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Brief Report

Persistent Human Pegivirus Infection across Two Pregnancies without Evidence of Vertical Transmission

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Abstract: Human pegivirus (HPgV) is best known for persistent, presumably non-pathogenic, infection and a propensity to co-infect with human immunodeficiency virus or hepatitis C virus. However, unique attributes, such as the increased risk of malignancy or immune modulation, have been recently recognized for HPgV. We have identified a unique case of a woman with high levels HPgV infection in two pregnancies, which occurred 4 years apart, without evidence of human immunodeficiency virus or hepatitis C virus infection. The second pregnancy was complicated by congenital heart disease. A high level of HPgV infection was detected in maternal blood from different trimesters by RT-PCR and identified as HPgV type 1 genotype 2 in both pregnancies. In the second pregnancy, the decidua and intervillous tissue of the placenta were positive for HPgV by PCR but not the chorion or cord blood (from both pregnancies), suggesting no vertical transmission despite high levels of viremia. The HPgV genome sequence was remarkably conserved over the 4 years. Using VirScan, sera antibodies for HPgV were detected in the first trimester of both pregnancies. We observed the same anti-HPgV antibodies against the non-structural NS5 protein in both pregnancies, suggesting a similar non-E2 protein humoral immune response over time. To the best of our knowledge, this is the first report of persistent HPgV infection involving placental tissues with no evidence of vertical transmission. Our results reveal a more elaborate viral-host interaction than previously reported, expand our knowledge about tropism, and opens avenues for exploring the replication sites of this virus.

Keywords: prenatal infection; virome; viral antibody; VirScan; ViroCap; maternal viral infection; viral protein; GBV-C; placenta; fetal viral infection

1. Introduction

Human pegivirus (HPgV) is a member of the *Pegivirus* genus within the *Flaviviridae* family (1). It is an enveloped virus, containing a positive-sense, single-stranded RNA genome of ~9,500 nucleotide, like that of hepatitis C virus (HCV), another member of the *Flaviviridae* family. HPgV has two structural proteins (E1 and E2), two predicted proteins of undetermined function (X and p* protein), and six non-structural (NS) proteins (1–4). To date, seven genotypes of HPgV-1 (species *Pegivirus C*, formerly GBV-C) have been classified, along with many variants (1,5,6), which vary in their distribution across the globe.

Knowledge regarding the biology and behavior of this virus remains scarce. HPgV-1 is a lymphotropic virus with a positive association between viremia with a risk of adult lymphomas (7,8). The prevalence of HPgV-1 infection in developed countries ranges from

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0.5 to 5% (9,10). Transmission of HPgV-1 primarily occurs through exposure to infected blood or sexual contact, and has been documented among intravenous drug users (11), blood transfusions recipients (3), patients with parenteral exposure (12), and transplant recipients (12–14). After HPgV-1 infection, 20-30% of people develop chronic infections (15). Viremia is typically cleared within 2 years in the majority of immune competent individuals (16,17). Antibodies directed toward the envelope HPgV glycoprotein E2, which is thought to be the immunodominant antigenic site, are detected as viremia is cleared (18). While the pathogenicity of this virus remains unclear, HPgV-1 has a propensity to co-infect individuals with other viral infections, particularly human immunodeficiency virus (HIV) (19,20) and HCV (6,21). HPgV interferes with the pathogenicity of HIV (22) and slows disease progression (7,9,19,23,24).

In pregnant women, the prevalence of HPgV infection is reported to be 1.1-6%, with a presumed rate of vertical transmission of up to ~65%, without HCV or HIV co-infection. These reports, however, were all from small cohorts and the route of transmission was not determined and only presumed to have been trans-placental based on detection of the virus in the newborn infant (8 positives out of 12 children, or 66.7%, born from HPgV positive mothers (25); and 13 positives out of 36 children, or 65%, born from HPgV positive mothers (26)). This presumed mother-to-infant transmission rate exceeds that of other viruses; for examples the rate of transmission for HIV is 23% (27), and the rate of transmission for HCV in mothers co-infected with HIV is 36% (28). HPgV infection in infants can persists up to 12 months with no reported adverse health effects (29). To the best of our knowledge, no one has clearly demonstrated vertical transmission by testing maternal and fetal placental tissues, and umbilical cord blood.

Here we present HPgV infection in a woman, spanning four years and two pregnancies with no evidence of vertical transmission. We used two comprehensive technologies to characterize the infection: 1) ViroCap to detect and genotype the virus (30,31), and 2) VirScan, to profile anti-viral IgG responses at epitope resolution (32). We then tested for the presence of HPgV nucleic acids by end-point PCR in first-trimester maternal plasma and umbilical cord blood samples from both pregnancies as well as maternal and fetal placental specimens from the second pregnancy.

2. Materials and Methods

2.1. Study Samples

We obtained samples from pregnant women who provided informed consent for the collection of biological samples and clinical data (Washington University in St. Louis; IRB#202002043; University of Iowa Maternal Fetal Tissue Biobank; IRB#200910784).

2.2. Pegivirus Sequence Analysis

Nucleic acid isolated from samples with Quick DNA/RNA Viral Kit (Zymo Research, # D7020) was used was used as input for the ViroCap assay [31]. Viral sequences were analyzed with the ViroMatch pipeline (33). After identifying HPgV sequences in the two maternal samples, HPgV genomes were assembled with IDBA-UD (34) and contigs were extended with PRICE software (35). The assemblies were manually reviewed with Tablet (36). Genomes were compared with NCBI BLAST (37) and MUSCLE (38) programs. For genotyping, phylogenetic trees were constructed using the NIAID Virus Pathogen Database and Analysis Resource online through the web site at www.viprbrc.org (39). Phylogeny was estimated using the maximum likelihood method with RAXML with 100 bootstraps and annotated with iTOL (40).

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2.3. RT- PCR Validation

The presence of HPgV was analyzed by nested-PCR (41) and one-step RT-qPCR (42) from the extracted nucleic acid. The HPgV RNA quantitation standard used was kindly provided by Dr. Jack Stapleton. For the nested-PCR reactions, primer sequences for PCR1 were as follows: HGV1 forward 5'-AGGTGGTGGATGGGTGAT-3′; HGV2 reverse 5′-TGCCACCCGCCCTCACCCGAA-3′. Primer sequences for PCR2 were as follows: HGV3 forward 5′-TGGTAGGTCGTAAATCCCGGT-3′; HGV4 reverse 5′-GGAGCTGGGTGGCCCCATGCAT-3′.

2.4. Viral Antibody Analysis

VirScan assay was performed as previously described (43). Sequences were aligned to the Vir3 reference genome (obtained from Dr. Steve Elledge) using Bowtie2 (version 2.2.6) with the option –very-sensitive-local. Samtools (version 1.6) was used to index and sort the BAM files and raw counts generated using the option -idxstats. All counts were normalized to 1 million reads prior to additional downstream analyses. The VirScan analysis pipeline was performed as previously described (44). To address cross-reactivity, a peptide is deemed specific, if against all other enriched peptides - 1) the calculated mean similarity score by Damerau-Levenshtein distance (R package stringdist, method='dl') is <0.125 and 2) shares < 7 contiguous amino acid sequence. Alignment of significantly enriched HPgV peptides was performed with UniProt Align tools (/www.uniprot.org/align/) to determine each peptide's starting and ending position. Then we manually aligned the peptides with the polyprotein sequence.

3. Results

3.1. Maternal Medical History

The first pregnancy of the individual resulted in a normal spontaneous vaginal delivery and the infant and postpartum course were normal. The second pregnancy 4 years later was complicated by congenital heart disease (CHD) (multiple muscular ventricular septal defects). The mother had a history of human papillomavirus (HPV) infection. Prior to her second pregnancy, the mother developed an eye melanoma. She also had 3 liver lesions (hemangioma/benign cysts) during her second pregnancy which was also complicated by iron-deficiency anemia. The mother was negative for HIV and hepatitis B surface antigen by serology testing in the clinic (data not shown). There are no other covariates known to affect HPgV infection.

3.2. Viral Sequence Analysis

By viral sequence analysis of first trimester maternal plasma from both pregnancies, we identified the complete coding sequences of the HPgV genomes. The consensus sequences from both genomes were identical and without mutations. The sequence was ~91% identical to the best match in the NCBI nucleotide database (Sequence ID MN551063.1), and the virus was determined to be most similar to HPgV-1 genotype 2 based on comparison with representative 5′-untranslated region (UTR) sequences from known genotypes (**Figure 1**).

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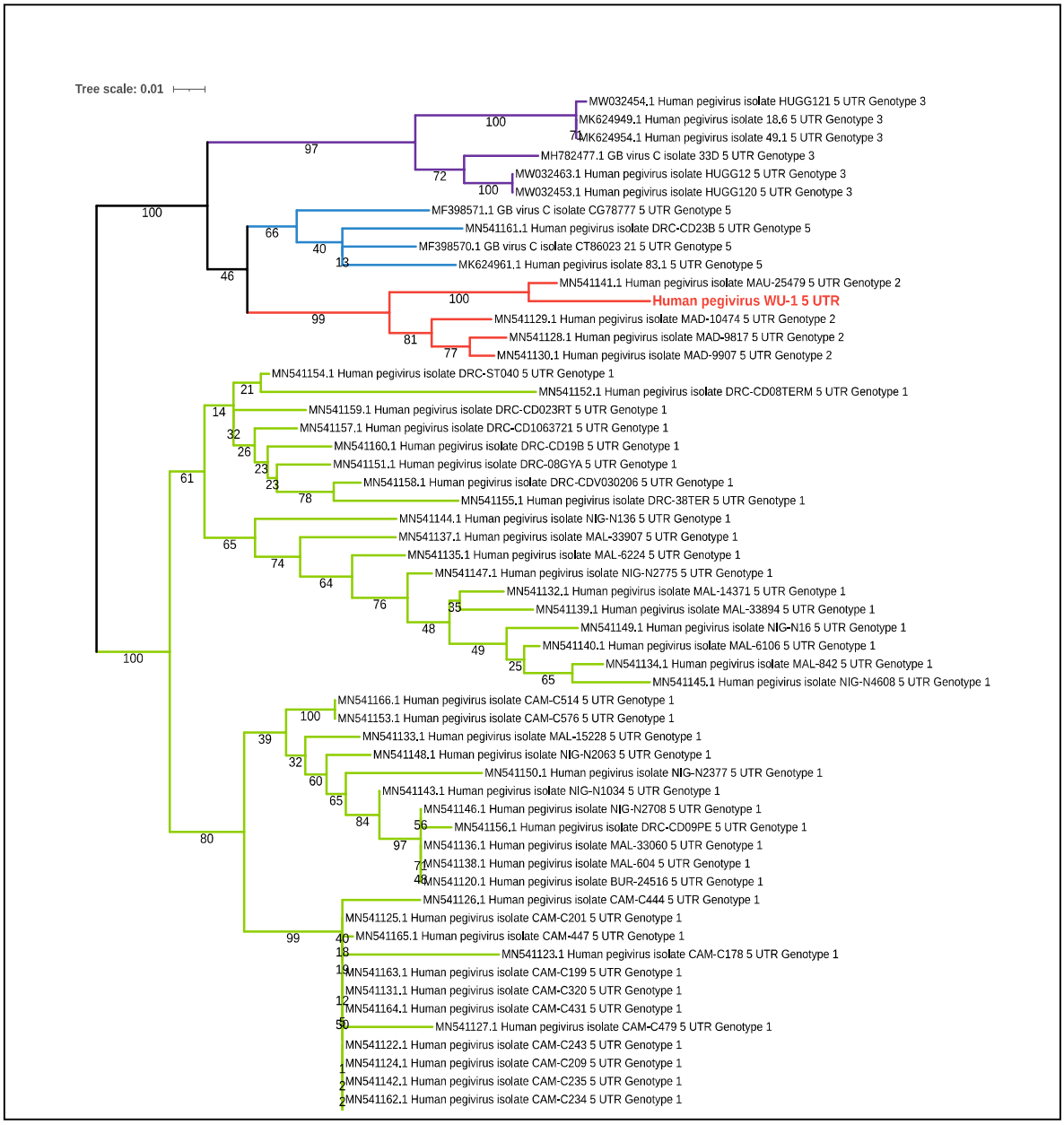

Figure 1. The human pegivirus sequenced in the first trimester maternal plasma samples shows high homology to HPgV-1 genotype 2. The 5′ UTR of the newly sequenced pegivirus genomes were compared to publicly available, curated genotyped 5′ UTR sequences (MN541120-MN541174) and supplemented with and additional sequences from GenBank to add genotype representation (MW032463.1, MW032454.1, MW032453.1, MK624961.1, MK624954.1, MK624949.1, MF398571.1, MF398570.1, and MH782477.1). Clades representing genotypes 1 (green), 2 (red), 3 (purple), and 5 (blue) are shown. The sample from this study is in the genotype 2 clade and is labeled in red font. Bootstrap values are shown on each branch.

3.3. VirScan Analysis

Using VirScan, we observed evidence of antibody responses against several viral species, including commonly observed group of viruses such as human herpesviruses, rhinoviruses, and adenoviruses, which corroborates findings from other groups (44,45). Relevant to this study, VirScan detected the presence of antibodies to HPgV peptides in the two maternal samples. There were several reactive HPgV peptides significantly enriched in those samples, but one passed through all our filtering and was considered specific (entry Q9QPC6-118095, **Figure 2**).

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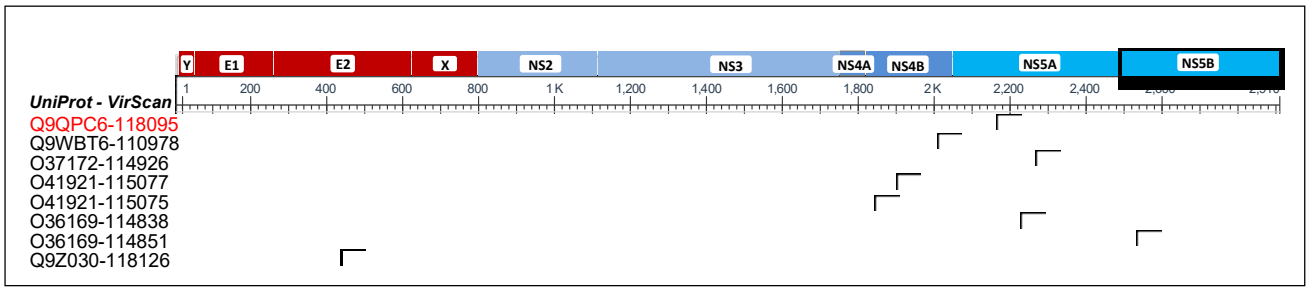

Figure 2. Alignment of HPgV significantly enriched peptides. Alignment positions of the peptide that passed through all our filtering (red text) and the other peptides (black text) are denoted by grey boxes. The row labels indicate the identifier from the VirScan library and consist of a UniProt entry ID and a VirScan peptide ID. Each peptide is aligned to the different portion of the polyprotein represented at the top of the figure by red and blue boxes, and the amino acid position is denoted just below.

3.4. Detection of HPgV by PCR

From both pregnancies, the maternal plasma samples consistently tested positive for HPgV by end-point RT-PCR, but the cord blood samples were negative (**Table 1**). Taking into consideration the possibility of low copy number or cross-contamination, RT-PCR assays were repeated several times. The detection in the maternal peripheral blood leukocytes was inconsistent (positive in 2 out of 8 independent RT-PCR reactions of the same sample preparation). In the second pregnancy, the decidua and intervillous tissue of the placenta were consistently positive for HPgV (positive in 8 out of 8 independent RT-PCR reactions of the same sample preparation); however, the chorion was negative. Results from the amnion was also inconsistent and showed a faint band in 2 out of 5 independent RT-PCR reactions of the same sample preparation.

4. Discussion

Prolonged persistence of the HPgV have been noted (7,13,46), including in infants born to mothers positive for HPgV (25,29). However, none have documented persistence of the virus over years and in multiple pregnancies. Our study, to our knowledge, is the first to show the presence of HPgV infection during two pregnancies from the same woman, 4 years apart. The patient had no prior molecular evidence for HIV or HCV infection. The first pregnancy resulted in a normal baby, with no detectable HPgV in the cord blood. In contrast, the second pregnancy was complicated by CHD. The decidua and intervillous tissue of the placenta from this second pregnancy were strongly positive for HPgV, while the cord blood and chorion showed no evidence of this virus. Altogether, the findings suggest the absence of vertical transmission in both pregnancies. The inconsistent (2/5) faint PCR bands in the amnion most likely reflects maternal tissue or blood contamination as HPgV cDNA was found to be present in high abundance in the maternal blood and placental tissues. Surprisingly, detection in maternal peripheral blood leukocytes was also inconsistent, given the reported lymphotropic nature of the virus. These data suggest low copy number of the virus in leukocytes and support the idea that we detected mostly free, circulating virus in the plasma. It is also conceivable that the endothelial cells may be an alternative site for persistence of the virus.

The second pregnancy was complicated by CHD; however, there was insufficient evidence from our data to suggest a causative effect of the persistent maternal HPgV infection. The absence of evidence for vertical transmission of the virus suggests direct fetal HPgV infection is unlikely to be a contributing factor in this case of CHD. In addition, since the infection persisted in both pregnancies but only one was affected with CHD suggests the virus is not the cause. We cannot, however, rule out the possibility of transient vertical transmission at an earlier point during pregnancy (e.g., 1st trimester) since we only examined the placenta at delivery.

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Detection of antibodies targeting the HPgV E2 protein is associated with viral clearance. There have been no reported antibody responses to the NS proteins in HPgV-1 infections (1). We were able to detect the presence of anti-HPgV-1 antibody responses simultaneously with evidence of viremia during both pregnancies. The notable peptide identified, from our VirScan analysis, in the mother as part of this immune response is a 56 aa fragment of the non-structural protein NS5, encoded by the NS5 gene of HPgV-1. This 56-aa peptide sequence maps to amino acid positions 2142-2197 of the HPgV-1 genotype 2 polyprotein (E-value: 1.1e-38; score = 324, UniProtKB: A0A895ZPP4_9FLAV). The molecular function of the NS5 protein includes RNA binding, and RNA-directed 5′-3′ RNA polymerase activity and is involved in viral RNA genome replication (47). Interestingly, the peptide fragments of this NS5 protein have been proposed as serological markers for HPgV-2 infection, a new HPgV species recently discovered (48). Perhaps the antibody profile we are seeing is a marker for those with persistent pegivirus viremia, which occurs in about 25% of those infected. Of note, a study found that amino acid polymorphisms in NS5A sequence (but not in E2 sequence) affected the sensitivity to interferon therapy and proposed this mechanism as a evasion method used by HPgV (49). Further studies are warranted to further delineate these immune responses. Importantly, our results show the potential utility of VirScan technology where no commercial antibodies are available, as in the case with HPgV.

Several genotypes of HPgV-1 have been identified world-wide by genome sequencing (3,6,12,50,51). HPgV-1 genotyping in our subject showed high homology to HPgV-1 genotype 2 and remained remarkably unchanged over the 4-year interval between the collections of samples. Thus, the VirScan, ViroCap and PCR results appear to be concordant with a maintained pattern of infection by our HPgV-1 isolate. We are not aware of other studies that have evaluated HPgV genotypes or antibody response over time in the same patient, so whether this lack of change is unusual for HPgV-1 is an interesting question for future studies.

In summary, we have identified a unique case of persistent HPgV-1 in a woman with two pregnancies in 4 years. Although the mother had HPV infections, the etiology of the HPgV-1 infection in our patient is not known and did not coincide with common co-infections (HIV and HCV). To the best of our knowledge, we are the first group to directly assess vertical transmission by testing fetal and placental tissues, and umbilical cord blood for the presence of HPgV. We saw no evidence of vertical transmission of the virus in two consecutive pregnancies. Our observation of this long-term persistent HPgV-1 infection and humoral response to NS5 epitopes during pregnancies highlights a complex interplay between virus and host and suggests putative effects on maternal health and fetal development to be explored in future studies.

Table 1. Detection of Pegivirus (HPgV) nucleic acid by end-point PCR in maternal (first trimester plasma, peripheral blood leukocytes (PBL), decidual and intervillous tissue of the placenta (IVTP), and fetal (cord blood, amnion, and chorion) samples.

Pregnancy	Maternal Plasma	PBL	Decidua	IVTP	Cord Blood	Amnion	Chorion
1st	+	?			-		
2nd	+	?	+	+	-	?	-

"+" indicates detected, "-" indicates not detected, and "?" indicates inconsistent detection. RT-PCR assay was repeated at least 5 times from the same sample preparation for all tissues.

List of abbreviations

CHD: congenital heart disease
HCV: hepatitis C virus
HIV: human immunodeficiency virus
HPgV: human pegivirus
HPV: human papillomavirus

IVTP: intervillous tissue of the placenta
PBL: peripheral blood leukocytes
UTR: untranslated region

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards of Washington University in St. Louis (IRB#202002043) and the University of Iowa Maternal Fetal Tissue Biobank (IRB#200910784).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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