Molecular diagnosis of CMV infection in fetal aborted tissues in the region of Thrace

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Summary

Purpose: To detect the incidence of CMV infection in spontaneous abortion in Thrace. *Methods:* Genetic material from 143 fetuses aged from 11 to 39 weeks was examined. The material originated from various regions of Thrace. All fetuses and the respective placentas underwent routine histopathology. DNA was isolated from sections of paraffinized tissues. Detection of CMV in the DNA genomic samples was performed using a commercial PCR-based detection kit. *Results:* From the 143 fetuses that were examined, two were found to be CMV positive. Pathological findings related to inflammatory corruptions were observed in the placentas of 97 embryos, including the CMV infected ones. *Conclusions:* This study indicates CMV-DNA infection in 1.4% of aborted fetuses. CMV infection incidence in aborted fetuses is similar to this reported in other European regions. The molecular technique of PCR applied on paraffin-embedded biopsy material is proven to be an accurate, valid and fast method for investigating the CMV infection in aborted fetuses.

Key words: Spontaneous abortion; Fetus; Cytomegalovirus - CMV; PCR.

Introduction

Cytomegalovirus (CMV) (originating from the Greek cyto "cell" and megalo "large") was isolated from Weller and his collaborators in 1956 in liver biopsies and in the urine of different patients [1]. It is the most common cause of congenital infection in humans worldwide with an incidence of 0.5-2.5% of live births [2, 3].

Microscopically, formation of big cells (cytomegalia) and inclusion bodies (owl's eye) in the nucleus and protoplasm of cells *in vitro* and *in vivo* are the two major characteristics of CMV. The virus is developed only in cultures of human fibroblasts manifesting cytopathological corruption after one to six weeks [4].

In terms of clinical manifestations of the infection, CMV causes a wide spectrum of symptomatology ranging from minor illness up to heavy mental retardation and other critical damage including petechiae, hepatosplenomegaly, jaundice, microcephaly, chorioretinitis, and typical lymphocytosis with a mortality rate ranging from 10-30% [4, 5].

This virus can be transmitted in utero or perinatally, person-to-person via close non-sexual contact, through sexual activity, organ transplantation, blood transfusions and breastfeeding [6, 7]. Furthermore, recent molecular epidemiological studies prove that a risk factor for CMV transmission is close interaction with young children that have been infected because they confect high concentrations of the virus in urine and salivary secretions [8, 9].

The virus can be transmitted in the fetus after primary infection of the mother (0.7-4.1% of pregnancies with 40% average rate of transmission) or due to the activation of latent infection during gestation (0.15-3%) [3, 4].

Indisputably, CMV constitutes the most common cause of congenital infection, as proven by several reports, over against controversial studies regarding the correlation of CMV and intrauterine fetal death [2, 3, 10]. The association of spontaneous abortion and CMV has not yet been clarified, whereas, CMV infection is an important agent of adverse outcome in infants and moreover, the nucleic acid and viral agent are often detected in aborted material [10].

In this study we examined human fetuses of spontaneous abortions for CMV infection, using the accurate and sensitive molecular method of polymerase chain reaction (PCR) in order to investigate whether CMV could provide the basis and reason behind these abortions.

Materials and Methods

Tissue specimens

In the present study 143 fetuses were examined in the Laboratory of Histology and Embryology of Democritus University of Thrace, deriving from spontaneous abortions in the Department of Obstetrics and Gynecology of University General Hospital of Alexandroupolis. The fetuses' age ranged from the 11th to 39th gestational week. The gestational week was estimated using developmental anatomical criteria.

The experimental procedure included the preparation of the tissue and its fixation in paraffin, standard histopathological examination, isolation of DNA, PCR procedure and electrophoresis of the respective PCR reaction products.

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Fig. 1

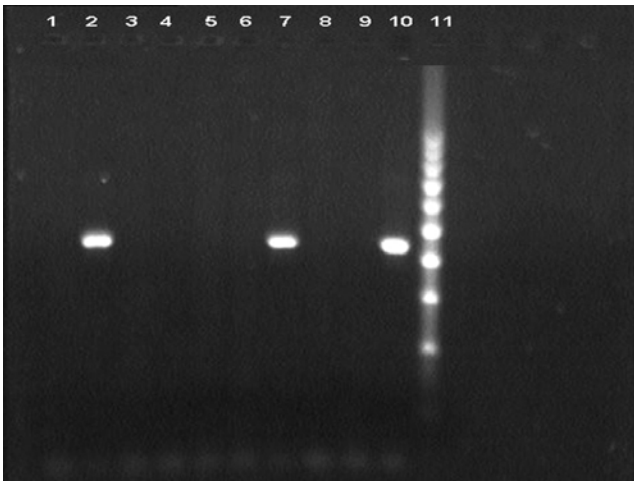

Fig. 2

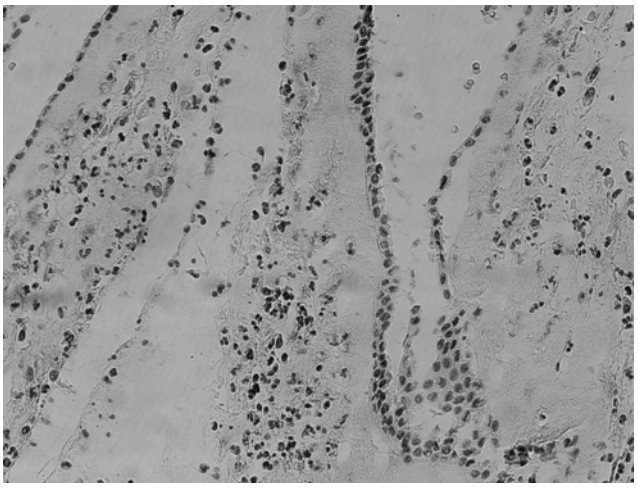

Fig. 3

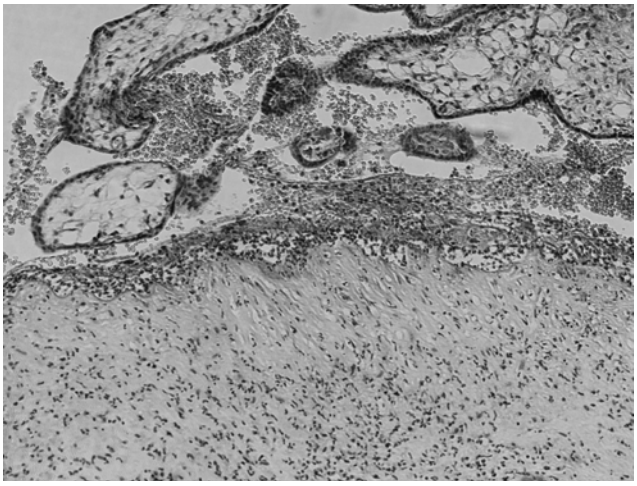

Figure 1. — Detection of CMV DNA by polymerase chain reaction.
Lines 1-3-4-5-6-8: negative samples, Line 7: positive sample, Line 2: positive control of DNA extraction, Lines 9-10: negative and positive controls of PCR respectively. Line 11: MW marker.

Figure 2. — Tissue section. Acute chorioamnionitis X 200.

Figure 3. — Tissue section. Acute placentitis X 100.

Sections from fetal liver, placenta and membranes of each embryo were employed. Each tissue was dehydrated and fixed in paraffin. Two μm sections were cut from paraffin blocks of the placenta and membranes and stained with hematoxylin and eosin for histopathological evaluation and 20 μm tissue sections of placenta and fetal liver were cut for the PCR procedure, respectively.

The tissue used for PCR amplification in the majority of fetuses was the placenta, except for multiple gestations where the examined tissue was the fetal liver of each fetus.

Extraction of DNA was performed using the Macherey-Nagel nucleospin tissue kit (GmbH & Co. KG, Germany), according to the manufacturer's protocol for DNA extraction from paraffin-embedded tissues. A positive control was included in each assay using 10 μl of control cDNA supplied in the PCR kit.

PCR was performed using CMV major immediate early gene, primer set kit (Maxim Biotech Inc, San Francisco, USA).

The primers used to amplify the sequence of CMV according to the manufacturer were: 5' Oligo: CCAAGCGGCCTCTGATAACCAAGCC, 3' Oligo: CAGCAC-CATCCTCCTCTTCCTCTGG. (alignment on database M21295) that were available as pre-mixed primers. PCR amplification of DNA was performed in a 50 μl total volume reaction using 1U of Taq polymerase, 10 μl of DNA and 40 μl of optimized buffer (including chemicals, enhancer, stabilizer, dNTPs). The amplification protocol was as follows: 96°C (1 min) for one cycle, 94°C (1 min), 58°C (1 min), 72°C (1 min) for 40 cycles, followed by a 10 min extension at 72°C. Presence of PCR products in the specimens was visualized on a 2% agarose gel stained with ethidium bromide. A negative control containing water instead of DNA was included in all assays. The positive control was also included using 10 μl of control cDNA supplied in the kit.

Data of serological examination from maternal sera of some cases were given by obstetricians.

Results

Molecular detection of CMV infection revealed two positive fetuses out of 143, as indicated by the appearance of a 435 bp DNA band, as a PCR product deriving from CMV DNA amplification of placenta tissues (Figure 1). Both positive fetuses were male aged 20 and 23 weeks, while the mothers' ages were 21 and 41 years, respectively (Table 1).

A male fetus positive to specific CMV antibodies indicated by serological examinations, proved negative for the presence of CMV genome by PCR (line 4 - Figure 1).

No other pathological findings related to congenital CMV infection were found by gross examination in the CMV positive fetuses (Table 1).

Histological examination of the placental samples revealed acute chorioamnionitis (Figure 2) in 86 of the 143 investigated fetuses (including the two positive for CMV) and acute placentitis (Figure 3) in 11 of these fetuses (Table 1), which may correlate to fetal death (Table 2).

Table 1. — *Summary of the candidate gene variants and genotyping methodology.*

Serial number	Gender	Gestational age	Mother's age	Clinical features/pathological findings	Chorioamnionitis (+, ++, +++)	Placentitis (+, ++, +++)	CMV (+, –)
1	♀	18 w	30 y	Cystic hygroma	–	–	–
2	♂	21 w	34 y	dropsy	–	–	–
3	♂	15 w	24 y		+++	–	–
4	♂	19 w	24 y	Cheiloschisis	–	–	–
5	♀	32 w		Cyclopia	–	–	–
6	♂	14 w	28 y		+++	–	–
7	♂	21 w	24 y	Dropsy	–	–	–
8	♀	14 w	32 y		+++	–	–
9	♂	17 w	34 y		+++	–	–
10	♂	18 w	32 y	Congenital abnormalities	+	–	–
11	♀	27 w	21 y	Recessive development	–	–	–
12	♀	25 w	36 y	Recessive development, oligohydramnios	–	–	–
13	♂	24 w	30 y	Gastroschisis	–	–	–
14	♂, ♂	28 w	38 y	Lung aplasia	+	–	–
15	♀	39 w	23 y	Lung abnormalities, parencephalis hemmorrhage	–	–	–
16	♂	20 w	21 y		+++	–	+
17	♂	23 w	27 y	Congenital abnormalities	–	–	–
18	♂	36 w	36 y		+++	–	–
19	♂	27 w	21 y		+++	–	–
20	♀	19 w	19 y	Congenital abnormalities	+++	–	–
21	♂	16 w	16 y	Gastroschisis, cheiloschisis	–	–	–
22	♂	28 w	28 y		+	–	–
23	♂	22 w	20 y	Heart abnormalities	+	–	–
24	♂	22 w	26 y	Anencephaly, meningocele	++	–	–
25	♂, ♂	23 w	26 y		+	–	–
26	♂	17 w	24 y	Anencephaly, omphalocele	–	–	–
27	♂	20 w	22 y	Anencephaly	–	–	–
28	♀	17 w	31 y	Down's syndrome	–	–	–
29	♀	21 w	43 y	Encephalocele, meningocele	–	–	–
30	♂	23 w	27 y		+	–	–
31	♂	20 w	17 y		++	–	–
32	♂	17 w	24 y	Anencephaly	–	–	–
33	♀	18 w	21 y	Anencephaly, spina binida aperta	–	–	–
34	♀	34 w	28 y	Dropsy	–	–	–
35	♀	20 w	24 y	Gastroschisis	+	–	–
36	♂	16 w	35 y	Down's syndrome	–	–	–
37	♂	23 w	24 y	Heart and lung abnormalities	+	–	–
38	♀	22 w	25 y	Procephaly	+	–	–
39	–	17 w	24 y		++	–	–
40	♀	21 w	40 y	Down's syndrome	–	–	–
41	♂	18 w	25 y	Nuchal cord	+++	–	–
42	♂, ♂, ♂	22 w	35 y		+++	–	–
43	♂	12 w +3d	26 y		+++	–	–
44	♀	23 w	33 y	Immature malignant sacrococcygeal teratoma	–	–	–
45	♂	18 w	22 y	Umbilical cord ischemic necrosis	++	–	–
46	♂	24 w	33 y		++	–	–
47	♂	19 w	29 y		+	–	–
48	♀	23 w	39 y	Umbilical cord ischaemic necrosis	++	–	–
49	♀	22 w	19 y	Anencephaly	++	–	–
50	♂	23 w	26 y		+++	–	–
51	♀	24 w	32 y	Edward's syndrome	–	–	–
52	♀, ♀	32 w	27 y	Hyaline membrane syndrome, atelectasis	–	–	–
53	♂	32 w 3 d	29 y	Hyaline membrane syndrome	–	–	–
54	♂	22 w	32 y	Fallot tertalogy, kidney abnormalities	–	–	–
55	♂	20 w	21 y	Kidney abnormalities	++	–	–
56	♂	20 w	41 y		+	–	–
57	♀	20 w	40 y	Anencephaly, meningomyelocele	+	–	–
58	♀	14 w	31 y	Head edema	++	–	–
59	♂	12 w			+	–	–
60	♂	25 w	32 y	Heart abnormalities	–	–	–

Table 1. — *Summary of the candidate gene variants and genotyping methodology*.

Serial number	Gender	Gestational age	Mother's age	Clinical features/pathological findings	Chorioamnionitis (+, ++, +++)	Placentitis (+, ++, +++)	CMV (+, –)
61	♀	26 w	28 y	Heart abnormalities	–	–	–
62	♂	17 w	30 y		++	–	–
63	♂	20 w	16 y	Total edema	++	–	–
64	♂	15 w 4 d	30 y		++	–	–
65	♂	17 w	29 y		+	–	–
66	♂	20 w		Syndrome XXY	–	–	–
67	♂	20 w	26 y		++	–	–
68	♂	16 w	36 y	Procephaly	+	–	–
69	♂	16 w	19 y		+	–	–
70	♀	38 w	33 y	Aspiration of amniotic fluid	++	–	–
71	♂	18 w	30 y	Devisceration, sull dissection	++	–	–
72	♂	14 w	30 y	Organ autolytic corruptions	–	–	–
73	♂	21 w	39 y	Down's syndrome	–	–	–
74	♂	20 w	33 y		++	–	–
75	♀, ♀		23 y		+++	–	–
76	♂	11 w	31 y		+	–	–
77	♂	20 w	28 y		++	–	–
78	♂	18 w	29 y	Dropsy	++	–	–
79	♂	20 w	19 y		++	+++	–
80	♀, ♂	23 w	31 y		++	–	–
81	♂	14 w	28 y	Organ autolytic corruptions	+++	–	–
82	♂				+++	++	–
83	♀	25 w	30 y		+++	+++	–
84	♀	18 w	31 y		+	–	–
85	♀	23 w	20 y	Heart abnormalities	–	–	–
86	♂	20 w	31 y	Cysts of lung and kidneys	–	–	–
87	♂	22 w	34 y		+++	+++	–
88	♂	25 w	29 y		+++	–	–
89	♀	19 w	26 y		++	–	–
90	♀	23 w	16 y		++	+++	–
91	♀	23 w 5 d	21 y		++	–	–
92	♀	23 w	32 y		++	–	–
93	♀	26 w	44 y		+++	–	–
94	♂	20 w	33 y		+++	–	–
95	♀	19 w	32 y	Procephaly, organ autolytic corruptions, umbilical cord ischemic necrosis	++	–	–
96	♀	21 w	28 y		+++	+++	–
97	♂	13 w	26 y	Dropsy, heart abnormalities	+	–	–
98	♀	21 w	25 y	Dropsy, heart abnormalities	+	–	–
99	♂	31 w	28 y	Lung abnormalities	+	–	–
100	♂	26 w	24 y		++	–	–
101	♀	22 w	31 y		++	–	–
102	♀	18 w	38 y	Meningomyelocele	++	–	–
103	♂	19 w	41 y	Down's syndrome	–	–	–
104	♂	17 w	34 y		++	–	–
105	♂	19 w	17 y		++	++	–
106	♀	25 w	27 y	Clubfoot, talipes	–	–	–
107	♀	17 w	34 y	Down's syndrome	+	–	–
108	♂	20 w	27 y	Anencephaly, neck failure	+	+	–
109	♂	13 w	37 y		++	+	–
110	♂	16 w	34 y	Organ autolytic corruptions	++	–	–
111	♂	27 w	35 y		+++	–	–
112	♂, ♂	19 w	28 y		+++	++	–
113	♀	22 w	21 y		++	–	–
114	♂	23 w	41 y		+++	–	+
115	♀, ♂	19 w	28 y		+++	–	–
116	♂	19 w	37 y		+++	–	–
117	♂	27 w	30 y		++	+++	–
118	♀, ♀	18 w	22 y	Hemosiderine deposition	+++	–	–
119	♂	12 w	24 y	Encephalomeningocele	+	–	–
120	♂	23 w	32 y		+	–	–

Table 1. — *Summary of the candidate gene variants and genotyping methodology.*

Serial number	Gender	Gestational age	Mother's age	Clinical features/pathological findings	Chorioamnionitis (+, ++, +++)	Placentitis (+, ++, +++)	CMV (+, –)
121	♂	35 w	32 y	Organ autolytic corruptions, circumvolution of umbilical cord	–	–	–
122	♂	20 w	32 y	Kidney aplasia	–	–	–
123	♂	14 w	25 y	Dropsy	–	–	–
124	♀	19 w	21 y	Skin dye abnormality	–	–	–
125	♂	14 w	30 y	Dropsy	–	–	–
126	♀	36 w	28 y	Hemolytic corruptions	–	–	–
127	♂	16 w	28 y	Congenital abnormalities	–	–	–
128	♂	24 w	34 y	Congenital abnormalities	–	–	–
129	♂	19 w	32 y	Brain abnormalities	–	–	–
130	♀	17 w	24 y	Organ autolytic corruptions, nuchal cord	–	–	–
131	♂	18 w	32 y	Thalassemia	–	–	–
132	♂	30 w	33 y	Intrauterine death	–	–	–
133	♂	27 w	32 y	Pre-eclampsia	–	–	–
134	♂	29 w	29 y	Head edema	–	–	–
135	♂	19 w	35 y	Phocomelia	–	–	–
136	♂	13 w	26 y	Dropsy	–	–	–
137	♀	23 w	30 y	Dropsy, meningomyelocele	–	–	–
138	♀	24 w	36 y	Congenital abnormalities	–	–	–
139	♂, ♂	17 w	35 y	Gastroschisis, devisceration	–	–	–
140	♂	22 w	38 y	Skeletal abnormalities	–	–	–
141	♂	18 w	25 y	Intrauterine death	–	–	–
142	♀	25 w	36 y	Intrauterine death	–	–	–
143	♂	18 w	29 y	Heart abnormalities	–	–	–

(+, ++, +++ = Gradation of chorioamnionitis and placentitis); (+, - = Positive and negative samples).

Table 2. — *Possible etiologies of fetal death.*

Etiologies of fetal death	No. of examined incidents
CMV	2
Chorioamnionitis	86
Placentitis	11
Other reasons	46

Discussion

In this study we examined the incidence of CMV in intrauterine fetal death in the region of Thrace by localizing the genetic material of the virus in aborted tissues using the PCR technique. Of 143 samples of aborted fetuses that were examined, two were found positive for the presence of CMV genome. Furthermore, the placentas of these two fetuses were positive for acute chorioamnionitis diagnosed with Hm-E staining, while no other pathological findings related to congenital CMV infection were observed.

For the majority of the cases no sufficient data about the maternal sera were provided to us, except for certain ones. For that reason we could not investigate whether the presence of specific antibodies against the virus, in correlation with PCR, would provide more composite results.

In order to ensure accurate results, PCR amplification of CMV genomic DNA was the method of choice. Published data have proven that experimental methods based on isolation of DNA from paraffin-embedded tissues and amplification of virus DNA with PCR have been successful [11, 12].

Alternative methods that can be used for CMV diagnosis consist of microscopic examination, immunohistochemistry, the culture of cells (CC and SV) [13, 14], the CMV pp65 antigenemia test (AGC)", the determination of CMV-specific IgG and IgM and others [10]. It is however reported that the culture of cells (CC and SV) and CMV pp65 antigen test have failed to provide the effectiveness of PCR [13, 14]. Niubo J. *et al*. presented data comparing results on peripheral blood samples of patients with heart transplantation for prognosis of CMV disease, and the reliability documented for each of the methods employed was: 89.9% reliability for PCR, 33.4% for CC (tube culture) culture, 42.6% for SV (shell vial culture) and 68.1% for pp65 antigen test. The numerous variants of PCR are applied internationally in the laboratories of molecular diagnostics for the detection of CMV [15].

Considering previous studies that proved the focal distribution of CMV [10, 16] and that the virus may not present in all different tissues of the same case, as well as the fact that placentas of CMV infected incidents show various pathological findings from absence of abnormalities up to variant inflammatory corruptions [10, 17], we performed the experimental procedure in different aborted tissues (placenta, membranes, liver) in order to have more reliable results.

The idea of using the placenta as an accessory tissue for DNA extraction is based on the knowledge that CMV or other pathological agents are transmitted by mother to the fetus, transplacentally [18-21]. Further studies have established that the CMV genome is localized on the villi

of the placenta, including the mesenchyme, trophoblasts and decidual cells [16].

Regardless of the above-confirmed facts, the cases of multiple gestations examined in the present study were treated with special caution because the type of placenta cannot determine which of the fetuses the virus may infect. Furthermore, it has not even been proven whether transmission by one fetus to the other is effected [22]. For that reason, and in order to insure reliability of results for such cases, we chose to use the fetal liver from each fetus body rather than the placentas. Alternatively, similar studies have employed biological fluids such as amniotic fluid [23, 24], peripheral blood [25, 26] or urine [27].

It is of great value to note that in our study the examination of a male fetus aged 12 weeks by PCR was proven negative for CMV infection, while it was expected to be positive, as the mother had appeared positive following serological examinations. This phenomenon is possible due to the fact that the presence of CMV-specific IgM is not always indicative of primary infection because of its production in low levels in reactivated CMV infection. Furthermore, the sensitivity of serological CMV IgM assay is 70% [28]. This has also been observed by other research [26] including a recent study in Turkey describing the opposite case where serological analysis was negative for CMV IgM and the CMV-DNA were found to be positive by PCR for two newborns [28].

CMV is the most common agent of congenital infection in humans [2] and the incidence of its specific antibodies in the adult population is high ranging – basen on several studies conducted in Greece and worldwide – from 40-90% [2, 10]. It is also known that the incidence of congenital CMV infection depends on socioeconomic conditions of the population. Consequently in our study we extrapolated that a part of the examined fetuses derived from the minority population of Thrace.

After the successful application of PCR and the electrophoresis of products in agarose gel, we estimated that frequency of appearance of the virus in the examined aborted fetuses was 1.4% (2 out of 143). This percentage appears to be in line with the percentage of CMV-infected samples that were spontaneously aborted in other respective studies where the percentage ranged from 3-16% [29, 30]. Furthermore, the high rate of chorioamnionitis that was seen is obviously basal but it is not clear whether CMV caused it in the two positive fetuses.

It is important to note that although several relevant studies have been conducted in various regions of Europe [8, 29] to estimate the relation to CMV infection with intrauterine fetal death, the exact percentage for incidence of the infection has not been defined.

Conclusion

In this study the molecular technique of PMR was employed in an attempt to determine whether CMV infection has a potential involvement in fetal death. Even though the fact that two out of 143 aborted fetuses that were examined and found positive in CMV gives an abortional role to CMV in Thrace with a percentage of 1.4%, we propose that this field of study involving the role and effect of CMV in embryology should be further investigated, as other studies – in accordance with ours – point out the incidence of CMV infection and its relation to congenital abnormalities and fetal death.

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