Welcome to Module 3 of this program on the laboratory approach to the diagnosis of smallpox. This module comprises the approach to Laboratory Diagnosis. Module 3 of this series provides an overview of laboratory methods for detecting and identifying orthopoxviruses. Additionally, laboratory algorithms for the selection of appropriate tests and biosafety will be discussed. At the conclusion of this module, participants will be able to complete the following learning activities:

- List the symptoms associated with acute, generalized vesicular or pustular rash illness that categorize the risk of smallpox
- Utilize appropriate safety procedures for virus propagation.
- List methods available for orthopox testing.
- Utilize laboratory algorithms to select appropriate tests based on clinical presentation.
Slide 2 of 22:

Module 3 of this series begins by providing an overview of optimal laboratory methods for detecting, identifying, handling, and containing viruses. Additionally, laboratory algorithms for the selection of appropriate tests based on clinical presentation will be discussed in detail.

Due to concerns that smallpox virus could be used as an agent of bioterrorism, health-care providers should be familiar with the disease and methods for its control. In addition to the information presented in Module 2, the CDC video “Smallpox, What Every Clinician Should Know” presents information on the virology, epidemiology, clinical features and diagnosis of smallpox, and the characteristics and use of the smallpox vaccine.

After reviewing the link, click the “Next” button to continue.

Slide 3 of 22:

A variety of laboratory methods can be used to confirm a clinical orthopoxvirus diagnosis. Because orthopoxviruses are rarely seen in the United States and smallpox has been eradicated worldwide, most of these tests are available only in a few reference laboratories.

However, highly sensitive real-time PCR assays for detecting orthopoxvirus DNA have been deployed to every state through the Laboratory Response Network. Real-time PCR methods can detect DNA even from non-viable virus particles. These PCR assays are typically considered the most sensitive of all available assays. Three unique assays can be used separately or in combination to facilitate diagnosis.

The orthopox real-time PCR detects all Eurasian orthopoxviruses including variola (the agent that causes smallpox), and the LRN non-variola orthopoxvirus real-time PCR will detect all Eurasian orthopoxviruses except for variola. A third assay is specific for variola virus alone. In addition to the LRN real-time PCR methods, CDC provides confirmatory testing using single-gene PCR with confirmation by restriction fragment-length polymorphism, or RFLP. This confirmatory procedure has a specificity of 99%. PCR detection methods will be discussed in more detail in Module 5.
Virus culture for virus isolation can be an important and sensitive method for detection of orthopoxviruses. Orthopoxviruses grow in a number of commonly used cell lines, and infection will produce varying patterns of cytopathic effects. Growing the virus provides a good source of additional material for more extensive analysis such as molecular characterization. Virus culture is considered the gold standard for comparison with other methods. Note that in the current setting where smallpox does not exist naturally anywhere in the world, viral isolation from high risk specimens SHOULD NOT be attempted at laboratories outside of CDC, where appropriate containment facilities are available. Additional information regarding the utility of virus culture is covered in Module 5.

Although several serologic methods have been used for antibody detection, these have limited diagnostic utility because orthopoxviruses are closely related and there is significant cross-reactivity. Serologic methods may help to evaluate the extent of an immune response. Appropriate use of these tests is discussed in Module 5.

Negative-stain electron microscopic analysis, known as EM, of suspect orthopoxvirus specimens can be very helpful to rule in or rule out poxviruses. The EM process for poxvirus identification may be accomplished relatively quickly by a skilled observer and can often be used to differentiate poxviruses from other groups of viral agents. The sensitivity of electron microscopy for clinical samples, performed by a skilled observer, is 95% for variola and 75% for vaccinia. However, EM may not be as sensitive as real-time PCR and cannot differentiate among variola and vaccinia and several other poxviruses. EM can provide extremely valuable confirmation of a poxvirus that is completely independent of the parameters of testing and potential sources of false-positive test results for orthopoxvirus DNA by PCR. Electron microscopy is detailed in Module 6.

Histopathologic evaluation and immunohistochemistry are especially important for successful diagnosis of several of the syndromes that look like orthopox infections. These methods are further described in Module 6.
Slide 4 of 22:

All laboratory tests are subject to false-positive and false-negative results. When determining which tests to request on various patient samples, and in interpreting results, it is important to understand the test-performance parameters of sensitivity, specificity, and predictive value.

Sensitivity describes the ability of the test to identify all those individuals with the disease. A highly sensitive test has a low false-negative rate. Specificity is the ability to correctly identify all those without the disease; therefore, tests with high specificity have low false-positive rates.

Predictive value is dependent on the prevalence of disease in the population. The predictive-value negative is the proportion of true negatives among those testing negative. The predictive-value positive is the proportion of the true positives among those testing positive.

Slide 5 of 22:

Calculating sensitivity, specificity, and predictive value can be done with a simple table based on both test results and true presence or absence of disease.

The predictive value of a test is very important for the clinician to understand when interpreting test results.

The predictive value of a positive lab test for smallpox is extremely low when the prevalence of smallpox is zero. Laboratorians should be familiar with the criteria for the diagnostic algorithm used for evaluating rash-illness cases and the various laboratory tests needed, especially to confirm diagnoses for smallpox look-alike illnesses. Clinically compatible criteria and multiple laboratory tests with independent sources of potential error improve the predictive-value positive of a laboratory diagnosis of variola.

Slide 6 of 22:

Again, the predictive value of a test is very important for the clinician to understand when interpreting test results. Even when using tests that are highly sensitive and specific, the predictive-value positive of a laboratory test for smallpox is extremely low in the current setting, where the prevalence of smallpox is zero. It is therefore essential that these tests be used judiciously. The predictive value of any one test is increased when several different confirmatory methods are used. Clinicians and laboratorians need to understand the utility and limitations of the available tests. As we have said, clinically compatible criteria and multiple
laboratory tests with independent sources of potential error must be used to improve the predictive value positive of a laboratory diagnosis of variola.

**Slide 7 of 22:**

Were smallpox ever to reemerge, early, specific smallpox diagnosis would be essential in implementing public-health responses to the disease. As previously described, in the current environment where the prevalence of naturally occurring smallpox is non-existent, it is important to apply laboratory testing for smallpox virus only to cases that have a real suspicion of clinical smallpox. In the absence of naturally occurring disease, the risk of getting ‘false-positive’ laboratory results, which potentially might trigger a misguided public-health response and public panic, is statistically much greater than obtaining an accurate ‘true-positive’ laboratory result. For this reason, carefully evaluating cases of febrile vesicular or pustular-rash illness, in accordance with the clinical febrile vesicular-rash algorithm that was discussed in detail in Module 2 is crucial to increase the predictive value of smallpox laboratory test results. A protocol and questionnaire to evaluate a rash illness suspicious for smallpox is also available from CDC.

After reviewing the link, click the “Next” button to continue.

**Slide 8 of 22:**

Laboratory-testing guidelines have been developed by CDC to complement the clinical febrile vesicular-rash algorithm. These laboratory-testing protocols consist of three algorithms. The first algorithm provides testing guidelines based on clinical assessment for smallpox. The second outlines appropriate testing for patients with suspect vaccine adverse events or monkeypox. The last algorithm presents the appropriate testing recommendations for environmental samples, if such testing is indicated.

All of the laboratory algorithms can be found at this CDC site.

After reviewing the link, click the “Next” button to continue.

**Slide 9 of 22:**

These clinical and laboratory algorithms are designed to facilitate rapid and accurate diagnosis of a nefarious reintroduction of smallpox virus into the human population, and to provide a series of public-health measures to control any reintroduction. If smallpox were to become reestablished in the human population, these diagnostic clinical and laboratory testing criteria would be much less strict; that is, if smallpox were known to exist within a community,
laboratory testing would in all likelihood not be required to initiate vaccination and control measures.

Slide 10 of 22:

As discussed in Module 2, the ultimate goal of the clinical febrile vesicular-rash algorithm, hereafter known as the clinical-rash algorithm, is to provide early and specific diagnosis of possible smallpox disease and to rule out smallpox look-alike diseases. The clinical-rash algorithm helps triage cases with varying levels of concern for smallpox-like disease, coupling appropriate testing and public-health responses to each level of suspected disease. This significantly decreases the odds of a false-positive smallpox virus laboratory result, and assures that clinical and laboratory resources are used prudently. The clinical-rash algorithm also prompts health-care providers to consider alternative diagnoses, particularly chickenpox, thus providing a framework for appropriate diagnostic testing. An added value of this process has been diagnosis of several cases of smallpox look-alike disease that may have otherwise remained undiagnosed.

Slide 11 of 22:

This poster, “Evaluating Patients for Smallpox”, contains images of cases of varying stages of chickenpox and smallpox, highlights clinical features to help differentiate smallpox from chickenpox or other look-alike diseases, and finally presents a method for classifying cases according to risk of smallpox using major and minor smallpox criteria. This poster can be found at the CDC site indicated.

After reviewing the link, click the “Next” button to continue.

Slide 12 of 22:

This is an algorithm for laboratory testing of specimens from patients presenting with acute generalized vesicular or pustular-rash illness. The two arms of the testing algorithm at the top provide alternate testing recommendations based on the risk of smallpox. Testing for specimens from individuals with low- and moderate-risk smallpox-like symptoms is shown on the left, and testing for specimens from individuals with symptoms compatible with a high risk for smallpox is shown on the right.

This two-armed algorithm assists in rapidly determining appropriate testing and providing timely results. It also helps ensure that testing of high-risk specimens is confined to laboratories with appropriate biosafety levels and expertise.
Slide 13 of 22:

The LRN or Laboratory-Response Network includes public-health, military, and other governmental laboratories that are equipped to perform standardized assays for agents of bioterrorism and other public-health threats. Most LRN Reference laboratories can perform PCR assays to screen samples for all orthopoxviruses including variola, as well as assist in confirming vaccine adverse events. A subset of LRN laboratories can perform a variola-specific PCR assay.

More information on collecting vesicular lesion specimens for laboratory testing is discussed in Module 4.

Slide 14 of 22:

This laboratory algorithm can help you decide how most efficiently to test patient samples based on clinical rash evaluation.

If the patient is at low-to-moderate risk for smallpox, and the diagnosis of varicella zoster, commonly known as chickenpox, has not been ruled out, then the sentinel or reference LRN laboratories should test the samples for smallpox look-alike diseases.

Using biosafety level 2 conditions, conduct direct-fluorescence antibody and polymerase chain-reaction or PCR assays for varicella and other herpesviruses. If possible, perform electron microscopy and viral cultures.

If results of any of these assays are positive, no further testing is required. However, if results for all of these assays are negative, the LRN reference laboratories may proceed to testing for non-variola orthopoxviruses, including the vaccinia virus.
Clues to a potential non-v variola orthopoxvirus infection include recent vaccination, exposure to another person who was recently vaccinated, or other circumstances in which there could be high risk of vaccinia or another orthopoxvirus infection; this is discussed more thoroughly in Module 2. If the non-v variola orthopoxvirus PCR is positive, laboratories may also test using the Orthopoxvirus PCR for additional verification. Again, the LRN non-v variola orthopoxvirus real-time PCR will detect all Eurasian orthopoxviruses except for variola, while the orthopox real-time PCR detects all Eurasian orthopoxviruses including variola. If you see positive assay results for both of these assays, the patient may have either a vaccine adverse reaction or a monkeypox infection, and we advise you to contact the Centers for Disease Control and Prevention (CDC).

If the assays are negative, re-evaluate the patient’s condition, and consider dermatologic and histologic testing. Importantly, if symptoms progress to a point where risk of smallpox is high, immediately contact the state public health department and CDC, and sequester all viral cultures and specimens.

When a patient is suspected of having smallpox, take digital photos of clinical features, as these can be shared electronically with CDC and other experts. Digital photos are especially important in high-risk cases.

Also initiate specimen chain-of-custody documentation. DO NOT perform virus culture on high-risk specimens. Initial smallpox testing for patients with a high risk of clinical smallpox can be done by the nearest LRN variola laboratory or the CDC. Results must NOT be released without CDC confirmation. Electron microscopy can be conducted under biosafety level 3 conditions at a local facility. The LRN variola laboratories will conduct three unique real-time PCR assays for detection of variola virus, for all orthopoxviruses, and for non-variola orthopoxviruses. All other specimens should be sent to the CDC.
Slide 17 of 22:

If all orthopoxvirus assays are negative, further testing for the presence of herpesviruses and culture can be done.

If the variola-specific assay is negative but the other orthopoxvirus assays are positive, the patient most likely has an adverse reaction to the smallpox vaccine or is infected with another orthopox virus such as monkeypox.

Finally, positive results for the variola-specific assay and the orthopoxvirus assay, coupled with compatible clinical symptoms, are highly suggestive of smallpox. In that case, contact the CDC for consultation before releasing any results.

Slide 18 of 22:

This chart presents a testing algorithm to use when you suspect a smallpox vaccine adverse event, monkeypox virus, or other orthopoxvirus infection.

When a patient presents with symptoms consistent with a vaccinia adverse event, refer specimens, such as touch preps of vesicular fluid and roofs of lesions, to LRN-reference laboratories with biosafety level-3 capacity that can perform orthopox virus PCR-detection assays. Some LRN laboratories can also examine specimens by electron microscopy or can assist in locating facilities with EM capabilities.

Slide 19 of 22:

Based on results of the orthopox testing in the LRN lab, an adverse reaction can be confirmed when consistent with exposure history, and the specimen may then be referred to CDC for confirmatory testing. PCR assays provided to the LRN laboratories are also useful in assessing patients suspected to have monkeypox disease. In addition to test results, also consider travel history, exposure to exotic animals, or other appropriate epidemiologic links. Refer specimens to CDC for further monkeypox-specific confirmatory testing.

Slide 20 of 22:

If orthopox testing is negative, then re-evaluate the patient and perform additional testing for other rash illnesses. If the combination of orthopox assays indicates that smallpox may be present, your state health department and CDC should be contacted immediately and
arrangements made for variola testing. Remember to take digital photos of clinical features in all suspected cases, as these can be shared electronically with CDC and other experts.

**Slide 21 of 22:**

This chart presents an orthopoxvirus-testing algorithm for environmental or non-clinical samples. Environmental samples should not be processed by clinical laboratories. Testing of environmental samples should be performed only after consultation with law enforcement and public-health officials. After explosives, radiation, and toxins have been ruled out, send environmental samples to the LRN Reference Labs for PCR testing and, if available, Electron Microscopy.

If orthopoxviruses are ruled out, the need for further testing should be assessed in conjunction with law-enforcement agencies and public-health officials.

If testing results suggest a non-variola orthopoxvirus, please refer to CDC for confirmatory testing.

If test results indicate the sample may contain variola virus, refer immediately to CDC and to the nearest LRN Variola testing laboratory for confirmatory testing.

Details for performance and interpretation of each assay are specified in each LRN procedure.

**Slide 22 of 22:**

As noted previously in discussions about the application of clinical and laboratory algorithms for smallpox evaluation, testing environmental samples absent suspicion of clinical disease increases concerns about the predictive values of apparently positive laboratory results. In addition, exercise special care to ensure that a test inhibitor is not responsible for a negative orthopoxvirus DNA result from a poorly characterized environmental sample. Understand that quality assurance and control criteria for heterogeneous environmental samples can be complex and are not well standardized.
Slide 2 of 10:

In the second unit of module 3, we discuss biosafety.

Slide 3 of 10:

Laboratory personnel may unexpectedly encounter orthopox viruses such as vaccinia in specimens from vaccinees or their contacts, monkeypox in specimens from people traveling to endemic areas or exposed to infected animals, and other poxviruses from patients with unusual travel or animal exposures. Therefore, consideration must be given to understanding and implementing proper biosafety and biocontainment practices.

After reviewing the links, click the “Next” button to continue.

To create a safe laboratory environment, the first step is to develop an effective risk-management plan, identifying risks and devising containment barriers to control hazards. Risk is the probability that an adverse event will occur. There are no risk-free activities. The goal of a risk-management plan is to identify risk factors and minimize harm. Public perception of risk can be important in a high-profile activity such as smallpox testing. Factors that influence the public’s perception of risk include familiarity with the hazard, degree of control, and catastrophic potential.
Several orthopoxviruses cause human infections. These include vaccinia, the live smallpox vaccine; monkeypox; cowpox; and variola viruses.

Working with orthopoxviruses can span a biosafety spectrum from relatively harmless to serious, depending on the activity. Careful consideration to appropriate laboratory-safety methods is the responsibility of laboratory supervisors as well as to anyone directly involved in manipulation of virus-containing samples. Safety recommendations for handling pathogenic microbes such as vaccinia are covered in detail in the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (known as the BMBL) biohazard safety manual. The BMBL recommends that all persons who routinely and knowingly work with non-highly attenuated vaccinia should be vaccinated and should adhere to Bio-Safety Level 2 (or BSL-2) biocontainment practices to prevent unexpected accidental infections.

Excerpt from the BMBL (Agent Summary Statements Section VIII-E: Viral Agents):
“Biosafety Level 2 practices and facilities are recommended for all activities involving use or manipulation of poxviruses, other than variola, that pose an infection hazard to humans. All persons working in or entering laboratory or animal-care areas where activities with vaccinia, monkeypox, or cowpox viruses are being conducted should have documented evidence of satisfactory vaccination within the preceding ten years.

Activities with vaccinia, cowpox, or monkeypox viruses, in quantities or concentrations greater than those present in diagnostic cultures, may also be conducted at Biosafety Level 2 by immunized personnel, provided that all manipulations of viable materials are conducted in Class I or II biological safety cabinets. Immunosuppressed individuals are at greater risk of severe disease if infected with a poxvirus.”

In general, handling diagnostic specimens is considered less risk-prone than working with large quantities of virus, such as may be encountered in a research setting.

After reviewing the link, click the “Next” button to continue.
Slide 5 of 10:

Smallpox was successfully eradicated as a naturally occurring entity in the mid-1970’s. Unlike monkeypox, cowpox, or vaccinia, humans were the only known host for variola virus; there were no other natural hosts and no animal reservoirs. Vaccination with smallpox vaccine—the vaccinia virus—was key to the successful eradication of smallpox, and also provided significant protection from challenge with variola virus. Prior to the eradication of naturally occurring smallpox, variola virus was handled in what would now be considered BSL-2 facilities by vaccinated personnel. Today smallpox live-virus research must be conducted under BSL-4 containment restrictions and is permitted only at two repositories sanctioned by the World Health Organization. The primary reason for these requirements is to prevent unintentional release of virus back into the human population, more so than as a protective measure for those persons working with the virus who are well trained and vaccinated.

Slide 6 of 10:

Vaccinia is used not only as smallpox vaccine but also as a research tool of laboratory virologists and immunologists. Some strains of vaccinia, such as MVA, have been attenuated to the extent that they can no longer support multiple rounds of viral replication in vertebrate host cells. Such viruses pose a minimal level of risk of infection, even in unvaccinated, immune-impaired individuals. Other strains, including those used for vaccine formulations, can replicate to a limited extent in otherwise healthy individuals but typically, as during vaccination, do not cause disseminated disease. Very importantly, persons with impaired immunity or with a variety of other risk factors for disseminated vaccinia infections are prone to more serious disseminated vaccinia disease. More information about vaccinia contraindications is available on the CDC website. Like vaccinia, cowpox is typically associated with limited lesion counts and disease. The BMBL recommends that all persons who routinely and knowingly work with non-highly attenuated vaccinia should be vaccinated and should adhere to BSL-2 biocontainment practices to prevent unexpected accidental infections. Accidental infections in unvaccinated workers can manifest as more serious conditions such as ocular disease or disseminated vaccinia instead of a localized lesion of the deltoid region, normally associated with vaccination. Note that infectious accidents may go unrecognized, and hence the resulting disease may not initially be associated with vaccinia infection.

After reviewing the link, click the “Next” button to continue.
Laboratory Approach to the Diagnosis of Smallpox: Module 3 – Approach to Laboratory Diagnosis

Slide 7 of 10:

Although rarely encountered outside of central Africa, monkeypox virus causes disseminated orthopoxvirus disease that is similar in appearance to smallpox. However, monkeypox is a zoonotic disease, meaning one that may be transmitted from animal hosts to humans. The natural hosts for monkeypox are thought to be certain African rodents. While several generations of transmission of monkeypox between humans has been documented, the virus does not appear to be adapted to continued virus-infection cycles in humans. There are two recognized strains of monkeypox virus; the strain typically associated with coastal Western Africa appears to produce less severe human disease with a case fatality rate of <1%, compared with the monkeypox strains isolated from the Central Congo Basin, which can have case fatality rates as high as 15%. Vaccination with smallpox vaccine is thought to provide protection from serious monkeypox-virus infections. Smallpox vaccination is a requirement for working with monkeypox virus on an experimental research basis. Working with monkeypox virus is considered by the BMBL to be a BSL-2 activity for persons with a smallpox vaccination history within the last 10 years. However, given the potential mortality associated with at least one strain of monkeypox virus in non-immunized personnel, it may be prudent to confine research applications of high-titer monkeypox virus to a BSL-3 environment. As with smallpox virus, monkeypox virus is considered a “select agent.” Select agent classification imposes administrative and additional biosecurity responsibilities on laboratories that knowingly and routinely work with the virus. It also requires that clinical laboratories transfer or destroy agents once identification is confirmed.

Slide 8 of 10:

In October 2002, the Advisory Committee on Immunization Practices or ACIP, recommended that enhanced bioterrorism preparedness should include vaccination of Smallpox Public-Health Response and Health-Care Teams. These revised recommendations regarding vaccinia vaccine, published in the Morbidity and Mortality Weekly Report or MMWR, update the previous 1991 ACIP recommendations. They include current information regarding the nonemergency use of vaccinia vaccine among laboratory and health-care workers occupationally exposed to vaccinia virus, recombinant vaccinia viruses, and other orthopoxviruses that can infect humans. In addition, this report contains ACIP's supplemental recommendations for use of vaccinia vaccine if variola virus were used as an agent of biological terrorism or if a smallpox outbreak were to occur for another unforeseen reason.
Visit the CDC MMWR website for more information. After reviewing the links, click the “Next” button to continue.

**Slide 9 of 10:**

All of the orthopoxviruses that can cause human infections can be safely handled, especially as diagnostic specimens, with appropriate laboratory-safety equipment and practices by staff who have received appropriate training. The laboratory-safety protocols should include criteria for entry and restricting access, staff-training and certification requirements, and systems to encourage or ensure compliance with security requirements.

Process all potentially infectious specimens within a HEPA-filtered biosafety cabinet or BSC. Persons working with the specimens should operate using standards for handling pathogenic microbes including, but not limited to, wearing disposable gloves, gown or lab coat, and wearing eye protection. If aerosol transmission is likely, add respiratory protection such as an N-95 mask or greater or a PAPR. Follow BMBL guidelines for handling and decontaminating lab equipment, including hood space. Vaccinating laboratory staff provides significant additional protection.

**Slide 10 of 10:**

Preparing tissue or other substances for laboratory DNA analysis would naturally begin within a biosafety cabinet. After treatment with heat and/or ionic detergents such as SDS, poxviruses can be considered essentially non-infectious. Once specimen is rendered non-infectious, further steps for preparing DNA assays can be performed outside of the BSC environment.
The module that you have just finished is an introduction to the laboratory methods for detecting and identifying orthopoxviruses and the appropriate biosafety practices for working with clinical specimens and viruses. In the event of reintroduction of smallpox virus into the human population, it would be critical to rapidly diagnose and respond to the first cases of smallpox and to invoke measures to limit further disease. In the meantime, we need to develop and encourage the use of methods for robust clinical evaluation and laboratory testing of specimens from patients with smallpox-like diseases that utilize resources responsibly and minimize false alarms, which could lead to public anxiety and temporary disruption of health care systems. In addition, the use of tools such as the febrile-rash algorithm and laboratory algorithm for human orthopoxviruses enhances diagnosis of diseases that may initially be confused with smallpox. More detailed discussion of each of the laboratory tests is provided in Modules 5 and 6.