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Viral invasion of the amniotic cavity (VIAC) in the midtrimester of pregnancy

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Introduction: **The prevalence of viral infections in the amniotic fluid (AF) has not yet been ascertained. The aim of this study was to determine the prevalence of specific viral nucleic acids in the AF and its relationship to pregnancy outcome.** ***Study design:*** **From a cohort of 847 consecutive women undergoing midtrimester amniocentesis, 729 cases were included in this study after exclusion of documented fetal anomalies, chromosomal abnormalities, unavailability of AF specimens and clinical outcomes. AF specimens were tested by quantitative real-time PCR for the presence of genome sequences of the following viruses: adenoviruses, herpes simplex virus (HSV), varicella zoster virus (VZV), human herpesvirus 6 (HHV6), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), parvovirus B19 and enteroviruses. Viral nucleic acid testing was also performed in maternal blood and cord blood in the population of women in whom AF was positive for viruses and in a control group of 29 women with AF negative for viral nucleic acids. The relationship between the presence of viruses and pregnancy and neonatal outcome was examined. The correlation between the presence of nucleic acids of viruses in the AF and the concentration of the cytokine interleukin-6 (IL-6) and the T cell chemokine CXCL-10 (or IP-10) in AF and maternal blood were analyzed.** ***Results:*** **Viral genome sequences were found in 16 of 729 (2.2%) AF samples. HHV6 was the most commonly detected virus (7 cases, 1.0%), followed by HCMV (6 cases, 0.8%), parvovirus B19 (2 cases, 0.3%) and EBV (1 case, 0.1%), while HSV, VZV, enteroviruses and adenoviruses were not found in this cohort. Corresponding viral DNA was also detected in maternal blood of six out of seven women with HHV6-positive AF and in the umbilical cord plasma, which was available in one case. In contrast, viral DNA was not detected in maternal blood of women with AF positive for parvovirus B19, HCMV, EBV or of women with AF negative for viruses. HHV6 genome copy number in AF and maternal blood was consistent with genomic integration of viral DNA and genetic infection in all women. There was no significant difference in the AF concentration of IL-6 and IP-10 between patients with and without VIAC. However, for HCMV, there was a significant relationship between viral copy number and IP-10 concentration in maternal blood and AF. The group of women with AF positive for viral DNA delivered at term healthy neonates without complications in 14 out of 16 cases. In one case of HHV6 infection in the AF, the patient developed gestational hypertension at term, and in another case of HHV6 infection in the AF, the patient delivered at 33 weeks after preterm premature rupture of membranes (PPROM).** ***Conclusion:*** **Viral nucleic acids are detectable in 2.2% of AF samples obtained from asymptomatic women in the midtrimester. HHV6 was the most frequently detected virus in AF. Adenoviruses were not detected. Vertical transmission of HHV6 was demonstrated in one case.**

Keywords: Midtrimester AF, neonatal outcome, prevalence, pregnancy outcome, viral DNA

Introduction

A role for microbial invasion of the amniotic cavity (MIAC) with bacteria in the etiology of complications of pregnancy, such as preterm labor with intact membranes [1–18], preterm premature rupture of membranes [19–30], and cervical insufficiency [31–36] is well established. Moreover, bacteria can invade the human fetus, elicit a fetal inflammatory response, and be associated with short term neonatal morbidity [37–46] and long term complications, such as cerebral palsy and chronic lung disease [47–55]. During the last 20 years, it has become clear that the frequency of intra-amniotic inflammation is higher than the frequency of MIAC [56–58] (bacteria identified with cultivation techniques). Since cultivation techniques only identify a fraction of bacteria, several investigators have used broad range [59–65] or specific PCR [28,36,66–70] to detect bacterial genomes.

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Even though the frequency of MIAC associated with bacteria is larger when molecular microbiologic techniques are used (either broad range PCR and/or PCR with specific primers) in combination with cultivation techniques [71–75], there is still a large fraction of patients with intra-amniotic inflammation of unknown etiology. One possibility is that some of these inflammatory processes are due to viruses [76–79] (e.g. herpesviruses [80,81]), since these microorganisms can cross the placenta [82–92] and infect the human fetus or ascend from the lower genital tract [93–99].

Previous studies have examined the presence of viruses in the amniotic cavity using PCR-based methods. The results have varied from the finding that no viral DNA was detected in 277 patients [100] to a report in which 6.4% (44/686) of AF samples obtained in the midtrimester contained viral DNA [29]. Moreover, this group of investigators reported a seasonal variation in the frequency of viral detection in the amniotic cavity, which was more frequent in summer and late winter [101]. In a subsequent study, Miller et al. reported that fetal viral infection was not associated with adverse perinatal outcome [102]. The authors refer to the outcome of viral invasion of the amniotic cavity, because fetal infection was not ascertained, and the clinical implications of a test for positive molecular viral footprints in the absence of sonographic evidence for fetal involvement is still unclear [103].

The purpose of this study was to examine the prevalence and clinical significance of VIAC in patients undergoing midtrimester amniocentesis for clinical indications, using PCR for the detection of nucleic acids of viruses which have been involved in VIAC [i.e., adenoviruses, herpes simplex virus (HSV), varicella zoster virus (VZV), human herpes virus 6 (HHV6), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), parvovirus B19, and enteroviruses].

Materials and methods

Study design

This was a prospective, cohort study of 847 women undergoing midtrimester amniocentesis for clinical indications. Patients were invited to donate AF for research purposes, as well as a sample of maternal blood obtained at the time of amniocentesis. The clinical outcome was obtained by chart review or by contacting the referring physician.

The collection of samples as well as clinical data was approved by the Institutional Review Boards of the participating Institutions (Padova, Azienda Ospedaliera and Treviso, Azienda Ospedaliera, Veneto Region, Italy). All women provided written informed consent.

Collection and processing of AF

AF was obtained by transabdominal amniocentesis and 4–5 mL were obtained for research purposes. AF samples were centrifuged at 1300×*g* for 10 min and stored at −80 °C until use. The AF underwent Gram stain examination [104–108], AF white blood cell count [108,109], AF glucose [110], culture for aerobic and anaerobic bacteria as well as Mycoplasma species. Plasma was collected immediately in EDTA evacuated tubes, centrifuged in a refrigerated centrifuge (2500*g*) and stored at −80 °C until use. After exclusion of 26 cases of chromosomal abnormalities and documented fetal anomalies and 92 cases of AF not adequate for nucleic acid testing, 729 cases were included in this study for viral nucleic acid detection.

PCR for viral detection

Total DNA was purified from 200 µl AF, maternal plasma, or umbilical cord blood by using the QIAmp DNA blood kit on a BioRobot 9604 Workstation (QIAGEN GmbH, Hilden, Germany). Sample adequacy was tested by real-time PCR amplification of the β-globin (*BGL*) gene [111].

Quantitative real-time PCR was used to detect the following panel of viruses: adenoviruses, HSV, VZV, HHV6, parvovirus B19, HCMV, enteroviruses and EBV. These viruses were selected considering their frequency in the general population, potential role in adverse pregnancy outcome and the lack of precise information about their prevalence in the midtrimester AF. About 100 ng of total DNA was used for quantitative real-time PCR detection of viral nucleic acids, which was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using previously reported oligonucleotide primers (Sigma-Aldrich, St. Louis, MO, USA) and TaqMan probes (Applied Biosystems) [111–114] and, for adenovirus DNA detection, a commercially available CE-marked real-time PCR kit (Adenovirus R-gene™, Argene). Adenovirus DNA was also detected by end-point PCR by using the following oligonucleotide primers: forward 5′-TGACTTTTGAGGTGGATCCCATGGA-3′ and reverse 5′ GCCGAGAAGGGCGTGCGCAGGTA-3′. PCR conditions were 40 cycles at 95° for 60 s, 60° for 60 s, and 72° for 60 s. Amplicons were revealed by agarose gel electrophoresis. The limit of detection of the test was 100 copies/mL, the limit of quantification was 300 copies/mL, the intra-day, inter-day and inter-laboratory coefficient of variation varied with the viral load. The total mean coefficient of variation was 0.03%.

Detection of maternal antibodies against specific viruses

Samples of maternal serum were collected at the time of amniocentesis and at the time of delivery. IgM and IgG antibody testing for HCMV and HSV infection was performed by ELISA (Enzygnost; Dade Behring - Siemens Healthcare Diagnostics Inc., Tarrytown, NY). Testing for IgM antibodies against EBV-viral capsid antigen (VCA) and IgG antibodies against EBV-early antigen, EBV VCA, and EBV-nuclear antigen was performed by ELISA (DiaSorin). B19 IgM and IgG were detected by enzyme immunoassay (Biotrin, Dublin, Ireland). Anti-HHV6 IgM and IgG antibody testing was performed using an immunofluorescence test (EUROIMMUN AG, Lubeck, Germany).

Viral DNA and specific antibodies were also studied in the three available umbilical cord plasma samples collected at birth. Concentrations of Il-6 and IP-10 in amniotic fluid and maternal plasma were measured by a specific immunoassay according to the manufacturers' instructions (R&D Systems, Minneapolis, MN, USA). The sensitivity of the assay was 0.1 and 4.9 pg/mL, respectively. The intra-assay coefficient of variation was 1.2 and 3.7%, respectively. The inter-assay coefficient of variation was 3.7 and 3.8%, respectively. The relationship among the presence of viruses in AF and IL-6 and IP-10 concentrations in AF and maternal serum, pregnancy outcome, and neonatal outcome at 1 year of age were analyzed.

Results

Amniocenteses were performed because of the following indications: advanced maternal age (437/729, 59.9%), abnormal serum screening (203/729, 27.8%), suspected fetal anomaly (46/729, 6.3%), previous child affected with aneuploidy (28/729, 3.8%) suspected viral infection (11/729, 1.5%) maternal request (10/729, 1.4%) and other indications (8/729, 1.1%).

There were no differences in demographic and clinical characteristics between the groups with positive and negative PCR for viruses in the AF. The demographic data and clinical

characteristics of the cohort according to the presence or absence of VIAC are displayed in Table I.

The prevalence of viral genome sequences in AF was 2.2% (16/729). HHV6 was the most commonly detected virus (7/729, 1%), followed by HCMV (6/729, 0.8%), parvovirus B19 (2/729, 0.3%) and EBV (1/729, 0.1%), while HSV, VZV, enteroviruses, and adenoviruses were not detected. All cases of VIAC were due to a single virus. The highest rate of VIAC was among women who had an amniocentesis due to advanced maternal age (1.2%, 9/437).

In all cases with VIAC, the presence of the specific virus detected in AF was investigated in maternal plasma collected at amniocentesis and, when available, at delivery and in the 29 controls with absence of viral nucleic acids in AF.

HHV6 DNA was detected in maternal blood of six out of seven women with HHV6-positive AF, and in the umbilical cord plasma, which was available in one case. In all AF, maternal blood, cord blood, and placenta samples, the HHV6 genome copy number was high and similar to the copy number of the *BGL* gene (which is a single copy gene in the human genome), consistent with HHV6 genome integration into cellular chromosomes and genetic infection. In two of the HHV6 DNA-positive AF cases, serum samples were available for antibody analysis, and HHV6-specific IgG, but not IgM, were detected.

In women with AF positive for parvovirus B19, HCMV and EBV, maternal plasma was negative for viral nucleic acids. However, in one case of AF positive for HCMV at amniocentesis, cord blood was also positive for HCMV DNA (2399 genome copies/mL). In patients without VIAC, viruses were not detected in maternal blood. The presence of viral genome sequences, including the number of copies in AF and maternal blood, is displayed in Table II.

The median AF and maternal plasma IL-6 and IP-10 concentrations were not significantly different between patients with and without VIAC (Table III). An elevated AF IP-10 concentration (≥2200 pg/ml; above the 95th percentile) was more frequently observed in patients with VIAC than in those without VIAC (18.8%, 3/16 vs. 5.7%, 40/704; $p = 0.06$); however, the difference did not reach statistical significance. In contrast, there was no significant difference in the prevalence of elevated concentrations of IL-6 (≥2935 pg/ml; above the 95th percentile) between the two groups (with VIAC: 6.3%, 1/16 vs. without VIAC: 5.3%, 38/713; $p = 0.6$).

To examine the relationship between viral load and the intra-amniotic inflammatory response, we focused on two viruses: HCMV and HHV6. For HCMV, the higher the viral copy number, the higher the maternal plasma concentration ($n = 6$; Spearman Rho 0.9; $p = 0.008$) and AF concentration of IP-10 ($n = 6$; Spearman Rho 0.8; $p = 0.05$). There was no relationship between HHV6 copy number and maternal plasma or AF concentration of IP-10. There was no relationship between viral copy number and the AF and maternal plasma concentration of IL-6.

Fourteen patients with VIAC had a normal term delivery of a healthy neonate. Two patients had complications of pregnancy. One patient with VIAC due to HHV6 developed gestational hypertension. The other case of VIAC due to HHV6 delivered at 33 weeks after preterm premature rupture of membranes (PPROM). The neonate was admitted to the Intensive Care Unit and discharged after 3 weeks without complications. Interestingly, in this case, HHV6 was found in maternal blood at amniocentesis, admission for PPROM and at delivery after three days of admission. A high number of viral genome copies (3500 copies/mL) for HHV6 was also present in umbilical cord plasma. The placenta showed evidence of chorioamnionitis and funisitis. The Gram stain of the membranes was negative for bacteria.

There was no correlation between VIAC and adverse neonatal outcome at one year of age. All neonates were healthy.

Table I. Clinical characteristics of the study population.

	Viral PCR in amniotic fluid		
	Negative ($n = 713$)	Positive ($n = 16$)	p
Maternal age (years)	36 (35–38)	36 (33–37)	0.4
Ethnicity			
Caucasian	699 (98%)	16 (100%)	0.9
African American	6 (0.8%)	--	
Asian	3 (0.4%)	--	
Others	5 (0.7%)	--	
Nulliparous	292 (41%)	8 (50%)	0.5
Previous spontaneous preterm delivery	19 (2.7%)	--	1.0
GA at amniocentesis (weeks)	16.3 (15.9–17.0)	16.4 (15.8–20.4)	0.6
AF white blood cell count (cells/mL)	3 (2–6)	3 (2–7.5)	0.9
AF glucose (mg/dL)	48 (44–52)	50 (44–54)	0.3
Amniocentesis-to-delivery interval (weeks)	23 (21–24)	22.5 (18–24)	0.3
GA at delivery (weeks)	39.6 (38.6–40.6)	39.5 (38.8–40.9)	0.8
Birth weight (g)	3340 (3060–3600)	3320 (3071–3780)	0.7

Value expressed as median (inter-quartile) or number (percent).
GA, gestational age; AF, amniotic fluid.

Table II. Number of viral copies in the amniotic fluid and maternal blood of the 16 cases positive for viral DNA in the amniotic fluid.

Virus	AF copies/mL	AF copies/cell	Maternal plasma copies/mL	Maternal plasma copies/cell
HHV6	13,000	1.5	25,300	2.0
	2700	0.8	Negative	Negative
	280	0.7	150	0.6
	30,000	0.8	25,300	0.7
	2600	0.9	13,100	1.7
	15,000	1.1	50,000	2.5
	765,000	2.9	3500	1.5
PV B19	1450		Negative	
	15		Negative	
HCMV	20		Negative	
	60		Negative	
	1485		Negative	
	91,950		Negative	
	99,700		Negative	
	36,650		Negative	
EBV	15		Negative	

Table III. Amniotic fluid and maternal blood cytokine concentrations.

		Viral PCR in amniotic fluid		
		Negative ($n = 713$)	Positive ($n = 16$)	p value
Amniotic fluid	IL-6 (pg/mL)	413 (214-880)*	1031 (209-1696)	0.09
	IP-10 (pg/mL)	595 (410-972)†	736 (428-1577)	0.6
Maternal blood	IL-6 (pg/mL)	0.70 (0.55-0.97)‡	0.66 (0.49-0.88)	0.4
	IP-10 (pg/mL)	85.4 (67.3-110.7)‡	93.5 (70.9-146.8)	0.2

Values are expressed as median (inter-quartile).
*$n = 710$.
†$n = 704$.
‡$n = 632$.

Discussion

Principal findings of the study: (i) the prevalence of VIAC in the midtrimester with the panel of viruses tested in this study was 2.2% (16/729); (ii) the presence of viral nucleic acids was not associated with a detectable inflammation as judged by significant changes in the AF white blood cell count, glucose and IL-6 concentrations. However, VIAC with HCMV was associated with an increased concentration of IP-10, which was related to the viral load; (iii) mothers with VIAC were asymptomatic; (iv) the most common virus identified in midtrimester AF was HHV6, followed by HCMV; (v) VIAC with adenovirus, enterovirus, and herpes simplex virus types 1 and 2 were not detected in any cases; (vi) vertical transmission of HHV6 was demonstrated in one case in which in maternal plasma and umbilical cord serum at the time of delivery were also available and tested positive for viral DNA; (vii) pregnancy outcome of patients with VIAC was largely normal (14/16 cases). Two cases of VIAC with HHV6 had complications: one patient had preterm PROM, and the other had gestational hypertension. However, these complications may not be due to VIAC; (viii) all infants born to mothers with VIAC were followed until the age of 1 year, and there was no evidence of disease or developmental handicap.

Viral invasion of the amniotic cavity in the midtrimester

The available evidence from decades of targeted studies suggests that viruses are not present in the AF in most cases. Thus, we consider that the detection of viruses using culture techniques or molecular methods is unlikely to be a normal finding. We propose the term "viral invasion of the amniotic cavity", or VIAC, to define a condition in which a virus (or nucleic acid for viruses) is present even though there is no clinical, pathological or immunological evidence that the organism is causing disease.

Table IV displays previous studies in which viruses have been searched in AF in the midtrimester. MacLean et al. [100]. examined 277 AF specimens collected prospectively to determine by end-point PCR the frequency of detectable adenovirus, HCMV, HSV, and parvovirus B19 DNA in the AF of low-risk patients, and all tested negative. The mean gestational age of patients included in the study was 17.1 weeks (range 12.3–32 weeks).

Wenstrom et al. [115] reported a nested case-control study based upon samples of midtrimester AF collected between 1988 and 1995. Cases were defined as spontaneous pregnancy loss within 30 days of amniocentesis (excluding aneuploidy and anomalies), while controls were randomly selected samples from patients who delivered at term without complications matched for the year of the test, gestational age, maternal age or indication of amniocentesis. PCR was conducted for adenovirus, parvovirus B19, HCMV, EBV, HSV, enterovirus, influenza A. The frequency of detectable viruses was 8% (5/62) of the cases and 15% (9/60) of the controls. This difference was not statistically significant. The viruses identified were adenovirus (in 4 cases and 5 controls), HCMV (in 3 controls) and HSV (in 1 case). There was no difference in the mean IL-6 concentration in AF between patients with a positive or negative PCR for viruses. The authors concluded that the presence of VIAC (for the viruses studied) did not play a role in early pregnancy loss.

Another case-control study by Van den Veyver et al. [116]. included 303 cases and 154 controls. Cases were defined as patients at risk for fetal viral infection (non-immune hydrops, pleural effusion, ventriculomegaly, echogenic bowel, polyhydramnios, etc.). Patients undergoing genetic amniocentesis between 14 and 22 weeks were chosen as controls. Specimens of AF, fetal blood, pleural fluid and placental and fetal tissues were analyzed for the presence of viruses using PCR primers for HCMV, HSV, parvovirus B19, adenoviruses, enterovirus, EBV,

Table IV. Summary of studies reporting recovery of viral nucleic acids in the amniotic fluid in the midtrimester.

Reference	Population	*n*	Viral prevalence *n* (%)	Virus Recovered (PCR or culture)	Complications
McLean et al. (1995 [100])	Low-risk	277	0	--	--
Wenstrom et al. (1998 [115])	Low-risk: normal outcome	60	9 (15)	Adenovirus (5), CMV (3), Parvovirus B19 (1)	No significant difference in the prevalence of viruses between normal and poor outcome groups
	Low-risk: poor outcome†	62	5 (8)	Adenovirus (4), HSV (1)	
Van de Veyver et al. (1998 [116])	Low-risk	154	4 (2.6)	Adenovirus (3), CMV (1)	--
	High-risk	303*	124 (41)	Adenovirus (74), CMV (30), enterovirus (22), HSV (9), parvovirus (8), EBV (4), RSV (2)	Non-immune hydrops, ventriculomegaly, growth restriction, stuck-twin syndrome, hepatic calcifications, ascites, myocarditis, pericardial effusion, hydramnios
Burguete et al. (1999 [117])	Fetuses with and without anomalies	238	64 (26.9)	Papillomavirus (25/208, 12%) Adenovirus (64/238, 27%) CMV (32/183,18%)	Higher prevalence of viruses in amniotic fluid in women with preterm labor and premature rupture of membranes
Baschat et al. (2000 [272])	Low-risk	240	20 (8.9)	Among all pregnancies: adenovirus (39), enterovirus (5), CMV (3), RSV (1), EBV (1), parvovirus (1)	Higher prevalence of viruses in CNS malformations (7), SGA (4), multiple malformations (4)
	Fetal complications‡	138	27 (19.6)		
Petrikovsky et al. (2003 [119])	Fetuses with and without anomalies	474	9 (1.9)	Cultures positive for CMV (5), adenovirus (2), enterovirus (2)	Higher prevalence of viruses in the presence of fetal anomalies
Baschat et al. (2003 [101])	Low-risk	686	44 (6.4)	Adenovirus (37), CMV (5), EBV (2), enterovirus (2), RSV (1)	--
Reddy et al. (2005 [118])	Normal US	287	24 (8.4)	Adenovirus, enterovirus, CMV, PV B19	Higher prevalence of viruses in fetal anomalies, SGA, hydrops
	Abnormal US	136	33 (24)		

*371 specimen samples: amniotic fluid (*n* = 253), fetal blood at cordocentesis (*n* = 42), cord blood at the time of delivery (*n* = 18), fetal ascites (*n* = 8), pleural effusion (*n* = 40).
†Pregnancies lost within 30 days of genetic amniocentesis.
‡Fetal anomalies, SGA, proven maternal infections; samples obtained from amniotic fluid, fetal blood or pleural fluid.

and respiratory syncytial virus (RSV). The presence of viruses was detected in 41% (124/303) of cases and 2.6% (4/154) of controls. The most common isolates were adenoviruses (74/303, 24%), HCMV (30/303, 10%) and enteroviruses (22/303, 7%). The authors concluded that adenoviruses and enteroviruses may cause fetal infection and disease. An important observation is that the prevalence of positive PCR in the control group was 2.6% (4/154), suggesting that some asymptomatic patients have viral infection in the AF [116].

Subsequently, an observational study of 238 non-selected amniotic fluid samples from 14 to 25 weeks of gestation reports a higher prevalence of viral nucleic acids in women who experienced preterm labor [117]. The number of patients with delivery information was, however, very small ($n = 32$). The overall prevalence of viral nucleic acids was 27% for adenoviruses (64/238), 12% for papillomavirus (25/208) and 18% for CMV (32/183) [117].

In a prospective observational study of 686 patients at low risk for viral infection who underwent midtrimester genetic amniocentesis, AF was analyzed by multiplex PCR for HCMV, parvovirus B19, adenoviruses, enteroviruses, HSV, RSV and EBV [101]. All fetuses had normal anatomy on ultrasound and karyotype. Forty-four (6.4%) AF samples were positive for viral nucleic acids. Of the 44 positive AF samples, 41 (93%) had a single viral infection and two were positive for two viruses. Adenovirus was the most frequent virus (37), followed by HCMV (5), EBV (2), enteroviruses (2) and RSV (1). Parvovirus B19 and HSV were not found. Moreover, this group of investigators reported a seasonal variation in the frequency of viral detection of the amniotic cavity, which was more frequent in summer and late winter [101]. The authors emphasized that the presence of a viral genome in the AF should not be equated with viral disease.

In a subsequent case-control study [102] based upon the cohort of 686 pregnancies studied at the University of Maryland, cases were defined as those with a positive result for viral nucleic acids. Controls were patients with negative AF results. The ratio of cases to controls was 1:2, and the samples were matched for maternal age, race, gestational age at amniocentesis and reason for referral. The frequency of pregnancy complications was similar. Miller et al. reported that fetal viral infection was not associated with adverse perinatal outcome [102]. The authors were referring to the outcome of VIAC, because fetal infection was not ascertained.

Reddy et al. [118] reported a case-control study in which the frequency of CMV, parvovirus B19, adenovirus (ADV), enterovirus, HSV, EBV and RSV was examined in patients with sonographically abnormal findings and normal pregnancies. The authors reported that 24% (33/136) of patients with abnormal sonographic findings had a positive AF PCR compared to only 8.4% (24/287) of normal pregnancies ($p < 0.001$). The abnormal sonographic findings included fetal structure malformations, intrauterine growth restriction and hydrops. Based on these findings, the authors proposed that a positive AF PCR is consistent with fetal infection [118].

Similarly, Petrikovsky et al. [119], in a cohort of 474 high-risk and low-risk women undergoing genetic amniocentesis, used viral culture for HCMV, adenovirus and enterovirus and found a higher prevalence of viruses in the amniotic fluid of women with fetal anomalies [119].

Despite the limited number of reports, it seems that VIAC is found in a small proportion of patients in the midtrimester of pregnancy. The frequency reported in our study is 2.2%, while that reported by Baschat et al. [101] was 6.9%. Similarly, our results, and those of Baschat et al. [101] (both cohort studies of low-risk patients), indicate that there is no evidence that the presence of a virus in the AF in the midtrimester is associated with an excessive rate of pregnancy complications. However, the data of the study of Reddy et al. [118] suggest that VIAC may be found more frequently in patients who have an abnormal ultrasound examination (including SGA) in the midtrimester. The role of VIAC in other complications of pregnancy (such as preterm labor, preterm PROM, preeclampsia, cervical insufficiency, etc.) at the time of presentation remains to be determined.

Pathways for viral invasion of the amniotic cavity and fetal involvement

Two main pathways can result in VIAC: hematogenous and ascending invasion from the lower genital tract. Maternal viral infection can result in transplacental passage of the virus. This has been well-described with rubella [90,91,120–125], cytomegalovirus [90,91,126–154], HIV [155–172], parvovirus [173–176] and others [143,177–189]. Ascending viral infection from the cervix into the amniotic cavity can also occur, as is the case with herpes virus type 2 [183,190], human papilloma virus (HPV) [191–195] and others [99,142]. If the infection is ascending from the lower genital tract, viral invasion of the amniotic cavity would occur before fetal involvement. On the other hand, if the mechanism of transmission is hematogenous through the placenta, the virus invades the fetal systemic circulation, and secondarily, the amniotic cavity. The typical example is fetal infection with CMV [154,196–198] during a primary maternal infection. The virus is excreted in fetal urine, which is the main constituent of AF.

Intra-amniotic inflammation in cases of viral invasion of the amniotic cavity

The immune response to viruses in the amniotic cavity has not been studied rigorously. Westrom et al. [115] examined the concentrations of IL-6 in the AF of patients with positive and negative PCR assays for viruses and reported that there was no difference in the mean concentration of IL-6 between the two [115]. Our study is consistent with the observations of Westrom et al. [115], since there was no difference in the median concentration of IL-6 in AF between patients with and without positive PCR for viruses.

It is possible that the cytokine and chemokine response to VIAC is different than that of bacteria. To address this question, we measured the chemokine CXCL-10, which is mainly a T cell chemokine implicated in chronic chorioamnionitis [199–201]. We found that 19% (3/16) of patients with VIAC had a CXCL-10 (or IP-10) concentration above the 95th percentile (2.2 ng/mL). An elevated CXCL-10 tended to be more frequently observed in patients with positive PCR for viruses than in those with a negative result ($p = 0.06$). We interpret this observation as suggesting that there is a need to characterize the full complement of chemokine and cytokine profiles in the AF in VIAC and MIAC due to bacterial infections. It is possible that IL-6 concentrations may not be the optimal method to assess the inflammatory response in the context of viral infection.

The occurrence of an intra-amniotic inflammatory response [25,202–205], as well as a fetal inflammatory response [42,206–210], is important because this is a mechanism of host defense [30,211–218], and also, because fetal injury can result from an inappropriate inflammatory response [48,206,219] to the presence of viruses.

Maternal systemic inflammation in cases of VIAC

It is well established that some viral infections can elicit a robust maternal inflammatory response Indeed, the higher mortality rate of mothers during epidemics of viral infections (e.g. pandemic influenza of 1918 and 2009-10 H1N1 [189,220–260]) has been attributed to the exaggerated inflammatory response that occurs during pregnancy [261,262]. In the current study, we only examined the concentrations of IL-6 and IP-10 in the maternal circulation as reflecting the maternal inflammatory response. Neither of these showed evidence of changes when the relationship was explored in the entire dataset. When the analysis focused on HCMV, we found a correlation between the copy numbers of the virus and the concentration of IP-10 in both AF and maternal blood, suggesting that the type of virus may be important in determining the characteristics of the host response.

However, we recognize that studies with a broader range of cytokines, chemokines and cytomics of the peripheral circulation will be required to characterize the maternal host response to viruses. Of interest is that most women with VIAC did not have viral DNA detected in the maternal circulation (10/16, 62.5%). This can be interpreted as suggesting that a viral infection was present sometime in the past and a viremia allowed passage of the virus through the placenta, but the mother eradicated the virus from her circulation and the virus remained located in the amniotic cavity.

Fetal involvement in VIAC

Among 16 cases with VIAC, three had umbilical cord blood collected at the time of delivery; HHV6 and HCMV were detected in two of these cases. In the HHV6 cord blood positive case viral DNA was also detected in the maternal circulation. This is evidence that VIAC in the midtrimester may result in the presence of detectable nucleic acids in the fetal circulation. It is noteworthy that we found evidence of pathology in only one case – this patient had preterm PROM (33 weeks). Yet, it is not possible to attribute preterm PROM to VIAC or fetal involvement.

Human herpes virus 6, the most common virus found in VIAC

Human herpes virus 6 (HHV6) is a double-stranded DNA virus which has been implicated in cases of fetal hydrops [263]. Ashshi et al. detected HHV6 type A DNA in the tissues of two of eight fetuses with hydrops and in none of the 10 deceased non-hydropic fetuses [263].

Previous studies of viruses in the AF have not included HHV6. This study represents the first evidence that HHV6 is probably a frequent virus in the AF of asymptomatic women in the midtrimester. The prevalence of VIAC due to HHV6 was 0.9% (7/729). Evidence of transmission of HHV6 from mother to fetus using specific antibodies and viral nucleic acids was reported by Dunne et al. [81] and Aubin et al. [80]. in 1992. Subsequently, several authors reported the presence of HHV6 in umbilical cord blood in healthy pregnancies at term [264–266]. The prevalence of nucleic acid detection for HHV6 was 1.6% (5/305), a rate similar to that of the prevalence of HCMV [264].

One patient with HHV6-positive in the AF developed preterm PROM. It is not possible to draw any causal inference from this observation. It is important to note, however, that there is recent evidence that viral infections may predispose pregnant mice to the effect of microbial products and lead to preterm labor [77–79].

HHV6 has the potential, however, to cause direct harm to the fetus. It is capable of establishing a state of latency in humans and, under a variety of conditions, to cause central nervous system disease [267,268], in particular, encephalitis, recurrent seizures and epilepsy [268]. The association of HHV6 and multiple sclerosis remains controversial. The virus is clearly neurotropic and the ability to reactivate periodically could mirror the relapsing/remitting form of the disease.

An interesting aspect of HHV6 is that this virus can integrate into the genome of the host, and therefore, can be inherited. Viral integration does not lead to serologic signs of active infection. Transmission could happen via parental germ line DNA containing the integrated viral genome. This occurs by insertion into telomeres of chromosomes during latency rather than forming episomes, and the integrated viral genome is capable of producing virions [269,270].

The lack of detection of adenoviruses in AF

Previous studies have reported that adenovirus is the most common virus found in AF in patients in the midtrimester of pregnancy [101,115,116]. We did not detect a single case of adenovirus in this study. The difference between studies may relate to the population and also to the methods used to detect adenoviruses.

Strengths and limitations of the study

The strengths of this study are its cohort design, the sample size and the study of some viruses which have not been previously examined in a large cohort (such as HHV6). Limitations include the inherent difficulties of searching for viruses and the limited panel of viruses tested in this study. With the development of high-sequencing technologies, we anticipate that it may be possible to characterize the human virome in different sites [271], and this may provide a more comprehensive understanding of the frequency of VIAC and its clinical significance. Further studies are required to study the host immune response (fetus and mother) to different viruses.

Viral Metagenomics

Viruses are widely considered the most abundant biological entities on earth; yet, a comprehensive catalogue of the human virome remains a challenge. The traditional method for viral discovery consisted of the multiplication of a virus in cell culture, which has many limitations. Viral metagenomic analysis suggests that less than 1% of viral diversity has been characterized. Metagenomic Koch's postulates have been proposed to link the identification of a viral sequence with disease [273–275]. A frontier in reproductive biology is the characterization of the virome of amniotic fluid, the reproductive tract, fetal blood, and other biological fluids.

Conclusion

We detected viral genomes in 2.2% of AF samples obtained from women in the midtrimester of pregnancy using a panel of specific viruses. The most commonly detected virus was HHV6, followed by HCMV. The presence of a viral genome in AF was subclinical, and not associated with adverse pregnancy outcome. An intra-amniotic inflammatory response was detected with IP-10 in a small number of cases. Vertical transmission of HHV6 was detected in one case. The role of viruses in the genesis of pregnancy complications, long term sequelae and the host immune response requires further study.

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