Chapter 5. Virus isolation and identification of measles and rubella in cell culture

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Chapter 5 overview

Although rarely used for diagnosis of measles or rubella due to the lengthy procedure for virus isolation, the ability to successfully produce a viable virus stock from clinical specimens collected from cases of measles or rubella is important for additional testing, including sequence analysis. Occasionally, the viral RNA extracted directly from the clinical specimen provides a positive signal by RT-PCR that is useful for case confirmation (chapter 6) but subsequent efforts to utilize the directly extracted RNA to sequence the region necessary for identification of the genotype prove to be unsuccessful. In addition to providing a source of viral RNA for genotyping, studies such as virus neutralization assays could not be undertaken without the availability of live virus.

The ability to recover virus from clinical specimens and maintain viable virus stocks is also important since a virus sample should be submitted to the measles or rubella virus strain bank if a novel genotype is identified. Also, larger quantities of viral RNA may be required for molecular epidemiologic investigations of outbreaks, particularly those that involve additional gene targets or extended sequencing.

The successful isolation of live virus from clinical specimens depends on the timing and type of specimens as well as other variables that have little or no impact on the ability to detect RNA. For example, a well-timed specimen that was positive by RT-PCR may be unsuitable for virus

growth due to bacterial contamination or the presence of substances that are toxic to the cell culture. The optimal timing of collection for the different types of samples for virus isolation is described in detail in chapter 3. In general, clinical specimens that are appropriate for RT-PCR testing for measles and rubella can serve as good sources of virus. Oropharyngeal or nasopharyngeal swabs are recommended for both purposes, and the availability of a urine sample has proven useful as a back-up sample. However, both measles and rubella viruses are difficult to culture from oral fluid (OF). Other types of clinical specimens (*e.g.*, throat swabs) that provide a reasonably good opportunity for successful virus isolation should be collected in addition to OF from representative cases in a chain of transmission.

Note: *The abbreviation, RT-PCR, may be used herein to refer to both conventional (end-point) reverse transcription PCR and to real-time (kinetic) RT-PCR, when describing characteristics of RT-PCR in general. Because real-time RT-PCR can be used for quantification of RNA in clinical specimens, real-time RT-PCR is sometimes referred to as quantitative real-time RT-PCR and abbreviated as RT-qPCR. However, in the context of the diagnostic use of RNA detection from measles and rubella samples specifically employing the use of real-time RT-PCR protocols, the term real-time RT-PCR will be used.*

5.1 Recommended cell line for measles and rubella virus isolation

With the integration of surveillance for measles and rubella, the ideal cell line will be permissive for robust growth of both viruses and is safe and relatively easy to work with. The Vero/hSLAM cell line has been adopted for use in most laboratories in the GMRLN [1]. These cells were developed by transfecting the Vero cell line with a plasmid that encodes the gene for the human signalling lymphocyte-activation molecule (hSLAM) [2].

Previously, many of the network laboratories had used the B95a cell line and measles virus stocks made in the 1990s were often obtained by culturing measles virus on the B95a cells. While highly sensitive for measles virus, the B95a cell line is persistently infected with Epstein-Barr virus and must be handled as infectious material. After the Vero/hSLAM cell line was

demonstrated to have an equivalent sensitivity for measles virus as that of the B95a cell line, the transition to Vero/hSLAM was recommended as the preferred cell line for the GMRLN.

Although hSLAM was identified as a cellular receptor for measles virus, the Vero/hSLAM cell line is also sensitive for rubella viruses. However, as with other cell lines used for rubella virus isolation, the cytopathic effect (CPE) is rarely apparent and the presence of rubella virus must be confirmed by RT-PCR or other viral detection methods. A disadvantage of the Vero/hSLAM cell line is the cost of the culture medium. In order to retain the expression of SLAM, Geneticin® must be added to the culture medium. However, once the cell stocks are prepared in the presence of Geneticin®, SLAM expression will be retained for at least 15 subsequent passages in culture medium prepared without addition of Geneticin®.

Note: *Cell stocks of the Vero/hSLAM cell line, developed by Dr.Yusuke Yanagi and colleagues at Kyushu University, Japan, have been provided to the GMRLN laboratories by the National Institute of Infectious Diseases, Tokyo, Japan, and are available in regional cell repositories*. Vero/hSLAM cells can be obtained on request (free of charge) through WHO.*

By acceptance of the Vero/hSLAM cell line, the laboratory agrees to the following provisions:

- The cell line Vero/hSLAM is used only for laboratory diagnosis of measles and rubella viral infection by virus isolation and/or investigation of measles or rubella strains for molecular epidemiological purposes
- The cell line is not used for commercial purposes
- The cell line is not distributed to laboratories outside the WHO Laboratory Network without Dr Yanagi's and WHO's permission
- Any publication of work using the Vero/hSLAM cell line acknowledges the original publication (Ono et al. J. Virol. 2001, 75:4399-4401)

*For those countries that are bound by the restrictions outlined by the CITES treaty, the transportation of products derived from endangered species including the African green monkey (Vero cells) are not allowed. Efforts are underway to transfect other types of cell lines that are sensitive for rubella growth (RK13, BHK21, JEG3) with hSLAM that would not be affected by the CITES restrictions.

5.2 Propagation of Vero-hSLAM cells

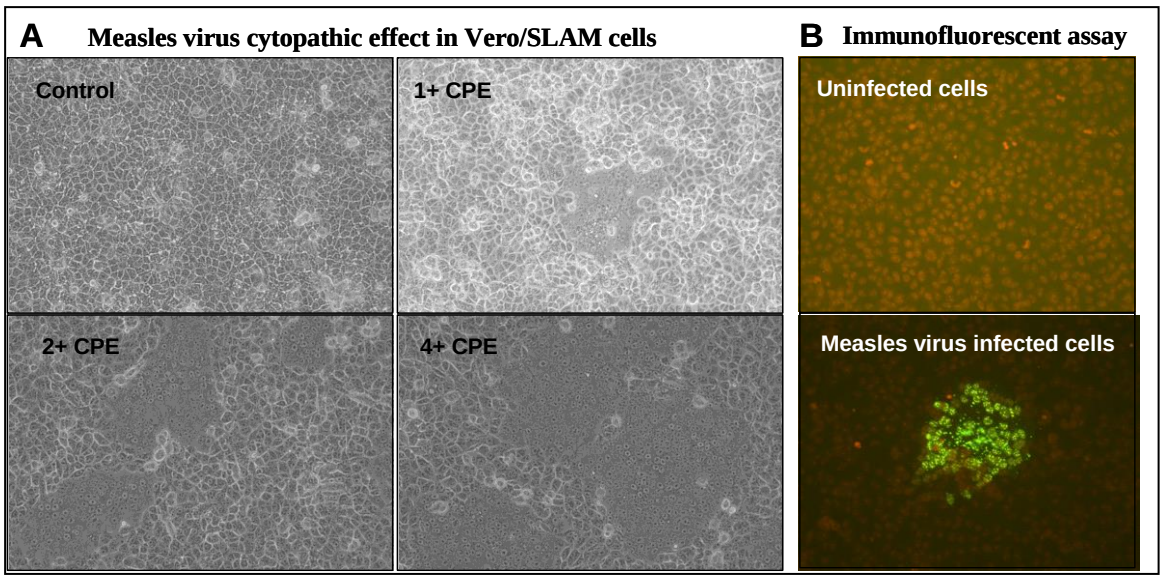
The successful propagation of any cell line requires adherence to established protocols. Preparation of frozen cell stocks and the successful recovery and expansion of the cells for virus isolation are critical techniques to acquire. Training for laboratories in the GMRLN is available and all laboratories should have SOPs in place for all steps in cell culture propagation and processing for virus isolation. Annex 5.1 includes protocols and guidelines that can be used to develop the laboratory SOPs for propagation, maintenance and preparation of frozen cell stocks of Vero/hSLAM cell culture.

Network laboratories should only accept Vero/hSLAM cells from a WHO-approved source (RRL or GSL). These RRL or GSL laboratories follow the protocols for the propagation of the Vero/hSLAM cell culture that maintains and verifies the expression of SLAM. In addition, the laboratories that provide the cell line will ensure that the cell lines are susceptible to infection with wild-type measles viruses and are free of mycoplasma contamination. Upon receipt, the cells should be passaged in cell culture medium containing 400 µg/ml Geneticin®. The cells are passaged forward (usually 2 times), increasing the number of flasks to provide a sufficient quantity of cells to prepare 20-50 vials of cell stock for liquid nitrogen storage (refer to annex 5.1).

5.3 Measles virus isolation and confirmation

The procedure for inoculation of clinical samples for measles virus isolation on Vero/hSLAM cells is provided in Annex 5.2. The morphological changes to the Vero/hSLAM cells, or the cytopathic effect (CPE), produced by measles virus replication in cell culture is easily observed. The typical CPE consists of the formation of syncytia, which appear as large multinucleated cells (giant cells), caused by fusion of infected cells (plaques). See Figure 5, below. These plaques can be seen by the naked eye in the monolayer. Light refracts differently through the lysed cells in the plaques compared to the uninfected cells in the monolayer which allows the visualization of the plaques.

Figure 5. Cytopathic effect of measles virus growth in Vero/hSLAM cells. Top left (Panel A) shows uninfected Vero/hSLAM cells; other views in Panel A show development of CPE (1+ to 4+ syncytium formation) after infection with wild-type measles virus. Top right (Panel B) shows uninfected cells (control); lower view shows immunofluorescence stained measles-infected syncytium.



Under the microscope, individual syncytia may consist of 50 or more nuclei encapsulated within a single cytoplasmic membrane. Verification of the isolation of measles virus should be carried out by immunofluorescence, immunohistochemistry or RT-PCR (chapter 6). Because the virus titre is often very high after passaging the measles virus-infected cells in cell culture, the manipulation of the cell lysate to confirm measles virus isolation should be conducted in a manner to avoid contamination of clinical specimens or other cell cultures.

5.4 Rubella virus isolation and confirmation

The optimal timing for collection and the types of clinical samples recommended for rubella virus isolation for postnatal rubella and for congenital rubella syndrome (CRS) are provided in chapter 3. The procedure for the inoculation of cell culture with clinical specimens from suspected cases of rubella is provided in Annex 5.2.

In additional to rubella virus detection by RT-PCR (chapter 6), a method for the detection of rubella E1 glycoprotein in infected cells using monoclonal antibodies in either an

immunofluorescent or an immunocolorimetric assay (ICA) has been developed by CDC, Atlanta [3,4]. These methods detect viral antigen in monolayers of Vero/hSLAM (or Vero cells) that have been infected with rubella virus. The optimal temperature for rubella virus growth in cell culture is 35°C, although incubators set at 35°C -37°C can be utilized for rubella virus cultures. Details of the ICA procedure and the procedure for an IFA for rubella virus detection are available in Annex 5.4.

5.5 Provision of virus isolates for molecular surveillance and the strain bank

Clinical specimens from suspected cases of measles and rubella for virologic surveillance may be submitted to any network laboratory. The specimens should be kept cold after the package is opened and throughout the accessioning process. Upon arrival, the specimens should be processed promptly and then stored appropriately according to the type of virologic specimen (refer to chapter 3). Laboratories that do not conduct virus isolation or molecular testing should forward the original clinical specimen in accordance with the recommended method of transport to the appropriate network laboratory to perform the necessary testing.

Laboratories with the facilities and trained staff to perform virus isolation should attempt to provide a representative virus isolate from outbreaks, or from each chain of transmission in elimination settings. If real-time RT-PCR is conducted in the receiving laboratory, an aliquot of the original clinical material is normally retained for an attempt to isolate virus. Under some circumstances, such as outbreaks that continue over several months or if cases are detected outside of the original population affected in the outbreak, collection of additional clinical specimens for genotyping and virus isolation may be required for molecular surveillance purposes (refer to Chapter 7).

The procedures and facilities for virus isolation are included in reviews of laboratories in the GMRLN (WHO accreditation). Laboratories in the GMRLN should either maintain frozen stocks of representative virus strains that have been successfully grown in cell culture or, if facilities are not available for long-term storage at -70°C, an aliquot of the frozen virus stock should be shipped to one of the strain banks or to the appropriate RRL. The two strain banks are located at

the CDC in Atlanta and the PHE in London. When sequences are submitted to the MeaNS and RubeNS databases, the submitter should indicate whether a virus isolate corresponding to the submitted sequence is available. This 'virtual' virus strain bank has become the most efficient way for the network laboratories to assure that virus isolates from a range of strains are available for studies, assay development and for external quality assessment.

5.6 Methods to ship virus isolates

When a viable virus culture must be shipped, the available options will depend on national and international regulations. For intra-country shipments between laboratories, a live culture (in a cell culture tube or flask) shipped at room temperature may be an acceptable option. The alternative is to ship an aliquot of frozen virus stock (lysate). Shipment of the frozen lysate requires less processing by the sender and the receiver, but dry ice must be available, and the cost of a dry ice shipment may be prohibitive. Refer to Annex 5.2 for detailed instructions on the preparation of virus stocks.

A flask of Vero/hSLAM cells must be precisely timed to be ready when the shipment is arranged, and advance notification and coordination with the recipient is critical. An aliquot of the virus isolate is used to infect Vero/hSLAM cells that have been seeded into a 25cm^2 tissue culture flask. The cells should be infected when the cell monolayer is 50-75% confluent (and incubated for the full inoculation period) just prior to shipment. Refer to Annex 5.2 for detailed protocols for inoculation and propagation of cell culture.

Prior to shipment, MEM or DMEM (plus antibiotics and 2% FBS) is added to the flask of infected Vero/hSLAM cells. The flask should be filled with the medium, leaving some space at the neck of the flask (~0.5ml) to allow for liquid expansion during shipping. The top of the flask should be screwed on tightly and sealed with parafilm (or other similar product, but not sticky tape). Sufficient absorbent material should be wrapped around the flask to absorb the entire liquid contents of the flask, should the flask become damaged. The flask and absorbent material is placed in a leak-proof container such as a zip-lock plastic bag. The sealed bag or container is then placed inside a leak-proof outer container that meets the United Nations Packing Instruction 620 standard for packaging and documentation and the package is shipped at room temperature.

An overview of the requirements of the packaging and shipping containers for Category A infectious substances is provided in chapter 3, section 3.1, Guidelines for the preparation and transport of clinical specimens. However, because shipping regulations and labelling instructions may be subject to modification, the publication prepared by WHO (*Guidance on Regulations for the Transport of Infectious Substances* [5], or most current update, should be consulted.

If a viable virus culture is not required, viral lysates or viral RNA extract can be applied to FTA® Cards, which can then be transferred to another laboratory. Chemicals impregnated in the paper card inactivate virus while preserving DNA and RNA in the fibre matrix of the card. Because the cards inactivate the virus, the cards may be shipped without the packaging and documentation required for an infectious substance. The nucleic acids extracted from the card can be utilized as a template for RT-PCR. Virus culture lysates or purified RNA from clinical specimens or from cell culture may be applied to the cards. The protocol for preparation of these samples is available in Annex 5.3; the protocol for RNA extraction is provided in Annex 5.4.

Bibliography to Chapter 5

1. Featherstone DA et al. Expansion of the global measles and rubella laboratory network 2005-09. Journal of Infectious Diseases, 2011, 204(Suppl 1):S491-S498.

2. Ono N et al. Measles viruses on throat swabs from measles patients use signalling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. Journal of Virology, 2001, 75:4399-4401.

3. Chen MH et al. An indirect immunocolorimetric assay to detect rubella virus infected cells. Journal of Virologic Methods, 2007, 146:414-418.

4. Zhu Z et al. Comparison of four methods using throat swabs to confirm rubella virus infection. Journal of Clinical Microbiology, 2007, 45:2847-2852.

5. World Health Organization. Guidance on regulations for the Transport of Infectious Substances 2017-2018. 2017. http://apps.who.int/iris/bitstream/10665/254788/1/WHO-WHE-CPI-2017.8-eng.pdf?ua=1.pdf