

Review

# Pathogenesis and prenatal diagnosis of human cytomegalovirus infection

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## Abstract

Congenital human cytomegalovirus (HCMV) infection is the leading infectious cause of mental retardation and sensorineural deafness. Intrauterine transmission and adverse outcome are mainly related to primary maternal infection. Mechanisms of intrauterine transmission are slowly being unraveled and compelling evidence of the importance of using HCMV clinical strains rather than laboratory-adapted strains for in vitro studies is growing. In the absence of a vaccine or a specific antiviral therapy which could be safely administered to pregnant women with primary HCMV infection, the option of prenatal diagnosis has a crucial role in the management of pregnancy complicated by primary HCMV infection. Reliability of prenatal results, however, is still a major concern presenting the risk of either false-negative or false-positive results. However, as more light is shed on the natural history of HCMV infection during pregnancy and fetal life, the predictive value of negative prenatal diagnosis results is becoming more defined, thus improving the quality of counseling. In addition, the availability of different assays for detection of HCMV in both fetal blood and amniotic fluid samples will eventually reduce the risk of false-positive results. Finally, the identification of reliable prognostic markers of fetal disease remains the ultimate goal and a major challenge.

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## 1. Introduction

Human cytomegalovirus (HCMV) or human herpesvirus 5 (HHV-5) is a member of the Herpesviridae family, sub-family Betaherpesvirinae. HCMV is ubiquitous and highly species-specific. HCMV establishes a lifelong relationship with the infected host (once infected, always infected) resulting in primary infection, lifelong latency, and intermittent excretion following reactivation. Such a remarkable property constitutes the basis for the peculiar natural history of HCMV in pregnancy. Well known additional biological features of HCMV include the ability to infect in vivo a broad spectrum of cells (fibroblasts, epithelial and endothelial cells, macrophages, muscle cells) (Sinzger et al., 1995), whereas in vitro human fibroblasts represent the cell system of choice for diagnostic and research purposes. In addition, HCMV can be detected in and isolated from peripheral blood polymorphonuclear leukocytes and monocytes during the viremic phase of primary infection in immunocompetent subjects (Revello et al., 1998a; Rinaldo et al.,

1977), and of both primary and recurrent infections in immunocompromised patients (Gerna et al., 1991). However, leukocytes do not seem to support complete viral replication which, in these cells, does not proceed beyond the expression of immediate early (IE) genes (Gerna et al., 2000). On the other hand, circulating endothelial cells fully permissive to HCMV infection can be detected in peripheral blood of immunocompromised patients undergoing severe disseminated HCMV infection (Grefte et al., 1993; Percivalle et al., 1993).

HCMV replication cycle in vitro is relatively long compared to other herpesviruses such as herpes simplex, and proceeds through an ordinate cascade of subsequent phases (IE, early and late), each one controlling the progression into the next step. These biological features, i.e. a slow and step-wise replication cycle, lead to two practical implications, the first being that the development of a visible cytopathic effect in cell cultures, particularly in the presence of low amounts of infectious virus, may require a considerable period of time (even weeks), thus slowing diagnosis based on conventional virus isolation. On the other hand, the availability of monoclonal antibodies (MAb) to the IE viral protein p72 in conjunction with low-speed centrifugation of clinical specimens onto fibroblast cell monolayers, has reduced the time

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required for virus isolation to 16–18 h following inoculation of clinical samples (Gleaves et al., 1984).

HCMV genome is among the largest and most complex animal virus genomes encoding for more than 200 potential proteins. Thus far, the only HCMV strain that has been completely sequenced is the laboratory-adapted AD169 strain. However, AD169 can hardly be considered a prototype. In fact, the virus was highly passaged for the specific purpose of obtaining an attenuated strain to be used for vaccination, resulting in extensive genetic and phenotype modifications. In particular, large deletions in the ULb' region have been reported with respect to clinical strains (Cha et al., 1996). Importantly and very recently, the genetic determinants of endothelial cell and leukocyte tropism of HCMV (two properties absent in AD169) have been mapped in the ULb' 131–128 locus (Hahn et al., submitted for publication). These findings indicate that results obtained *in vitro* by using AD169 may not be entirely representative of the behavior of wild strains.

## 2. HCMV infection in pregnancy

Intrauterine transmission of HCMV occurs as a result of maternal infection. Quite remarkably and unlike other infectious agents such as rubella or toxoplasma, fetal infection can ensue following both primary and recurrent (either reactivation of endogenous virus or reinfection with a new virus strain) HCMV maternal infection, albeit the difference in the incidence of transmission is remarkable. While during primary infections the antiviral immune response starts following virus transmission to the fetus, during reactivated infections virus replication occurs in the presence of both humoral and cell-mediated immune response. As a result, viremia occurs as a rule only in primary infections. As a consequence, the transmission rate is about 40% following primary infection of the mother and only about 1% in case of recurrent maternal infection (Stagno, 2001). Primary HCMV

infections carry the highest risk for symptomatic congenital infection, whereas congenital infections in infants born to mothers with preconceptual immunity are less likely to be symptomatic at birth (Fowler et al., 1992). However, there is increasing evidence that the incidence of symptomatic infection in infants born to immune mothers may be higher than previously thought. Recently, the possibility that recurrences and unfavorable outcome might be related to reinfection by a new viral strain has been suggested (Boppana et al., 2001). Overall, the incidence of congenital HCMV infection in all newborns varies between 0.2% and 2.2% depending on the seroprevalence of the population examined (Stagno et al., 1982).

Another intriguing aspect of the natural history of HCMV infection during pregnancy concerns the transmission rate during gestation. In particular, while primary HCMV infection acquired either before or around conception carries the lowest risk of transmission (Revello et al., 2002), maternal infections acquired during the first and second trimester of gestation can be transmitted at a similar rate (Fig. 1). On the other hand, during the third trimester, maternal infection has the highest probability of being transmitted to the fetus. These data clearly indicate that: (i) the virus is efficiently transmitted from mother to fetus despite the presence of an innate barrier; (ii) mechanisms of protection are more effective during the first two-thirds of gestation becoming less effective late during pregnancy. Since placental infection has been detected either in the presence or absence of fetal infection, placenta is considered the most important site of either protection (by sheltering the fetus from HCMV infection) or transmission (by acting as a viral reservoir and allowing the infection to reach the fetal compartment).

The present review will focus on most recent advances in the pathogenesis and prenatal diagnosis of congenital HCMV infection, following fetal blood sampling and amniocentesis. The diagnostic value of different assays will be examined with respect to virus transmission to the fetus.

### Transmission of HCMV infection according to gestational age

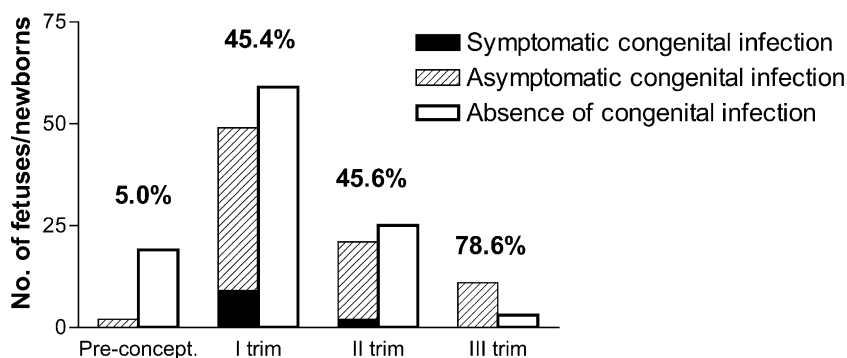


Fig. 1. Frequency of intrauterine transmission following primary maternal infection at different stages of gestation (Revello and Gerna, unpublished).

### 3. Mechanisms of intrauterine HCMV transmission

Maternal viremia, placental infection, and hematogenous dissemination to the fetus is the most likely sequence of events leading to congenital HCMV infection after primary maternal infection. Indeed, viremia has been detected only during primary infection being consistently absent or undetectable in immunocompetent subjects undergoing HCMV reactivation (Revello et al., 1998a). On the other hand, no data are available concerning virus presence in blood of immunocompetent subjects undergoing HCMV reinfection. During the viremic phase, the virus circulates and disseminates carried by leukocytes. While viral DNA can be detected in plasma, cell-free infectious virus is not found in blood. HCMV viremia, as assessed by DNA or IEmRNA determination, can be detected for several months following primary infection in both pregnant (Revello et al., 1998a, 2001) and non-pregnant immunocompetent subjects (Zanghellini et al., 1999) despite the presence of substantial humoral immunity (Fig. 2).

Little is known about the mechanism by which HCMV crosses the placental barrier. It has been reported that placental but not fetal infection can be detected in about 15% of women with primary infection who abort spontaneously early during pregnancy. On the other hand, at later gestational times, placental infection is consistently associated to fetal infection (Mühlemann et al., 1992). In human placenta, floating villi, bathed by maternal blood in the intervillous space, are the sites of maternal fetal-exchange. Villi are covered by a double layer of epithelial cells (the inner cytotrophoblast (CT) and the outer syncytiotrophoblast (ST), derived from CT). Villi cores contain blood vessels and a relatively large amount of connective tissue together with mesenchymal cells and macrophages (Hofbauer cells). As pregnancy proceeds, the CT layer of villi disappears, connective tissue decreases, and villi consist of little more than capillaries surrounded by a thin layer of ST. Some of the

villi (anchoring villi) extend into the uterine wall. CT of anchoring villi invade the endometrium and the first-third of myometrium (interstitial invasion) as well as maternal arterioles (vascular invasion) that span these regions. By 20 weeks' gestation, the endothelial and most of the smooth muscle cells of these arteries are replaced by a hybrid vasculature composed of fetal and maternal cells. It is currently speculated that passage of HCMV through the placenta may occur through the CT columns of anchoring villi or across the ST in the intervillous space.

In vitro studies have demonstrated that CT cells can be productively infected, albeit with low efficiency, by HCMV (Table 1). However, the experimental conditions adopted in some studies may have somewhat flawed the results. In particular, the use of: (i) laboratory-adapted strains; (ii) cell-free virus; (iii) CT cells obtained from term placentae and grown in non-polarized conditions, may not fully reflect in vivo conditions. Indeed, in the only study in which leukocytes infected with a clinical HCMV strain (thus reproducing the in vivo phase of acute viremia) were used to infect either explants of floating and anchoring villi or differentiating CT cells, no infection was observed (Maidji et al., 2002). On the other hand, the same study showed that HCMV-infected leukocytes could productively infect uterine endothelial cells which, in turn, were able to transmit the infection to CT cells. These data indicate that the development of suitable in vitro models and conditions are crucial for achieving results that can be transferred in vivo and, ultimately, for the understanding of the pathogenesis of transplacental HCMV transmission.

In conclusion, possible mechanisms of HCMV transmission in vivo following primary infection are the following: (i) maternal leukocytes carrying infectious virus may infect uterine endothelial cells which, being in partially modified vessel segments in close contact with CT, can transmit the infection to contiguous CT cells. The infection can then spread to fibroblasts of the villous core and to endothelial

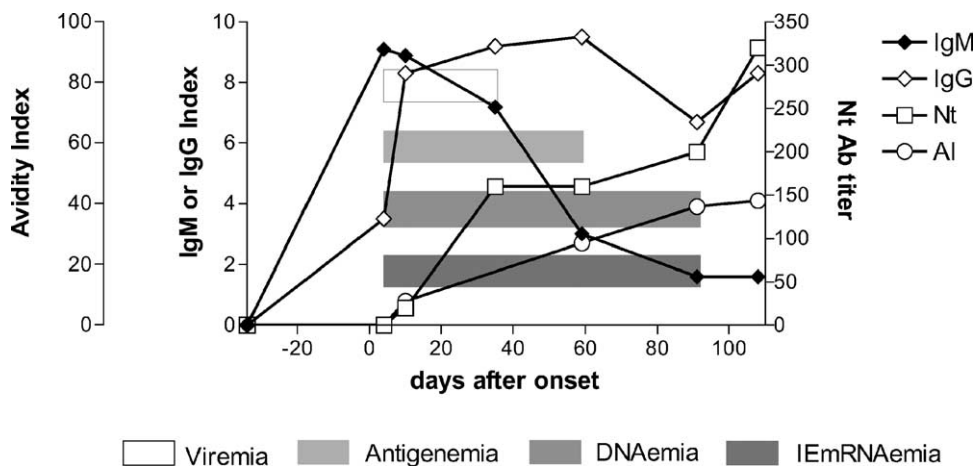


Fig. 2. Kinetics of HCMV-specific antibody response and viral load in blood of a pregnant woman suffering from primary HCMV infection at 19 weeks of gestation (Revello and Gerna, unpublished).

Table 1  
Reports on in vitro infection of placental trophoblast by HCMV

HCMV strain	Infecting virus	Placental cell substrate	Result	Reference
AD169, Towne, and three clinical strains	Cell-free	CT (term placentae)	Productive infection	Halwachs-Baumann et al., 1998
AD169, Towne, Davis, and one clinical strain	Cell-free	CT (term placentae, first trimester placentae)	Productive infection	Hemmings et al., 1998
AD169 mutants	Cell-free	First trimester chorionic villi explants and isolated CT	Infection of CT	Fisher et al., 2000
Toledo	Cell-free	First trimester placenta explants	Complete replication in trophoblast and stromal fibroblasts	Gabrielli et al., 2001
AD169 and one clinical strain	Cell-free	Polarized ST, term placentae	Productive infection	Hemmings and Guilbert, 2002
Clinical strain VR1814	Virus-infected peripheral blood leukocytes	Explants of floating and anchoring villi, differentiating CT, early placentae	Absence of infection	Maidji et al., 2002

CT: cytotrophoblast; ST: syncytiotrophoblast.

cells of fetal capillaries with hematogenous dissemination to the fetus. (ii) Alternatively, infected leukocytes may directly reach fetal endothelium through breaches of ST layer (particularly in the last part of gestation). (iii) An additional possibility is that the virus coated by specific antibody may cross the ST layer by transcytosis and be released still infectious to the underlying CT cells (Fisher et al., 2000).

In addition, in the case of vertical transmission from immune mothers, it must be considered that the placenta is a hemiallograft inducing local immunosuppression in the uterus (Fisher et al., 2000; Roth et al., 1996). As a consequence, HCMV may reactivate locally in macrophages which can infect invading CT cells. Then, virus could spread in a retrograde manner to anchoring villi and eventually to the fetus. Supporting evidence to this hypothesis comes from the observation that HCMV can be reactivated in vitro by allogeneic stimulation of monocyte-derived macrophages from healthy donors (Söderberg-Nauclér et al., 1997).

Once HCMV reaches the fetal compartment, then hematogenous dissemination ensues. Indeed, HCMV can be detected in blood of infected fetuses by a number of assays (see below), and viremia has been shown to persist until birth and for many months afterwards, particularly in symptomatic infants (Revello et al., 1999a). In addition, HCMV-infected circulating endothelial cells have been detected in blood of infected fetuses with ultrasound abnormalities, thus confirming this parameter as a prognostic marker of severe disseminated infection (Gerna et al., 1998; Percivalle et al., 1993).

Finally, why only a minority of HCMV-infected fetuses will eventually show clinical signs at birth or develop sequelae is not clear. Although it has been reported that the risk for congenital defects is present throughout gestation, compelling evidence demonstrates that maternal infections acquired (and transmitted) early during gestation are at higher risk of worst outcome (Pass et al., 1994; Stagno et al., 1986).

#### 4. Diagnosis in the fetus

More than 30 years have elapsed since the first reported case of fetal HCMV infection diagnosed antenatally (Davis et al., 1971). Notwithstanding some concerns have been arisen in the past about the utility of prenatal testing, given the undefined predictive values of positive and negative results and the absence of prognostic markers and fetal therapy (Pass, 1992), nowadays prenatal diagnosis represents a fundamental option in the management of pregnancy complicated by primary HCMV infection (Revello and Gerna, 2002).

Three requisites should, however, be satisfied before planning a prenatal diagnosis procedure. The first is relevant to the diagnosis of primary infection in the mother. This issue has been extensively covered in a recent review (Revello and Gerna, 2002) and will not be discussed here. Nevertheless, it is important to stress that the option of prenatal diagnosis should be offered only to those mothers with ascertained or highly suspected primary HCMV infection and/or in the presence of ultrasonographic abnormalities. Most frequently reported sonographic manifestations of fetal HCMV infection include fetal growth restriction, cerebral ventriculomegaly, ascites, intracranial calcifications and abnormality of amniotic fluid (AF) volume (usually oligohydramnios). In addition, microcephaly, hyperechogenic bowel, cardiomegaly, hepatomegaly, splenomegaly have also been documented (Crino, 1999). However, it must be considered that the invasive procedure carries some potential complications, including fetal loss (Grose et al., 1989). Although the overall risk of fetal loss is currently estimated about 1%, and in our experience it is about the same for infected (1.9%) and uninfected (1.5%) fetuses, it must be considered that pregnancies carrying severely infected fetuses are often terminated soon after the invasive procedure.

The second issue concerns dating of maternal infection, which is important for at least two reasons. The first con-

cerns the assessment of risks and, consequently, whether the offer of prenatal diagnosis is justified. In this respect, we have recently shown that primary infections acquired before last menstrual period carry a low risk of transmission, and therefore women in this situation can be substantially reassured and procedures for prenatal diagnosis omitted (Revello et al., 2002).

The other reason is relevant to timing of the procedure with respect to the onset of maternal infection. It has been repeatedly reported that prenatal diagnosis procedures performed too close to the onset of maternal infection carry a substantial risk of false-negative results (Bodéus et al., 1999; Lipitz et al., 1997; Mulongo et al., 1995; Nicolini et al., 1994; Revello et al., 1995; Ruellan-Eugene et al., 1996). Our experience is in keeping with these observations. Indeed, sensitivity of prenatal diagnosis results increases from 50% to 76.2% and 91.3%, when <8, 9–12 and >13 weeks, respectively, elapse between the onset of maternal infection and the procedure (Table 2). Negative predictive values (NPV) improve accordingly from 44.4% to 80% and 92.3% for the three time intervals, respectively. However, sensitivity of the techniques employed may be a confounding factor. In fact, the influence of time lapse becomes less prominent when highly sensitive techniques for HCMV detection are used. This appears particularly true in the <8 weeks time interval in which sensitivity of prenatal diagnosis results increases to 80% when the most sensitive PCR technique for detection of viral DNA in AF samples is employed (Table 2). Finally, it is interesting to note that when the highest time interval (>13 weeks) is considered, the use of very sensitive

PCR techniques does not increase the overall sensitivity of prenatal diagnosis, thus confirming that the unpredictability of HCMV transmission remains a basic obstacle to 100% sensitivity (Revello et al., 1998b).

The third issue is relevant to the gestational age at which the procedure is performed. In fact, gestational age at time of amniocentesis has been shown to be an additional variable affecting sensitivity of prenatal diagnosis. In a recent study, prenatal diagnosis showed a sensitivity of 30% if the first AF sample was taken before 21 weeks of gestation, whereas after 21 weeks of pregnancy sensitivity increased to 71% with tests performed on AF, and to 61% with tests on fetal blood (Liesnard et al., 2000). Other groups have reported similar results (Donner et al., 1994; Enders et al., 2001; Lipitz et al., 1997). In general, we can confirm these observations, albeit in our experience, the increase in sensitivity appears less dramatic, particularly when standard techniques are employed (Table 3). Once again, however, sensitivity of the technique plays an important role. In fact, by using an improved PCR technique for HCMV DNA detection in AF obtained beyond 21 weeks of gestation, a significant increase in sensitivity is obtained compared to AF obtained before 21 weeks' gestation and examined with standard assays. In conclusion, the use of a very sensitive PCR assay in conjunction with a procedure performed beyond 21 weeks of gestation provides the best combination for the highest sensitivity.

Cordocentesis and amniocentesis are the most common procedures by which fetal blood and AF samples, respectively, are obtained.

Table 2

Diagnostic value (with respect to virus detection at birth) of different assays for HCMV detection in amniotic fluid in relation to time interval between maternal infection and prenatal diagnosis procedure

Time interval between maternal infection and prenatal diagnosis (weeks)	Assay	Result	Congenital infection		$P^a$	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	
			Present	Absent						
≤8	SVA + CC + nPCR <sup>b</sup>	Positive	5	0	0.02	50.0	100	100	44.4	
		Negative	5	4						
	Improved nPCR <sup>c</sup>	Positive	8	0						
		Negative	2	4						
9–12	SVA + CC + nPCR	Positive	16	0		0.02	76.2	100	100	80.0
		Negative	5	20						
	Improved nPCR	Positive	19	0						
		Negative	2	20						
≥13	SVA + CC + nPCR	Positive	21	0	0.02		91.3	100	100	92.3
		Negative	2	24						
	Improved nPCR	Positive	21	0						
		Negative	2	24						

NPV: negative predictive value; PPV: positive predictive value; ns: not significant.

<sup>a</sup> Chi square test.

<sup>b</sup> SVA: shell vial assay; CC: conventional cell culture; nPCR: nested PCR.

<sup>c</sup> Nested PCR performed on multiple 100 µl aliquots of amniotic fluid (Revello et al., 1998b). (Revello and Gerna, unpublished).



Table 3

Diagnostic value (with respect to virus detection at birth) of different assays for HCMV detection in amniotic fluid samples obtained before and after 21 weeks of gestation

Weeks of gestation at prenatal diagnosis	Assay	Result	Congenital infection		$P^a$	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
			Present	Absent					
14–20 (median 18)	SVA + CC + nPCR <sup>b</sup>	Positive	9	0	ns	64.3	100	100	77.3
		Negative	5	17					
	Improved nPCR <sup>c</sup>	Positive	12	0					
		Negative	2	17					
21–35 (median 22)	SVA + CC + nPCR	Positive	34	0	0.03	85.0	100	100	83.8
		Negative	6	31					
	Improved nPCR	Positive	36	0					
		Negative	4	31					

NPV: negative predictive value; PPV: positive predictive value; ns: not significant.

<sup>a</sup> Chi square test.

<sup>b</sup> SVA: shell vial assay; CC: conventional cell culture; nPCR: nested PCR.

<sup>c</sup> Nested PCR performed on multiple 100 µl aliquots of amniotic fluid (Revello et al., 1998b). (Revello and Gerna, unpublished).

#### 4.1. Fetal blood

Fetal blood can be used for both determination of HCMV-specific IgM and quantification of viral load as well as for the assessment of biochemical and hematologic parameters. Determination of HCMV-specific IgM has long been the only widely used approach for prenatal diagnosis of congenital HCMV infection, notwithstanding its limited (20–75%) sensitivity (Donner et al., 1993; Lamy et al., 1992; Lynch et al., 1991; Revello et al., 1995, 1999b). More recently, an interesting observation concerning the potential use of IgM determination as a prognostic marker of congenital HCMV disease has been reported (Revello et al., 1999b). In more detail, it has been observed that frequency and levels of virus-specific IgM were significantly higher in fetuses with abnormal ultrasound and/or biochemical findings compared to fetuses with normal findings. These results were confirmed by Enders et al. (2001) who also found a significant correlation between high levels of virus-specific IgM and adverse fetal outcome.

Virus presence in blood can be ascertained by means of different conventional, rapid, or molecular assays. The most widely used assays include determination of: (i) viremia, i.e. infectious HCMV in leukocytes; (ii) antigenemia, i.e. presence of pp65-positive leukocytes (both polymorphonuclear leukocytes and monocytes, Fig. 3A and B); (iii) DNAemia, i.e. detection of viral DNA, and more recently, (iv) IEmRNAemia, i.e. detection of IE-messenger RNA in whole blood. Most of these assays allow quantification of HCMV in blood, thus providing the basis for their potential use as prognostic markers of fetal disease (see below).

Thus far, very few studies have addressed the issue of HCMV detection in fetal blood. In addition, great variations in results have been reported. Indeed, for viremia, sensitivities of 0% (Hohlfeld et al., 1991), 7% (Liesnard et al., 2000), 30% (Ahmad-Zalmai et al., 2001), 41% (Revello

et al., 1999b), and 55% (Revello and Gerna, 2002) have been reported. Similarly, sensitivities of pp65 antigenemia varied from 16% (Lazzarotto et al., 1998) to 58–64% (Revello and Gerna, 2002; Revello et al., 1999b). Higher, but again quite variable sensitivities have been reported for DNAemia, with percentages ranging from 41% (Liesnard et al., 2000) to 66.6% (Lazzarotto et al., 1998), 83–84.6% (Revello and Gerna, 2002; Revello et al., 1999b), and 92.6% (Enders et al., 2001). Specificities of these assays have been unanimously reported to be 100% with the exception of one study (Lazzarotto et al., 1998), in which DNA detection in fetal blood by PCR showed 85% specificity and a positive predictive value (PPV) as low as 33% (8 out of 12 PCR-positive fetuses were found to be uninfected at birth). With respect to the specificity problems raised by the last study, we have recently shown that determination of IEmRNA and pp67 mRNA by the nucleic acid sequence-based amplification (NASBA) assay in fetal blood provides sensitivities comparable to PCR (84.6%) and antigenemia (63.6%), respectively, with 100% specificities (Revello and Gerna, 2002). Prospective use of IEmRNA determination in fetal blood has confirmed previous results (Table 4). As both NASBA assays are commercially available and less demanding than antigenemia in terms of expertise required for reading test results, they should be included in the panel of diagnostic assays for confirmation of PCR and antigenemia results (or as alternatives to either assay).

Although detection of virus or virus components in fetal blood does not represent a diagnostic approach sensitive enough to significantly improve prenatal diagnosis of intrauterine transmission with respect to virus detection in AF (see below), tests performed on fetal blood may be confirmatory of results obtained on AF and, when performed quantitatively, may provide important prognostic information. In this respect, in a retrospective study performed by our group, it was observed that fetuses with abnormalities,

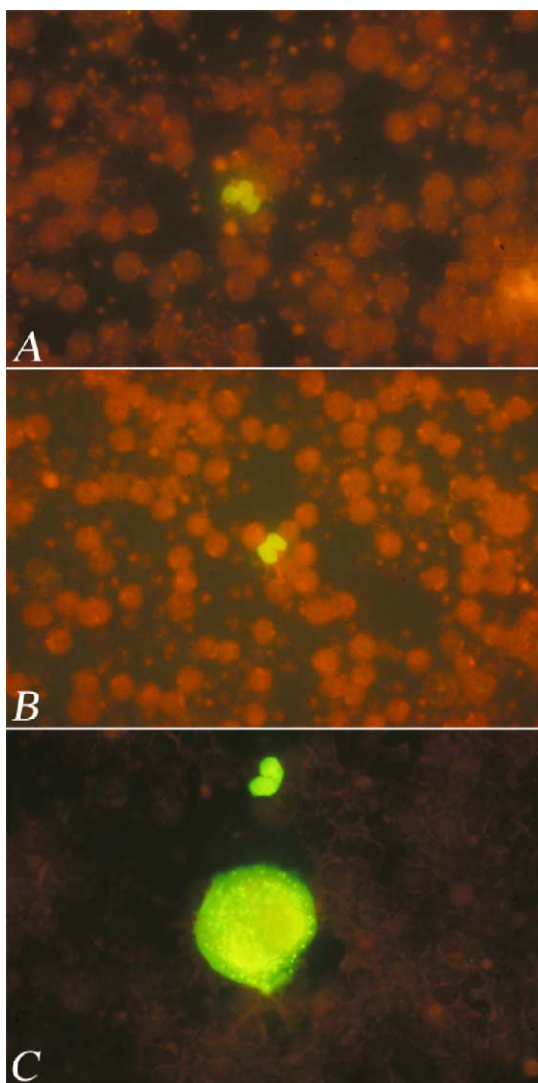


Fig. 3. HCMV-infected leukocytes and a circulating cytomegalic endothelial cell detected by indirect immunofluorescence and a pool of pp65-specific monoclonal antibodies in blood of a HCMV-infected fetus. (A) pp65-positive polymorphonuclear leukocyte; (B) pp65-positive monocyte; (C) circulating cytomegalic endothelial cell with a pp65-positive monocyte (Revello and Gerna, unpublished).

either detected antenatally or at birth, had a viral load (as determined by antigenemia, viremia, and DNAemia), that was higher than that observed in fetuses with normal findings (Revello et al., 1999b). In addition, as reported above, the level of virus-specific IgM was significantly lower in fetuses with normal findings. Recent surveys have confirmed previous results (Revello and Gerna, 2002) by clearly showing that in fetal blood of symptomatic fetuses or newborns, all virologic parameters as well as IgM antibody levels were significantly higher than those observed in asymptomatic fetuses or newborns (Fig. 4).

Cytomegalic endothelial cells circulating in peripheral blood of fetuses with high viral load and ultrasound abnormalities have been detected (Fig. 3C). The finding of these cells in infected fetuses indicates disseminated infec-

tion comparable to those reported in immunocompromised patients (Gerna et al., 1998; Salzberger et al., 1997).

Finally, biochemical and hematological parameters that can be usefully assessed in blood of fetuses with suspected HCMV infection include alanine aminotransferase, aspartate aminotransferase,  $\alpha$ -glutamyl transferase, blood cell count and total IgM determination.

#### 4.2. Amniotic fluid

HCMV presence in AF can be detected by conventional, rapid, and molecular assays. Given its absolute specificity, virus isolation has been recognized as the gold standard for prenatal diagnosis (Grose et al., 1992; Hogge et al., 1993; Hohlfeld et al., 1991; Mulongo et al., 1995). In addition, as reported above, thanks to the availability of MAb to the major IE protein p72 and the shell vial technique (Gleaves et al., 1984), HCMV can be detected and identified within 16–24 h after AF collection, thus allowing a very rapid diagnosis of fetal infection. With respect to rapidity, our most recent experience with the shell vial technique indicates that HCMV can be reliably detected as early as 6 h p.i. of AF samples (unpublished observation) (Fig. 5).

HCMV identification by the shell vial assay relies on the specificity of the MAb employed. Therefore, it is noteworthy to emphasize that very recently we isolated from AF a HCMV strain which was not recognized in the shell vial assay by an “in house” developed IE-specific MAb (Gerna et al., 2003). This observation underscores the importance of using a pool of MAbs with different p72 epitope specificity rather than a single MAb in the routine diagnostic setting.

Apart from technical reasons, the actual sensitivity of HCMV isolation remained undefined for a while, given a series of studies reporting false-negative results of AF cultures (Donner et al., 1993, 1994; Mulongo et al., 1995; Nicolini et al., 1994). The possibility that molecular techniques such as PCR could actually increase the sensitivity of HCMV detection in AF was, therefore, explored. In a retrospective study, we showed that sensitivity of prenatal diagnosis was only slightly increased (from 69.2% to 76.9%) by the use of either single-step or nested PCR (nPCR) (Revello et al., 1995). Similar results have been obtained by others (Enders et al., 2001; Gouarin et al., 2001). However, a more substantial increase in sensitivity was observed when a modified nPCR protocol was adopted. In this procedure, multiple (instead of single) and 100 (instead of 20)  $\mu$ l aliquots of AF were individually amplified and tested (Revello et al., 1998b). By using the improved nPCR assay, low (1–10 genome equivalents, GE) amounts of viral DNA were detected in a variable number of replicates of six AF samples taken from four fetuses previously diagnosed as uninfected. However, the same study showed that the new assay did not anticipate a positive prenatal diagnosis in a retrospective case that required two procedures for correct diagnosis,

Table 4

Diagnostic value (with respect to virus detection at birth) of different assays for prenatal diagnosis of congenital infection in 104 fetuses of 102 mothers with primary HCMV infection in pregnancy

Clinical specimen	HCMV assay	Test result	No. of fetuses		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
			Infected	Uninfected				
Fetal blood	Antigenemia	Negative	15	42	63.4	100	100	73.7
		Positive	26	0				
	Viremia	Negative	21	38	38.2	100	100	64.4
		Positive	13	0				
	DNAemia	Negative	6	34	84.2	100	100	85.0
		Positive	32	0				
IE mRNA	Negative	3	18	82.3	100	100	85.7	
	Positive	14	0					
pp67 mRNA	Negative	4	11	63.6	100	100	73.3	
	Positive	7	0					
IgM antibody	Negative	19	43	52.5	100	100	69.3	
	Positive	21	0					
Amniotic fluid	Virus isolation	Negative	10	53	80.4	100	100	84.1
		Positive	41	0				
	DNA	Negative	5	47	90.2	100	100	90.4
		Positive	46	0				
	IE mRNA	Negative	3	41	90.9	97.6	96.8	93.2
		Positive	30	1				
	pp67 mRNA	Negative	3	42	90.3	100	100	93.3
		Positive	28	0				

PPV: positive predictive value; NPV: negative predictive value; IEmRNA: immediate-early messenger RNA; pp67 mRNA: pp67 (late) messenger RNA. (Revello and Gerna, unpublished).

nor avoided one false-negative diagnosis when used prospectively.

Altogether, in our experience, 5 out of 53 (9.4%) newborns, who had been found uninfected antenatally by the AF testing, were congenitally infected when examined at birth, whereas fetal HCMV infection was confirmed in 100% of cases either at birth or following voluntary termination of pregnancy (Table 5). Our most recent data of sensitivity, specificity, PPV and NPV of DNA detection in AF obtained in a series of 102 pregnant women (104 fetuses) are 90.2%, 100%, 100%, and 90.4%, respectively (Table 3), thus confirming previously reported findings (Revello and Gerna, 2002). Comparable results have been found by other groups. In particular, Enders et al. (2001) and Liesnard et al. (2000) in two large series of pregnancies (189 and 237 pregnant women, respectively) reported sensitivity of

about 80% and specificity of 98–100% for prenatal diagnosis based upon PCR on AF. High specificity of PCR results have been confirmed by other groups (Donner et al., 1993; Gouarin et al., 2001; Lipitz et al., 1997; Ruellan-Eugene et al., 1996) except one. In fact, in a series of reports from a single group, specificity of PCR was 67.3–83.3% with a PPV of 48–48.5% (Guerra et al., 2000; Lazzarotto et al., 1998; Maine et al., 2001). The authors suggested that the highly sensitive PCR technique employed detected small quantities of virus later cleared by the defenses of the mother or fetus. Alternative explanations may be contaminations during PCR performance or insensitivity of the shell vial assay used for detection of HCMV in urine of newborns, considering that the same authors reported 50% sensitivity for rapid isolation assay from AF samples (Lazzarotto et al., 1998). Our experience as well as that of

Table 5

Outcome of 113 pregnancies (two twin pregnancies) following prenatal diagnosis of congenital HCMV infection

Prenatal diagnosis	No. of fetuses	Outcome				No. of congenital infection/examined (%)
		Term	TOP	Miscarriage	Lost	
Absence of congenital infection	69	53	0	1	15	5/53 (9.4)
Presence of congenital infection	46	28	16	2	0	46/46 (100)

TOP: termination of pregnancy (Revello and Gerna, unpublished).



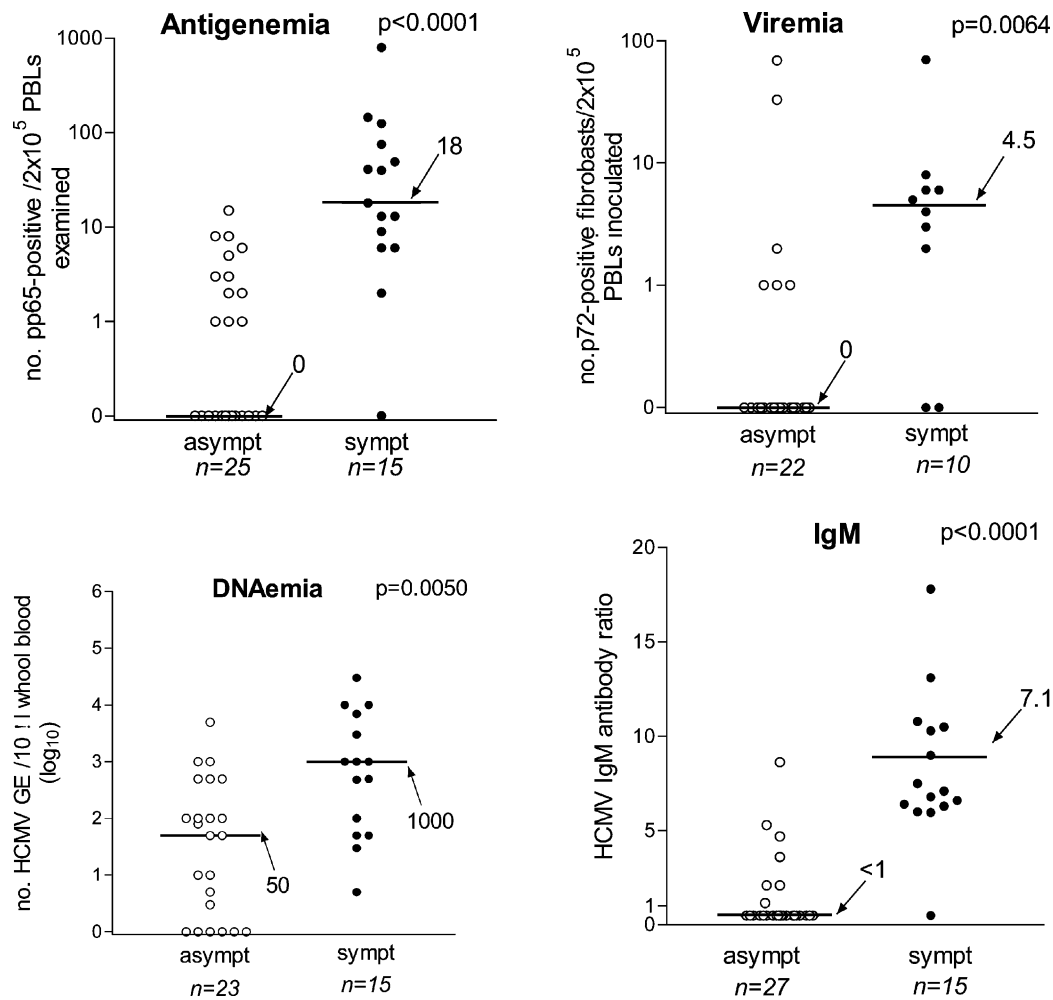


Fig. 4. Median levels (horizontal bars) of HCMV antigenemia, viremia, DNAemia, and IgM ratio in fetal blood of symptomatic (symp) and asymptomatic (asympt) congenitally infected fetuses. Fetuses were considered symptomatic if they showed ultrasound abnormalities at the time of prenatal diagnosis or symptoms at birth.  $P$  indicates levels of significance by Mann–Whitney  $U$ -test. PBLs: peripheral blood leukocytes; GE: genome equivalents (Revello and Gerna, unpublished).

others indicate that the detection of even small amounts of viral DNA in AF correlate with congenital infection at birth (Gouarin et al., 2002; Revello et al., 1999c). In conclusion, when appropriate measures of containment are adopted, the detection of HCMV in AF must be considered a consistently reliable marker of fetal infection (Gouarin et al., 2002; Liesnard et al., 2000; Revello and Gerna, 1999).

It should, however, be kept in mind that if amniocentesis is performed in the presence of maternal DNAemia, then a theoretical source of viral DNA in AF may be represented by maternal DNA. Since the risk of transmission during antenatal procedures performed in the presence of maternal DNAemia cannot be ruled out (Revello et al., 1998b), it appears highly advisable to check the mother for viral DNA in blood (in order to interpret possible low PCR positivity in AF), and, whenever feasible, to postpone diagnostic procedures until the viral DNA is no longer detected in blood (to avoid any risk of iatrogenic transmission).

Prenatal diagnosis is a very delicate task and it should never be based on the result of a single assay. We have recently evaluated commercially available molecular assays other than PCR to be used as confirmatory assays for HCMV detection in AF samples (Revello et al., 2003). In this retrospective study, 69 AF samples previously characterized by virus isolation and PCR were tested for HCMV IE and pp67mRNA by the NASBA technique. Identical sensitivities (93.5%) were observed with the two NASBA assays, PCR and virus isolation, whereas specificity was 100% for all assays with the exception of IEmRNA NASBA which detected one false-positive result (98.5% specificity). The study showed that both NASBA assays can be used as first choice assays or assays to confirm results obtained by PCR in those laboratories where virus isolation cannot be performed. Results collected and updated for this review confirm the above conclusion (Table 4).

The clinical significance of HCMV load in AF has been recently investigated. Higher levels of viral DNA were found

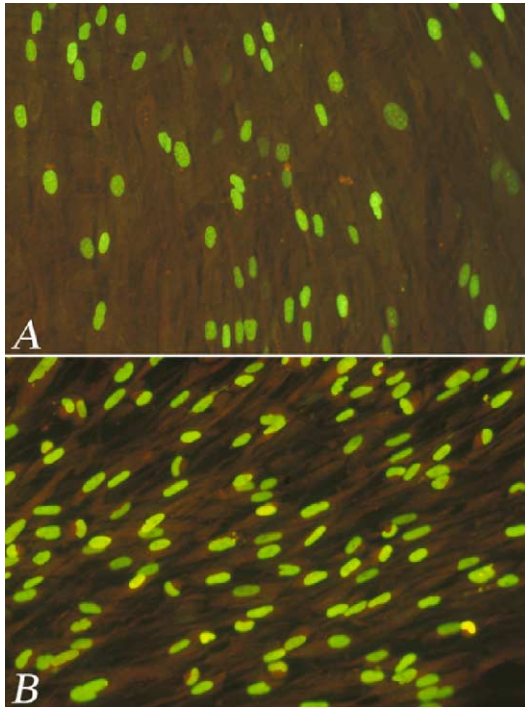


Fig. 5. Rapid HCMV identification 6 h (A) and 24 h (B) after inoculation of the amniotic fluid sample from a pregnant woman with primary HCMV infection onto human embryonic lung fibroblasts grown in shell vials. Although the number and intensity of fluorescing IE-positive cells increases from 6 to 24 h p.i., an early identification can be attempted as early as 6 h p.i. in specimens such as AF (or urine of newborns with suspected congenital infection), given the high amount of infectious virus usually present in these clinical samples (Revello and Gerna, unpublished).

in AF samples of fetuses with abnormal ultrasound findings or with symptomatic infection at birth (Revello et al., 1999c) compared to fetuses with normal ultrasound findings at time of amniocentesis and subclinical infection at birth. In that study, however, the difference was not found to be statistically significant. In particular, very high levels of viral DNA could be detected in both symptomatic and asymptomatic fetuses and the DNA amount was found to increase in one asymptomatic fetus during two procedures performed 5 weeks apart. Very recent data obtained by our group on a larger series of fetuses have confirmed previous results in that median DNA levels were higher ( $1.0 \times 10^7$  GE/ml) in AF of symptomatic fetuses compared to asymptomatic fetuses ( $5.1 \times 10^5$  GE/ml), the difference becoming significant ( $P = 0.0038$ ) when all cases in the asymptomatic group were considered, including those fetuses with very low or undetectable DNA levels (Fig. 6). However, as observed previously, most (18/28, 64.3%) asymptomatic fetuses showed DNA levels  $>10^5$  GE/ml, i.e. in the range observed in symptomatic fetuses.

Similar results have been recently published by Gouarin et al. (2002). In this study, HCMV DNA load values in AF samples, as determined by real-time PCR, were significantly ( $P = 0.014$ ) higher in the group of symptomatic ( $2.8 \times 10^5$  GE/ml) than in the group of asymptomatic ( $8 \times$

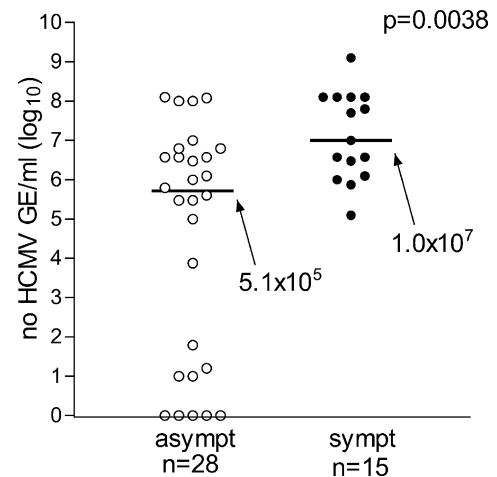


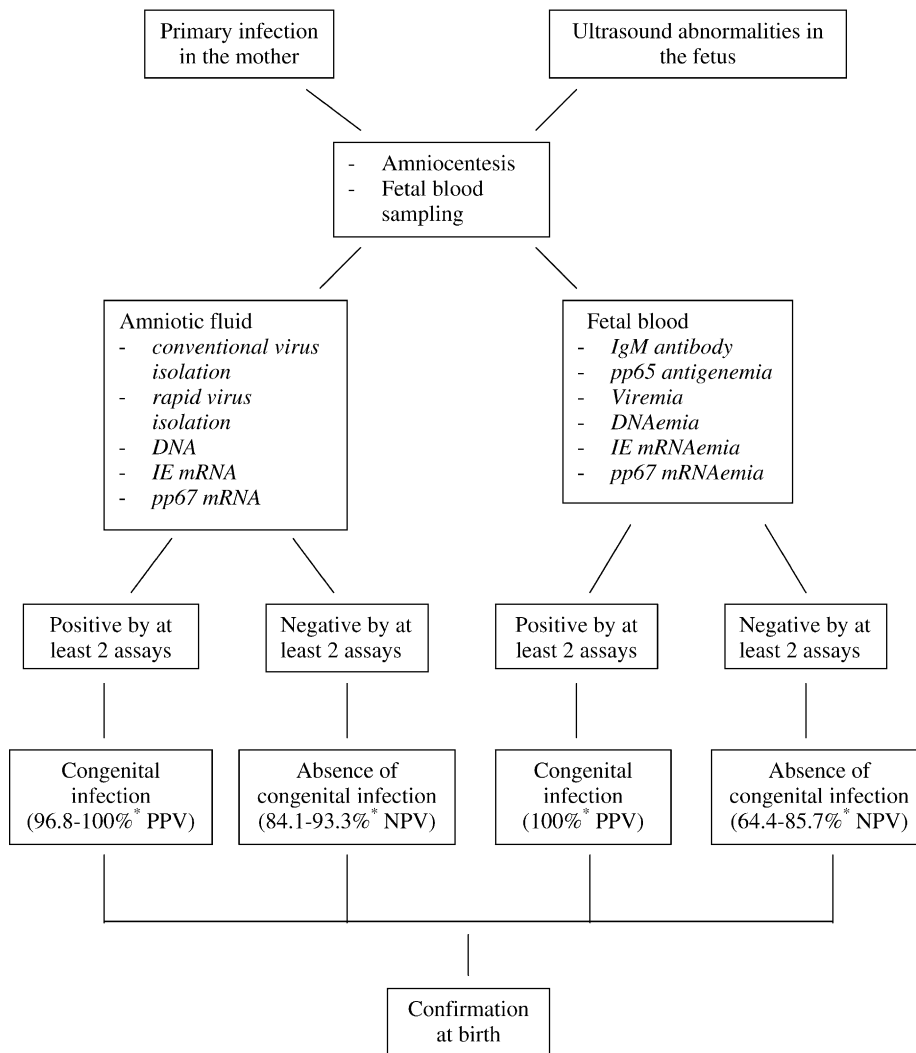
Fig. 6. Median HCMV DNA levels in amniotic fluid samples of 43 congenitally infected fetuses grouped according to presence or absence of symptoms at the time of amniocentesis or at birth. Median value (horizontal bar) of HCMV load was significantly higher in symptomatic (sympt) compared to asymptomatic (asympt) fetuses (Revello and Gerna, unpublished).

$10^3$  GE/ml) fetuses. However, a great number of values were found to overlap between the two groups also in that study. In addition, other variables such as gestational age at time of amniocentesis and the time elapsed since maternal infection were found to possibly influence viral load irrespective of fetal outcome. Indeed, viral DNA appears to accumulate in the amniotic fluid rather than being cleared, as indirectly confirmed by the lack of its degradation in an amniotic fluid sample stored at 37 °C for more than 6 months (personal observation).

Neither study could establish a threshold value predictive of fetal outcome. On the other hand, Guerra et al. (2000) reported that an HCMV viral load  $>10^3$  GE/ml was 100% predictive of fetal infection, whereas a level  $>10^5$  GE/ml was predictive of symptomatic HCMV infection. At the moment, these results are still awaiting confirmation. An additional recent report showed no correlation between HCMV load in AF and fetal outcome (Nedelec et al., 2002).

## 5. Conclusions

While in vivo and in vitro findings relevant to the interaction of maternal and fetal circulation are accumulating, thus contributing to the elucidation of some pathogenetic aspects of vertical transmission of HCMV infection from mother to fetus, the offer of prenatal diagnosis represents a major breakthrough in the management of HCMV infection during pregnancy. Both amniocentesis and cordocentesis are useful and may be complementary to each other. The risks of these procedures are minimal. Although a low number of false-negative results cannot be avoided due to the unpredictability of HCMV transmission, the diagnostic value of tests available at the moment assures a sensitivity

**Diagnosis of fetal HCMV infection. Flow chart**

\* Refer to Table 4 for individual figures for each test.

Fig. 7. Flow chart for prenatal diagnosis of congenital HCMV infection.

of about 90% and a specificity close to 100%. A flow chart for diagnosis of fetal HCMV infection is shown in Fig. 7. Counseling is critical and should be done by experienced medical personnel following an accurate anamnestic examination aimed at defining: (i) the presence of apparently insignificant clinical symptoms, such as fever, headache, asthenia during pregnancy; (ii) the presumed time of onset of HCMV infection with respect to gestation time; (iii) the opportunity to offer prenatal diagnosis. The first and most important issue to address is whether the mother has been affected by primary HCMV infection. Simultaneous analysis of serological and virological laboratory findings allows to solve this issue in the great majority of cases. Then, prenatal diagnosis, when required, allows pregnant women to know whether the infection has been transmitted (10% of risk of false results). At this time, the woman, who has been informed that clinical symptoms are present in 10% only of

congenitally infected babies, can consciously decide how to proceed with her pregnancy.

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