Review

Laboratory assessment and diagnosis of congenital viral infections: Rubella, cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV), parvovirus B19 and human immunodeficiency virus (HIV)

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Abstract

Viral infections during pregnancy may cause fetal or neonatal damage. Clinical intervention, which is required for certain viral infections, relies on laboratory tests performed during pregnancy and at the neonatal stage. This review describes traditional and advanced laboratory approaches and testing methods used for assessment of the six most significant viral infections during pregnancy: rubella virus (RV), cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV), parvovirus B19 and human immunodeficiency virus (HIV). Interpretation of the laboratory results according to studies published in recent years is discussed.

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Keywords: Laboratory diagnosis; Congenital viral infections; Rubella; Cytomegalovirus (CMV); Varicella-zoster virus (VZV); Herpes simplex virus (HSV); Parvovirus B19; Human immunodeficiency virus (HIV)

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1. General introduction

Viral infections during pregnancy carry a risk for intrauterine transmission which may result in fetal damage. The consequences of fetal infection depend on the virus type: for many common viral infections there is no risk for fetal damage, but some viruses are teratogenic while others cause fetal or neonatal diseases ranging in severity from mild and transient symptoms to a fatal disease. In cases where infection during pregnancy prompts clinical decisions, laboratory diagnostic tests are an essential part of the clinical assessment process. This review describes the six most important viruses for which laboratory assessment during pregnancy is required and experience has been gained over many years. Rubella virus and CMV are teratogenic viruses, while VZV, HSV, parvovirus B19 and HIV cause fetal or neonatal transient or chronic disease.

The ability of viruses to cross the placenta, infect the fetus and cause damage depends, among other factors, on the mother’s immune status against the specific virus. In general, primary infections during pregnancy are substantially more damaging than secondary infections or reactivations.

Laboratory testing of maternal immune status is required to diagnose infection and distinguish between primary and secondary infections. Assessment of fetal damage and prognosis requires prenatal laboratory testing primarily in those cases where a clinical decision such as drug treatment, pregnancy termination or intrauterine IgG transfusion must be taken.

This review describes basic virological facts and explains the laboratory approaches and techniques used for the diagnostic process. It aims at familiarizing physicians with the rational behind the laboratory requests for specific and timely specimens and with the interpretation of the tests results including its limitations.

The laboratory methods used for assessment of viral infections in general are of two categories: serology and virus detection. Serology is very sensitive but often cannot conclusively determine the time of infection, which may be critical for risk assessment. Traditional serological tests, which measure antibody levels without distinction between IgM and IgG, usually require two samples for determination of seroconversion or a substantial rise in titer. The modern tests can distinguish between IgG and IgM and may allow diagnosis in one serum sample. However, biological and technical difficulties are common and may cause false positive and false negative results. The properties of all serological assays used for each of the viruses will be described in detail in the following chapters.

Virus detection is used primarily for prenatal diagnosis. Invasive procedures must be used to obtain samples representing the fetus such as amniotic fluid (AF), cord blood and chorionic villi (CV). The traditional “gold standard” assay for virus detection used to be virus isolation in tissue culture, but other, more rapid and sensitive methods were developed in recent years. Among the new methods are direct antigen detection by specific antibodies and amplification and detection of viral nucleic acids. The general characteristics of all laboratory assays described in this article are summarized in Table 1.

Since the algorithm for maternal and fetal assessment and the interpretation of tests results vary from one virus to another, we have described the approach to each viral infection in a separate chapter. Figs. 1–6 depict the most common algorithms used for the laboratory diagnosis of each of the viral infections.

2. Rubella virus

2.1. Introduction

Rubella is a highly transmissible childhood disease which can cause large outbreaks every few years. It is a vaccine preventable disease and in developed countries outbreaks are mostly confined to unvaccinated communities [1]. Rubella reinfection following natural infection is very rare. Rubella virus (RV) is classified as a member of the togaviridae family and is the only virus of the genus rubivirus [2]. Hemagglutinating activity and at least three antibody neutralization domains were assigned to the virus of the genus rubivirus [2]. Rubella vaccination induces immunity that confers protection against the disease for the majority of individuals [10,11]. Rubella vaccination induces immunity that confers protection from viremia in the vast majority of vaccinees, which usually persists for more than 16 years [10–12]. A small fraction of the vaccinees fail to respond or develop low levels of detectable antibodies which may decline to undetectable levels within 5–8 years from vaccination [13–17].

Several methods are used to determine immunity (Table 1). Neutralization test (NT) and hemagglutination inhibition test (HI) correlate well with protective immunity, but since they are difficult to perform and to standardize, they were replaced by the more rapid, facile and sensitive enzyme-linked immunosorbant assay (ELISA) [5,18]. In our experience (unpublished data), there is a clear distinction between antibody levels measured using ELISA, and antibody levels measured using functional assays such as NT and HI. Moreover, standardization of anti RV antibody assays using different techniques and a variety of antigens (i.e., whole virus, synthetic peptides, recombinant antigen, etc.) has not been achieved, leading to uncertainties regarding the antibody levels that confer immunity and protection against reinfections and against virus transmission to the fetus [19–26]. Most of the reinfection cases (9 out of 18 cases;
Table 1: Summary and characteristics of the laboratory tests used for assessment of viral infections in pregnancy

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Test principles</th>
<th>Clinical samples</th>
<th>Technical advantages</th>
<th>Technical disadvantages</th>
<th>Interpretation of positive results</th>
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<tbody>
<tr>
<td>Serology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralization (NT)</td>
<td>Inhibition of virus growth by tissue culture by Ab§</td>
<td>Maternal blood</td>
<td>Corresponds with protection</td>
<td>Laborious, not very sensitive, not Ab class-specific</td>
<td>Neutralizing antibodies are present at a certain titer</td>
</tr>
<tr>
<td>Hemagglutination inhibition (HI)</td>
<td>Prevention of hemagglutination by binding of Ab to viral Ag§</td>
<td>Maternal blood</td>
<td>Accurate and corresponds with protection</td>
<td>Laborious, not Ab class specific</td>
<td>HI antibodies are present at a certain titer</td>
</tr>
<tr>
<td>ELISA IgM</td>
<td>Detection of virus specific Ab bound to a solid phase by a labeled secondary anti-IgM Ab</td>
<td>Maternal blood, fetal blood, newborn blood</td>
<td>Fast and sensitive, commercialized, automated</td>
<td>None</td>
<td>False positive and false negative</td>
</tr>
<tr>
<td>ELISA IgG</td>
<td>Detection of virus specific Ab bound to a solid phase by a labeled secondary anti-IgG Ab</td>
<td>Maternal blood, newborn blood</td>
<td>Fast and sensitive, commercialized, automated</td>
<td>None</td>
<td>IgM antibodies are present</td>
</tr>
<tr>
<td>IgG avidity (ELISA)</td>
<td>Removal of low avidity IgG Ab which results in a reduced signal</td>
<td>Maternal blood</td>
<td>Fast and sensitive, commercialized, automated</td>
<td>Not many available commercially</td>
<td>Low avidity: recent infection; medium avidity: not known; high avidity: probably old infection. Antibodies are present at a certain titer</td>
</tr>
<tr>
<td>Immunofluorescence (IFA; IFAMA, etc.)</td>
<td>Detection of IgG or IgM Ab which binds to a spot of virus infected cells on a slide by a labeled secondary Ab</td>
<td>Maternal blood, fetal blood, newborn blood</td>
<td>Can yield titer, short time</td>
<td>Manual, reading is subjective</td>
<td>Unsuitable for testing large numbers</td>
</tr>
<tr>
<td>Western blot (WB)</td>
<td>Separated viral proteins attached to a nylon membrane react with patient’s serum and detected by labeled anti-human Ab</td>
<td>Maternal blood infant’s blood</td>
<td>Detects antibody specific to a viral protein</td>
<td>Laborious</td>
<td>Antibodies specific to certain viral antigens are present</td>
</tr>
<tr>
<td>Virus detection</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Virus isolation in tissue culture</td>
<td>Innoculation of specific tissue cultures with clinical samples and watching for CPE</td>
<td>Any clinical sample which may contain virus</td>
<td>Detects and isolates live virus</td>
<td>Very labourious, Slow</td>
<td>Live virus is present in the clinical sample</td>
</tr>
<tr>
<td>Direct antigen detection</td>
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<td></td>
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</tr>
<tr>
<td>Shell-vial assay</td>
<td>Innoculation of specific tissue cultures with clinical samples, then fixation and detection of viral cell-bound antigen by IFA</td>
<td>Any clinical sample which may contain virus</td>
<td>Detects live virus; rapid: results within 16–72 h</td>
<td>Labourious, requires high skills; uses expensive monoclonal Abs</td>
<td>The sample most likely contains live virus</td>
</tr>
<tr>
<td>Molecular assays</td>
<td></td>
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</tr>
<tr>
<td>PCR, RT-PCR</td>
<td>Enzymatic amplification of viral nucleic acid and detection of amplified sequences</td>
<td>Any clinical sample which may contain virus</td>
<td>Fast, sample can be automated; very sensitive</td>
<td>Very prone to contaminations</td>
<td>False positive by contamination; may detect latent virus</td>
</tr>
</tbody>
</table>

§ (Neutralizing antibodies)
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Test principles</th>
<th>Clinical samples</th>
<th>Technical advantages</th>
<th>Technical disadvantages</th>
<th>Limitations</th>
<th>Interpretation of positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time PCR/RT-PCR</td>
<td>Detection of accumulating PCR products by a fluorescent dye or probe in a specialized instrument</td>
<td>Any clinical sample which may contain virus</td>
<td>Very fast, simple not prone to contaminations, can be quantitative</td>
<td>Expensive instruments</td>
<td>Sometimes too sensitive, interpretation of very low result questionable</td>
<td>Viral nucleic acid is present in the sample (at a certain amount), not known if live virus is present</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>Detection of viral nucleic acid in smears or tissue sections by labeled probes</td>
<td>Cells or tissue from clinical samples</td>
<td>Sensitive and specific</td>
<td>Difficult to perform</td>
<td>Done only in specializing labs</td>
<td>Viral nucleic acid is present in the sample, not known if live virus is present</td>
</tr>
<tr>
<td>In situ PCR</td>
<td>Detection of viral nucleic acid in smears or tissue sections by PCR using labeled primers</td>
<td>Cells or tissue from clinical samples</td>
<td>Sensitive and specific</td>
<td>Difficult to perform</td>
<td>Done only in specializing labs</td>
<td>Viral nucleic acid is present in the sample, not known if live virus is present</td>
</tr>
</tbody>
</table>

50%) which were detected during an outbreak in Israel in 1992 occurred in the presence of low neutralizing antibody titers of 1:4 (cut off levels), and sharp decline in the reinfection rate correlated with the presence of higher titers of neutralizing antibodies (unpublished data). Reinfection rates following vaccination are considerably higher than following natural infection, ranging between 10% and 20% [19]. Many developed countries adopted the infant routine vaccination policy using MMR (mumps measles and rubella) vaccine designed to provide indirect protection of child-bearing age.

Fig. 1. Algorithm for assessment of rubella infection in pregnancy: the algorithm shows a stepwise procedure beginning with testing of the maternal blood for IgM and IgG. If the maternal blood is IgM negative the IgG result determines if the woman is seropositive (immune) or seronegative (not immune). If not immune the woman should be retested monthly for seroconversion till the end of the 5th month of pregnancy. If the maternal blood is IgM and IgG positive the next step would be an IgG avidity assay on the same blood sample to estimate the time of infection. Low avidity index (AI) indicates recent infection while high AI indicates past or recurrent infection. Medium AI is inconclusive and the test should be repeated on a second blood sample obtained 2–3 weeks later. If the results remain the same (IgM+ IgG−), then the IgM result is considered non-specific, indicating that the woman has not been infected (however she is seronegative and should be followed to the end of the 5th month as stated above). If the woman has seroconverted (IgM+ IgG+), recent primary infection is confirmed and prenatal diagnosis should take place if the woman wishes to continue her pregnancy. Determination of IgM in cord blood is the preferred method with the highest prognostic value: Post natal diagnosis is based on the newborn’s serology (IgM for 6–12 m and IgG beyond age 6 m) and on virus isolation from the newborn’s respiratory secretions.
women regardless of vaccination status. However, in Israel and in other countries with high vaccination coverage, RV still circulates and may cause reinfections in vaccinated women whose immunity has waned [19,20,23, unpublished data].

2.1.3. Laboratory assessment of primary rubella infection in pregnancy

Assessment of primary rubella infection in pregnant women relies primarily on the detection of specific maternal IgM antibodies in combination with either seroconversion or a >4-fold rise in rubella specific IgG antibody titer in paired serum samples (acute/convalescent) as shown in Fig. 1. Today, due to the high sensitivity of the ELISA-IgM assays low levels of rubella specific IgM are detected more frequently, leading to an increase in the number of therapeutic abortions and reducing the number of CRS cases. However, frequently the low level of IgM detected is not indicative of a recent primary infection for several reasons: (a) IgM reactivity after vaccination or primary rubella infection may sometimes persist for up to several years [27–29]; (b) heterotypic IgM antibody reactivity may occur in patients recently infected with Epstein Barr virus (EBV), cytomegalovirus (CMV), human parvovirus B19 and other pathogens, leading to false positive rubella IgM results [30–35]; (c) false positive rubella specific IgM response may occur in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) or juvenile rheumatoid arthritis, etc., due to the presence of rheumatoid factor (RF) [36,37]; (d) low level of specific rubella IgM may occur in pregnancy due to

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Fig. 2. Algorithm for assessment of CMV infection in pregnancy: the algorithm shows a stepwise procedure which begins with detection of IgM in maternal blood. If the maternal blood is IgG positive, an IgG avidity assay on the same blood sample should be performed to estimate the time of infection. Low avidity index (AI) indicates recent primary infection and prenatal diagnosis should follow. Medium or high AI is mostly inconclusive, especially if the maternal blood was obtained on the second or third trimester. Continuation of the assessment is based on either maternal blood or fetal prenatal diagnosis. If the first maternal blood was IgM positive but IgG negative, a second blood sample should be obtained 2–3 weeks later. If the IgG remains negative then the IgM is considered non-specific. If the woman has seroconverted and developed IgG, primary infection is confirmed and prenatal diagnosis should follow. For prenatal diagnosis amniotic fluid (AF) should be obtained not earlier than the 21st week of gestation and 6 weeks following seroconversion. Fetal infection is assessed by virus isolation using standard tissue culture or shell-vial assay, and/or by PCR detection of CMV DNA. Positive result by either one of these tests indicates fetal infection.

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Fig. 3. Algorithm for assessment of VZV infection in pregnancy: two situations are shown: (1) clinical varicella in a pregnant woman (top left) should be assessed by serology (IgM and IgG in maternal blood) and by virus isolation or detection in early dermal lesions. If either of these approaches confirms maternal VZV infection (positive virus isolation/detection test and/or maternal seroconversion), then fetal infection can be assessed by virus detection in amniotic fluid using direct antigen detection or PCR. (2) Exposure of a pregnant woman to a varicella case (top right) should prompt maternal IgG testing within 96 h from exposure. If the mother has no IgG (not immune) she should receive VZIG within 96 h from exposure.
Fig. 4. Algorithm for assessment of HSV infection in pregnancy and in neonates: the algorithm shows two complementary approaches to the confirmation of genital HSV infection in pregnant women. (1) If genital lesions are present (top right), virus isolation and typing is the preferred diagnostic approach. A positive woman should be examined during delivery for genital lesions. If normal delivery has taken place, the newborn should be examined for HSV infection symptoms and tested by virus isolation or PCR using swabs taken from skin, eye, nasopharynx and rectum or CSF. Detection of IgM in the newborn’s blood also confirms the diagnosis. Negative infants should be followed for 6 months. (2) Serology (top left) is a stepwise procedure beginning with maternal IgM and IgG testing. The interpretation of the results is shown: low positive or negative IgM in the presence of IgG indicates previous infection with the same virus type (reactivation), or recurrent infection with the other virus type. Type-specific serology may resolve the issue. If the IgG test is negative, in the presence or absence of IgM, a second serum sample should be obtained to observe seroconversion. If the IgG remains negative then no infection occurred. If the woman seroconverted, type specific serology can identify the infecting virus type.

Fig. 5. Algorithm for assessment of parvovirus B19 infection in pregnancy: the algorithm shows a stepwise procedure beginning with maternal serology following clinical symptoms in the mother or in the fetus or maternal contact with a clinical case. Negative IgM and positive IgG indicate past infection, but if the IgG is high recent infection cannot be ruled out. In all other cases a second serum sample should be obtained and tested. Only in the case of repeated negative results for both IgG and IgM recent infection with B19 can be ruled out. Positive results are usually followed by fetal infection. In all other cases the fetus should be observed for clinical symptoms and if present tested for B19 infection by nested PCR or rt-PCR performed on amniotic fluid or fetal blood. Positive result confirms fetal infection while negative result suggests that the fetus was not infected with B19.
polyclonal B-cells activation triggered by other viral infections [33,35,38].

False negative results may also occur in samples taken too early during the course of primary infection. Thus, the presence or absence of rubella specific IgM in an asymptomatic patient should be interpreted in accordance with other clinical and epidemiological information available and prenatal diagnosis may be required.

A novel assay developed recently to support maternal diagnosis is the IgG avidity assay (Table 1) which can differentiate between antibodies with high or low avidity (or affinity) to the antigen. It is used when the mother has both IgM and IgG in the first serum collected (Fig. 1). Following postnatal primary infection with rubella virus, the specific IgG avidity is initially low and matures slowly over weeks and months [39–41]. Rubella specific IgG avidity measurement proved to be a useful tool for the differentiation between recent primary rubella (clinical and especially subclinical infection), reinfection, remote rubella infection or persistent IgM reactivity. This distinction is critical for the clinical management of the case, since infection prompts a therapeutic abortion, reinfection requires fetal assessment, while remote infection or non-specific IgM reactivity carry no risk to the fetus [39–42].

2.1.4. Pre- and postnatal laboratory assessment of congenital rubella infection

Maternal primary infection prompts testing for fetal infection (Fig. 1). The preferred laboratory method for prenatal diagnosis is determination of IgM antibodies in fetal blood obtained by cordocentesis [27,43]. Other options include virus detection in chorionic villi (CV) samples or amniotic fluid (AF) specimens. The laboratory methods used for virus detection are virus isolation in tissue culture or amplification of viral nucleic acids by RT/PCR (Table 1). However, using those methods for detection of rubella virus in AF and CV might be unreliable, particularly in AF samples due to low viral load. Studies showed that rubella virus may be present in the placenta but not in the fetus, or it can be present in the fetus but not in the placenta, leading to false negative results [6,43,44]. Thus, according to one opinion, detection of rubella virus in AF or CV does not justify the risk of fetal loss following these invasive procedures [45], while according to another opinion, laboratory diagnosis of fetal infection should combine a serological assay (detection of rubella specific IgM) with a molecular method (viral RNA detection) in order to enhance the reliability of the diagnosis [46]. A recent study showed 83–95% sensitivity and 100% specificity for detection of RV in AF by RT/PCR [47].

Postnatal diagnosis of congenital rubella infection [9,27,36] is based on one or more of the following:

a. Isolation of rubella virus from the infant’s respiratory secretions.

b. Demonstration of rubella specific IgM (or IgA) antibodies in cord blood or in neonatal serum, which remain detectable for 6–12 months of age.

c. Persistence of anti-rubella IgG antibodies in the infant’s serum beyond 3–6 months of age.

The principles, advantages and disadvantages of each laboratory test, are described below.
2.2. Laboratory assays for assessment of rubella infection and immunity

2.2.1. Rubella neutralization test (NT)

Virus neutralization is defined as the loss of infectivity due to reaction of a virus with specific antibody. Neutralization can be used to identify virus isolates or, as in the case of rubella diagnosis, to measure the immune response to the virus [24,36,48]. As a functional test, neutralization has proven to be highly sensitive, specific and reliable technique, but it can be performed only in virology laboratories which comprise only a small fraction of the laboratories performing rubella serology.

Rubella virus produces characteristic damage (cytopathic effect, CPE) in the RK 13 cell line that was found most sensitive and suitable for use in rubella neutralization test. Other cells such as Vero and SIRC lines can be used if conditions are carefully controlled [36]. Principally, 2-fold dilutions of each test serum are mixed and incubated with 100 infectious units of rubella virus under appropriate conditions. Then cell monolayers are inoculated with each mixture and followed for CPE. Control sera possessing known high and low neutralizing antibody levels and titrations of the virus are included in each test run. The neutralization titer is taken as the reciprocal of the highest serum dilution showing complete inhibition of CPE [25,36].

2.2.2. Hemagglutination inhibition test (HI)

Until recently, assessment of rubella immunity and diagnosis of rubella infection has been carried out mainly by the HI test which is based on the ability of rubella virus to agglutinate red blood cells [49]. HI test is labor intensive, and is currently performed mainly by reference laboratories. HI is the “gold standard” test against which almost all other rubella screening and diagnostic tests are measured. During the test, the agglutination is inhibited by binding of specific antibodies to the viral agglutinin. Titers are expressed as the highest dilution inhibiting hemagglutination under standardized testing conditions [50-53].

The HI antibodies increase rapidly after RV infection since the test detects both, IgG and IgM class-specific antibodies. A titer of 1:8 is commonly considered negative (cut off level: 1:16) and a titer of ≥1:32 indicates an earlier RV infection or successful vaccination and immunity. Seroconversion is interpreted as primary rubella infection, and a 4-fold increase in titer between two serum samples (paired sera) in the same test series, is interpreted as a recent primary rubella infection or reinfection [52]. Considerable experience has been accumulated over the years in the interpretation of the clinical significance of HI titers [52-54], and the test results accurately correlate with clinical protection [5,18]. Although HI is generally considered as not sensitive enough, in certain situations it is still in use for resolution of diagnostic uncertainties.

Detection of rubella specific IgM class antibodies by HI test which requires tedious methods for purification of IgM or removal of IgG [55,56], are no longer in use due to the development of a variety of rapid, easy to perform and sensitive methods, of which ELISA is the most vastly used [18,57].

2.2.3. Rubella specific ELISA IgG

The ELISA technique was established for detection of an increasing range of antibodies to viral antigens. In 1976, Vollet et al. [58] developed an indirect assay for the detection of anti-viral antibodies. The technique has been successfully applied for the detection of rubella specific antibodies.

Almost all commercially available ELISA kits for the detection of rubella specific IgG are of the indirect type, employing rubella antigen attached to a solid phase (microtiter polystyrene plates or plastic beads). The source of the antigen (peptide, recombinant or whole virus antigen) affects the sensitivity and specificity of the assay. After washing and removal of unbound antigen, diluted test serum is added and incubated with the immobilized antigen. The rubella specific antibodies present in the serum bind to the antigen. Then, unbound antibodies are removed by washing and an enzyme conjugated anti-human IgG is added and further incubation is carried out. The quantity of the conjugate that binds to each well is proportional to the concentration of the rubella specific antibodies present in the patient’s serum. The plates are then washed and substrate is added resulting in color development. The enzymatic reaction is stopped after a short incubation period, and optical density (OD) is measured by an ELISA-reader instrument. The test principle allows the detection of IgM as well by using an appropriate anti-human IgM conjugate [53,57].

In most commercial ELISA IgG assays the results are automatically calculated and expressed quantitatively in international units (IU). When performed manually, the procedure takes approximately 3 h but automation has reduced it to about 30 min [57-59]. It is important to note that in order to obtain reliable results, determination of a significant change in specific IgG activity in paired serum samples should always be performed in the same test run and in the same test dilution.

The correlation between the ELISA and HI or NT titers is not always high. This may be explained by the fact that the three methods detect antibodies directed to different antigenic determinants [54]. Certain individuals fail to develop antibodies directed to protective epitopes such as the neutralizing domains of E1 and E2 due to a defect in their rubella specific immune responses [21] but they do develop antibodies directed to antigenic sub-regions of rubella virus proteins. ELISA assays utilizing whole virus as antigen may fail to distinguish between these different antibody specificities. Thus, seroconversion determined by ELISA based on a whole virus antigen does not necessarily correlate with protection against infection [52].

2.2.4. Rubella specific ELISA IgM

Commercially available ELISA kits for the detection of IgM are mainly of two types:

a. Indirect ELISA: The principle of the assay was described above for rubella IgG except for using enzyme labeled anti-human IgM as a conjugate. In this assay, false negative results may occur due to a competition in the assay between specific IgG antibodies with high affinity (interfering IgG) while the specific IgM have lower affinity for the antigen [31,32]. In the
new generation ELISA assays this is avoided by the addition of an absorbent reagent for the removal of IgG from the test serum. False positive results may occur if rheumatoid factor (RF: IgM anti-IgG antibodies) is present along with specific IgG in the test serum. Absorption or removal of RF and/or IgG is necessary prior to the assay to avoid such reactions [30–32,60].

b. IgM capture ELISA: In these assays anti-human IgM antibody is attached to the solid phase for capture of serum IgM. Rubella virus antigen conjugated to enzyme-labeled anti-rubella virus antibody is added for detection. This type of assay eliminates the need for sample pretreatment prior to the assay [32,61]. As for the rubella virus antigens, most assays are based on whole virus extracts, but recent developments led to production of recombinant and synthetic rubella virus proteins [5,62].

2.2.5. Rubella specific IgG-avidity assay

This assay is based on the ELISA IgG technique and applies the elution principle in which protein denaturant, mostly urea (but also diethylamine, ammonium thiocyanate, guanidine hydrochloride, etc.) is added after binding of the patient’s serum. The denaturant disrupts hydrophobic bonds between antibody and antigen, and thus, low avidity IgG antibodies produced during the early stage of infection are removed. This results in a significant reduction in the IgG absorbance level [63]. The avidity index (AI) is calculated according to the following formula [57]:

\[
AI = \frac{100 \times \text{absorbance of avidity ELISA}}{\text{absorbance of standard ELISA}}
\]

The AI is a useful measure only when the IgG concentration in the patient’s serum is not below 25 IU [39]. Low avidity (usually below 50%) is associated with recent primary rubella infection while reinfection is typically associated with high avidity as a result of the stimulation of memory B cells (immunological memory) [39–41].

In infants with CRS the low avidity IgG continues to be produced for much longer than in cases of postnatal primary rubella, where it lasts 4–6 week after exposure [39]. This may be used for retrospective assessment of initially undiagnosed CRS cases.

2.2.6. Rubella virus isolation in tissue culture

Diagnosis of prenatal or postnatal rubella infections are essentially based on the more reliable and rapid serological techniques. However, virus isolation is useful in confirming the diagnosis of CRS (Fig. 1) and rubella virus strain characterization required for epidemiological purposes. Rubella virus can be isolated using a variety of clinical specimens such as: respiratory secretions (nasopharyngeal swabs), urine, heparinized blood, CSF, cataract material, lens fluid, amniotic fluid, synovial fluid and products of conception (fetal tissues: placenta, liver, skin, etc.) obtained following spontaneous or therapeutic abortion [6,36,44,64]. In order to avoid virus inactivation, specimens should be inoculated into cell culture immediately or stored at 4 °C for not more than 2 days, or kept frozen (–70 °C) for longer periods [36].

Rubella virus can be grown in a variety of primary cells and cell lines [36,65], but RK-13 and Vero cell lines are the most sensitive and suitable for routine use. In these cell systems rubella virus produces characteristic CPE. Since the CPE is not always clear upon primary isolation, at least two successive subpassages are required [66]. When CPE is evident the identity of the virus isolates should be confirmed using immunological or other methods [36,65,67].

2.2.7. Rubella RT-PCR assay

Reverse transcription followed by PCR amplification (RT-PCR) is a rapid, sensitive and specific technique for detection of rubella virus RNA in clinical samples using primers from the envelope glycoprotein E1 open reading frame [45,46,68,69]. Coding sequences for a major group of antigenic determinants are located between nucleotides 731 and 854 of the E1 gene of RV strain M33. This region is highly conserved in various wild type strains and is likely to be present in most clinical samples from rubella infected patients. Specific oligonucleotide primers located in this region were designed for amplification by RT-PCR [70–72]. Following rubella genomic RNA extraction from clinical specimens and RT-PCR amplification, the product is visualized by gel electrophoresis. Positive samples show a specific band of the expected size compared to size markers [68,69,72].

A nested RT-PCR assay, in which the RT-PCR product from the first amplification reaction is re-amplified by internal primers, was developed and shown to provide a higher level of sensitivity for the detection of rubella virus RNA [72]. However, the risk of contamination is markedly increased. The detection limit of the RT-PCR assay is approximately two RNA copies.

Clinical specimens for rubella virus genome detection include: products of conception (POC), CV, lens aspirate/biopsy, AF, fetal blood, pharyngeal swabs and spinal fluid (CSF) or brain biopsy when the central nervous system (CNS) is involved [68,69,73–75]. An additional advantage of RT-PCR is that it does not require infectious virus [74]. RV is extremely thermo-labile and frequently is inactivated during sample transportation to the laboratory.

Finally, it should be noted that clinical samples may contain PCR inhibitors (such as heparin and hemoglobin), and the extraction procedure itself may cause enzyme inhibition [72,76,77]. This underscores the need and importance for strict internal quality control during each step of the RT-PCR procedure and participation in external quality assessment programs is of a high value.

2.3. Summary

Rubella infection during pregnancy, although rare in countries with routine vaccination programs, is still a problem requiring careful laboratory assessment. The laboratory testing should confirm or rule-out recent rubella infection in pregnant women and identify congenital rubella infections in the fetus or neonate. Maternal infection is currently assessed by serological assays, primarily by ELISA IgM and IgG. Borderline results for the IgG assay can be further assessed by the HI or NT assays available
3.1. Introduction

3.1.1. The pathogen

CMV is a common pathogen which can cause primary and secondary infections. CMV is a member of the herpesvirus family possessing a 235 kb double stranded linear DNA genome, a capsid and a loose envelope. Membranal glycoproteins embedded in the envelope carry neutralization epitopes. CMV can infect all age groups usually causing mild and self-limited disease. Its sero-prevalence in women of child-bearing age varies. CMV is a 235 kb double stranded linear DNA genome, a capsid and a loose envelope. Membranal glycoproteins embedded in the envelope carry neutralization epitopes. CMV can infect all age groups usually causing mild and self-limited disease. Its sero-prevalence in women of child-bearing age varies from 50% to over 80%, with inverse correlation to socio-economic levels. Primary CMV infection during pregnancy carries a high risk of intrauterine transmission which may result in severe fetal damage, including growth retardation, jaundice, hepatitis, pneumonia and CNS abnormalities. Those who are asymptomatic at birth may develop hearing defects or learning disabilities later in life. It is now recognized that intrauterine transmission may occur in the presence of maternal immunity [78]. Pre-conceptional primary infection carries a high risk identical to the risk of infection during early gestational weeks [79].

CMV, like other members of the herpesvirus family, establishes a latent infection with occasional reactivations as well as recurrent infections in spite of the presence of immunity. However, reactivation or recurrent infections carry a much lower risk for fetal infection and damage is much lower in such events. The infectious cycle in vitro takes 24–48 h while in vivo the incubation period for postnatal infection can last for 4–8 weeks. The incubation period for congenital infection is not known and the gestational age of congenital infection is currently defined by the maternal seroconversion, if known, which does not necessarily reflect the actual timing of the fetal infection. The host defense against CMV infection in immune-competent individuals combines cellular and humoral immune responses which together prevent a severe CMV disease in the vast majority of infections. Antibodies of the IgM class are produced immediately after primary infection and may last for several months. IgM can be produced in secondary infections in some cases. Antibodies of the IgG class are also produced immediately after infection and last for life.

3.1.2. Laboratory assessment of CMV infection in pregnant women

CMV was recognized as the cause of fetal stillbirth following a cytomegalic inclusion disease (CID) in the mid 1950s when it was first grown in tissue cultures in three laboratories [80–82]. Since then demonstration of CMV infection of the mother or fetus by laboratory testing has become an essential part of the assessment of pregnancies at risk [76,83]. Assessment of congenital CMV infection begins with maternal serology which should establish recent primary or secondary infection (Fig. 2).

Not all maternal infections result in fetal transmission and damage. Only 35–50% of maternal primary infections and 0.2–2% of secondary infections lead to fetal infection, out of which only 5–15% in primary infection and about 1% in secondary infections are clinically affected [84–87]. Therefore, following maternal diagnosis, and if early pregnancy termination was not chosen, subsequent prenatal diagnosis should take place using methods for virus detection in AF samples. Demonstration of maternal infection relies on ELISA IgM and IgG assays and on CMV IgG avidity assay (Fig. 2). Unlike HI and NT for rubella, for CMV there are currently no serological “gold standard” assays which can be used for confirmation and reassurance. Recently an attempt to find association between viral load in maternal blood and the risk for fetal infection did not yield positive results [88].

3.1.3. Prenatal assessment of congenital CMV infection

Maternal infection during pregnancy prompts testing for fetal infection as outlined in Fig. 2. Prenatal CMV diagnosis cannot rely on detection of fetal IgM since frequently the fetus does not develop IgM [76,89–94]. On the other hand, because CMV is excreted in the urine of the infected fetus, detection of virus in the AF has proven to be a highly sensitive and reliable method. Numerous studies have focused on the most appropriate timing for performing amniocentesis which will yield the best sensitivity for detection of fetal infection [76,83,97–99]. These studies clearly indicated that amniotic fluid should be collected on 21–23 gestational week and at least 6–9 weeks past maternal infection. If these requirements are met then the sensitivity of detection of intrauterine infection can reach over 95% while the general sensitivity is only 70–80%. One study measured the sensitivity for AF obtained at gestational weeks 14–20 and reported only 45% [100]. Most of the studies state that the timing of the amniocentesis is more critical for sensitivity than the laboratory methods used to detect the virus in the AF.

Initially, virus isolation in tissue culture and its more sophisticated variant “Shell-Vial” technique (Table 1) were the leading laboratory methods for detection of CMV in amniotic fluid. However, during the late 1980s highly sensitive molecular methods were developed for detection of specific viral DNA in clinical specimens such as dot-blot hybridization [101–103]. These methods were much faster, less laborious and repeatable compared to virus culturing. Performance of the biological and molecular techniques in parallel assured that the precious amniotic fluid sample will not be wasted and that false negative results will not be obtained by a technical problem in any of these “home-made” assays.
Since the early 1990s the polymerase chain reaction (PCR) has become the preferred method for CMV detection in amniotic fluid [95,96,104–106]. Problems with molecular contamination leading to false positive results and the need to address prognostic issues, led finally to the development of quantitative PCR assays with the highly advanced real-time PCR (rt-PCR) as the most updated method (Table 1). Current studies deal with the correlation between the “viral load” in the amniotic fluid and the pregnancy outcome, in an attempt to establish the prognostic parameters of this powerful technique.

The laboratory methods used for assessment of maternal and fetal CMV infection are described in detail below.

3.2. Laboratory assays for assessment of CMV infection

3.2.1. CMV IgM assays

IgM detection is a hallmark of primary infection although it may also be associated with secondary infections [90,107–109]. Major efforts were put into developing sensitive and reliable assays for IgM detection using ELISA. The technical and biological obstacles and their solutions which were described for rubella IgM assays apply for CMV as well, including long-term persistence of IgM antibodies [110–114].

The source of the viral antigen affects sensitivity and specificity [113,115–122], but in the absence of a gold standard assay, comparisons between various commercially available assays were based on multi-variant analyses of “consensus” results between several assays. These studies demonstrated high variability in specificity and sensitivity among assays and a high rate of discordance [123–126]. Thus, testing for IgM, particularly in asymptomatic pregnant women, may frequently create a problem rather than solving it: borderline results or conflicting results among two or more commercial kits are interpreted as inconclusive and require further testing as described below. Other methods, such as immunoblotting and IF assays (Table 1) were developed to confirm positive IgM results and to distinguish between specific and non-specific reactions [88]. However, these assays did not gain vast usage because of lowered sensitivity [127] and the lack of automation.

3.2.2. CMV IgG assays

IgG assays which are currently based on ELISA, are generally used for determination of immune status but, unlike rubella, there is neither definition of a CMV-IgG international unit (IU) nor of the protective antibody level. IgG assays may also help to establish diagnosis of current CMV infection in suspected secondary infections, or when the IgM result is inconclusive, by demonstration of IgG seroconversion or a significant IgG rise between paired sera taken 2–3 weeks apart. This ability is limited in cases when women initially present with a high titer of IgG or when it is impossible or too late to obtain a second serum sample. Commercial ELISA IgG assays are relatively simple, correlate well with each other and most of them are quantitative but are not yet internationally standardized. Commercialized assays use arbitrary units (AU) which differ from one assay to another and thus, to demonstrate an increase in antibody level, it is critical to run the two samples in parallel in the same test. Additionally, since a “significant increase” is rarely defined for commercial ELISA assays, it is up to the laboratory to define it.

3.2.3. CMV IgG avidity assays

The IgG avidity assay was developed to circumvent diagnostic problems as described for rubella [128–130]. It is performed when both IgM and IgG are positive on initial testing, but cannot be performed on sera with very low IgG titers. Various commercial assays are calibrated in different ways for determination of the diagnostic threshold: some assays exclude recent infection if the AI reaches a certain threshold level, yet others approve recent infection if the AI is lower than a certain threshold level. However, none of these assays can exactly determine when the infection occurred or give any interpretation of results falling outside of its exclusion or inclusion criteria. Numerous studies published in recent years aimed at evaluating IgG-avidity assays (by commercial kits or “in-house” methods) for their ability to identify or exclude recent primary CMV infection, and to predict congenital infection. Concordance between different commercial assays for determination of low avidity was high (98–100%), but not for determination of high avidity (70%). Because the use of this assay is relatively new, some of these studies are described in detail below.

One set of studies evaluated the ability of the assay to assess the risk for fetal infection [127,131–133]. In a cohort of women considered at risk for transmitting CMV to their fetuses based on demonstration of IgM or seroconversion, low avidity was strongly associated with fetal infection (100% sensitivity) if the serum sample tested was collected at 6–18 weeks gestation. Moderate or high AI levels were associated with 33% and 11%, respectively, of cases with CMV genome-positive amniotic fluid, but with no fetal infection. Lowered sensitivity (60–63%) for detection of primary infection was found for sera collected at 21–23 weeks gestation, since some of the mothers, infected early in pregnancy, already developed moderate or high avidity.

Another set of studies [134–136] examined the ability of the IgG-avidity assay to exclude those with past infection and therefore with low risk of fetal transmission. Women with positive or equivocal IgM but without documented seroconversion were tested. High avidity was interpreted as remote infection which did not occur within the last 3 months. The results of this series of studies also showed that congenital infection was strongly associated with low avidity, while moderate or high avidity were associated with uninfected fetus. Additional studies further confirmed the strong association between low avidity and primary infection, and thus risk for fetal infection, and between high avidity and past infection [130,136,140]. One study showed the lack of full concordance between different commercial IgG avidity assays [141]. It showed that the ability of a commercial kit to exclude recent infection by high avidity was restricted to AI of >80% and to determine recent infection to AI of <20%. Any result in between those limits was inconclusive since sera with AI of 50–80% included 48 out of 257 (18%) women with a history of past infection and 3 sera from 2 patients with a history of recent infection. Testing the latter three samples with a different kit yielded low avidity (30%).
In conclusion, the IgG-avidity assay is a powerful tool but it should be used and interpreted properly. The association between low AI and recent primary infection with a high risk for congenital infection is stronger than the association between moderate or high AI and past infection with low risk. Interpretable results can be achieved mainly for sera obtained within the first 3–4 month of pregnancy. However both the inclusion and the exclusion approaches can be used and the IgG avidity assay is now implemented in a testing algorithm following the IgG test [142] as shown in Fig. 2.

3.2.4. CMV neutralization assays

Neutralizing antibodies appear only 13–15 weeks following primary infection, thus the presence of high titer of neutralizing antibodies during acute infection indicates a secondary rather than a primary infection. The neutralization assays have not reached a wide use as they are labor intensive, very slow and cannot be commercialized. Therefore, they are rarely performed by specialized reference laboratories. Attempts to correlate neutralization with specific response to the viral glycoprotein gB by IF using monoclonal antibodies directed against early viral proteins synthesized shortly after infection. This method gained wide acceptance and is now used by most laboratories. Its sensitivity and specificity are highly comparable to virus isolation except for rare cases in which the monoclonal antibody does not recognize the viral antigen [147–149].

Today, virus isolation from AF remains a key method for demonstration of fetal infection and has been described extensively in many studies either exclusively or in conjunction with molecular methods, particularly PCR [97,99,132,150–155]. The main subject under investigation in recent years has been the comparative sensitivity and specificity of the PCR and the virus isolation methods.

3.2.6. Detection of CMV by PCR

Detection of viral DNA in clinical samples involves DNA extraction and analysis. PCR has become the preferred method for rapid viral diagnosis in recent years. Its main disadvantage is the possible contamination leading to false positive results. The PCR assay includes several components which can vary from test to test. Viral DNA can be extracted using in-house methods or various commercial kits. The primers used can be derived from different viral genomic sites and the reaction conditions can be altered. For CMV, most assays utilize the early (E) or immediate-early (IE) genes which are highly conserved compared to the structural matrix or glycoprotein genes presenting higher variability among wild-type isolates. To increase specificity [95,156] some assays include a second round of amplification using nested or hemi-nested primers. The nested PCR is however more prone to molecular contamination and false-positive results.

The comparative specificity and sensitivity of PCR and virus isolation is dependent upon technical parameters which vary from one laboratory to another with relation to the overall medical set-up in which they are placed and the technical skills of the laboratory personnel. However, it is generally agreed that for CMV, PCR is more sensitive than tissue culture isolation. PCR is also a repeatable assay which is of great advantage in controversial cases. Original samples kept frozen at or below −70 °C can be re-processed and extracted DNA can be re-tested or sent to another laboratory for confirmation.

3.2.7. Quantitative PCR-based assays

Many previous studies have shown that detection of CMV DNA in AF by itself does not predict the outcome of fetal infection. Clinical measures such as ultrasonographic examinations are a key component in fetal assessment, but might also fail to detect affected fetuses. In an attempt to address prognostic issues it was suggested that symptomatic fetuses can be distinguished from asymptomatic ones based on the viral load in the amniotic fluid. Quantitative PCR methods were developed as “in-house” assays or are available as commercial kits using various technologies. The most up-to-date technology is the real-time PCR assay in which the amplified sequences are detected by a fluorescent probe in a real-time and quantitative manner [157–159]. These assays, performed by dedicated instruments, carry the advantages of high sensitivity and specificity conferred by the hybridization probe, and the lack of contamination by amplification products, since the reaction tubes are never opened after amplification.
Few recent publications have addressed the prognostic value of determination of viral load in AF with controversial results. Three studies [160–162] found no statistically significant difference in viral load between symptomatic and asymptomatic fetal infections. Yet other two studies reported predictive values for viral load [163,164]. In one of these two studies [164] the presence of $10^3$ or more CMV genome-equivalents per millilitres (GE/ml) predicted mother to child transmission with 100% probability, and $10^2$ GE/ml or more predicted symptomatic infection.

In the second report [163] CMV DNA load with median of $2.8 \times 10^3$ GE/ml was associated with major ultrasound abnormalities while median values of $8 \times 10^3$ GE/ml was associated with normal ultrasound and asymptomatic newborn. The slight discordance between the two studies calls for further evaluations on a larger scale and underscores the need for standardization, since the quantitative assays may vary by orders of magnitude using different methods or primers derived from different genomic regions [165,166].

### 3.5. Summary

Laboratory testing for determination of intrauterine CMV infection involves several steps. Maternal primary or recurrent infection is assessed by serology using IgM, IgG and IgG-avidity assays. In controversial cases a second blood sample should be sought to demonstrate antibody kinetics typical of current infection and not of remote infection or a non-specific reaction. If maternal primary infection was established and the pregnancy was not terminated, prenatal diagnosis follows at 21–23 weeks gestation and 6–9 weeks after seroconversion (if known). Detection of CMV in AF is done by virus culturing and/or PCR. Quantitative PCR is still not established for assessment of fetal damage and prognosis.

During the diagnostic process, which may last for several weeks, collaboration between the laboratory and the physician is of utmost importance. Appropriate timing of sampling, sample treatment, usage of validated assays under quality assurance conditions, and correct interpretation of the results are all essential for obtaining a reliable diagnosis. The algorithm describing the laboratory diagnostic process for CMV is shown in Fig. 2.

### 4. Varicella-zoster virus (VZV)

#### 4.1. Introduction

#### 4.1.1. The pathogen

Varicella-zoster virus (VZV) is a common pathogen belonging to the herpesvirus family which can establish latent infections and subsequent reactivations. Primary infection, chickenpox, is a common childhood disease. Reactivation is manifested as zoster and occurs in the presence of anti VZV antibodies. Approximately 90% of the adult population is positive for VZV antibodies and studies on pregnant and parturient women found between 80% and 91% seropositivity [167–170].

Primary infection with VZV (chickenpox) during pregnancy carries a risk for clinical complications for both the mother and the fetus. Complications and sequelae include pneumonia, increased rate of prematurity abortions, congenital varicella syndrome (CVS), neonatal varicella and herpes zoster during the first year of life [171–177]. Rarely VZV may cause a life threatening CNS infection. The risk of adverse effects for the mother is greatest in the third trimester of pregnancy, while for the fetus the risk is greatest in the first and second trimesters. The risk of CVS for all pregnancies continuing for 20 weeks is about 1%, but is lower (0.4%) between weeks 0 and 12 and is higher (2%) between weeks 13 and 20. Maternal infection after 20 weeks and up to 36 weeks is not associated with adverse fetal effect, but may present as shingles in the first few years of infant’s life indicating reactivation of the virus after a primary infection. If maternal infection occurs 1–4 weeks before delivery, up to 50% of the newborns are infected and 23% of them develop clinical varicella. Severe varicella occurs if the infant is born within seven days of the onset of maternal disease.

#### 4.1.2. Assessment of VZV infection in pregnancy

Laboratory diagnosis of VZV in pregnancy is required in two situations: (a) the pregnant woman has developed clinical symptoms compatible with chickenpox or herpes zoster (Shingles) (b) The pregnant woman was exposed to a chickenpox or a zoster case (Fig. 3).

If the pregnant woman has developed clinical symptoms the infection should be confirmed by laboratory testing using serology or virus detection by culturing, antigen detection or molecular methods. Assessment of VZV IgM which remains in the blood for 4–5 weeks is diagnostic. However, false positive results are common in the presence of high VZV IgG antibodies and virus reactivations may also induce IgM. Therefore, determination of IgG seroconversion or a 4-fold rise in VZV IgG titer should accompany the IgM test. Virus isolation or PCR from dermal lesions can be attempted and, if positive, confirm the diagnosis (Fig. 3).

If the pregnant woman has reported exposure to a case of chickenpox or zoster, prompt assessment of her immune status by testing for IgG within 96h from exposure should be done since varicella-zoster immunoglobulin (VZIG) given as a prophylactic measure at the time of exposure is known to prevent or reduce the severity of chickenpox [178,179].

Serological screening for IgG of women with negative or uncertain histories of illness, who are planning a pregnancy, or of women who give history of recent contact with chickenpox, has been suggested as a strategy for preventing CVS and neonatal VZV [180,181]. In a study conducted in our laboratory 52 pregnant women were assessed for immunity to VZV following exposure to chickenpox and 25% of them were found susceptible. The attack rate among the susceptible women was 85% [181]. In another study, Linder et al. [182] reported that in 327 pregnant women assessed for VZV immunity, 95.8% of the women who recalled chickenpox in themselves and 100% of women who recalled chickenpox in their children were seropositive, and only 6.8% of the women with a lack or uncertain history of exposure were seronegative. The screening strategy
might gain momentum due to the availability of VZV vaccine, as seronegative women can be vaccinated.

4.1.3. Prenatal and perinatal laboratory assessment of congenital VZV infection

If VZV infection of the mother during the first or the second trimester has been confirmed, the need to diagnose the fetus arises. Unfortunately, laboratory methods for fetal assessment are of limited value. Assessment of fetal infection by determination of VZV IgM in fetal blood is not widely performed. IgM may be manifested in the fetus only after 24 weeks of gestation, thus in case of intrauterine infection during early gestation, functional immunity may not be present in the fetus [183]. Alternatively, determination of fetal infection can be done by demonstration of VZV DNA in AF using PCR, but its presence is not synonymous with development of CVS [183]. Only one in 12 infected fetuses will develop pathological signs, so interpretation of a positive VZV DNA result is problematic if the fetus appears normal upon ultrasonographic examination [180].

Diagnosis of clinical varicella in neonates is based on serology (IgM) and virus isolation or detection in vesicle fluid or in CSF (in case of CNS infection).

4.2. Laboratory assays for assessment of VZV infection and immunity

4.2.1. VZV IgG assays

Several methods were applied for determination of VZV antibodies. In the past, specific IgG antibodies were measured by the complement fixation (CF) assay, which can be used only for diagnosis of recent infection or by the latex agglutination assay [184–188]. Today, highly sensitive and specific ELISA and immunofluorescence (IF) methods are used for determination of VZV IgG antibodies. Several versions of the IF assay exist (Table 1): fluorescent antibody to membrane antigen (FAMA) and indirect fluorescent antibody to membrane antigen (IFAMA) detect binding of antibodies to membrane antigens in fixed VZV infected cells, and are highly specific and sensitive [169,181,186,189]. ELISA is comparable to IF using monoclonal or polyclonal antibodies directed to VZV antigens [203]. Stained smears, in which multicleonial antibodies are those directed against the cell-membrane associated viral antigens [203].

4.2.2. VZV IgM assays

Both the IF and ELISA methods are used for determination of VZV IgM in the same manner as for IgG, except that the conjugate is an anti-human IgM rather than anti-human IgG. Commercial ELISA IgM kits may give false positive results as was described for RV and CMV.

4.2.3. Virus detection in clinical specimens

Virological methods for the diagnosis of VZV infection include detection of infectious VZV, viral antigens and viral DNA in clinical specimens. These include vesicular fluid, swabs or smears, AF in pregnant women or cerebrospinal fluid (CSF) in encephalitis cases [183,190–195].

4.2.4. Virus isolation in tissue culture

VZV isolation in tissue culture is not a very sensitive assay and the isolation of the virus from skin lesions is possible only from early vesicles. VZV is a very labile virus which grows slowly in selected tissue cultures. Specimens for cell culture (vesicle fluid, skin-lesion swabs and AF) should be transported to the laboratory as soon as possible after collection [190–198]. Swabs should be put in a vial containing virus transport medium and AF should be placed in a sterile container. Both should be kept at 4–8 °C during transportation. Inappropriate conditions during transportation may easily reduce virus viability [199].

Upon receipt, the specimen is inoculated into semi-continuous diploid cells such as human diploid fibroblasts (MRC-5) and continuous cell lines derived from tumors of human or animal tissue such as A549. CPE may appear within 2 weeks. AF should be sampled after 18 weeks of pregnancy and after complete healing of skin lesions of the mother. Confirmation of virus isolation can be done by immunofluorescent staining using monoclonal anti-VZV antibody. Shell vial cultures as described for CMV improve the sensitivity of VZV detection and allow rapid identification of positive samples within 1–3 days [196,200].

4.2.5. Direct detection of VZV antigen

Immunofluorescence or immunoperoxidase assays use monoclonal or polyclonal antibodies directed to VZV antigens to detect VZV infection in epithelial cells isolated from AF, or from suspected lesions (Table 1). This simple method allows rapid diagnosis (approximately 2 h) of VZV infection and is available to most laboratories [201,202]. It is highly specific and has sensitivity ranging from 73.6% to 86%. The preferred monoclonal antibodies are those directed against the cell-membrane associated viral antigens [203].

4.2.6. Molecular methods for detection of viral DNA

PCR and hybridization methods to detect VZV DNA sequences are very sensitive and specific [183,191,205–208]. Modifications of the basic PCR technique have been used to increase the sensitivity by using nested PCR assays. However, this assay is highly susceptible to contamination, leading to false positive results. Rapid laboratory diagnosis is important when the CNS is involved, especially in cases with clinically confused dermal manifestations, and is crucial in neonates to prevent lethal outcome of disease. In recent years real-time PCR assays were developed for VZV as for CMV and other viruses. Real-time PCR assays appear to have equal sensitivity as VZV nested PCR assays but are faster, easier and have markedly reduced risk for molecular contamination. Because of its high sensitivity compared to other methods, PCR has become the most appropriate method for detection of VZV DNA in AF and other specimens [183,206,209,210]. However, standardization is a major problem and should be done in order to avoid false positive or false negative results.

In a study conducted in our laboratory AF samples were analyzed either by PCR or real-time PCR in parallel to tissue culture isolation. The DNA amplification target was located in...
the viral UL gene. VZV DNA was detected in the amniotic fluid of two (7.4%) out of 27 women who developed chickenpox in the second trimester of pregnancy. One gave birth to a normal child while no follow-up was available for the second woman. All amniotic fluid cell cultures were negative. No case of CVS occurred following negative PCR results (unpublished data).

4.3. Summary

VZV infection during pregnancy poses a risk to the mother and fetus. Laboratory assessment of maternal infection is based on serology (both IgG and IgM), and on virus detection in skin lesions. Exposure of a pregnant woman to a varicella case prompts immediate assessment of her immune status to determine the need for VZIG administration. The availability of a VZV vaccine may encourage the screening of women for VZV immunity before conception.

There are no fully reliable methods to assess fetal infection and damage. Fetal IgM and virus detection in AF are used, but false negative results are common and positive results do not necessarily correlate with fetal damage.

An algorithm describing the laboratory diagnostic assays for VZV infection in pregnancy is shown in Fig. 3.

5. Herpes simplex virus (HSV)

5.1. Introduction

5.1.1. The pathogen

Herpes simplex virus (HSV) establishes latent infection following primary infection which may lead to reactivation. It is a neurotropic member of the herpesvirus family and the genus consists of two types: HSV type 1 (HSV-1) and HSV type 2 (HSV-2).

The consequences of infection with HSV can vary from asymptomatic to the life-threatening diseases manifested as HSV-encephalitis and neonatal herpes [211]. Recurrent infections with one type after primary infection with the other type are common. HSV-1 is usually transmitted through the oral route and causes disease in the upper part of the body, while HSV-2 is a sexually transmitted virus which tends to cause primarily genital herpes. However, both HSV-1 and HSV-2 can cause genital herpes which can be a severe disease in primary episodes [211–214]. An increase in the prevalence of genital herpes infection has been documented worldwide. HSV-2 was the main cause of genital herpes during the 1980s but since the 1990s HSV-1 constitutes an increasing proportion of the cases. This shift from HSV-2 to HSV-1 may have implications for prognosis [215]. Assessment of risk and diagnosis of HSV infections may involve testing for both HSV-1 and HSV-2.

HSV infection has serious consequences for the fetus and neonate. Before 20 weeks of gestation, transplacental transmission can cause spontaneous abortion in up to 25% of the cases [216,217]. Later in pregnancy HSV infections are not associated with increase in spontaneous abortions but intrauterine infections may occur. Symptomatic and asymptomatic first episodes of genital herpes but not asymptomatic recurrent infections are associated with prematurity and fetal growth retardation.

Herpes simplex is a devastating infection in the neonate, with primary asymptomatic and symptomatic maternal infection near delivery carrying a greater risk to the newborn than recurrent infections [218,219]. Forty percent of women who acquired genital HSV during pregnancy but did not complete their seroconversion prior to the time of delivery will infect their newborns. Transmission of genital herpes during vaginal delivery is high in neonates exposed to asymptomatic shedding [220–223], since serial HSV genital cultures during the last few weeks of gestation are no longer recommended as a method to prevent neonatal herpes. Instead, women are examined at the time of labour and caesarean section is carried out only if there is an identifiable lesion. The use of fetal scalp electrodes may also increase the risk for neonatal infection [224,225]. HSV infections should be suspected as the cause of any vesicle appearing in the neonate.

5.1.2. Laboratory assessment of HSV infection in pregnancy and in neonates

Clinical symptoms compatible with genital herpes during pregnancy require laboratory assessment (Fig. 4). Diagnosis of HSV infection relies on both serological and virological methods, but the diagnostic process may be complicated due to the nature of the viral infection. Recurrent infections and reactivations pose a special challenge to serology, and therefore viral detection in genital lesions is more reliable as a diagnostic mean.

The standard laboratory method to confirm current HSV infection is virus isolation and typing in cell culture since HSV grows readily in tissue culture. The virus may be isolated within 2–4 days from swabs taken from herpetic skin and laryngeal or genital lesions (Fig. 4). PCR techniques improved the correct diagnosis of HSV infections especially in cases without clear overt manifestations [226–229] and can detect viral DNA from lesions that are culture negative. For genital swabs it might be too sensitive to reflect true reactivation, and it is not clear whether a positive PCR in a patient with a negative HSV culture reflects a true risk of transmission of the virus. Therefore physicians should interpret results with caution and according to additional criteria. Supplemental serological testing can be used when genital lesions are not apparent.

In primary infections antibodies appear within 4–7 days after infection and reach a peak at 2–4 weeks. Antibodies persist for life with minor fluctuations. Specific IgM antibodies appear after primary infection but may be detectable during recurrent infections as well [226]. HSV-1 and HSV-2 share cross reactive epitopes of the surface glycoproteins which are the major targets of serum antibodies. Therefore it is difficult to identify a newly acquired infection with one HSV type on the background of pre-existing immunity to the other type (usually infection with HSV-1 precedes infection with HSV-2). Type specific serological assays were developed [230] which may be valuable for the management of a pregnant woman and her partner: these assays can identify previous infections, seroconversions and discordant couples. The results may provide information to the pregnant
woman about her risk of acquiring and transmitting HSV to her infant (Fig. 4). The cost-benefit value of general screening for type-specific antibodies in pregnant women and their partners were recently assessed [231,232].

Rapid and sensitive diagnosis of neonatal HSV disease is of utmost importance for initiation of acyclovir treatment and improvement in the outcome of neonatal herpes has been achieved due to the application of PCR as a diagnostic tool [233]. Neonatal HSV infections are confirmed through positive viral culture or PCR from skin, eye lesion, nasopharynx, rectum or CSF and detection of IgM in a neonate is highly significant [234]. Neonatal HSV infection can occur in newborns without the presence of any vesicular skin disease [229,235,236].

5.2. Laboratory assays for assessment of HSV infection and immune status

5.2.1. HSV IgG assays

Several techniques are used for detection of HSV specific IgG, among them CF, NT, IF and ELISA. The principles of these assays were described above for VZV and are shown in Table 1. These assays are used to determine HSV specific IgG directed to HSV-1 or HSV-2. These assays are useful in primary infection in the absence of prior immunity, and they use crude preparations of cells infected with HSV-1 and/or HSV-2. They may detect infection with HSV-2 on the background of HSV-1 [237]. More accurate type-specific assays were developed in order to detect recurrent infections with either type.

5.2.2. HSV type-specific IgG assays

Type specific serological assays vary in their abilities [230]. NT antibody level can be measured specifically for each type by the plaque reduction assay using serial dilutions against a fixed dose of HSV-1 or HSV-2, with 50% reduction in the plaque number as an endpoint. However these assays are accurate only in patients infected with only one virus type. NT assays were used for determination of HSV-2 infection based on the ratio between the antibody titers to the two viruses. Positive cases were those with HSV-2 to HSV-1 ratio of ≥ 1 [237,238].

IF can be used for detection of antibodies to HSV cell surface and internal proteins. IF correlates well with NT titers but subtyping is difficult. ELISA assays utilize a variety of conditions and antigens. These tests are more sensitive than NT and IF and can detect antibodies within the first week of infection. Manufacturers of these tests use mathematical calculations to infer the presence of HSV-1 or HSV-2 antibodies based on their relative ratios. New type-specific tests, which distinguish HSV-2 from HSV-1 are based on glycoproteins gG1 and gG2 [239] and can provide useful information on the etiology of the genital herpes in the symptomatic patient when viral culture and PCR are not helpful. These tests allow the identification of patients with unrecognized genital herpes [240,241]. There are few good commercial ELISA kits for type specific serology which were approved by the FDA. Nevertheless, HSV type specific antibody tests are interpreted in the context of the clinical history, clinical presentation and other laboratory test results for herpes or other possible etiological agents. Finally, Western blotting (WB) for HSV is the most accurate method for type-specific serology [241]. This method is the serology gold standard and is performed only by few laboratories. WB is expensive to perform and requires 2–5 days for completion. New approaches to antigen preparation include the application of recombinant gG1 and gG2 from a mammalian expression systems to nitrocellulose, or purification of glycoprotein gG2 by lectin chromatography [242].

5.2.3. HSV IgM assays

These assays are based primarily on ELISA and IF methods as described for VZV. Due to the persistent nature of the viral infection and the frequency of recurrent infections, the presence of IgM is of high value primarily in neonates and infants. In most cases testing for IgM must be accompanied by testing for IgG, since a negative IgM result does not rule out recent infection.

5.2.4. Virus isolation in tissue culture

HSV is a labile virus, and successful virus culturing from clinical specimens depends on appropriate sample collection and maintenance of the cold chain. Swabs should be placed into viral transport medium and other samples should be placed in a sterile container. Rapid transportation of specimens to the laboratory and avoiding freeze-thawing cycles results in more than 90% sensitivity of culturing from skin lesions. However, poor sample quality due to inappropriate sampling or compromised transport conditions may reduce sensitivity by 20% for primary episodes and down to 50% for recurrent episodes even if herpetic lesions are present. When an appropriate sensitive cell line (such as Vero cells) is used, appearance of a characteristic CPE within 18–96 h after inoculation points to the presence of active HSV [237]. Direct immunofluorescence detection of HSV in cultures with CPE is regarded as the standard for confirmation of HSV presence.

The so-called “Shell vial” method, described earlier for CMV isolation (chapter 2.5 and Table 1) shortens the time required to identify HSV in specimens to 24–48 h. An innovated commercially available diagnostic test for rapid detection of HSV in tissue culture is the ELVIS HSV test kit. It utilizes a recombinant cell system in which a reporter enzyme is quickly accumulated inside the infected cells and is detected by intense blue color. The test is sensitive and specific and detects both HSV-1 and HSV-2 [243]. The kit is expensive and is used only by few laboratories.

5.2.5. Direct antigen detection of HSV

Other rapid assays such as the direct fluorescent assay (DFA) or enzyme immunoassays (EIA) performed directly on smears of cells recovered from lesions are rapid but less sensitive than virus culturing. Accordingly, for detection of virus in genital swabs they may have similar sensitivity to viral culture for symptomatic shedding but have reduced sensitivity for detecting asymptomatic viral shedding.

5.2.6. Detection of HSV DNA by PCR

Methods for detection of HSV DNA, particularly PCR techniques (PCR, nested PCR and real-time PCR) were described above for other viruses. They are highly sensitive especially for
samples with very low concentration of virus, as was demonstrated by numerous research studies. The performance of PCR analysis is dependent upon the quality of the specimen. The reported sensitivity for PCR is 75–100% for the diagnosis of CNS HSV infection [228,244,245]. The reason for the broad range in sensitivity is the differences in methodologies and variability in performance of PCR between laboratories. Standardization using reference samples to assure identical results is not yet in place [227].

5.5. Summary

HSV-2 is the main type involved in congenital and neonatal infection, although genital HSV-1 infections have become more common in recent years. Primary maternal symptomatic and asymptomatic infections may cause a serious risk of abortion and fetal growth retardation, while infection during delivery may cause a devastating neonatal disease. Laboratory assessment of maternal infection and immune status as well as neonatal infection is critical for timely management and treatment. Virus isolation from genital lesions is the primary tool for assessment of maternal infection. Serological assays are useful but are complicated by pre-existing heterotypic antibodies. Type-specific IgG assays must be applied for determination of recurrent maternal infections.

Virus isolation, antigen detection or DNA detection by PCR in skin lesions samples are additional tools for rapid and sensitive diagnosis of symptomatic current infection.

Prenatal diagnosis is not common and has not been assessed for sensitivity and specificity. Neonatal infection is diagnosed by virus culturing or PCR in skin lesions or other clinical specimens in case of a disseminated disease, and by detection of IgM antibodies.

An algorithm describing the laboratory diagnostic assays for HSV infection in pregnant women and neonates is shown in Fig. 4.

6. Parvovirus B19

6.1. Introduction

6.1.1. The pathogen

Human parvovirus B19 (B19) can infect humans at all ages. It is a member of the family Paroviridae, genus Erythrovirus which comprise small, non-enveloped viruses containing linear single-stranded DNA genomes. Most of the humoral immune response is directed to the two capsid proteins VP1 and VP2 [246]. The seroprevalence is 50% by age 15 and exceeds 80% by age 70 [247]. Childhood infection is mild leading to the disease erythema infectious (“fifth disease”) [248–251]. In adults the disease is not yet in place [227].

Diagnosis of parvovirus B19 is critical for timely management and laboratory testing is an essential part of the diagnosis [274–279].

6.1.2. Laboratory assessment of parvovirus B19 infection in pregnancy

Laboratory diagnosis of parvovirus B19 infection has been complicated by the nature of the virus, the viral infection process and the immune response. Parvovirus B19 is a fastidious virus which cannot be grown in regular continuous cell lines. In those cells where it grows it replicates poorly and the virus yield is minimal, thus viral culturing is not a diagnostic option and cannot be used for NT assays or for preparation of native viral antigens for serology [261,280,281].

Assessment of parvovirus B19 infection in pregnant women relies primarily on serology (Fig. 5). During acute infection IgM antibodies appear 7–14 days from infection with Parvovirus B19 and may last for 6 month or even longer. IgG antibodies appear several days after IgM and persist for life. The antibodies produced specifically react with both structural and non-structural viral proteins and recognize linear and conformational epitopes of the capsid proteins VP1 and VP2 [282–288]. The antibodies recognizing conformational epitopes last longer for both IgM [285] and IgG [282,284,286], and IgG antibodies recognizing linear epitopes disappear around 6 month post infection. This creates technical problems for serological assays as described below.

Maternal infection during pregnancy is frequently asymptomatic and by the time of fetal infection and symptoms, 2–12 weeks after maternal infection, she may have high IgG and no IgM responses [280,275,289,290]. Viral DNA detection by highly sensitive molecular assays such as dot-blot hybridization and PCR can be applied to maternal serum or whole blood [276,291]. However, parvoviruses are known for their ability to integrate into the host genome and to establish persistent infections [292–296]. Recent studies [297–299] showed that B19 DNA can be detected in serum samples by highly sensitive methods up to 6 months after acute phase viremia, although quantitative assays showed that the amount of DNA present decreased with time [300]. It has also been shown that 0.01–0.03% of the healthy blood donors have B19 DNA detected in their serum [301–303]. Thus, detection of B19 DNA in maternal blood by PCR might not be related to recent infection.

6.1.3. Prenatal laboratory assessment of congenital B19 infection

In many cases congenital B19 infection is suspected only when typical fetal abnormalities are observed. Since the fetus...
does not always develop IgM it is necessary to detect the virus itself in fetal samples. B19 cannot be cultivated and therefore detection of viral DNA is sought. The type of specimen and the detection methods are still controversial: fetal blood and AF are the most common specimens obtained and the most highly sensitive molecular methods are employed. However, 10–25% of the specimens from asymptomatic fetuses may have B19 DNA [289,290]. This drives the need to develop quantitative molecular assays which can determine viral load and possibly associate it with the risk to the fetus. Those assays include quantitative PCR-ELISA, in situ hybridization and rt-PCR. The latter has advantages which will be discussed below [304–308].

6.2. Laboratory assays for assessment of parvovirus B19 infection

6.2.1. B19 IgM and IgG assays
Several commercial tests have been developed on the basis of recombinant and synthetic antigens [309–311]. These recombinant antigens frequently lack important conformational epitopes reducing the sensitivity and specificity of the assays. Moreover, “gold standard” methods like virus NT or HI assays are absent, and it is difficult to assess the sensitivity and specificity of the serological assays. The variation in specificity and sensitivity among commercial assays is high, and discordant results are often obtained indicating false positive and false negative responses [314–317].

Studies aimed at evaluating the sensitivity and specificity of various such assays were mostly based on comparative evaluation, using samples selected on the basis of clinical diagnosis. In a study conducted in Norway [315], five commercial ELISA and IF IgM kits were comparatively evaluated in three groups of patients. The calculated specificities for the kits were 70.1–94.8%, but the authors refrained from determining sensitivity because of the absence of suitable reference methods. In an another study conducted in Sweden [316], four commercial IgM assays were evaluated in comparison to an IgM antibody capture radioimmunoassay as a reference method [318]. The calculated sensitivity varied between 90% and 97% and the specificities varied from 88% to 96%.

In a third study conducted in the USA [314] three ELISA systems utilizing one or more conformational antigens for detection of B19 IgM or IgG in sera of 198 pregnant women were comparatively evaluated. Agreement with the consensus results varied from 92.3% to 100% for IgM and from 97.9% to 99.5% for IgG. The authors note the high agreement in this study compared to their previous study [312] relating to the fact that the antigens utilized in the two studies were substantially different: the earlier study used a linear VP1 antigen while the latter one used a conformational VP2 antigen.

A study designed to evaluate the most appropriate assays for IgG detection was conducted in Italy [313] which compared the performance of three commercial assays using different antigens: (a) an ELISA assay using VP1 + VP2 recombinant native conformational antigens, (b) an ELISA assay using VP2 recombinant native conformational epitopes, (c) a Western blot assay using denatured linear antigens. Four hundred and forty six serum samples from blood donors with no IgM were tested. Overall, 353 sera were found positive by all methods combined. Of those 98.6%, 94.6% and 89% were positive by assays a, b and c, respectively. Some sera reacted only with conformational epitopes. The results of this study underscore the need to use ELISA-IgG assays which include conformational epitopes of both VP1 and VP2 or VP2 alone, and demonstrate the lower sensitivity of the Western blot assay.

In conclusion, when evaluating recent or past infections in pregnant women it is important to use well-established ELISA assays, and in cases of discrepancy between the test results and the clinical and epidemiological circumstances, confirmation must be sought using other commercial assays as well as supportive molecular assays discussed next.

6.2.2. Detection of viral DNA in maternal and fetal specimens
Due to the limitations of the serological assays described above and since viremia is long-lasting in B19 infections, molecular assays for detection of B19 viral DNA were developed early. The methods most frequently used are DNA hybridization and PCR.

Molecular hybridization assays employing DNA probes derived from most of the viral genome are labeled with 32P, biotin or digoxigenin [319–322] and are capable of detecting approximately 10⁻⁶–10⁻¹⁰ genome copies/ml [275,323]. This assay is sensitive enough to detect viral DNA in serum during peak viremia since the viral load exceeds 10¹⁰ genome copies/ml, but low levels of viremia may be missed [275,296,298]. The PCR assays, developed later relied on a variety of primer sets derived from different genomic regions and were capable of detecting 10⁻⁵–10⁻¹⁰ genome copies/ml [324,325]. The nested PCR, the PCR-ELISA and the PCR-hybridization assays increased sensitivity and moved the detection limit down to 1–10 genome copies/ml [291,292,296,297,299,326–328]. By using those highly sensitive methods it was revealed that in many cases serology failed to detect maternal or fetal infections, as some seronegative mothers as well as fetuses turned out positive in the DNA detection assays. This finding was not surprising in view of the known limitations of the serological assays. The ability to detect maternal and fetal infection by the highly sensitive molecular methods allowed correct diagnosis of previously unresolved cases, particularly cases of intrauterine infection which did not result in fetal hydrops or were completely subclinical [275,276,280,282,296,297,324,327,329]. However, as stated above, low levels of B19 DNA in maternal blood can be unrelated to recent infection.

The use of various techniques without standardization, the epidemiologically variable circumstances (epidemic vs non-epidemic years), and the use of different primer sets and clinical specimens led to a high variability in the sensitivity, specificity and interpretation of the assays results. Moreover, new genotypes discovered recently were missed by common primer sets. Comparison of IgM and DNA detection by PCR in fetal blood was conducted in a study done on 57 pregnant women and their fetuses who had abnormal ultrasonography [327]. Viral DNA was found by PCR in 16 out of 58 fetuses (27%) while IgM was
detected only in 7 (12.3%). Two fetuses had false-positive IgM result, not supported by any other findings. Other researchers [276] compared maternal IgM to fetal serum or AF PCR results in 56 women at high risk for B19 infection. They found positive PCR in 24 IgM-negative/IgG-positive and 4 seronegative (total 50%) out of, in addition to positive PCR in 15 (26%) of IgM-positive women. Another group [282] reported detection of viral DNA in fetal serum or AF by a PCR-hybridization assay in 11 out of 80 cases of fetal hydrops (14%) while maternal IgM antibodies were detected only in 3 (3.7%). Finally, a comprehensive prospective study in 18 fetal hydrops cases conducted in Italy [275] examined maternal serum, fetal cord blood and amniotic fluid using nested PCR, dot-blot hybridization and in situ hybridization (ISH). The results showed that the ISH assay in fetal blood cells was 100% sensitive while the other methods missed few to many cases. The conclusion drawn from this study is that various assays have complementary roles, and reliable diagnosis can be achieved only by a combination of serological and molecular assays done on maternal and fetal samples as outlined in Fig. 5.

6.2.3. Quantitative assays for detection of viral DNA

The need to determine the quantity of B19 viral DNA present in clinical samples (viral load) arose because B19 can establish long-lasting persistent infection in immunocompetent individuals. It is not clear if low viral loads reflect whole genomes or viable infectious virus particles, or only pieces of viral DNA. Studies in seronegative plasma-pool recipients showed that only recipients of plasma containing >10^7 genome copies/ml became infected or seroconverted [305]. B19 DNA can also be detected in solid organ tissues by PCR for years [323,330,331]. As noted earlier viral DNA can be detected in fetal tissue or blood in the absence of any detectable congenital abnormalities, and the outcome of detectable fetal infection, including fetal hydrops, varies from spontaneous resolution to still-birth.

Real-time PCR assays developed recently are equivalent to or more sensitive than nested PCR and PCR-ELISA but are much less prone to molecular contamination and produce a quantitative result which can be standardized and automated [304,306–308,332]. In a retrospective study, Knoll et al. [332] used real-time PCR to investigate the viral load in paired samples from mothers and their abnormal fetuses, and from mothers with normal fetuses who were exposed to B19. The viral load in the maternal serum ranged from 7.2 × 10^3 to 2.6 × 10^3. The authors did not report statistically significant correlation between maternal or fetal viral load and fetal condition. However, they noted that they could not exclude a correlation between peak maternal viremia levels and fetal condition since most of the mothers were not aware of their infection until onset of fetal symptoms, and their serum was collected after peak viremia. Although the viral load in fetal sera was higher than in AF samples, the rt-PCR assay detected all positive cases using either one of these fetal specimens. The authors recommend testing AF rather than fetal blood because AF can be drawn earlier, is simpler to obtain and less risky to the fetus. Clearly, more prospective studies are necessary on larger groups of patients to learn more about the association between viral load and pregnancy outcome.

6.3. Summary

Parvovirus B19 infection during pregnancy may cause fetal damage resulting in fetal loss. Early diagnosis of maternal infection will allow fetal assessment and treatment by intravascular blood transfusion. Unfortunately, mothers often are not aware of their infection until fetal damage is observed.

Confirmation of B19 infection requires laboratory assessment, which is complicated by the nature of the viral infection and immune response. Serology, performed by using ELISA assays rely on recombinant antigens and concordance is low among all commercial assays available. In the absence of a “gold standard” assay false positive and false negative results prevail. Virus culturing is impossible and virus detection is based on various molecular assays.

In spite of several studies there is no consensus regarding the most appropriate clinical specimen and method for detection of viral DNA. Currently, on practical grounds, it is recommended to use ELISA IgM and IgG assays based on recombinant conformational epitopes of VP1 and VP2 or VP2 alone, and to use AF or fetal serum for detection of fetal infection by the most sensitive molecular methods available (nested PCR or rt-PCR). Since B19 may establish long lasting infection in the absence of symptoms, interpretation of viral DNA detection in maternal blood is difficult. Assessment of fetal infection and risk should rely on the clinical situation and other prenatal diagnostic means.

An algorithm describing the most practical approach to laboratory assessment of B19 infection in pregnancy is shown in Fig. 5.

7. Human immunodeficiency virus

7.1. Introduction

Human immunodeficiency virus (HIV) is a retrovirus that infects helper T cells of the immune system causing a progressive reduction in their numbers, and eventually acquired immunodeficiency syndrome (AIDS). HIV is a member of the Retroviridae family [333], genus Lentivirus (or “slow” viruses). The course of infection with these viruses is characterized by a long interval between initial infection and the onset of serious symptoms. The single-stranded RNA viruses exploit their reverse transcriptase enzyme to synthesize DNA using their RNA as a template. The DNA is then incorporated into the genome of infected cells.

AIDS was first diagnosed in European sailors with African connections [334–336]. The first AIDS virus, HIV-1, was initially identified in 1983 [337–341]. A second AIDS virus, HIV-2, was discovered in 1986 [342–344]. Forty million people were estimated to be infected with HIV at the end of 2004 [345]. The highest prevalence is found in Sub-Saharan Africa and it is rising mainly in Asia and some of the former Soviet Union countries like Ukraine and the Russian Federation [345,346]. During its spread among humans, group M HIV-1 (one of three groups: M, O and M), has evolved into multiple subtypes that differ from one another by 10–30% along their genomes [347–350].
HIV is passed on primarily via four routes: unprotected sexual intercourse (both homosexual and heterosexual), sharing of needles by IV drug users, medical procedures using HIV-contaminated blood, tissues or equipment, and mother-to-child transmission (MTCT). The likelihood of transmission is increased by factors that may damage mucosal linings of exposed tissues, especially by other sexually transmitted diseases that cause ulcers or inflammation.

HIV can be transmitted from infected mothers to infants during pregnancy (intrauterine) through placental passage of the virus [351], during labor and delivery (intrapartum) through exposure to infected maternal fluids (blood or vaginal secretions) [352–355] and during the post partum period through breastfeeding [356–359]. Infants who have a positive virologic test (see below) at or before age 48 h are considered to have early (i.e., intrauterine) infection, whereas infants who have a negative virologic test during the first week of life and subsequent positive tests are considered to have late (i.e., intrapartum) infection [360]. In the absence of breast-feeding, intrauterine transmission accounts for 10–35% of infection, and 60–75% of transmission occurs during labor and delivery [361–363]. Without intervention, maternal infection leads to about 25–30% of babies being infected [364–369].

Use of anti-retroviral therapy (ART) during pregnancy in HIV-infected women is associated with improved obstetric outcome of reduced infection rates of babies to less than 2% [370–373] and little maternal toxicity [374–376]. The current US guidelines are to offer all pregnant HIV-1-infected women highly active antiretroviral therapy (HAART) to maximally suppress viral replication, reduce the risk of prenatal transmission, and minimize the risk of development of resistant virus. In addition, HIV-infected women are offered an elective cesarean section delivery [355]. The results also suggest that special attention should be given to women belonging to previously identified risk groups.

Because testing has proven very successful in helping to prevent the spread of the disease to babies, a US federal panel has recommended that all pregnant women, not just those considered at high risk, be screened for the AIDS virus [377–381]. No prenatal (intrauterine) diagnosis is performed because: (a) the infection can occur during delivery and (b) the testing intervention may increase the chance of virus transmission to the baby.

Assessing the infection status of the mother is critical [381] and late (i.e., intrapartum) infection leads to about 25–30% of babies being infected [382]. Knowledge of maternal HIV infection during the antenatal period enables HIV-infected women to receive appropriate antiretroviral therapy during pregnancy, during labor, and to newborns to reduce the risk for HIV transmission from mother to child [370,383,384]. It also allows counseling of infected women about the risks for HIV transmission through breast milk and advising against breast feeding in countries where safe alternatives to breast milk are available [385]. Early diagnostic evaluation of HIV-exposed infants permits early initiation of aggressive antiretroviral therapy in infected infants and institution of prophylaxis against Pneumocystis carinii pneumonia (PCP) in all HIV-exposed infants beginning at age 4–6 weeks in accordance with Public Health Services (PHS) guidelines [386].

7.1.2. Importance of laboratory assessment of HIV infection in pregnancy

Identification of HIV-infected women before or during pregnancy is critical to providing optimal therapy for both infected women and their children and to preventing perinatal transmission (see below). For women with unknown HIV status during active labor, ART can still be effective when given during labor and delivery, followed by treatment of the newborn [382]. This expedited intervention requires the use of rapid diagnostic testing during labor or rapid return of results from standard testing.

7.1.3. Prenatal laboratory assessment of HIV infection

Prenatal laboratory assessment of congenital HIV infection in AF or cord blood is not recommended as the invasive procedures increases the risk of transmitting the virus from the maternal to the fetal blood stream.

7.2. Laboratory assessment of HIV infection

7.2.1. HIV antibody assays

The initial screening for HIV infection in adults and children is done by testing for antibodies. Viral load assays are not intended for routine diagnosis but could be used in clinical management of HIV-infected persons in conjunction with clinical signs and symptoms and other laboratory markers of disease progression. Detection of HIV-1 p24 antigen (Table 1) is used for routine screening in blood and plasma centers but their routine use for diagnosing HIV infection in individuals has been discouraged because the estimated average time from detection of p24 antigen to detection of HIV antibody by standard enzyme-immuno-assay (EIA) is 6 days, and not all recently infected persons have detectable levels of p24 antigen [387]. In the USA several FDA approved tests are available that enable the testing of HIV antibodies in different body fluids such as whole blood, serum, plasma, oral fluid and urine.

The standard testing algorithm for HIV-1 consists of initial screening with an EIA to detect antibodies to the virus. Reactive specimens undergo confirmatory testing with a more specific supplemental test, usually a Western blot assay (WB) or, less commonly, IFA (Table 1) [388]. Using both tests increases accuracy of the results while maintaining their sensitivity [389–391]. Only specimens that are repeatedly reactive by EIA and positive by IFA or reactive by WB are considered HIV-positive and indicative of HIV infection [389,390,392,393].

Incomplete antibody responses that produce negative or indeterminate results on WB tests can occur among persons recently infected with HIV who have low levels of detectable antibodies (i.e., seroconverting), persons who have end-stage HIV disease, and perinatally exposed but uninfected infants who are seroreverting (i.e., losing maternal antibody). Non-specific reactions producing indeterminate results in uninfected persons have occurred more frequently among pregnant women than among other persons [389–391,394].

False-positive WB results are rare [395].
7.2.2. Detection of viral DNA in maternal and newborn specimens

7.2.2.1. Diagnosis of HIV infection in maternal specimens. Pregnant women are screened for the presence of antibodies using the standard testing algorithm for adults (EIA plus WB or IFA) as shown in Fig. 6. For women with unknown HIV status during active labor, rapid diagnostic tests are used if rapid return of results from standard testing is not available.

7.2.2.2. Diagnosis of HIV infection in newborns. Infant HIV testing should be done as soon after birth as possible so appropriate treatment interventions can be implemented quickly [377,384]. The standard antibody assays used for older children and adults are not useful for diagnosing children younger than 18 months as the presence of maternal antibodies makes serologic tests uninformative. Therefore, a definitive diagnosis of HIV infection in early infancy requires viral diagnostic assays, including HIV-1 p24 antigen assays, nucleic acid amplification (e.g., PCR) or viral culture. HIV infection can be definitively diagnosed in most infected infants by age 1 month and in virtually all infected infants by age 6 months. HIV infection is diagnosed by at least two positive assays using two separate specimens [378].

HIV DNA PCR is the preferred virologic method for diagnosing HIV infection during infancy. It has 99% specificity to identify HIV proviral DNA in peripheral blood mononuclear cells (PBMC) obtained from whole blood samples collected in EDTA-containing tubes [396].

A meta-analysis of published data from 271 infected children indicated that HIV DNA PCR was sensitive for the diagnosis of HIV infection during the neonatal period. Thirty-eight percent of infected children had positive HIV DNA PCR tests by age 48 h, 93% by age 14 days and 96% by age 28 days. No substantial change in sensitivity during the first week of life was observed, but sensitivity increased rapidly during the second week.

Quantitative assays that detect HIV RNA in plasma (see Section 7.2.2.3 below) appear to be as sensitive as HIV DNA PCR for early diagnosis of HIV infection in HIV-exposed infants [397–402]. The specificity is comparable between the two tests, but results of HIV RNA load below 10^3 copies/ml should be interpreted with caution [403]. Some clinicians use HIV RNA assay as the confirmatory test for infants testing HIV DNA PCR positive since it provides viral load measurement which guides treatment decisions. Available quantitative RNA tests include the Amplicor HIV-1 monitor test 1.5 (Roche Diagnostics), the NASBA EasyQ HIV-1 (BioMerieux), the Quantiplex HIV RNA 3.0 (bDNA) (Bayer) and the LCx HIV RNA quantitative assay (Abbott Laboratories) assays [416–421]. However, special attention should be taken where non-B HIV is expected.

HIV culture for the diagnosis of infection has a sensitivity that is similar to that of HIV DNA PCR [404]. However, HIV culture is more complex and expensive to perform than DNA PCR, and definitive results may not be available for 2–4 weeks. Both standard and immune-complex-dissociated p24 antigen tests are highly specific for HIV infection and have been used to diagnose infection in children. However, the use of p24 antigen testing alone is not recommended because of its substantiality-related reduced sensitivity and specificity compromising the critical need for timely diagnosis [405].

Whether the current, more intensive antiretroviral combination regimens women may receive during pregnancy for treatment of their own HIV infection will affect diagnostic test sensitivity in their infants is unknown. Similarly, if more complex regimens are administered to HIV-exposed infants for perinatal prophylaxis, the sensitivity of diagnostic assays will need to be re-examined [401].

7.2.2.3. Different subtypes. HIV subtype B is the predominant viral subtype found in the U.S. and western Europe. Non-subtype B viruses predominate in other parts of the world, such as subtype C in regions of Africa and India and subtype E in much of southeast Asia.

Currently the available HIV DNA PCR commercial tests are less sensitive for detection for non-subtype B HIV, and false negative HIV DNA PCR assays have been reported in infants infected with non-subtype B HIV [406–410]. Caution should be exercised in the interpretation of negative HIV DNA PCR test results in infants born to mothers who may have acquired an HIV non-B subtype. Some of the currently available HIV RNA assays have improved sensitivity for detection of non-subtype B HIV infection [411–413], although even these assays may not detect some non-B subtypes, particularly group O HIV strains [414]. In cases of infants where non-subtype B perinatal exposure may be suspected and HIV DNA PCR is negative, repeat testing using one of the newer RNA assays shown to be more sensitive for non-subtype B HIV is recommended for example, the Amplicor HIV-1 monitor test 1.5, Nuclisens HIV-1 qt or Quantiplex HIV RNA 3.0 (bDNA) assays. In children with negative HIV DNA PCR and RNA assays but in whom non-subtype B infection continues to be suspected, the clinician should consult with an expert in pediatric HIV infection and the child should undergo close clinical monitoring and definitive HIV serologic testing at 18 months of age.

7.2.2.4. Test algorithm for neonates. HIV infection is diagnosed by two virological tests performed on separate blood samples, regardless of age. The testing rules are summarized below and shown in Fig. 6:

1. Initial testing is recommended by age 48 h. As many as 40% of infected infants can be identified at this time. Blood samples from the umbilical cord should not be used for diagnostic evaluations, because of concerns regarding potential contamination with maternal blood.

2. Repeated diagnostic testing can also be considered at age 14 days in infants with negative tests at birth. The diagnostic sensitivity of virological assays increases rapidly by age 2 weeks. Early identification of infection would permit discontinuation of neonatal ZDV chemoprophylaxis and a further evaluation of the need for more aggressive drug combination therapy.

3. Retest infants with initially negative virological tests at age 1–2 months. Using ZDV monotherapy to reduce perinatal
transmission did not delay the detection of HIV in infants in PACTG protocol 076 [370,409–415].

(4) At age 3–6 months retest HIV-exposed children who have had repeatedly negative virological assays at birth and at age 1–2 months.

(5) HIV infection can be reasonably excluded in non-breast fed infants with two or more negative virologic tests performed at age >1 month, with one of those being performed at age >4 months [386].

(6) Two or more negative HIV immunoglobulin G (IgG) antibody tests performed at age >6 months with an interval of at least 1 month between the tests can also be used to reasonably exclude HIV infection in HIV-exposed children with no clinical or virologic laboratory evidence of HIV infection.

(7) Serology after 12 months is recommended to confirm that maternal HIV antibodies transferred to the infant in utero have disappeared if there has not been previous confirmation of two negative antibody tests.

(8) If the child is still antibody-positive at 12 months, then testing should be repeated between 15 and 18 months [387]. Loss of HIV antibody in a child with previously negative HIV DNA PCR tests definitively confirms that the child is HIV uninfected.

(9) A positive HIV antibody test at >18 months of age indicates HIV infection [378].

7.3. Summary

Identification of HIV-infected women before or during pregnancy is critical to providing optimal therapy for both infected women and their children and to preventing perinatal transmission. Pregnant women are screened for the presence of antibodies using the standard testing algorithm for adults (EIA plus WB or IFA). Prenatal laboratory assessment of congenital HIV infection is not recommended as it increases the risk of infecting the fetus, but extensive testing is performed to assess the infection status of the baby following delivery. A definitive diagnosis of HIV infection in early infancy requires repeated testing using virological assays, with HIV DNA PCR being the currently preferred method. All infected infants can be definitively diagnosed or IFA). Prenatal laboratory assessment of congenital HIV infection is not recommended as it increases the risk of infecting the fetus, but extensive testing is performed to assess the infection status of the baby following delivery. A definitive diagnosis of HIV infection in early infancy requires repeated testing using virological assays, with HIV DNA PCR being the currently preferred method. All infected infants can be definitively diagnosed or excluded with clinical or virologic laboratory evidence of HIV infection.

(6) Two or more negative HIV immunoglobulin G (IgG) antibody tests performed at age >6 months with an interval of at least 1 month between the tests can also be used to reasonably exclude HIV infection in HIV-exposed children with no clinical or virologic laboratory evidence of HIV infection.

(7) Serology after 12 months is recommended to confirm that maternal HIV antibodies transferred to the infant in utero have disappeared if there has not been previous confirmation of two negative antibody tests.

(8) If the child is still antibody-positive at 12 months, then testing should be repeated between 15 and 18 months [387]. Loss of HIV antibody in a child with previously negative HIV DNA PCR tests definitively confirms that the child is HIV uninfected.

(9) A positive HIV antibody test at >18 months of age indicates HIV infection [378].

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