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Unit 1 of this Module will discuss the methods and procedures of virus isolation used to detect and diagnose orthopoxvirus infections and rule out other rash-like illnesses.

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The laboratory algorithm (Laboratory Testing for Acute, Generalized Vesicular or Pustular Rash Illness in the United States) was introduced and discussed in detail in Module 3. It provides:

1) A standardized approach to rapidly triage and test specimens for the possible presence of variola virus, the infectious agent that causes smallpox, and

2) A logical progression of testing if the case patient is not considered to be at high risk for smallpox.

Virus isolation is not as rapid as other diagnostic methods such as PCR or electron microscopy. Nonetheless, it is a useful tool in the diagnostic process, especially to confirm the presence of viable virus, and to amplify unknowns for more complete analyses such as nucleic acid sequencing. Virus isolation from high-risk specimens SHOULD NOT be attempted at laboratories outside of the WHO collaborating centers for smallpox and other poxviruses. There are two WHO-designated centers. One is at the Centers for Disease Control and Prevention—also known as the CDC in Atlanta, Georgia. The other is at the State Research Center of Virology and Biotechnology, known as Vector, in the Novosibirsk Region of Russia.

Virus isolation from specimens considered low- and moderate-risk for smallpox, via viral infection of cell culture, can be done under biosafety level 2--BSL-2--conditions in Sentinel and Laboratory Response Network—LRN--Reference Laboratories. Direct fluorescent antibody--DFA; Polymerase Chain Reaction--PCR; and electron microscopy can be performed to evaluate specimens for the presence of varicella zoster virus, herpes simplex viruses, and enteroviruses which also cause vesicular rash illness.

If the case patient is considered to be at high risk for smallpox, no testing should be performed prior to consultation with the State or local Public Health Laboratory and CDC. Chain of custody documentation should be immediately initiated. For specimens from patients with a high-risk of smallpox, virus isolation should NOT be attempted until smallpox has been ruled out by variola-specific testing at CDC or an LRN reference laboratory.
Laboratory Approach to the Diagnosis of Smallpox: Module 5 – Laboratory Methods Part 1

Now, let’s take a closer look at virus isolation and its utility in orthopoxvirus diagnostics. This module will not cover all of the details of cell culture methods, or discuss isolation of other infectious pathogens that may have clinical manifestations similar to smallpox.

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Many different methods have been used to differentiate orthopoxvirus infections from other rash-like illnesses. Prior to the development of modern diagnostic techniques such as cell culture, electron microscopy, serologic antibody detection methods, and molecular detection assays, other viral phenotypes were invaluable laboratory diagnostic markers. These phenotypic characteristics included the ability to form lesions or pocks on chick chorioallantoic membranes (known as CAM), hemagglutination of specific types of red blood cells, observance of intracellular inclusions, and scarification reaction in rabbits.

When making a diagnosis, one would have looked for the following results: orthopoxviruses form characteristic pock-like lesions on CAM while herpes simplex viruses form small white-ish lesions. The yatapox, parapox, and molluscum contagiosum viruses all do not form lesions on CAM.

The orthopoxviruses exhibit a varied hemagglutination response while the other viral families listed lack hemagglutination activity.

Orthopoxviruses, yatapoxviruses, and parapoxviruses form B-type cytoplasmic inclusions and certain cowpox virus strains also form A-type cytoplasmic inclusions. Molluscum contagiosum virus forms cytoplasmic, acidophilic granular masses (commonly termed molluscum bodies), while the herpesviruses form nuclear rather than cytoplasmic inclusions.

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Historically, a panel of diagnostic markers was used to differentiate between strains of orthopoxviruses.

Morphologic and phenotypic differences in viral lesions upon chorioallantoic membranes, or CAMs, were one of the most useful methods for differentiating orthopoxviruses. Cowpox virus pocks on CAM tend to be flat, poorly defined, hemorrhagic lesions. Monkeypox virus forms small pocks that at late stages of growth have central hemorrhage. Variola virus forms monomorphic white, sharply defined dome-like pocks, whereas vaccinia forms large pocks that appear white or grey in color.

Both cowpox and variola viruses lack or have weakly expressed hemagglutination activity. In contrast, vaccinia has a marked level of hemagglutination activity, and monkeypox expresses a high level of hemagglutination under the appropriate experimental conditions.

With regard to intracellular inclusions, monkeypox, variola, and vaccinia viruses all produce cytoplasmic B-type inclusions. Cowpox virus produces both A- and B-type inclusions.
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These photographs reveal variola virus pocks on the chorioallantoic membrane of a developing embryonic chick. Notice the small, sharply defined, white dome-like pocks formed by variola infection. This procedure utilizes living embryonated eggs and is resource intensive. It is widely considered less amenable to modern laboratory biosafety procedures.

For increased speed, convenience and safety, most labs now utilize tissue culture-derived cell lines. Cell culture infections with various viruses frequently produce a distinctive cytopathic effect on the cell monolayers. However, cell culture infection with orthopoxviruses does not provide the distinctive pock phenotypic information that can be visualized when orthopoxviruses are inoculated onto chorioallantoic membranes.

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There are many other pathogens and diseases that can be mistaken for orthopoxvirus infections, as listed here and discussed in Module 2. The presence of orthopoxvirus cytopathic effects in cell culture allows us to rule out many of these non-infectious rash-like illnesses. Cell rounding and cytoplasmic protrusions are cytopathic effects characteristic of orthopoxvirus infection of cell culture. These cytopathic effects confirm the presence of viable virus in the clinical sample. However, they do not enable us to determine which orthopoxvirus pathogen is present.
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Biosafety regulations vary depending on the orthopoxvirus being investigated. If the patient is considered at high risk for smallpox, virus culture should NOT be performed. Recommendations from the 5th edition of the Biosafety in Microbiological and Biomedical Laboratories, also known as the BMBL, include the following: persons using biosafety level two conditions and practices can safely work with live vaccinia virus; all live virus manipulations are conducted within a biosafety cabinet. Smallpox vaccination is also recommended by the Advisory Committee on Immunization Practices, or ACIP, for persons working with vaccinia.

According to current BMBL recommendations, monkeypox virus must be contained under at least biosafety level two–BSL–2–conditions; most safety officers recommend the use of BSL–3 practices. All work with live monkeypox virus must be conducted within a biosafety cabinet. Smallpox vaccination is recommended by the ACIP. Further guidance is provided on the CDC website.

At present, laboratory investigation of variola virus, the causative agent of smallpox, can only be performed at a laboratory designated for this purpose by the World Health Organization. There are two WHO-designated centers. One is at the CDC in Atlanta, Georgia. The other is at the State Research Center of Virology and Biotechnology, known as Vector, in the Novosibirsk Region of Russia. All research utilizing viable virus is conducted by vaccinated personnel in the biosafety level 4 laboratory. Other than at these two locations, no one should attempt to culture virus from any sample if there is a high level of suspicion that it contains variola virus.

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Several specimen types are appropriate for virus isolation in cell culture. Lesion “roofs” and crusts usually contain high concentrations of virus, as does vesicular fluid. Shown here are several different tools used for collection of specimens. A more complete description of collection methods can be found in module 4 and at the CDC website.

The method of processing depends on the sample type. The tissue samples, including lesion crusts, roofs, and biopsies, must be ground in phosphate buffered saline—PBS before inoculation into cell culture. Vesicular fluid collected on a slide via touch prep may be reconstituted in PBS–for cell culture inoculation. Dry swabs of vesicular fluid must also be rehydrated in PBS. When a swab is sent in viral transport media, the media alone is sufficient for virus culture. (Note, however, that viral transport media is not optimal for electron microscopy analysis).

Cell cultures should be monitored daily for 14 days; any signs of cytopathic effect indicate the presence of live virus in the specimen.
Laboratory Approach to the Diagnosis of Smallpox: Module 5 – Laboratory Methods Part 1

Remember, under NO circumstances should anyone attempt to grow virus from any sample where there is a high level of suspicion that it contains variola virus.

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Orthopoxviruses frequently produce distinctive cytopathic effects, generally referred to as CPE, in infected tissue-culture cell lines. Some commercially available cell lines commonly used in diagnostic laboratories are also useful for growing orthopoxviruses. These include:

- **A549**, which are Human lung carcinoma cells,
- **Hep2**, which are Human larynx carcinoma cells with HeLa cell contamination,
- **FRHK4**, which are Rhesus monkey kidney cells. CPE seen in these cells is similar to the CPE that would be seen in Primary Rhesus Monkey Kidney cells, and
- **BSC-40**, an African Green monkey kidney cell line.

In the images that follow, these cell lines were infected at very low concentrations of virus (also known as multiplicity of infection) with vaccinia, monkeypox, and variola viruses to mimic conditions that would likely be found in a clinical sample.

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This slide demonstrates the cytopathic effect of orthopoxviruses in human lung carcinoma (A549) cells. The earliest obvious signs of characteristic CPE are demonstrated at forty-eight hours post-infection as seen by cell rounding and cytoplasmic projections or extensions. These characteristics become more evident at seventy-two hours post-infection. Note that the cytopathic effect of variola is somewhat delayed compared to that of vaccinia and monkeypox. The CPE shown here resulted from an inoculum of approximately $10^4$ infectious virus particles.

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Unfortunately, human larynx carcinoma (Hep2) cells do not display orthopoxvirus cytopathic effects as well as other cell lines do. You can still see rounded cells and the beginnings of cytoplasmic projections at forty-eight hours post-infection. However, at seventy-two hours post-infection, this cytopathic effect becomes less evident as compared to the uninfected control. Only the monkeypox-infected cells still clearly show orthopoxvirus cytopathic effect. Because Hep2 cells continue to grow after confluency, the new cells closely mimic the “rounded” infected cells. This often confusing effect only intensifies as the infection continues and the host cells age.
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Rhesus monkey kidney cells are extremely useful for visualizing orthopoxvirus cytopathic effect. This slide demonstrates CPE in FRHK4 cells. Similar patterns would be seen in primary rhesus monkey kidney cells used in most clinical settings. At forty-eight hours post-infection, the formation of cytoplasmic projections can be seen for vaccinia, monkeypox and variola. Furthermore, you clearly see fusion of cells caused by monkeypox or variola virus infections. At seventy-two hours post-infection, the cytopathic effect becomes more noticeable with “rounding” of the infected cells.

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The African green monkey kidney (BSC-40) cells are also very useful for visualizing orthopoxvirus cytopathic effect. These cells are used in many poxvirus laboratories. At forty-eight hours post-infection, cells infected with each of the three orthopoxviruses all show formation of cytoplasmic projections, fused cells, and “rounded” cells. At seventy-two hours post-infection, the cytopathic effect is at its late stages, with almost all of the infected cells displaying “rounded” CPE.

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As shown in the preceding photos of cell culture, you should always include a negative—that is, uninfected—control when trying to identify an infectious agent in a clinical sample. This uninfected control ensures that any cytopathic effect observed is specific to the sample and not to the cell line. However, we do not recommend that you include an orthopoxvirus-positive culture sample. There is too great a risk of a false-positive due to cross-contamination of the sample culture. It is important to remember that cytopathic effect alone cannot differentiate between different orthopoxvirus species, so supplemental testing by PCR or EM is required.

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Digital cameras make it much easier to exchange electronic images of unknown cytopathic effect. This can be a valuable tool if you need to contact others for assistance in diagnosing an unknown isolate, or getting consultative advice when clinical presentation and or CPE suggest an unexpected or unusual virus. If you have questions about cytopathic effect in your cell culture that suggests orthopoxvirus infection, take digital images of the cell culture, and if possible, the patient’s lesions, and send images to the WHO collaborating center at the CDC in Atlanta, Georgia for consultation.
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When CPE is suggestive of orthopoxvirus infection, contact the nearest LRN reference lab or state public health laboratory for consultation. If appropriate, specimens and cultures can be referred to the LRN or CDC for supplemental testing. LRN approved PCR methods will be used for virus identification. Additional information on PCR testing is available in Unit 2 of Module 5.

All cultures suspected of containing orthopoxviruses should be carefully harvested, under the appropriate biosafety conditions. The presence of orthopoxviruses should be confirmed by polymerase chain reaction or electron microscopy. If smallpox is suspected based on clinical presentation or concerns of bioterrorism, no manipulation of cell cultures should be performed. Properly contain all materials and contact CDC and the state public health laboratory to arrange for transfer of all culture and specimen materials.

Here are 2 CDC emergency contact numbers if smallpox infection is suspected:
CDC Emergency Operations Center 770-488-7100
Poxvirus Hotline 404-639-4129

The emergency operations center is staffed 24/7, the Poxvirus Hotline is staffed only during routine business hours.
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Unit 2 of this Module will discuss the nucleic acid detection methods used in the detection and diagnosis of orthopoxvirus infections.

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There are many other pathogens and diseases that can be mistaken for orthopoxvirus infections, as listed here and discussed in Module 2. Nucleic-acid based diagnostic tests – such as the Polymerase Chain Reaction or PCR - can help determine if the infectious agent found in a specimen from a patient with a rash-like illness is from an orthopoxvirus infection or from one of these other illnesses.
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The laboratory algorithm (Laboratory Testing for Acute, Generalized Vesicular or Pustular Rash Illness in the United States) was introduced and discussed in detail in Module 3. It provides:

1) A standardized approach to rapidly triage and test specimens for the possible presence of variola virus, the infectious agent that causes smallpox, and

2) A logical progression of testing if the case patient is not considered to be at high risk for smallpox.

The utility of nucleic acid-based diagnostics within the algorithm is invaluable for providing rapid diagnoses. This module will focus on the use of nucleic acid testing strategies to evaluate patients with suspect rash illness.

If the case patient is considered to be at low or moderate risk for smallpox, the specimen can be processed at a sentinel or reference LRN laboratory and evaluated with a standardized PCR assay for varicella; that is, chickenpox. Many Laboratory Response Network—LRN—reference labs, as well as sentinel labs, have nucleic acid testing capacity for enteroviruses and herpesviruses (other than varicella). The first step is to for labs to rule out varicella zoster virus, herpes simplex viruses, and enteroviruses by PCR (and/or some combination of viral culture, direct fluorescence assay, and electron microscopy techniques). Once this has been done, we can focus our attention on a smaller area of the laboratory algorithm: orthopoxvirus PCR testing. This includes both orthopoxvirus (exclusive of variola) and orthopoxvirus-generic PCR tests. These tests would be especially pertinent if there were a report of exposure to an orthopoxvirus other than variola. In these instances orthopoxvirus testing would be appropriate early in the laboratory workup.

If the case patient is considered to be at high risk for smallpox, no testing should be performed prior to consultation with the State or local Public Health Laboratory and CDC. Chain of custody documentation should be immediately initiated. Real-Time PCR testing for variola, as well as generic orthopox, and non-variola orthopoxviruses is performed at LRN Variola Testing Labs with appropriate biosafety and security facilities for specimens derived from high-risk smallpox patients. Once variola is ruled-out, testing for other causes of rash-like illness can be performed.

Now, let’s take a closer look at these various nucleic acid-based diagnostic tests used for orthopoxvirus testing of clinical samples.
Nucleic acid detection methods for orthopoxviruses generally require amplifications of viral DNA, often using polymerase chain reaction technology. Application of PCR has revolutionized diagnostic methods for infectious diseases. While this training module covers the general application of the technology as it pertains to orthopoxvirus detection and identification, it is not intended to be a primer on nucleic acid amplification.

Nucleic acid detection methods have been designed to detect and differentiate between various orthopoxviruses. These assays are available in LRN Reference laboratories, at the Centers for Disease Control and Prevention as well as at a number of research laboratories. Some clinical and commercial laboratories have also developed nucleic acid detection methods for other agents that cause rash-like illness, such as varicella and other herpes viruses. Real-time nucleic acid detection methods—Real-Time PCR—allow for quick identification of the infectious agent, usually within hours, depending on the number of specimens, the method of sample preparation, and the detection method. However, PCR can NOT confirm whether the virus in the sample is viable. Results from PCR analysis, along with isolation of virus through standard culture methods as discussed in Module 5 Unit 2, are used in combination to further aid in diagnosis and patient management.
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Since viral DNA is extracted from specimens potentially containing viable virus, the biosafety conditions are the same as for infection of cell culture. As mentioned in module 3, the biosafety regulations vary, depending on the orthopoxvirus being detected. Vaccinia requires biosafety level two—BSL-2—conditions, with all live virus manipulations conducted within a biosafety cabinet. In processing a specimen for nucleic acid-based testing, inactivation of the virus should be conducted within the biosafety cabinet. After completing inactivation, further testing can be conducted outside the biosafety cabinet. The Advisory Committee on Immunization Practices—ACIP—recommends smallpox vaccination for individuals working with live orthopoxviruses.

Current practices at the CDC for working with possible monkeypox specimens include vaccinations of laboratory personnel and biosafety level 2 or 3 facilities with BSL-3 work practices. There is further guidance at the Centers for Disease Control and Prevention website.

Variola virus can only be studied at two World Health Organization—WHO—collaborating centers. One is at the Centers for Disease Control and Prevention in Atlanta, Georgia; the other is at the State Research Center of Virology and Biotechnology, known as Vector, in the Novosibirsk Region of Russia. All research utilizing viable virus is conducted in the BSL-4 laboratory by vaccinated personnel. Currently no one outside of these two laboratories should attempt to grow virus from any sample considered to be high risk for containing variola virus. However, select LRN reference laboratories with enhanced biocontainment and biosafety practices have been approved to perform Real-Time PCR for variola virus.

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Several specimen types are appropriate for PCR analysis. Lesion "roofs" and crusts usually contain high levels of viral DNA, as does vesicular fluid. Several different tools used to collect samples are shown here. A more complete description of collection methods can be found in Module 4 and at the CDC website.

The method of processing depends on the sample type and the DNA extraction method to be used. The most common samples used for PCR analysis are tissue and vesicular fluid. The tissue samples, including lesion crusts, roofs, and biopsies, must be ground prior to DNA extraction. Vesicular fluid collected on a slide, generally referred to as a touch prep, must be reconstituted in Phosphate Buffered Saline—PBS. Dry swabs of vesicular fluid must also be rehydrated in PBS; the sample is subsequently extracted from the swab using a swab extraction system. When a swab is sent in viral transport media, the media alone can be sufficient for DNA extraction.
Several methods can be used to extract orthopoxvirus DNA from clinical specimens. Prior to DNA extraction, potential virus in the specimen must be inactivated. Until inactivation is complete, all manipulations must be conducted within the biosafety cabinet. There are many different commercially available DNA extraction kits to isolate viral DNA from clinical samples. Within the LRN Reference Laboratories, a number of these kits have been validated for orthopoxvirus DNA testing. These kits include a non-ionic detergent lysis and an inactivation step at fifty-five degrees celsius. Strong detergent buffers at high temperatures have been shown to inactivate most orthopoxviruses. After inactivation, further testing can be performed outside the biosafety cabinet.

As an alternative to manual extractions, robotic systems are becoming more common. These systems may be faster, increase specimen throughput, and limit potential contamination. Regardless of which method of extraction is used, it is critical to minimize any potential cross-contamination between samples. It is NOT recommended to include a positive control with your sample extractions due to the potential for false positives caused by cross-contamination. A control that detects human DNA, such as RNaseP, can be used to validate the integrity of the specimen and sample processing.

The targeted genes for detection of orthopoxvirus DNA are both non-essential and essential genes conserved within the orthopox genome.

The species-specific PCR assay targets variations in non-essential genes such as the A-type inclusion, or ATI; hemagglutinin, or HA; and the cytokine receptor, crmB genes. As with many nucleic acid based diagnostic tests, the sensitivity of the species-specific assay varies depending on the type of detection assay used and the target region.

One target that can be used for the detection of DNA from all orthopoxviruses is the essential DNA polymerase gene, E9L.

Publications on the detection of various orthopoxvirus target genes can be found in the journals listed.
Laboratory Approach to the Diagnosis of Smallpox: Module 5 – Laboratory Methods Part 1

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The development and advancement of methods for DNA analysis using the Polymerase Chain Reaction have revolutionized laboratory-based diagnostics. PCR-based methods can be sensitive, specific, and greatly reduce the amount of time necessary to arrive at an accurate diagnosis. Currently, three nucleic acid-based tools, in addition to sequencing, are utilized in the detection of orthopoxvirus DNA in clinical samples. These are Single gene PCR/restriction-endonuclease fragment length polymorphism, or RFLP, analysis; Extended PCR/RFLP, or E-PCR/RFLP; and Real-Time PCR, or RT-PCR. The laboratory timeline for completing the assays and obtaining results varies depending on both the assay performed and the specificity of the assay. This includes the time necessary for manual nucleic acid preparation from the clinical sample, but does not include the time to transport and accession specimens. We will now take a closer look at each of these DNA based diagnostic tools.

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Single gene Polymerase Chain Reaction/Restriction Fragment Length Polymorphism, or PCR/RFLP, combines standard PCR with RFLP. In this assay, the amplified viral DNA is digested with restriction enzymes and the resulting fragments are screened by gel mobility, thus providing a DNA footprint. This footprint can then be compared with known orthopoxvirus DNA footprints to determine the virus or strain of orthopoxvirus DNA present in a clinical sample. In this case, the DNA footprint of a sample from both a patient and a suspected infected prairie dog obtained during the 2003 U.S. Monekypox Outbreak, are compared with known DNA footprints of reference orthopoxvirus isolates including vaccinia, monkeypox, and variola. The patient sample exhibits the same DNA footprint as the monkeypox control reference isolate, so the lesion from this patient, as well as the prairie dog, contains monkeypox virus.

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Extended PCR/RFLP expands upon the standard PCR/RFLP analysis previously discussed. In E-PCR/RFLP, several primer pairs targeting conserved orthopoxvirus sequences are used separately to produce 20 amplicons, which collectively overlap and cover nearly the entire viral genome. The amplicons are subsequently digested with the appropriate restriction enzymes and resulting fragments are resolved on several 20% acrylamide gels under standardized electrophoresis conditions. The resulting DNA footprint is then further analyzed. A bioinformatics analysis software program such as BioNumerics is used to produce a cluster analysis and dendrogram showing the relationship of the viral DNA isolated from the clinical sample to other orthopoxvirus DNA isolates. This technique is currently available at the Centers for Disease Control and Prevention in Atlanta, GA.
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Here we have a closer examination of the E-PCR/RFLP DNA footprint and the resulting dendrogram of amplicon A9. Amplified A9 DNA from the species of orthopoxviruses listed here was digested with the Hinc II restriction endonuclease. As you can see, we are able to differentiate between species of orthopoxviruses based on the DNA footprint produced by the digestion and electrophoresis of the amplicon DNA.

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Real-Time PCR methods have revolutionized the ability of diagnostic laboratories to detect infectious pathogens. Real-Time PCR methods for the detection of orthopoxvirus DNA in clinical samples have been developed at CDC and made available to LRN reference laboratories. Real-Time PCR requires an instrumentation platform that consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software. These machines are available from several manufacturers. They vary in sample capacity; some are 96-well standard format while others process fewer samples or require specialized glass capillary tubes. They also differ in method of excitation; some use lasers, while others use broad spectrum light sources with tunable filters. There are also differences in overall sensitivity. In addition, there are platform-specific differences in how the software processes data. Real-Time PCR machines are not inexpensive, currently costing about $25,000 - $95,000, but are well within purchasing reach of core facilities or labs that have the need for high throughput quantitative analysis.

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Real-Time PCR methods have dramatically decreased the time required to detect orthopoxvirus DNA in clinical samples. Real-Time PCR uses a fluorescently-labeled probe or intercalating dye to visualize a PCR reaction product and actually views the increase in the amount of DNA as it is amplified. There are several types of Real-Time PCR assays available, which use different detection methods, such as:

1) Single Dual-labeled probe within the 5' exonuclease assay, commonly known as the TaqMan assay,
2) Minor Groove Binding Protein--MGB--which is a Single dual-labeled probe linked to MGB protein,
3) Linear Hybridization Probes or FRET Probes, consisting of 2 single dye-labeled probes, and
4) DNA Binding Fluorophores.
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Real-Time PCR reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected. This is in contrast to standard PCR, which indicates the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The light emitted from the dye in the excited state is received by a computer and displayed as an amplification plot (as seen here), which is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT, which refers to cycle threshold or crossing threshold, is defined as the cycle number at which the fluorescence passes the fixed threshold. The platform software can then quantify the amount of target DNA in unknown samples by comparing the sample CT value to the CT’s produced by known standards to determine starting copy number.

We will give a brief description of each Real-Time PCR method and a more detailed view of the three assays routinely utilized in the detection of orthopoxivirus DNA in clinical samples received by CDC: the 5' exonuclease, or TaqMan; Minor Groove Binding Protein, or MGB; and Linear Hybridization Probes, or FRET, assays. Additionally, the recent development of a Real-Time PCR assay utilizing SYBR Green I, a DNA Binding Fluorophore, in the detection of Brazilian orthopoxviruses will be discussed.

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In the 5' Exonuclease--TaqMan--assay, TaqMan probes are utilized in a Real-Time PCR assay. The TaqMan probes are oligonucleotides. The probes are longer than the primers used to amplify the DNA and contain a fluorescent reporter dye, usually on the 5' base, and a quenching dye on the 3' base. When the probe is intact and irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule; no signal is seen at the wavelength at which the fluorescent reporter dye normally releases light. Thus, the close proximity of the reporter and quencher prevents standard emission while the probe is intact. As the PCR reaction progresses, the polymerase replicates the template on which the TaqMan probe is bound. This causes a strand displacement of the reporter end of the probe. The 5' exonuclease activity of the DNA polymerase then cleaves the probe. This cleavage releases the fluorescent dye from the quencher and the reporter dye fluoresces. The fluorescent signal increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye.
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Minor Groove Binding, or MGB, molecules are based on a potent class of naturally occurring antibiotics that bind to the minor groove of double stranded DNA. When attached to either the 5' or 3' terminus of DNA probes, the hydrophobic binding of MGB molecules to the minor groove stabilizes the DNA duplexes formed by probes with their complementary targets. As a result, shorter probes can be used, which yields higher sensitivity to discern mismatches. This property is particularly beneficial when designing probes to analyze single nucleotide polymorphisms--SNPs--or short, conserved sequences of viral genomes or multi-gene families for which limited target sequence is available.

One method utilized at CDC has the quencher and the MGB™ moiety positioned at the 5'-end of the probe. The fluorescent reporter dye is located on the 3'-end of the probe. When the probe is in solution during the denaturation phase, the three-dimensional conformation brings the quencher into close proximity with the reporter dye and its fluorescence is quenched. However, when the probe anneals to a target sequence and the probe unfolds, the quencher becomes spatially separated from the reporter dye, allowing fluorescence.

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Linear Hybridization Probes, more commonly referred to as Fluorescence Resonance Energy Transfer or FRET Probes, have also been used by some laboratories for orthopoxvirus DNA detection. In FRET Probe analysis, two dye-labeled probes are used: One FRET probe--referred to here as the anchor probe--has an excitor fluorophore on the 3' end. The second probe--referred to here as a sensor probe--contains a reporter fluorophore at its 5' end as well as a quencher on the 3' end. When the FRET probes are free in solution and unbound to the target DNA, no fluorescence is observed. Similarly, if only one probe is bound to the target DNA, no fluorescence is observed. For fluorescence to occur, BOTH probes must bind in close proximity on the target DNA. As light excites the excitor fluorophore on the anchor probe, the energy is transmitted to the adjacent reporter fluorophore on the sensor probe, causing fluorescence. The excitor and reporter must differ in both their excitation and their emission wave lengths.
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A less expensive alternative to designing DNA probes, DNA Binding Fluorophores are often used as an alternative to TaqMan and MGB and FRET assays. Double-stranded DNA binding dye chemistries quantitate amplicon production through the use of a non-sequence specific fluorescent intercalating agent such as SYBR Green I or Ethidium Bromide. However, the absence of a sequence specific probe makes this method less diagnostically “specific” for the target DNA, so it is not routinely used for the detection of orthopoxvirus DNA in clinical samples. Recently, a rapid and inexpensive method for detecting Brazilian Orthopoxvirus infections using SYBR Green in a quantitative Real-Time PCR reaction was developed; it has proven useful in detecting orthopoxvirus viral DNA in lesion biopsy material without the need for DNA extraction. Further information on the use of SYBR Green in the real-time detection of orthopoxvirus DNA can be found in the journal listed.

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As previously mentioned in Module 3, highly sensitive Real-Time PCR assays for the detection of orthopoxvirus DNA have been deployed to every state through the Laboratory Response Network, or LRN. Currently, there are three unique assays that can be used separately or in combination to facilitate diagnosis. The orthopoxvirus generic real-time PCR assay detects all Eurasian orthopoxviruses, as well as, North American orthopoxviruses. Similarly, the LRN Orthopox non-variola Real-Time PCR assay will detect all Eurasian orthopoxviruses except variola. A few select LRN laboratories are currently capable of conducting a variola specific assay, which detects ONLY variola. The variola specific assays are performed at LRN Variola Testing Labs with appropriate biosafety and security facilities for specimens derived from high-risk smallpox patients.

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Due to the highly specific and sensitive nature of the orthopoxvirus Real-Time PCR assays, it is critical to minimize any potential cross-contamination between samples. Due to the risk of false-positives from cross-contamination, including a positive control when extracting DNA from clinical samples is NOT recommended. However, a control assay that detects human DNA, such as RNaseP, can be used to validate the integrity of the specimen and sample processing. It is important to include the proper controls, both positive and negative, when setting up the Real-Time PCR assays for the detection of orthopoxvirus DNA in clinical samples. For the Orthopox Generic and Orthopox Non-Variola assays, it is suggested that vaccinia virus DNA be used as the positive control, and water be used as the negative control for both assays. For the Variola specific assay, plasmids containing key regions of the variola genome are used in place of intact variola DNA. Water is used as the negative control for the Variola specific assay, as it is in the orthopox generic and orthopox non-variola assays.
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In general, there are many advantages to using Real-Time PCR over traditional PCR methods.

- Real-Time PCR is more sensitive, with detection capabilities down to 10 genome equivalents.
- A clear advantage of Real-Time PCR over standard PCR is the rapid return of results. For orthopoxvirus diagnostics, the time from sample preparation to results is typically 3-4 hours.
- Real-Time analysis allows for simultaneous amplification and detection. This eliminates the post PCR processing of amplified DNA sample. No electrophoretic separation is needed.
- Compared to PCR with restriction-endonuclease fragment length polymorphism, or RFLP, methods, there is a lower risk of amplicon carryover contamination since there is no post-PCR processing within Real-Time PCR reactions.
- Results from the real-time PCR assay are highly reproducible and can be quantitative. Reporter fluorescent signal increases in direct proportion to the amount of amplified viral DNA product.
- The ancillary reagents required for Real-Time PCR are commercially available.
- Available Real-Time PCR platforms make the technology user friendly and adaptable to meet the needs of clinical and research settings.

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While Real-Time PCR is very useful in the detection of Orthopoxvirus DNA in clinical samples, there are a few limitations to be considered:

- There is an increased potential for false positives due to the extreme sensitivity of the Real-Time PCR assays. Extra caution should be taken in preparation and set up of the PCR assay. The slightest amount of contamination will be detected and will cause a false positive result.
- There are also increased costs. Though Real-Time PCR reagents are commercially available, they are more expensive when compared to reagents used in Standard PCR analysis.
- Real-Time PCR platforms are also a costly financial investment. They often cost tens of thousands of dollars, much more than a Standard PCR platform.
Unit 4: Serology

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Unit 3 of this module will present diagnostic serology, which is the measure of pathogen-specific antibodies in serum, as a laboratory tool for the detection and diagnosis of exposure to orthopoxviruses.

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The laboratory algorithm (Laboratory Testing for Acute, Generalized Vesicular or Pustular Rash Illness in the United States) was introduced and discussed in detail in Module 3. It provides:

1) a standardized approach to rapidly triage and test specimens for the possible presence of variola virus, the infectious agent that causes smallpox, and

2) a logical progression of testing if the case patient is not considered to be at high risk for smallpox.

Laboratory testing such as PCR, virus isolation, and electron microscopy play a major role in rapid laboratory confirmation of viral infection by detecting the virus. At this time, serologic testing is not sufficient to detect the presence or absence of virus, nor does it provide information regarding the species of orthopoxvirus encountered. However, when no acute specimens for virus testing are available, serologic methods can be an important option.

Serology will provide evidence of exposure to orthopoxvirus. It has a diagnostic role in identifying such exposure, particularly in the absence of a suitable specimen for virus detection or isolation. In this manner the use of serology may provide useful input to the algorithm.

Note that if a case patient is considered to be at high risk for smallpox, no testing should be performed prior to consultation with the State or local Public Health Laboratory and CDC. Chain of custody documentation should be immediately initiated.

Now, let’s take a closer look at the various serology based diagnostic tests used for orthopoxvirus testing of clinical samples.
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Historically, serology has provided a method for surveillance of orthopoxvirus exposure. In contrast to the polymerase chain reaction--PCR assay-- it is not sufficient for rapid diagnosis of viral infection. However, in the absence of suitable samples for molecular testing, serology may play a valuable role in understanding an outbreak scenario and can provide evidence of exposure to an orthopoxvirus.

It is important to note that serologic testing does not reliably provide information on the “type” or species of orthopoxvirus in question. For example, these tests do not discriminate between detection of antibodies to vaccinia, monkeypox, or variola viruses. This is the major limitation of these assays to date. However, certain laboratories are working on serologic diagnostic methods for detecting specific orthopoxvirus species.

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Blood, sera, and plasma specimens from suspected cases utilized in serologic testing could all potentially contain viable virus. Therefore, the biosafety conditions are the same as for infection of cell culture and nucleic acid detection. As mentioned in module 3, the biosafety regulations vary, depending on the orthopoxvirus being studied. Vaccinia requires biosafety level two—BSL-2--conditions, with all live virus manipulations conducted within a biosafety cabinet. Smallpox vaccination is recommended by the Advisory Committee on Immunization Practices, or ACIP, for individuals working with live virus. It is important to remember that blood, sera and plasma specimens from suspected cases being studied for orthopoxvirus testing may also harbor other bloodborne pathogens, which also require that proper precautions be used. Additionally, any laboratorian exposed to or working with bodily fluids, especially blood, should receive Hepatitis B vaccination.

Current practices at the Centers for Disease Control and Prevention—CDC—for working with possible monkeypox specimens include vaccinations of laboratory personnel, and biosafety level 2 or 3 facilities with BSL-3 work practices. There is further guidance at the CDC website.

Variola virus can only be studied at two World Health Organization collaborating centers. One of the WHO collaborating centers is at the CDC in Atlanta, Georgia. The other is at the State Research Center of Virology and Biotechnology—known as Vector—in the Novosibirsk Region of Russia. All research utilizing viable virus is conducted in the BSL-4 laboratory by vaccinated personnel. Currently no one outside these two laboratories should attempt to grow any virus from a sample that is at high risk for containing variola virus.
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Sample types appropriate for serologic testing include serum, plasma, whole blood, and cerebral spinal fluid. Blood samples from persons with a severe, dense rash may be difficult to draw as the skin may slough off. A central line may be needed for access in cases such as these, where peripheral blood draw is difficult. A more complete description of collection methods and specimen types can be found in Module 4 and at the CDC website.

After reviewing the links, click the next button to continue.

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Before discussing serologic methods for detecting and diagnosing orthopoxvirus exposure, it is important to understand the kinetics of antibody responses. In general, the kinetics of antibody responses to orthopoxvirus infection are similar to those elicited by other viral infections. There are distinct windows of opportunity for virus isolation, acute-phase IgM serologic responses, and convalescent IgG serologic responses. These windows represent the basis for interpreting results of virus isolation and diagnostic tests based on immune responses.

As the acute-phase antibody response begins to wane, the convalescent response begins and maintains for a longer term. Serologic tests can exploit the kinetics of this response to provide information on the timing of virus exposure, based on antibody levels, virus isolation, and clinical presentation. For example, isolation of virus and positive tests for viral-specific IgM are indicative of an acute-phase infection.

Now, let’s review various serologic tests as they have been used for orthopoxvirus testing.
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As stated previously, serologic methods have limited diagnostic utility. Testing for anti-orthopoxvirus antibodies cannot be used for extremely rapid diagnosis of orthopoxvirus infections. However, these methods have proven useful in surveys to establish past outbreaks and epidemics of orthopoxviruses and to assess immune induction in vaccination efforts. These efforts include use in population exposure and vaccine trial efficacy testing. Additionally, these methods can be used to indicate past exposure to orthopoxviruses.

Methods of antibody detection include: immunoprecipitation in agar, the Ouchterlony test; immunofluorescence, or IFA; complement fixation, or CF; hemagglutination inhibition, or HAI; plaque-reduction neutralization testing, abbreviated as PRNT; and enzyme-linked immunosorbent assays, or ELISA. We will review each of these methods in more detail.

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The immunoprecipitation in agar test is also known as the Ouchterlony or double immunodiffusion test. It is based on the principle of concurrent migration of antigen and antibody toward each other through an agar medium and precipitation within the agar matrix. This test is considered a very precise immunologic diagnostic tool. However, it cannot readily differentiate between orthopoxviruses and relies on high titered antiserum and viral antigen such as a lesion. It is not routinely used in orthopoxvirus diagnostics today. In clinical settings, it was most often used to detect orthopoxvirus antigen in lesion tissues, primarily during the smallpox eradication campaign. Today, more sensitive serologic tests are used to detect orthopoxvirus specific antibodies.
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The Ouchterlony precipitation test is performed using a slide containing small holes or "wells" cut into a thin layer of solidified agar. A small sample of known positive antigen, such as an extract of variola crusts, is spotted into the top reservoir, followed by the unknown test extract. Antiserum, such as anti-vaccinia rabbit serum, is carefully placed into the opposing side wells. Normal rabbit serum is also included in the test against the extracts as a control for possible non-specific precipitation. The slide is then incubated in a covered, humidified petri dish to prevent the agar from drying out while the antigens and antibodies diffuse towards each other, eventually forming a visible immune complex. In a negative diagnostic test, precipitation lines fail to form between the patient antigen and hyperimmune sera. A precipitant does form between the reservoir containing the positive control antigen and the reservoir containing the immune serum. In a positive sample, visible precipitation lines will form within 2-4 hours between the test specimen reservoir and the antiserum reservoir as well as between the antiserum and positive antigen reservoirs. However, a test should not be considered negative until the precipitation has been allowed to stand for 24 hours. In recent years, the more sensitive ELISA techniques have largely replaced the Ouchterlony test. However, the Ouchterlony test remains useful in that the specificity of the observed reaction can be directly compared with a known positive control serum; most commercially available indirect ELISAs lack such a specificity control. In addition, the straightforward nature of the Ouchterlony test makes it amenable to application under conditions where more technology-intensive methods may be unrealistic.

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The immunofluorescence antibody test, or IFA, is a laboratory technique that uses a fluorescent antibody--that is, antibody indirectly conjugated with a fluorochrome--to detect a specific antigen or antibody in sera. For detection of anti-orthopoxvirus antibodies in sera, an indirect IFA staining method can be used. The primary antibody is unlabeled, and is detected with a fluorescently labeled secondary antibody. The fluorochrome can then be visualized with a fluorescence microscope equipped with a UV light source and excitation filters. The most commonly used fluorescent dyes are fluorescein and rhodamine. Florescein emits an intense yellow-green fluorescence while rhodamine emits a deep red fluorescence. IFA is a rapid and well utilized research diagnostic test for many infectious agents. Potential problems with the test include the requirement for a fluorescent microscope, standardization of antigen preparations, and standardization of user interpretation. Since the fluorescence is measured by visual observation, interpretation of results can be subjective.
Hemagglutination inhibition or HAI tests exploit the fact that orthopoxviruses have a hemagglutination protein—that is, an antigen—on their surface that can bind and subsequently agglutinate avian red blood cells or, in another form of the assay, latex beads. This agglutination produces a “shield” or “lattice” while negative hemagglutination produces a “pellet” in the bottom of the test well or tube. Incubation of patient sera with orthopoxvirus antigen prior to addition of red blood cells allows for detection of orthopoxvirus-specific antibodies that bind the virus; this prevents the virus from binding to the red blood cells, which results in the red blood cells “pelleting” at the bottom of the well. In the absence of orthopoxvirus-specific antibodies, the virus binds to the red blood cells to form a “lattice” or “shield” that yields a tinted well, uniform in color, and the absence of a “pellet” in the bottom of the well. Orthopoxvirus antigen-specific antibodies prevent agglutination in the following way.

Co-incubation of patient sera with orthopoxvirus antigen allows any orthopoxvirus-specific serum antibody to bind and form a complex with the antigen and prevent hemagglutination—that is, virus binding to red blood cells. In the presence of orthopoxvirus antibody in the serum, agglutination is inhibited and the red blood cells “pellet” at the bottom of the well, forming a “button.” In the absence of orthopoxvirus specific antibodies, the virus binds to the red blood cells and produces a “lattice” or “shield” in the well.

Disadvantages of the HAI tests include (1) the presence of inhibitors in some sera which also inhibit agglutination and (2) the necessity to standardize the antigen each time a test is performed to allow test-to-test comparison. Testing of red blood cell preparations is also labor intensive, requiring evaluation of optimal red blood cell preparations, as well as serum and antigen dilutions.
The complement-fixation, or CF assay, is used to detect the presence of anti-orthopoxvirus antibodies in patient serum. Similar to the Hemagglutination assay, CF assay also utilizes the fact that orthopoxviruses contain hemagglutination proteins that bind avian or sheep red blood cells--RBCs. Red blood cells and orthopoxvirus antigens are mixed together, forming a complex. The patient serum is then added at doubling dilutions in sequential tubes. Finally, complement is added to the solution. If the patient serum contains anti-orthopoxvirus antibodies, a complex forms between the orthopoxvirus antigen bound on the red blood cell and the anti-orthopoxvirus antibody; complement fixation and subsequent lysis of the red blood cell occur, indicated by a red tint to the solution and an absence of a red blood cell “pellet” at the bottom of the tube or well. Alternatively, if the patient serum lacks anti-orthopoxvirus antibodies, complement fixation and subsequent lysis of red blood cells do not occur. A “pellet” of red blood cells forms at the bottom of the tube or well.

CF is a cumbersome assay that requires labor intensive testing of animal red blood cell preparations as well as dilutions of antigen, serum and complement. The test has low sensitivity efficiency for diagnosis. Historical data suggest that CF identified anti-orthopoxvirus antibodies from only about 50% of smallpox patients.
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Plaque-reduction neutralization, or PRNT assays, provide significant information about the presence of orthopoxvirus-specific antibodies. They also provide information about the ability of those antibodies to neutralize virus particles \textit{in vitro}. Neutralization of virus is considered to provide some evidence of a protective immune response, as this test also evaluates the sera’s ability to prevent virus infection of cell culture substrate \textit{in vitro}.

In the plaque-reduction neutralization assay, cell monolayers are commonly grown in 6 well plates. A predetermined concentration of virus is added to each well. Also added are diluted sera from individuals suspected of being exposed to or infected with orthopoxviruses. Over the course of 48-72 hours, the virus particles adhere to and infect the cells. Infection then spreads to adjacent cells, resulting in the formation of plaques, or holes, in cell culture monolayers. The plaques are demonstrated by visible areas of clearing in the monolayer. When anti-orthopoxvirus antibodies are present in serum, plaque formation is inhibited. The reduction of those plaques can be seen and quantified. It should be noted that in studies of smallpox vaccination efficacy, a strong correlation is observed between ELISA reactivity and virus neutralization. PRNTs remain a crucial, but labor intensive laboratory test for determining the efficacy of smallpox vaccine. Recently, a rapid high-throughput vaccinia virus neutralization assay utilizing green fluorescent protein was developed for testing smallpox vaccine efficacy. More information on this novel assay can be found in the journal listed.
Perhaps the most widely used serologic test today is the ELISA, or Enzyme-linked immunosorbent assay. Two types of ELISA are routinely run at the CDC. Both are indirect ELISA and detect anti-orthopoxvirus antibodies by an indirect conjugate reaction, or by using enzyme-linked secondary antibodies against human immunoglobulin. ELISA to detect IgM- and IgG-class antibodies is used to identify such antibodies in vaccinated individuals, and in those suspected of being infected during orthopoxvirus outbreaks. These two ELISAs provide information for distinct aspects of potential diagnoses.

The necessary components for both the IgM and IgG ELISA tests are shown. First, the wells of 96-well plates are coated with either a coating antibody (for the IgM ELISA), or the orthopox antigen (for the IgG ELISA). Next, the serum antibody is added followed by the orthopox antigen and anti-orthopox antibody for the IgM ELISA test. Finally, the conjugate antibodies are added to the wells for both tests. The conjugate antibody used in the IgM ELISA is a goat anti-mouse IgG Horse Radish Peroxidase--HRP--conjugate. The conjugate antibody used in the IgG ELISA is a goat anti-human IgG HRP. Other immunologic assays to evaluate immune response to orthopoxvirus infection or vaccination may be currently available elsewhere.
An orthopox-specific IgM ELISA allows for detection of recent exposure to an orthopox virus, or primary vaccination against orthopox. The IgM test provides support for evaluation of orthopox infection during outbreaks, but it is not considered a stand-alone diagnostic test. The detection of virus specific IgM is a hallmark for acute or recently acquired infection.

IgG detection provides a long-term marker for orthopox immunity. This may represent residual immune induction from vaccination. Under specific circumstances, it may indicate exposure to an orthopox virus in non-vaccinated persons. Numerous studies have demonstrated a long-lived IgG response to vaccinia, smallpox vaccine, and orthopoxviruses – likely contributing to the efficacy of our vaccines.

Shown here are the kinetic antibody responses seen in confirmed human monkeypox cases during the 2003 US outbreak. These responses generally followed the classic “textbook” antibody response with an initial increase followed by a decline in IgM response and a continual increase in the IgG response. Although the assay detects orthopox antibodies in human serum, it does not determine the specific antibody type. Modified ELISA assays developed at CDC enable us to detect orthopox antibodies in animal species as well. The animal anti-orthopox ELISA may be a valuable tool for identifying susceptible animal populations or zoonotic sources of orthopox infection.

Serology has played a key role in the study of orthopoxviruses for over three decades. Recent work at the CDC has enabled refinement of the standard ELISA, allowing its utilization during the outbreak of monkeypox in the United States in 2003. ELISA clearly provided diagnostic support in conjunction with molecular testing and epidemiologic and clinical information. Both IgM and IgG ELISA continue to be used for assessment of smallpox vaccine trials and surveillance for orthopox exposure in humans and animal species.
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If serologic assays are not utilized for standalone diagnostic purposes, what can the results tell us? After infection, the immune system makes virus specific antibodies to help fight the infection. Antibodies are produced by B cells with help from T cells in response to infection. IgG antibodies develop within 21 days after infection; they are typically long-lived, remaining in the bloodstream for years. So detection of IgG by serologic tests indicates that the person was exposed to an orthopoxvirus in the past, such as through vaccination or other viral exposure. IgM antibodies develop rapidly and are detectable between day 7 and day 21 post infection. These antibodies are considered an early infection marker and are short-lived. Diagnostically, if you test positive for orthopoxvirus specific IgG, you likely have been exposed to an orthopoxvirus at some point in the past, either through vaccination with vaccinia, or other exposure. If you are negative for orthopoxvirus specific IgG, you most likely have never been vaccinated or exposed to an orthopoxvirus. If you test positive for orthopoxvirus specific IgM, you were likely exposed to an orthopoxvirus within the past 6 to 12 months. If the serologic test is negative for IgM, you were probably not infected with an orthopoxvirus in the past 6 months. Serologic tests are valuable for diagnostic support, but are best utilized in conjunction with clinical and epidemiologic data involving population or individual case knowledge.

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As with other assays used in the diagnosis of orthopoxvirus infections, it is always important to include the proper controls in serologic assays. Positive controls for orthopox serologic testing are derived from humans recently vaccinated with the smallpox vaccine—vaccinia—using either individual or pooled sera. Negative controls are derived from unvaccinated humans. The conjugates utilized in these tests include anti-human enzyme-labeled antibodies, specifically goat anti-human IgG peroxidase or goat anti-human IgM peroxidase and are available through commercial vendors.
Historically, serology has provided a method of surveillance for orthopoxvirus exposure. In contrast to the polymerase chain reaction assay, it is not sufficient for rapid diagnosis of viral infection. However, in the absence of suitable samples for molecular testing, serology may play a valuable role in understanding an outbreak scenario and can provide evidence of exposure to orthopoxvirus. The main drawback of using serologic assays for orthopoxvirus diagnostics is the inability to detect antibodies to specific orthopoxvirus species. For this reason, serologic assays are not generally utilized as a stand-alone diagnostic tool.
Module 5 review

The module that you have just finished presented information on virus isolation, nucleic acid detection and serologic assay methods used to detect orthopoxvirus infections and rule out other rash-like illnesses. The appropriate application of each of these techniques for testing specimens from patients at low risk and high risk for smallpox was discussed. We learned that virus isolation is a useful tool in the diagnostic process, especially to confirm the presence of viable virus, and to amplify unknowns for more complete analyses such as nucleic acid sequencing. However, virus isolation from high-risk specimens SHOULD NOT be attempted at laboratories outside of the CDC—where appropriate BSL-4 containment facilities are available. Several nucleic acid, PCR-based tools were described, these technologies are highly sensitive and specific and greatly reduce the amount of time necessary to detect infectious agents in clinical samples. Serologic markers of exposure to orthopoxviruses were described that can be used in conjunction with epidemiologic and clinical data. Module 6 will discuss histopathology, immunohistochemistry, and electron microscopy. Please keep in mind that as technology advances, new detection methods may become available. We highly recommend that you check the Centers for Disease Control and Prevention website periodically for updates.