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False human cytomegalovirus IgG-positivity at prenatal screening

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ABSTRACT

Background: An incorrect definition of immune status to human cytomegalovirus (HCMV) can lead to incorrect management of pregnant women.
Objectives: Aims of the study were: i) to describe 10 cases of unconfirmed HCMV IgG-seroconversion in pregnancy; ii) to develop a panel of confirmatory tests to define HCMV serostatus; iii) to investigate the frequency of false IgG-positive results in pregnant women screened with the LIAISON®CMVIgGII automated assay.
Study design: Blood samples from 10 pregnant women referred for HCMV IgG-seroconversion were examined to confirm/exclude a primary infection. In addition, samples were tested for HCMV IgG by immunoblotting, neutralization assay, and ELISA against gB, gH/gL/pUL128L and gH/gL/gO recombinant glycoproteins. LIAISON®CMVIgGII results obtained on 1158 pregnant women were reviewed and samples with low IgG titers were further investigated.
Results: A primary infection was excluded in the 10 women referred for HCMV IgG seroconversion. None of them was confirmed to be IgG-seropositive. Of the 1158 women prenatally screened by LIAISON®CMVIgGII, 678 (59%) were IgG-positive and, of these, 40 (5.9%) showed low levels of IgG (14–50 U/mL). Thirty-three women with low IgG-positivity were further tested by confirmatory tests and 11 (33.3%) were found to be non reactive to HCMV.
Conclusions: At least 1.6% (11/678) women who tested positive with LIAISON®CMVIgGII were found to be seronegative when tested with confirmatory tests. These women should be informed to reduce the risk of a primary HCMV infection. Furthermore, should a congenital infection occur in any of these women, a maternal non-primary infection could be erroneously diagnosed.

1. Background

Human cytomegalovirus (HCMV) prenatal screening is not recommended in any country at the moment. However, a variable proportion of pregnant women is tested for HCMV IgG and IgM antibody in Europe [1]. In particular, in Italy about 40% of pregnant women are tested for HCMV on their gynecologist's or general practitioner's recommendation (personal data). To this purpose, fully automatized assays with a short turnaround are commonly used. Previous studies evaluated the overall performance of HCMV IgG commercial assays and results were reported as relative agreement between methods [2–4]. However, no confirmatory assay have been used to define HCMV serostatus. Recently, discordant results have been reported in 6.67% samples, tested with 4 commercial IgG kits for the determination of IgG antibody to HCMV, leading to the worrying conclusion that up to 4 out of 100 pregnant women could get a false positive IgG result. [5]. Discrepancies between assays can lead to false seroconversion and confused clinical management of pregnant women, as recently observed for rubella virus (RV) [6]. In addition, HCMV serology is crucial in pre-transplantation management because donor and recipient serostatus are important to define the risk of the transplanted patients.

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2. Objectives

In this study, we describe 10 pregnant women referred to our Institution because of a suspected HCMV IgG seroconversion and the panel of assays developed to further investigate their immune status. Since all the seroconversions were diagnosed by using the fully automated chemiluminiscence assay LIAISON®CMVIgGII (DiaSorin, Saluggia, Italy), specificity of the assay was further investigated on a large population of pregnant women undergoing prenatal screening at our Institution.

3. Study design

3.1. Subjects and samples

Ten pregnant women were referred to our Institution over the period October 2015–November 2016 because of a suspected IgG seroconversion in the absence of detectable IgM antibody observed elsewhere. Overall, 31 sequential blood samples (median 2 samples/woman, range 1–7) were tested for virus-specific antibody by LIAISON®CMVIgGII, LIAISON®CMVIgMII, LIAISON®CMVAvidityII, (DiaSorin) according to the manufacturer's instructions. An in-house developed capture ELISA for IgM antibody was used as confirmatory assay, as previously described [7]. Viral DNA in whole blood samples was detected by real time PCR, as previously described [8].

In addition, results of 1158 women prenatally tested at our Institution for HCMV-specific IgG by the LIAISON®CMVIgGII assay over the same period of time, were reviewed. Serum samples showing test results in the range of 14–50 units (U)/mL were further investigated by using the confirmatory tests panel (see below).

3.2. Diagnosis of primary HCMV infection

A primary infection was confirmed on the basis of the following parameters: i) presence and/or kinetics of IgM, ii) kinetics of IgG, iii) avidity index values (low and intermediate results being suggestive of a recent primary infection, whereas high values being suggestive of a remote infection) and iv) presence of DNAemia [9].

3.3. Confirmatory tests panel for definition of HCMV immune status

The following tests were included in the panel: i) immunoblotting (IB) assay (*recom*Line CMV IgG, MIKROGEN Diagnostik, GmbH, Neuried, Germany). Strips with separate bands corresponding to IE1, CM2, p150, p65, gB1 and gB2 recombinant HCMV antigens were used. Test result was determined by adding the point values of antibody-antigen reactivities when greater than cut-off band. IgG reactivity to CM2 and p150 antigen corresponds to 5 and 6 points, respectively. Reactivities to the remaining antigens correspond to 1 point each. Test interpretation is negative, borderline or positive for HCMV IgG when the score (sum of points obtained) is ≤ 1, 2–5, or ≥ 6, respectively. ii) Neutralization (Nt) assay. The test was performed as previously described [10]. Briefly, four-fold serum dilutions (starting dilution 1:10) were mixed with 50–100 infectious units of VR1814 and mixtures inoculated in duplicate onto monolayers of epithelial cells (ARPE-19). The serum dilution inhibiting virus infectivity by 50% was reported as the neutralizing antibody titer. A neutralizing antibody titer $\geq 1:10$ was considered suggestive of the presence of HCMV-specific antibody. iii) ELISA for IgG antibody determination to recombinant glycoproteins gB, gH/gL/pUL128L and gH/gL/gO. ELISAs were performed as described [11], with minor modifications. Briefly, 96 wells polystyrene plates were coated overnight with a murine anti-gH or an anti-gB monoclonal antibody. Anti-gH-coated wells were then incubated for 90 min with 3 μg/ml soluble HCMV pentamer (gH/gL/pUL128L) or 4 μg/ml trimer (gH/gL/gO). Anti-gB-coated wells were incubated with 3 μg/ml gB recombinant glycoprotein. Alternate wells were incubated with dilution buffer alone to check for non specific reactivity of test samples. Following two washings, serum samples were added at a single 1:100 dilution and incubated for 1 h at 37 °C. A horseradish peroxidase-labeled goat IgG fraction to human IgG (Fc-chain-specific) was then added followed by substrate solution. Net optical density (OD) was obtained by subtracting the OD value without antigen from the value obtained in the presence of the antigen. Based on the reactivities of 25 sera from HCMV-seronegative donors, a 0.20 net OD was considered the cut-off value for IgG determination to the pentamer, the trimer and gB antigen. iv) Determination of HCMV-specific T-cells by intracellular IFNγ production following incubation of peripheral blood mononuclear cells (PBMC) with autologous, monocyte-derived, HCMV-infected (strain VR1814) dendritic cells [12]. Subjects with HCMV-specific CD4+ or CD8+ T cells ≥ 0.4/μL blood were considered as "responders", whereas subjects with HCMV-specific CD4+ or CD8+ T cells < 0.2/μL blood (mean value ± 2SD of HCMV-seronegative donors) were considered as "non responders". Subjects with an intermediate value (grey zone) were considered as "equivocal responder".

4. Results

4.1. Suspected IgG seroconversion in 10 pregnant women

Ten women were tested for HCMV antibody during pregnancy or before conception elsewhere and in all cases the first serum sample tested negative and the following one tested positive for IgG in the absence of IgM. At referral, a blood sample was collected from each woman and tested together with a leftover of the serum samples analyzed elsewhere, when available (Table 1). Absence of IgM was confirmed in all 10 cases whereas LIAISON®CMVIgGII antibodies were detected at levels ranging from 15.5 to 41.2 U/mL (median 23.4). The IgG avidity index was high in four women (#6-8, #10) and not evaluable in the remaining six (#1-5, #9). Viral DNA was undetectable in all women. In all but two women (#4, #10), a serological follow-up was performed and at least one additional blood sample was collected after 3–4 weeks. During follow-up, IgG titers and avidity index values remained stable and IgM and DNAemia remained undetectable (detailed results of follow-up of two women are shown in Table 2). Therefore, a primary HCMV infection was excluded in all these women.

However, in consideration that the suspected IgG seroconversion occurred in pregnant women and because of the low levels of IgG antibodies detected, further testing by additional assays was suggested to better define HCMV serostatus of those women.

4.2. Definition of HCMV immune status according to additional assays

Samples from all 10 women were further examined by the additional serologic tests, whereas T-cell specific immunity was performed only on 4 women (Table 1). All serological assays gave concordant negative results in eight women (#1–8). Moreover, four women (#1, #5, #6, #8) tested negative for T-cells HCMV-specific immunity. Based on the above results, eight women were considered IgG-seronegative and IgG reactivity observed when using the LIAISON®CMVIgGII assay was considered to be non-specific.

The remaining two women (#9, #10) showed IB reactivity to p150 only, in the absence of neutralizing antibodies and ELISA reactivity against recombinant glycoproteins. T-cells specific immunity testing was not performed in these two women. Therefore, their HCMV serostatus was considered undefined.

4.3. Frequency of non-specific IgG reactivity

Overall, 1158 women (1909 sera) were tested during the study period by the LIAISON®CMVIgGII assay and serological results are shown in Table 3. Of these, 480 (41%) tested IgG-negative (< 12 U/mL) and 678 (59%) women tested positive (> 14 U/mL). We

Table 1
Results of diagnostic and confirmatory assays for 10 women referred for suspected HCMV IgG seroconversion in the absence of IgM.

Woman #, age	Week of gestation (WG) at			Blood samples		Diagnostic assays at referral				Results of confirmatory tests to define CMV seropositivity					
										Immunoblot	Nt[a]	ELISA (OD[b])			IFNγ-producing
(years)	last IgG-negativity	first IgG-positivity	referral	N.	WG	IgG (U/mL)	IgM (U/mL)	Avidity (index)	DNAemia (copies/mL)	(protein, score)	titre	gB	gH/gL/pUL128L	gHgLgO	T cells(cells/μl)
1, 37	preconception (-2)	5	6	7	from 6 to 32	16.8	neg	NE[c]	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	< 0.2
2, 34	preconception (-4)	8	9	3	from 9 to 19	21.7	neg	NE	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	ND[d]
3, 37	11	22	23	2	from 23 to 26	15.5	neg	NE	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	ND
4, 37	30	36	37	*1*	37	23.5	neg	NE	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	ND
5, 25	5	13	26	2	from 26 to 31	15.7	neg	NE	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	< 0.2
6, 38	9	20	22	2	from 22 to 28	23.3	neg	0.3	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	< 0.2
7, 35	20	25	28	4	from 20 to 37	41.2	neg	0.26	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	ND
8, 36	7	12	17	7	from 12 to 36	37.1	neg	0.66	< 30	neg	< 1:10	< 0.2	< 0.2	< 0.2	< 0.2
9, 33	5	16	19	2	from 19 to 23	34.1	neg	NE	< 30	pos (150, 6)	< 1:10	< 0.2	< 0.2	< 0.2	ND
10, 35	7	12	22	1	22	19	neg	0.61	< 30	pos (150, 6)	< 1:10	< 0.2	< 0.2	< 0.2	ND

[a] Nt, neutralization assay.
[b] OD, net optical density.
[c] NE, not evaluable value.
[d] ND, not done.

retrospectively analyzed pregnant women with low levels (range 14–50 U/mL) of IgG detected in the absence of IgM. Fourteen U/mL represents the cut-off value of the LIAISON® CMVIgGII assay, whereas the upper value (50 U/mL) was chosen because it was the highest value observed at referral and during the follow-up of the 10 cases of false IgG seroconversion described above.

Among the 678 IgG-positive women, 40 (5.9%) showed levels < 50 U/mL (median 30.5 U/mL), and, of these, 33 had a serum sample available for further testing. In 11/33 (33.3%) women, the presence of HCMV-specific IgG was not confirmed by the additional tests. When the 33 women were stratified according to IgG levels, it was found that 9/16 (56.3%) women with IgG levels ranging from 14 to 30 U/mL, tested negative with the additional assays and they were considered IgG-seronegative. Furthermore, only 2/17 (12.8%) women with levels of IgG ranging from 31 to 50 U/mL tested negative with all the additional assays. Overall, at least 11/678 (1.6%) women tested by the LIAISON® CMVIgGII were erroneously identified as IgG-positive to HCMV.

5. Discussion

De novo appearance of virus-specific IgG in a previously seronegative subject is considered the most straightforward indicator of HCMV primary infection [13]. However, in the 10 pregnant women referred to our Institution during last year because of HCMV IgG seroconversion, not only a primary infection was excluded but the very presence of HCMV-specific IgG was firstly questioned and subsequently not confirmed.

In our cases, exclusion of primary infection was relatively simple, given the absence of IgM and DNAemia and the detection of stable low levels of IgG during follow-up. It is worth mentioning that the determination of IgG avidity did not prove to be very useful in our case series. In fact, four women showed a high avidity index which was hardly compatible with the short time elapsed between the last seronegative and the first seropositive serum sample. On the other hand, the remaining six women had undeterminable avidity index values, a finding that may be observed in the early phase of acute primary infection as well as in the presence of low IgG levels. According to the manufacturers' instruction, avidity determination can be performed by the LIAISON® CMVAvidityII on the entire HCMV IgG measurable positive concentration range (manufacturer's leaflet). However, Berth and colleagues [14] observed that the avidity index should be interpreted cautiously in cases of low HCMV IgG levels and absence of IgM.

Difficulties in defining serostatus by using commercial immunoassays were recently observed for RV in pregnant women with low level of IgG antibodies [6]. In that study, true positive and true negative samples were defined by the concomitant presence or absence, respectively, of IB and Nt antibody. Taking advantage of this experience, a panel of different commercial and in-house developed functional and diagnostic assays was used to define HCMV serostatus in the presence of low IgG levels. The reasons of the choice of the confirmatory assays used in the panel are briefly discussed: i) IB is considered a very specific assay, which is widely used for confirmation of positive or unclear screening results, allowing the identification of specific antibodies reacting with individual HCMV antigens; ii) Nt is the standard assay for defining immunity to many viral infections (e.g., measles). In a previous study, the standard serum containing the minimal amount of detectable neutralizing antibody was selected as a cut-off standard for an ELISA for HCMV-specific IgG determination [15]. Furthermore, it has been recently shown that antibodies neutralizing HCMV infection of epithelial cells appear early after the infection onset together with ELISA IgG antibodies to gB, gH/gL/pUL128L and, later, gH/gL/gO and these antibodies remain detectable lifelong [16]. iii) When clinical samples were available, the T-cell specific immunity against HCMV was considered as additional test because it is well known that HCMV specific T-cell response appears early

Table 2
Detailed results of follow-up of two women referred for suspected HCMV IgG seroconvesion in the absence of IgM.

Woman#, age	Week of gestation (WG) at			Blood samples		Results of diagnostic assays		
(years)	last IgG-negativity	first IgG-positivity	referral	WG	IgG (U/mL)	IgM (U/mL)	Avidity index	DNAemia (copies/mL)
1, 37	preconception (-2)	5	6	6	16.8	neg	NE[a]	< 30
				10	17.9	neg	NE	< 30
				15	12.9	neg	NE	< 30
				19	14.2	neg	NE	< 30
				23	12	neg	NE	< 30
				27	12.8	neg	NE	< 30
				32	12	neg	NE	< 30
8, 36	7	12	17	12	37.1	neg	0.66	< 30
				16	46.3	neg	ND[b]	< 30
				17	50.1	neg	ND	< 30
				21	50.8	neg	ND	< 30
				26	33	neg	ND	< 30
				31	41.7	neg	ND	< 30
				36	39.7	neg	ND	< 30

[a] NE, not evaluable value.
[b] ND, not done.

Table 3
Serological results on women prenatally screened by LIAISON®CMVIgGII.

	N (%)
Population of pregnant women	1158
Results of prenatal testing:	
IgG seronegative (< 12 U/mL)	480 (41)
IgG seropositive (> 14 U/mL)	678 (59)
IgG levels ranging from 14 to 50 U/mL	40 (5.9)
women with serum available for further testing	33
Results of confirmatory tests (IB[a], Nt[b] and ELISAs[c]):	
negative	11 (33.3)
positive	22 (66.7)

[a] IB, immunoblotting.
[b] Nt, neutralization assay.
[c] ELISAs, ELISA assays against gB, pentamer and trimer.

after the onset of infection in immunocompetent individuals and they maintain it lifelong. According to the concordance of all confirmatory assays, 8/10 women were defined as true seronegative. In the remaining two women, results were discordant, because only an IB-reactivity against one protein (p150) was observed and their serostatus was arbitrarily considered undefined. The reasons for this phenomenon of false-reactivity with LIAISON®CMVIgGII assay are not clear as neither the type of antigen nor the dilution used or other technical details are available from the manufacturer. We excluded a problem linked to a specific lot of LIAISON®CMVIgGII because several different lots were used during the period of the study.

When the frequency of false-positive IgG results in the population of pregnant women routinely screened at our Institution was investigated, it was found that at least 1.6% (11/678) of women who tested positive with LIAISON®CMVIgGII at prenatal screening proved to be seronegative when tested with confirmatory assays. All of these women had low (14–50 IU/ml) level of IgG, and the lower the IgG antibody level, the higher was the chance to be seronegative with confirmatory tests. Indeed, more than half of the women with IgG level < 30 U/mL were seronegative.

To our knowledge, this is the first report about the specificity of LIAISON®CMVIgGII in a large population of pregnant women undergoing prenatal testing. Our results support the preliminary findings reported by Huzly and colleagues [5]. The authors analyzed 1004 consecutive unselected serum samples, including 112 samples from pregnant women, with four commercial HCMV IgG assays including LIAISON®CMVIgGII. As many as 67 (6,67%) samples showed discrepant results and false positive measurements were observed with all the assays evaluated.

Three major conclusions can be drawn based on the above findings. First, reporting of laboratory results for HCMV IgG should include quantitative results, range, and technology used. This should improve and facilitate interpretation of results, particularly in the case a pregnant woman moves from one laboratory to another. Secondly, pregnant women with low levels of IgG antibodies with commercial automated assays and absence of IgM at prenatal screening should be further investigated with additional tests, in order to confirm the presence of HCMV-specific antibodies. False-positive results are of great concern, in consideration that seronegative women can actually lower the risk of acquiring a primary infection when properly counselled about hygiene measures [17]. Finally, when considering the current approach to retrospective diagnosis of non-primary infection [18], based on the presence of high avidity index values in the absence of IgM before or at the onset of pregnancy, should one of these women with false IgG seropositivity deliver a HCMV-infected newborn, a congenital infection due to a maternal non-primary infection would be erroneously diagnosed.

Performances of highly automated assays currently used for the determination of immune status to HCMV in pregnant women and in the pretransplant management of immunocompromised patients should be carefully assessed.

Author contributions

MF and AS conceived and designed the study; AA, MF, MZ, AS and MP recruited patients and collected data; AS and CF, performed the experiments; DL and LP contributed reagents; FB, PM, AS critically revised the paper.

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Competing interests

None declared.

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