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Diagnostic Methods of Viral Exanthemas in Children

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1. Introduction

To diagnose a causing agent of viral infections in clinical practice we can use a number of laboratory diagnostic methods and procedures. With different pathogens we use different methods. What is important is a quality of collected biological samples, time of delivery of samples to the laboratory (one hour is the best), appropriate transport in cooling box and proper medium for virological examination. Body sites and collection methods vary according to the type of infection and viral etiology. We have to take into consideration a stage of infection, incubation period, beginning of clinical appearance and dynamic of immune response. Storage temperature of samples depends on type of biological material and used medium and of course time you need to store the sample. Each type of biological sample, such as nasopharyngeal swab tampons, cerebrospinal fluid, content of skin blisters, skin biopsies, scabs, urine samples, stool samples and blood fractions such as leukocytes, plasma or sera, requires specific way of storage and transport. We introduce a review of the most commonly used diagnostic techniques in viral infections:

1. Growth and isolation of the virus in a cell culture from a specimen taken from the patient. Most commonly used are fertilized hen's egg or laboratory animals.
2. Detection of virus-specific antibodies in the blood, IgG, IgM, IgA etc. by serological testing like ELISA, hemagglutination, complement fixation, blotting, EIA and fluorescent antibodies. Levels of immunoglobulins can show us the kinetic of infection.
3. Viral antigen detection by ELISA in tissues and fluids, or by direct or indirect immunofluorescence, or immunoperoxidase.
4. Detection of virus encoded DNA and RNA (after Rt-RNA procedure) done by polymerase chain reaction (PCR)
5. Histological examination of biopsies taken from infected tissues, or lesion typical for viral infection. Looking for viral inclusion bodies, collections of replicating virus particles either in the nucleus or cytoplasm.
6. Visualization of viruses by electron microscopy or immune electron microscopy.

2. Isolation of virus from tissue cultures

Viral disease diagnosis has relied on the isolation of viral pathogens in cell cultures. This method is often slow and requires considerable technical expertise and equipment. Cell

cultures are more convenient and less expensive than eggs and lab animals. Cell cultures are suitable to be examined microscopically for evidence of viral proliferation, and, they have provided a desirable environment for the detection and identification of many human viral pathogens. Viruses reach high titres when grown within susceptible cells, and culture tubes are convenient to manipulate. Cell cultures in cell monolayer can be prepared in a variety of containers, the 16- by 125-mm glass or plastic round-bottom screw-cap tube is standard.

Clinical samples collected with a polyester swab from body sites such as skin and the genital tract, are usually contaminated with microbial flora, have to be placed in viral transport medium, which contain antibiotics, a buffered salt solution, a proteinaceous substance (such as albumin, gelatin, or serum), and a pH indicator. Stool samples or other highly contaminated samples have to be suspended and filtered through 0,45 µm membrane filters, before use. Respiratory tract samples include sputum, bronchial alveolar lavage specimens, nasopharyngeal washes, aspirates and swabs and oropharyngeal swabs have to be placed in viral transport medium. Specimens which are expected to be free of microbial contamination are collected in sterile containers and are not placed in transport medium. Preservation of the viral titre and viral infectivity until cell cultures can be inoculated is essential. Keeping the samples at 2 to 8°C or on wet ice until cell culture inoculation helps preserve viral infectivity and increases the virus recovery rate (Leland, 2007).

The transport medium tube is vortexed, the swab is discarded, the liquid medium is centrifuged, and the supernatant fluid is used to inoculate the cell cultures. Cells are cultivated in defined culture media with addition of antibiotics in sterile conditions and all handling must be done in laminar flow cabinate (boxes with laminar flow of sterile air). Usually after 24 – 48 hours of incubation at 37°C cell exhibit first cytopathic changes. These changes are best seen under the inverted microscope.

Various cell cultures are suitable for cultivation and identification of different viruses. Hundreds of cell lineages are available in international cell culture catalogues, most known is ATCC (American Type Culture Collection). Examples of well-known cell types are primary rhesus monkey kidney (RhMK) cells, primary rabbit kidney cells, human lung fibroblasts (MRC-5), human foreskin fibroblasts, human epidermoid carcinoma cells (HEp-2), human lung carcinoma cells (A549), and others (Leland, 2007).

Degenerative changes in monolayer cells provide evidence of viral presence. Viruses are quantified in suspension of infected cells, and are classified by number of viral plaques. The plaque is identified as a focus of cytopathic changes (swelling, shrinking, and rounding of cells to clustering, syncytium formation, and, in some cases, complete destruction of the monolayer), around the one infected cell.

3. Animal tissue cultures

In addition to use of cell cultures in virology, it's possible to use tissue cultures for virus isolation. Fertile hen's eggs and laboratory animals like newborn mice are very useful for the isolation of certain viruses. Brain cells of newborn mouse are convenient for replication of Coxsackie virus, for which cell cultures are not suitable. Methods for identification of replicating viruses in living organisms are same like in cell cultures, hemagglutination or immunofluorescence using print technique. Immunofluorescence can be used on tissue

sections, cultured cell lines, or individual cells and is only limited to fixed (i.e., dead) cells. Direct or indirect immunofluorescence can be used for the detection of virus antigen, whereas indirect immunofluorescence is virtually always used for the detection of antibody. Cells from the culture are immobilized onto glass slide. Specific monoclonal or polyclonal sera raised against the viral antigen can be used. Monoclonal sera offer the advantage of increased sensitivity and specificity. Samples have to be blocked with specific sera, than primary and potentially secondary antibody is added and detected with fluorescent microscope. Isolation and identification of unknown virus is very difficult. For ordinary isolation our material is sent to laboratory with specific request, for example to isolate enterovirus, etc. (Rajčáni ,Čiampor, 2006).

4. Polymerase Chain Reaction (PCR)

Detection of virus encoded DNA and RNA (after Rt-RNA procedure) is done with polymerase chain reaction. The polymerase chain reaction (PCR) is a scientific technique used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Almost all PCR applications use a heat-stable DNA polymerase. This DNA polymerase enzymatically assembles a new DNA strand from nucleotides, by using single-stranded DNA as a template and DNA primers, which are required for initiation of DNA synthesis.

PCR method uses thermal cycling, heating and cooling of the PCR sample to a defined series of temperature steps, necessary first to denature (physically separate) two strands in a DNA double helix at a high temperature 94°C.

Next step is the hybridization, when at a lower temperature of 50°C two complementary primers are attached to the 3´ends of separated strands of the target segment of DNA. PCR primers are usually short, chemically synthesized oligonucleotides, with a length not more than 30 (usually 18–24) nucleotides. They need to match the beginning and the end of the DNA fragment to be amplified and are then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA.

Third step is a proper synthesis of the new complementary DNA strand. As a starting point for DNA synthesis are used PCR primers, because DNA polymerases, can only add new nucleotides to an existing strand of DNA.

Another type of nucleic acid detection is nucleic acid hybridization with virus-specific probes detecting specific viruses. Molecular techniques such as dot-blot, Western blot and Southern-blot depend on the use of specific DNA/RNA probes for hybridization. The specificity of the reaction depends on the conditions used for hybridization. A mixture containing the molecule to be detected is applied directly on a membrane as a dot. This is then followed by detection by either nucleotide probes. Dot blots can only confirm the presence or absence of a viral nucleic acid which can be detected by the DNA probes.

5. Serology

When the immune system of a patient encounters a virus, it produces specific antibodies which bind to the virus and mark it for destruction. The presence of these antibodies is often

used to determine whether a person has been exposed to a given virus in the past. Serology is the detection of rising titres of antibodies between an acute and convalescent stage of infection, or the detection of IgM in primary infection. There are several serology techniques that can be used depending on the antibodies being studied. These include: ELISA, agglutination, precipitation, complement-fixation, fluorescent antibodies or Western blot.

In serologic diagnostic, concrete virus acts as a single antigen, although it represents wide antigen mosaic.

5.1 Agglutination reaction and virus neutralization reaction

Agglutination reaction and virus neutralization reaction measure effect of antibodies on infectivity of the virus, in determinate sera concentration (Rajčáni & Čiampor, 2006).

5.2 Agglutination test

When bacteria, antigen-coated particles, or cells in suspension are mixed with antibody directed to their surface determinants, reaction leads to agglutination of cells or particles carrying the antigen. All antibodies can theoretically agglutinate particulate antigens but IgM, due to its high valence, is particularly good.

Agglutination tests can be used in qualitative or quantitative manner, for presence of antibody or to measure the level of antibodies to particulate antigens.

Serial dilutions of a sample are made to be tested for antibody and then a fixed number of red blood cells or bacteria or other antigen is added. The maximum dilution that gives visible agglutination is called the titer.

When the antigen is an erythrocyte the term hemagglutination is used.

Hemagglutination tests can be subclassified depending on whether they detect antibodies against red cell determinants (direct and indirect hemagglutination) or against compounds artificially coupled to red cells (passive hemagglutination).

- Direct hemagglutination – red cells are agglutinated using IgM antibodies recognizing epitopes
- **Paul-Bunnell test**: diagnosis of infectious mononucleosis, detects circulating cross-reactive antibodies that combine with antigens of an animal of a different species induce the agglutination of sheep or horse erythrocytes
- Indirect hemagglutination – detects IgG antibodies that react with antigens present in the erythrocytes but which by them cannot induce agglutination. A second antibody directed to human immunoglobulins is used to induce agglutination.
- **Coombs test**: used to test for autoimmune hemolytic anemia.
- Passive hemagglutination - it is possible to coat erythrocytes with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten) and use the coated red blood cells in an agglutination test for antibody to the soluble antigen

5.3 Complement fixation reaction

Complement fixation reaction can be used to detect the presence of either specific antibody or specific antigen in a patient's serum. The complement system is a system of serum

proteins that react with antigen-antibody complexes resulting in the formation of membrane pores and therefore destruction of the cell. If the patient's serum contains antibodies against the concrete virus, they will bind to the antigen to form antigen-antibody complexes. The complement proteins will react with these complexes and be depleted. When commonly used sheep red blood cells with their own antibody complexes are added, there will be no complement left in the serum. If no antibodies against the concrete virus are present, the complement will not be depleted and it will react with the sheep red blood cells antibody complexes, lysing the red blood.

5.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) serves for detection and quantification of specific antibodies in sera. A liquid sample is added onto a solid phase with special binding properties and is followed by adding antibodies and other reagents, incubated and washed. The result is a color development by the product of an enzymatic reaction. The quantity of the analyte is measured by quantitative detection of intensity of transmitted light by spectrophotometric method.

A wide variety of assay principles can be used in ELISA techniques.

In competitive methods one component of the immune reaction is insolubilized and the other one labelled with an enzyme. The anti viral antibodies can then be quantified by their ability to prevent the formation of the complex between the insolublized and the labelled reagent.

Sandwich method is the method in which antigen is used in an insolubilized form to bind the viral antibody, which is subsequently determined by addition of labelled second antibody against the same class of antibody as the analyte antibody.

5.5 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) instead of measuring color changes, radioactive substances is used to visualize viral antigens. RIA technique is very sensitive and specific, but it requires specialized equipment and special precautions and licensing, since radioactive substances are used.

For serologic examination two blood samples (5-10 ml) are needed to confirm existence of specific antiviral antibodies. First sample must be taken in the shortest possible time after clinical manifestation of viral infection, the latest 5th -6th day. In this sample, levels of antibodies are low, but specific IgM antibodies are present. Second sample should be taken after appropriate interval depending on the type of infection. In general the most convenient is convalescence time, around 21 days after clinical manifestation (Rajčáni & Čiampor, 2006), on the other hand according to Sterling (2004) the best time for second blood sample is 10 – 14 days after first blood sample examination, when first antiviral antibodies start to be detectable.

The best way is to examine both samples at once to compare titres of antibodies. It is valuable also individual serological testing, in cases of high antiviral antibody titres.

Very important is to interpret the results in right way, by specialist. In case of immunodeficient or immunosupressed patients results are not valuable (Sterling, 2004).

5.6 Fluorescent antibodies or immunofluorescence

Fluorescent antibodies or immunofluorescence is used primarily on biological samples. Technique uses the specificity of antibodies to their antigen to target fluorescent dyes within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells. In virus diagnosis, we can use specific antibodies to concrete viral capsid proteins or to their nucleic acid specific sequences.

Primary, or direct, immunofluorescence uses a single antibody that is chemically linked to a fluorophore. The antibody recognizes the target molecule and binds to it, and the fluorophore it carries can be detected via microscopy.

Secondary, or indirect, immunofluorescence uses two antibodies; the unlabeled primary antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it. Multiple secondary antibodies can bind a single primary antibody. This provides signal amplification by increasing the number of fluorophore molecules per antigen.

6. Diagnosis of hand, foot and mouth disease

Hand, foot and mouth disease is a human syndrome caused by intestinal viruses of the Picornaviridae family. The most common strains causing hand, foot and mouth disease (HFMD) are Coxsackie A virus, and Enterovirus 71.

Biological material from infected individuals must be cultivated in tissue cultures, same as used for common virus cultivation. Samples are inoculated mainly on *Macacus rhesus* or *cynomolgus* kidney tissue, or on human embryonic kidney cells, or WI 38 cells (Chonmaitree et al., 1981). Cytopathic effect on cell cultures is monitored and identified using direct immunofluorescence, PCR, or RT-PCR (Ooi et al., 2003).

Using mentioned methods it is possible to identify viral genome directly in samples from infected tissues, or in biologic samples from infected persons. Biopsies from infected patient can be examined also immunohistochemicaly to prove viral antigens, or with direct and indirect immunofluorescence.

Molecular biology methods do not serve only for verification of the virus in the samples, but also for more accurate genotypization of viruses that cause infection (Shimizu et al., 2004).

Except these diagnostic methods to identify virus causing HFMD, serologic screening can be used in clinical practice. From serologic methods, mainly ELISA is used to prove existence of virus neutralization antibodies class IgG, IgA, IgM, and anti-gangliozide antibodies GM1, GA1, GDa, GDb and GQ1b. Other standard biochemical methods can be used too.

7. Diagnosis of parvovirus B l9 infection

Diagnosis of Parvovirus B 19 infection in immunocompetent patients is primarily confirmed by detection of anti-viral antibodies. Other assays based on antigen detection have been developed, including a receptor mediated hemagglutination assay (RHA), based on interaction of Parvovirus B l9 and P antigen on human erythrocytes. Because human parvovirus B19 is unable to replicate in culture systems, viral antigens were initially

obtained from acutely infected patients. Recently, human Parvovirus B 19 antigens have been expressed in different systems. However, bacterial systems for the viral antigens undergo denaturation. This phenomenon may be responsible for false negative results due to the absence of conformational epitopes. According to several authors these conformational epitopes are essential for accurate serologic diagnosis (Manaresi, 2001). In l982 Anderson et al., developed a radioimmunoassay (RIA) for detection of specific anti - Parvovirus B 19 IgM antibodies. Later, an enzyme - linked immunosorbent assay (ELISA) was developed for the detection of specific anti-Parvovirus IgM antibodies (Anderson, 1986). This assay used virus obtained from viremic patients as an antigen source.

Specific IgM antibodies are detectable in more than 90% of cases at the end of the first week of illness and the titer and positivity rate decreased after 1 month. The sensitivity and specificity of ELISA was confirmed by Schwarz et al. (Schwarz 1988) who found that anti - Parvovirus B19 IgM could be detected for up to 20 weeks post-viremia and non-specific reactions with rheumatoid factor or Rubella were not found. Using immunoblotting, some authors also observed that the immune response after acute infection was directed initially against virus protein 2 (VP2) and secondly against virus protein (VP1) (Schwarz, 1988). IgM response against VP1 linear epitopes is more prevalent and persistent than the one directed against the VP2 linear antigen (Palmer, 1996). When the IgM response declines, an IgG immune-response against structural proteins VP1 and VP2 becomes prominent and can be detected by IFA, Western blot and ELISA. Similarly to the IgM response, specific IgG antibodies are produced against both denatured and undenatured VP1 and VP2 epitopes.

Parvovirus B 19 infections are responsible for variety of disorders in humans. The clinical presentation is different accordingly to the period of life at which the infection or reactivation of the virus occurs. The virus has been extensively studied as causative agent of autoimmune diseases (Peterlana, 2006).

PCR and real time-PCR improve the sensitivity of detection of viral infection and many clinical laboratories complement serologic diagnosis with PCR. However, this molecular procedure may be necessary only in particular clinical setting because anti-Parvovirus B 19 VP2 IgM has been reported to correlate with Parvovirus B 19 DNA level (Beersma, 2005). Therefore the use of PCR procedure does not add any further information to a positive serologic test.
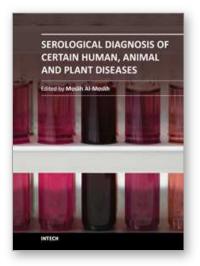
8. Detection of concomitant virus infections

New opinions in infections, the vaccinations and associated changes of viral antigenic qualities, influence clinical symptomatology. These changes in viral factors, including virulence and tropism are possible. Co-infection with a second virus has been suggested and this theory is supported by concomitant isolation of subgenus B adenovirus with an enterovirus from three persons who died during hand, foot and mouth disease (HMFD) outbreak in Sarawak (Cardosa, 1999). Among the Singaporean patients with HEV71 infection, three had second virus isolated concurrently. However, the presence of dual viruses did not result in severe disease, although a child with HEV71 (Human enterovirus 71) and CAV16 (Coxsackie A 16) co-infection died in Singapore in 1997. An epidemic outbreak of HFMD occurred in Singapore between September and November 2000. During the epidemic, there were four HFMD-related deaths and after the epidemic, another three HFMD-related deaths. Enterovirus 71 positive fatal (n = 4) cases and non fatal controls (n = 63) were also compared. Of the 131 non fatal cases three had concomitant infections

with virus bronchiolitis, right-side pneumonia (respiratory syncytial virus bronchiolitis, right-sided pneumonia, Haemophilus influenza type B meningitis), 2 had aseptic meningitis and l had transient drowsiness (Chong, 2003). Since June 2006 till September 2009 in Europe -in eastern region of Slovakia 295 children have been examined because of unknown exanthemas. According to complete history, physical examination, and specific viral and serological examinations, viral exanthemas were detected. Parvovirus B 19 infections were positive in 45 children, Coxsackie B3, B4, B6 serotypes of hand, foot and mouth /(HFMD) present in 25 persons. Concomitant dual infection occurred in 20 children. *Mycoplasma pneumonia* and Parvovirus B19 were the most frequent. Authors suggested that differences in clinical features of exanthemas, duration of diseases and severe diseases were a result of co-infections (Martinaskova, 2010).

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This book explains the concept of serological methods used in laboratory diagnoses of certain bacteria, mycoplasmas, viruses in humans, animals and plants, certain parasitic agents as well as autoimmune disease. The authors present up-to-date information concerning the serological methods in laboratory diagnosis of such infectious diseases. Section one deals with the serological methods for bacteria. Section 2 deals with serological methods in human, animal and plant viruses. Section 3 is concerned with the serological laboratory diagnosis of echinococcus and human toxocariasis agents. The last section deals with serological laboratory methods in the diagnosis of coeliac disease.

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