia Adverse Reactions

- **Test uses primers and probes designed to detect Eurasian Orthopoxvirus other than variola**
 - **Potential human diseases detected:**
 - **Vaccinia**
 - **Cowpox (zoonotic disease of European origin)**
 - **Monkeypox (zoonotic disease of central Africa)**



The specific real-time PCR assay that is currently deployed for analysis of vaccinia adverse reactions detects the presence of the DNA polymerase gene of vaccinia virus, the so-called E9L gene. This assay will also detect cowpox virus and monkeypox viruses, two viruses that do not occur naturally in North America. From a North American diagnostic perspective then, this assay can be considered diagnostic for vaccinia infections.

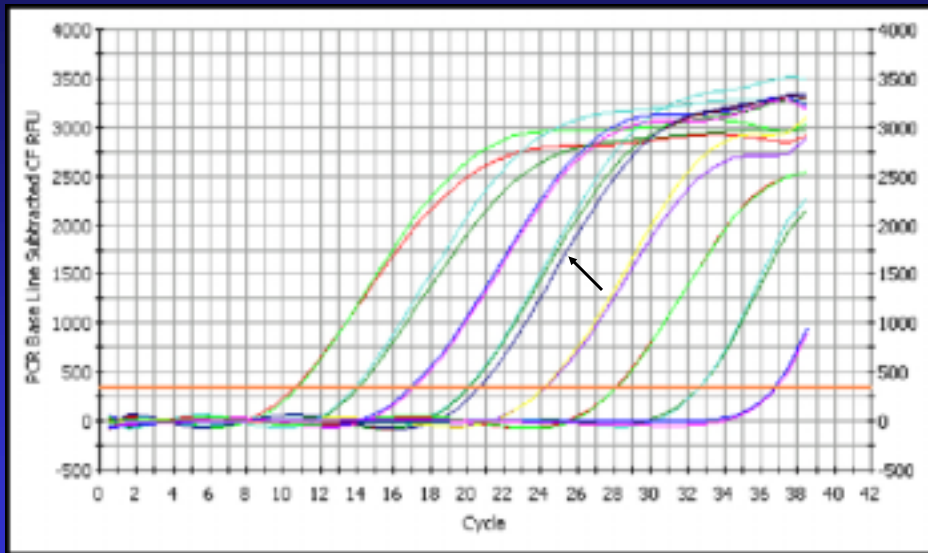
Vaccinia/Orthopox and Variola: Real-Time PCR Assays

- **E9L: VAC, MPX, CPV(TET); variola (FAM)**
 - Essential gene of poxviruses
 - 16S control for inhibitors
 - Can be used to detect vaccinia (adverse event monitoring); use TET portion
 - Can be used to detect variola use FAM and TET portions
- **Additional assays are being evaluated**



An almost identical assay will also detect the DNA of variola virus and therefore can be used for the diagnosis of smallpox. This is accomplished by simply changing specific fluorescent labeled probes. Both assays incorporate internal controls. A wide variety of similar assays, targeting a variety of poxvirus genes, are currently being evaluated and readied for deployment. This is very much an ongoing process.

Example of DNA titration, R-T PCR data



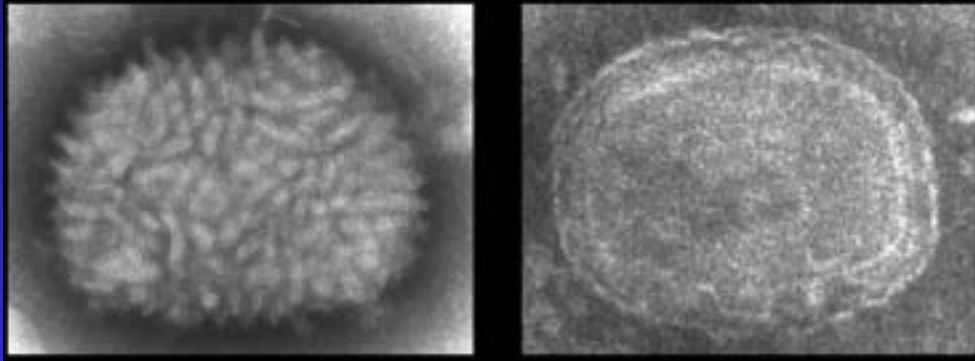
This is an example of experimental data from a real-time PCR test that incorporated calibrated amounts of DNA at increasing dilutions together with an unknown test sample containing orthopox DNA. Depending on the amount of original starting viral DNA, fluorescent signal develops at varying times over the duration of the experiment. Detection of virus DNA from a patient sample is indicated by the arrow.

**If smallpox were to re-emerge,
real-time PCR assay would be
modified for variola using an
alternate probe.**



Once again, just to emphasize, if smallpox were to re-emerge in the human population, the E9L real-time PCR assay would be modified for variola use simply by changing to an alternate probe.

Negative Stain Electron Microscopy



vaccinia

~1/2 hour per sample



This is an example of a negatively stained electron micrographic preparation of vaccinia virus. You can see the characteristic brick-shaped particle of the orthopoxvirus and the characteristic surface morphology (example seen on the left). The EM process for orthopoxvirus identification may be accomplished relatively quickly by a skilled observer and can be used to differentiate generic orthopoxviruses from other groups of viral agents. However, it may not be as sensitive as real-time PCR and cannot differentiate between variola and vaccinia.

Orthopoxvirus Cell Culture Isolation

HEK 293

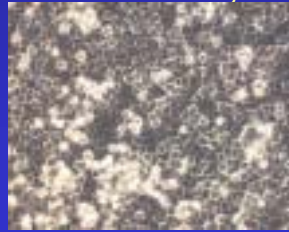
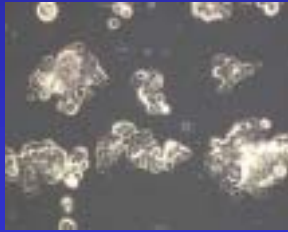
HeLa

BSC-40

Uninfected, 24 hpi



Variola infected, 24hpi



Cell culture isolation can be an important and very sensitive method for detection of vaccinia since it also amplifies the virus for further characterization. It should be noted that specimens with high suspicion for variola should not be subjected to culture in a pre-event setting due to concerns for biocontainment and laboratory safety.

Specimens for Vaccinia-Related Disease: Vesicular Rash

- Lesion ‘roofs’ and crusts
- Vesicular fluids:
 - Touch prep
 - EM grid
- Biopsy
- Serum
- Others (e.g., CSF)



The best specimens for many of the orthopox laboratory tests are the “roofs” or crusts from the lesions, which contain large amounts of orthopoxvirus material. Vesicular fluids from the lesions are also convenient sources of diagnostic material. Vesicular fluids are another 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 equally suitable for virus detection. Collection of biopsies can be done with local anesthetic if histopathologic exam is considered. Histopathologic evaluation is especially important for successful diagnosis of several of the orthopox look-alike syndromes.



Vaccinia lesion on foot



This is a good example of one of several vaccinia lesions associated with the laboratory-acquired case of disseminated vaccinia in a previously unvaccinated laboratory worker.

Specimen Collection

- **Vaccinia and variola specimen collection essentially the same**
- **Check CDC website for recent updates in orthopox specimen collection specifics:**
 - **www.bt.cdc.gov/agent/smallpox/response-plan/files/guide-d.pdf**



Collection procedures for vaccinia in the event of an adverse reaction to vaccination or variola in the event of a terrorist release of smallpox virus are essentially identical. Specifics for collection techniques can be found at the CDC website. Updates are expected in the future so check the website occasionally.

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**



Typical practices associated with collection of patient specimens are appropriate for collection of orthopoxvirus lesions as well. These include wearing personal protective equipment, including 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.



These are examples of lab materials useful for collection of orthopox specimens for laboratory testing including the plastic vials in which lesion crusts would be shipped to the LRN site for analysis.

Specimen Collection

- **Vesicles:**
 - **Use scalpel or 26 gauge needle to unroof vesicle**
 - **Roof goes to collection tube**
 - **Scrape base of vesicle with blunt edge scalpel, or wooden applicator; apply to microscope slide**
 - **Lightly apply em grid, shiny side down, against lesion. Repeat (X2) using more or less pressure**



For collection of vesicles, it's suggested to use a scalpel or needle to unroof the vesicle. The skin or scab that constitutes the roof goes to the collection tube and is sent otherwise dry. 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 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 a lesion. The slide and/or EM grid are allowed to air dry for ten minutes. Store in slide in a slide holder and an EM grid in the appropriate box.



Lifting a crust or 'roof' from the skin



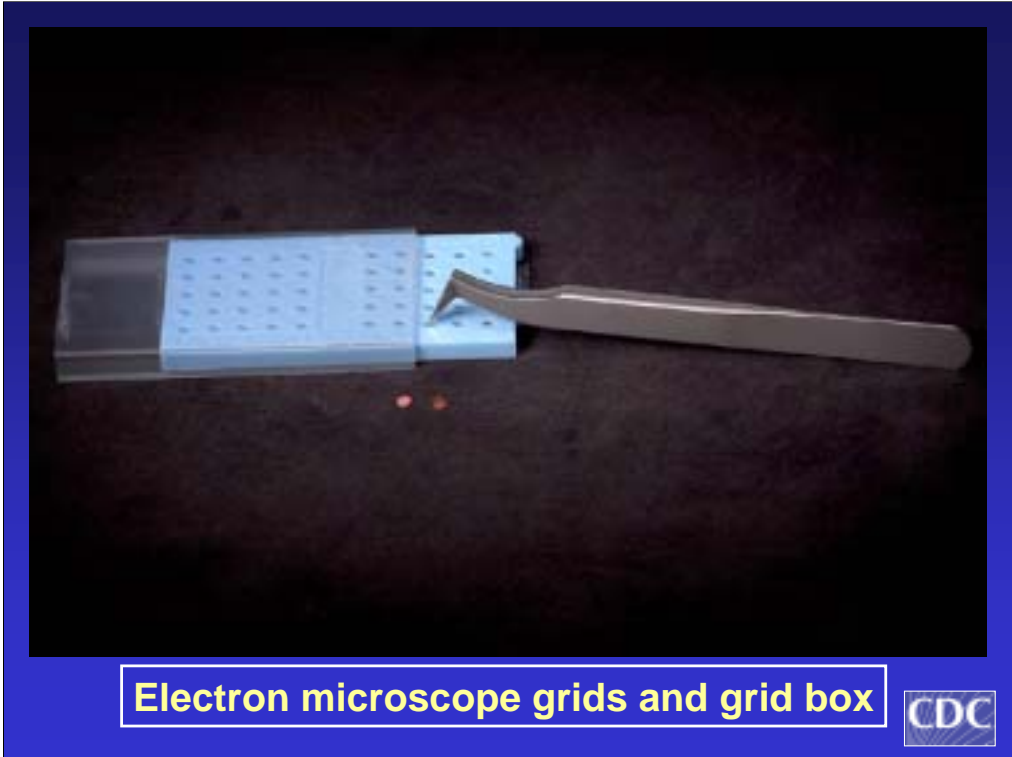
This is an example of lifting a crust – in this case an mature scab from a vaccination site.



This is the same scab being prepared to be put into a vial as sterilely as possible.



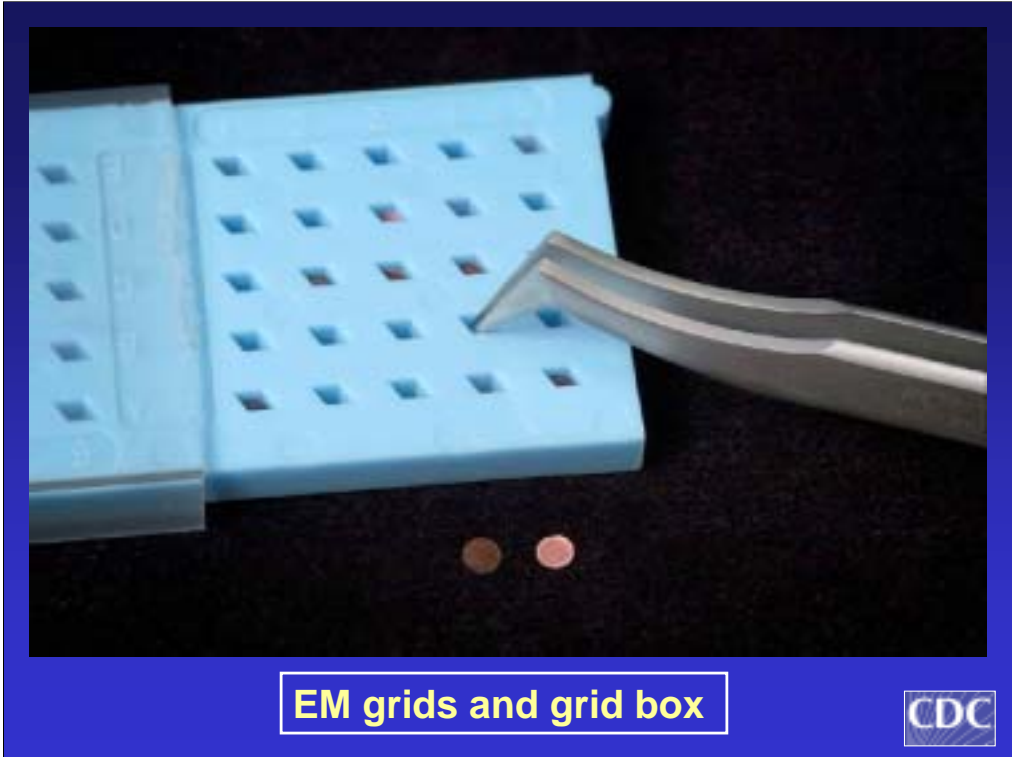
This is a simulated orthopox lesion and the making of a touch-prep with a glass or plastic microscope slide. The same lesion is touched 3 times with the same slide.



These are electron microscope grids, forceps for handling the grids, and the box for the grids.



Here's the electron microscopic grid is being used to touch down on the lesion.



EM grids and grid box



Close-up of the EM grids and the box.

Specimen Collection

- **Biopsy: 3.5 - 4 mm punch biopsy, bisect lesion, or obtain 2 biopsies.**
 - **Place 1 sample in specimen collector tube,**
 - **Place 1 sample in formalin**
- **Serum, if serology considered useful**



Biopsy specimens should either be split in two or taken in duplicate so that one specimen can be fixed in formaldehyde for histopathology, while the other is used for DNA detection or virus isolation. Serum, if necessary, can be collected as well.

Specimen Transport – How to Send

- **Standard diagnostic specimen shipping guidelines available:**

www.bt.cdc.gov/labissues/PackagingInfo.pdf

- **Serum, if collected, should be shipped frozen**
 - **If unable to separate serum from blood on-site, send whole blood refrigerated**



For vaccinia 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). If serum is collected, it is highly desirable to separate the serum from the blood on site, however, if this is not possible, one can send refrigerated whole blood to the LRN laboratory.

Specimen Transport – How to Send

- **Formalin fixed tissue must be shipped at room temperature - do not freeze**
- **EM grids must be shipped at room temperature**



Formalin-fixed tissue must be shipped at room temperature, not frozen. Electron microscopic grids must 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 must be stored and shipped frozen. However, if overnight transportation to the LRN lab can be arranged, freezing of fresh viral specimens is not necessarily required. Keep all virus-containing material out of direct sunlight.

Specimen Transport - Where to Send

- **Suspect vaccinia adverse events specimens go to closest Laboratory Response Network (LRN) laboratory**
- **Contact state Public Health Laboratory Director for specific shipping information**



Suspect vaccinia samples should be sent to the closest state or regional LRN laboratory. Contact your state's public health laboratory director's office for specific address information. It might be prudent to obtain this local contact information prior to onset of large-scale vaccination programs.

Specimen Transport - Where to Send

- **Specimens from persons with high suspicion of smallpox dx: Refer to Rash, Vesicular Disease Algorithm... specimens go to BOTH selected LRN with smallpox surge potential (contact CDC) and CDC simultaneously**
- **Contact CDC prior to shipment**



Specimens with high suspicion of smallpox diagnosis need to come directly to CDC and to selected LRN labs with smallpox surge potential. As part of the febrile vesicular rash disease algorithm, it is anticipated that state health departments would be in contact with CDC regarding such a specimen and its transport.

Past and Future

- **What about 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**



What about the past? It's 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 re-emerge in the future. Diagnostic electron microscopy capability was more common in the past, and relatively low-tech gel diffusion serological assays were available.

Past and Future

- **Future? (diagnostic development evolving rapidly)**
 - **Additional DNA-PCR targets**
 - **Antigen capture and DFA**
- **Following recognition of smallpox cases, expectations for diagnosis would likely change**



What about the future? Additional sensitive diagnostic tests are currently being developed and will be deployed. 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 point of care. It is also worth considering that in the event of a validated outbreak of smallpox, expectations for smallpox diagnosis would change considerably. As previously mentioned, it is likely that there would be an increased emphasis on clinical patient evaluation and subsequent initiation of vaccination even in the absence of laboratory confirmation.

I hope this brief introduction to vaccinia and variola diagnostic testing has been useful to you. Of course there's much more information available at the CDC website.

For More Information

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

