Antibodies to the Novel Human Pegivirus 2 Are Associated with Active and Resolved Infections

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A novel blood-borne human pegivirus (HPgV), HPgV-2, was recently identified in hepatitis C virus (HCV)-infected individuals and individuals who had received multiple transfusions. Robust serological assays capable of detecting antibodies in HPgV-2-infected individuals are needed to establish global seroprevalence rates and potential disease associations. The two objectives of this study were to determine the utility of mammalian cell-expressed HPgV-2 E2 glycoprotein or bacterium-expressed nonstructural protein 4AB (NS4AB) in detecting past or present infections and to compare the total prevalence (antibody and RNA positive) of HPgV-2 with that of the other human pegivirus, HPgV-1 (GB virus C [GBV-C]). HPgV-2 E2 antibodies were detected in 13 (92.86%) of 14 HPgV-2-viremic cases, and NS4AB antibodies were detected in 8 (57.14%) of 14 cases. The HPgV-2 seroprevalence was significantly higher (P < 0.0001) among HCV-infected individuals (3.31% [24 of 726 samples]) than among non-HCV-infected individuals (0.30% [4 of 1,348 samples]). Of 31 anti-E2-positive samples, 22 had supplemental supporting data; 12 samples were HPgV-2 RNA positive and 10 nonviremic samples were antibody positive for peptides or NS4AB. The total prevalence of HPgV-1 (35.00%) was significantly higher than that of HPgV-2 (1.33%) in all populations tested (P < 0.0001). For HPgV-1, codetection of antibodies to E2 and RNA was infrequent (5.88%). In contrast, antibodies to E2 were detected in most HPgV-2-viremic individuals (92.86%), as is observed among individuals chronically infected with HCV, most of whom are antibody positive for HCV E2. Our studies indicate that HPgV-2 circulates with HCV and displays a profile similar to the serological profile of HCV-infected persons, although the pathogenicity of this virus has yet to be established.

Two recent independent reports describe the discovery of a novel human pegivirus (HPgV) of the family Flaviviridae, which has been provisionally designated human hepegivirus 1 (HHpgV-1) (1) or human pegivirus 2 (HPgV-2) (2). HPgV-2 and HHpgV-1 are different strains of the same virus (96% identity) and are distinct from the only other known human pegivirus, HPgV-1 (GB virus C [GBV-C]). For this report, the HPgV-2 designation is used. Both studies indicated that HPgV-2 is a highly divergent virus, is blood borne, and exhibits a low prevalence of viremia in populations at risk for parenteral exposure. In the study by Kapoor et al., 4 (1.82%) of 220 samples in cohorts with transfusion transmission or hemophilia were found to be viremic; 3 of those 4 samples were also viremic for hepatitis C virus (HCV) (1). In the study by Berg et al., 11 (0.45%) of 2,440 samples were viremic for HPgV-2, with all cases being found among individuals with active HCV infections, despite screening of other high-risk groups (HIV and hepatitis B virus [HBV] groups) and volunteer donors (2). Given that approximately 75% of HCV infections are associated with intravenous drug use (3), these data suggest that HPgV-2 is likely transmitted by a parenteral route. Similar to findings for HCV, it was noted that HPgV-2 viremia persisted for up to 5.4 years, indicating an ability to establish chronic infections (1).

The first serological assay for detection of HPgV-2 antibodies used peptides designed for regions selected from the index case sequence (2). Three peptides from nonstructural proteins (NSs) were found to be useful for identification, identifying 9 (75.00%) of 12 HPgV-2 RNA-positive samples. An antibody test using peptide 16 (P16), encompassing overlapping regions of NS4A and NS4B, had the highest rate of detection, identifying 8 of 12 HPgV-2 RNA-positive samples and resulting in a positive predictive value of 66.70%. The design of P16 was based on the HCV 5-1-1 epitope within the C100-3 antigen of HCV, which also encompasses the NS4A/B region and is an immunodominant marker for HCV infection (reviewed in references 4 and 5). While no single marker emerged with a 100% predictive value for viremia, the data presented are similar to those for HCV, in that single peptides (core peptide, NS4, and NS5A) can be useful as markers for the identification of HCV-infected individuals (6). In contrast, the use of peptides or prokaryotically expressed antigens for nonstructural proteins had limited utility as serological markers for HPgV-1 (GBV-C) (7). In order to expand on the first serological study, a recombinant antigen was designed for the NS4AB region that included the P16 peptide sequence.

In the family Flaviviridae, the envelope proteins are the major structural components of the virion surface and are important targets of the humoral immune response (8, 9). For many flaviviruses that do not establish chronic infections, such as West Nile
virus, dengue virus, and yellow fever virus, the appearance of antibodies to envelope proteins is an indicator of resolving or resolved infections (8, 10–13). Notably, for HPgV-1, antibodies to mammalian cell-expressed envelope glycoprotein E2 are associated with resolving or resolved infections, and individuals who do not develop detectable antibodies to the envelope protein establish chronic infections (14–17). In contrast, during HCV infection, chronically infected individuals maintain active infections in the presence of envelope protein-specific antibodies (18–21). Mammalian expression constructs of HPgV-1 and HPgV-2 E2 were designed, to determine whether the appearance of antibodies to E2 served as a marker for resolving/resolved infections or active infections.

One of the key first steps in establishing the prevalence and potential pathogenicity of HPgV-2 is to develop serological and molecular tests that enable accurate identification of individuals who have been infected. In the current study, we designed improved serological assays for HPgV-2, using recombinant viral proteins, and we used them to compare the seroprevalence of HPgV-2 with that of the other human pegivirus, HPgV-1 (GBV-C).

MATERIALS AND METHODS

Expression and purification of HPgV-2 NS4AB. The Protein 3D program (DNASTAR, Madison, WI, USA) was used to predict that an 81-amino acid segment of HPgV-2 NS4A/4B (derived from GenBank accession number KT437414.1) would be localized to the cytoplasm. This region was chosen for expression in the pMAL-C5X vector, which, under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, allowed for the addition of a maltose-binding protein (MBP) tag at the amino terminus and a 6-histidine tag at the carboxyl terminus (GenScript, Piscataway, NJ, USA). The construct was expressed in Escherichia coli strain BL21 (DE3). Following IPTG induction for 4 h at 37°C, cells were lysed, and soluble protein was purified using the ProBond purification system (Invitrogen, Grand Island, NY, USA). Western blotting of the purified protein was performed using a WesternBreeze chromogenic kit (Invitrogen), and purified protein was detected using an anti-His antibody (Invitrogen). Protein was visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) staining (Novex by Life Technologies) and a Bio-Rad Gel Doc EZ imager, using Image Lab v4.0 software.

Expression and purification of E2 glycoproteins from HPgV-1 and HPgV-2. Two separate expression constructs were designed to express the HPgV-1 E2 glycoprotein and the HPgV-2 E2 glycoprotein (GenBank accession number KT427414.1). The predicted ectodomain of each glycoprotein was subcloned into a mammalian expression vector containing a cytomegalovirus (CMV) promoter and a signal sequence encoding a protein tag to the microparticles. Cross-linking was performed for 3 h at room temperature, followed by fixing using 4% paraformaldehyde. Cov- erslips were washed three times with phosphate-buffered saline (PBS), followed by blocking for 1 h at room temperature in PBS with 5% bovine serum albumin (BSA) and 0.3% Triton X-100, with rocking. Both primary (anti-His) and secondary (Alexa Fluor 488) antibody incubations were carried out in PBS with 1% BSA and 0.3% Triton X-100. Coverslips were mounted onto slides using ProLong Gold reagent plus 4’,-6-diamidino-2-phenylindole (DAPI) (Thermo Fisher). Images were obtained using Metamorph software and a 20× objective on a Nikon (TE2000) inverted microscope, with fluorescein isothiocyanate (FITC) and DAPI filter cubes.

Detection of HPgV-2 antibodies by slot blotting. Purified human IgG (Southern Biosciences), NS4AB, HPgV-2 E2, and HPgV-1 E2 were diluted in 50 mM 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO) buffer (pH 7.0) at concentrations of 0.5 mg/ml (IgG), 10 and 100 μg/ml (NS4AB), and 10 and 100 μg/ml (HPgV-1 E2 and HPgV-2 E2) and were applied to individual channels of the slot blot apparatus (Immune- nets Miniblotter 28SL) containing a nitrocellulose membrane. Protein was allowed to adhere for 1 h at room temperature, with rocking, followed by aspiration of unbound protein. Membranes were washed twice with TNT wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.3% Tween 20 [pH 8.0]) and twice with 1× PBS and then were blocked for 30 min at room temperature in 1× PBS with 5% nonfat dry milk, with rocking. Membranes were washed twice with 1× PBS, after which they were cut into strips.

For immunodetection, each strip was incubated with sample diluent (TNT wash buffer with 5% nonfat dry milk and 10% heat-inactivated calf serum) for 10 min at room temperature, with rocking. Fifteen microliters of sample was added to each well containing a strip and the samples were incubated for 2 h at room temperature, followed by aspiration and three washes with TNT wash buffer. Strips were incubated for 1 h with secondary antibody [alkaline phosphatase-conjugated goat anti-human IgG (H+L); Southern Biotechnologies] in sample diluent, followed by aspiration and two washes with TNT wash buffer. Bound antibodies were detected using SigmaFast BCIP/NBT tablets dissolved in distilled water. Strips were allowed to develop for 15 min and then washed three times with distilled water.

Sample collection. Samples evaluated by Berg et al. (2) were retested if adequate serological tests were available. Overall, the following samples were purchased for HPgV-2 and HPgV-1 prevalence studies: 456 HBV-positive samples (300 from the American Red Cross [Gaithersburg, MD, USA] and 156 from ProMedDx [Norton, MA, USA]), 434 HIV-positive samples (200 from the American Red Cross and 286 from ProMedDx), and 299 HCV RNA-negative/antibody-positive samples from the American Red Cross. A volunteer donor population consisting of 416 volunteer plasmapheresis donors from the southern Midwest region of the United States (Gulf Coast Regional Blood Center, Houston, TX) was used to establish a cutoff value. The negative control consisted of pooled plasma samples negative for HCV, HIV, HBV, HPgV-1 RNA, and HPgV-1 antibodies (Architect HPgV-1 E2 assay).

Molecular screening. A multiplex quantitative PCR (qPCR) assay using primers and probes directed against the HPgV-2 targets (5’-untranslated region [UTR] and NS4/3) and the HPgV-1 target (5’-UTR) was used to screen the samples described above for the presence of HPgV-2 and HPgV-1 viral RNAs (2).

Architect testing. Magnetic microparticles (Spherotech, Lake Forest, IL, USA) were coated with 50 μg of NS4AB or 100 μg of purified HPgV-2 or HPgV-1 E2, using N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDAC) (Sigma-Aldrich, St. Louis, MO, USA) to cross-link the protein to the microparticles. Cross-linking was performed for 3 h at room temperature with end-over-end mixing, after which unbound
Expression and purification of the NS4AB recombinant protein. (A) Design of the NS4AB recombinant protein. The NS4A sequence is italicized, and the sequence representing peptide 16 (P16) is underlined. The expected size of the recombinant protein is 54 kDa. AA, amino acids. (B) Western blot of fractions from the Ni⁺ column purification of the NS4AB recombinant antigen, detected using an anti-His antibody. Lane 1, induced cytoplasmic lysate; lane 2, flowthrough fraction; lane 3, wash 1; lane 4, wash 2; lane 5, eluted protein.
testing to confirm reactivity among samples that were reactive for screening.

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dually positive for both RNA and antibodies. Reactivity for HPgV-1 markers was mutually exclusive; no samples were strongly reactive for HPgV-2 peptides or E2 (Table 3). In parallel, samples were screened for antibodies to HPgV-1 E2 (Table 4). The cutoff value for the assays was set as the median signal from a HPgV-2-negative population plus 7 SDs, requiring samples to have a substantial signal to be identified as reactive (Table 4 and Fig. 3). Populations that had been tested previously for antibodies to HPgV-2 peptides and for HPgV-2 and HPgV-1 RNA were evaluated for antibodies to recombinant HPgV-2 E2. In total, 31/2,331 samples were repeatedly reactive for HPgV-2 E2, with 22/31 showing supplemental evidence of HPgV-2 exposure (Table 3). If we consider the remaining 9 samples to be nonconfirmed and potentially false-positive samples, then the specificity was 99.61%. Consistent with previous findings (2), the greatest prevalence of antibodies to HPgV-2 E2 was found among HCV-infected donors (3.31%). Of the 24 E2 antibody-positive samples, 18 samples had supporting evidence for exposure to HPgV-2 (Table 3). Among the HCV antibody-positive/HCV RNA-negative samples, 3 (1.00%) of 299 had supporting evidence of exposure. The seroprevalence among non-HCV groups was 0.30% (4/1,348 samples), with 2 samples having supporting evidence of HPgV-2 exposure. None of the samples from volunteer donors, HIV-infected subjects, or HBV-infected subjects was HPgV-2 RNA positive, including the 4 seroreactive samples.

The total prevalence (sum of reactivity for RNA and anti-E2 reactivity) for HPgV-1 was significantly higher ($P < 0.0001$) than that for HPgV-2 in all populations tested (Table 4). While HPgV-2 RNA was detected only in HCV-infected individuals, HPgV-1 RNA was detected in all populations tested (Table 4). Similarly, the prevalence of antibodies to HPgV-1 E2 was significantly higher ($P < 0.0001$) in all populations tested, whereas antibodies to HPgV-2 E2 were most frequently detected among HCV-positive individuals (Table 4). For HPgV-2, 12/31 anti-E2-reactive samples (38.71%) were RNA positive; in contrast, 8/664 HPgV-1 anti-E2-reactive samples (1.21%) were RNA positive, thus supporting earlier reports that the HPgV-1 E2 antibody response is a marker of resolved infections (17). With respect to the coassociation of RNA and antibodies, all 12 HPgV-2 RNA-positive samples (100.00%) were anti-E2 positive, whereas only 8/158 HPgV-1 RNA-positive samples (6.78%) were anti-E2 positive.

Testing for HPgV-2 antibodies in HCV and HBV seroconversion panels. In an effort to identify a longitudinal sample displaying HPgV-2 seroconversion, 68 HBV and 42 HCV seroconversion panels.

### TABLE 1 Slot blot reactivity of HPgV-2 RNA-positive samples with recombinant HPgV-2 NS4AB, HPgV-2 E2, or HPgV-1 E2

| Sample      | HPgV-2 NS4AB | HPgV-2 E2 | HPgV-1 E2 |
|-------------|--------------|-----------|-----------|
| ABT0096P.US | ++           | +         | +         |
| ABT0070P.US | ++           | +         | +         |
| ABT0188P.US | ++           | +         | +         |
| ABT0055A.US | +            | ++        | +         |
| ABT0029A.US | ++           | +         | +         |
| ABT0128A.US | ++           | +         | +         |
| ABT0239.AN.US | –          | –        | –         |
| ABT0030P.US | –            | +         | –         |
| ABT0011P.US | –            | +         | –         |
| ABT0116A.US | ++           | ++        | +         |
| ABT0118A.US | ++           | +         | –         |
| ABT0130A.US | ++           | ++        | –         |
panels were tested for HPgV-2 antibodies. The first and last bleeds (when available) were tested for antibodies to E2 or NS4AB; however, no seroconversion panels yielded positive results (see Fig. S3 in the supplemental material). Due to the high predictive value of anti-E2 for HPgV-2 RNA positivity, only selected panel members were tested for HPgV-2 RNA, and none had detectable HPgV-2 RNA.

Concordance between peptide- and recombinant protein-based antibody tests. Twenty-nine of the 36 peptide antibody-positive samples from the original study were available for antibody testing using the recombinant proteins (2). In total, 16 of the 29 samples reactive for peptides were reactive for one or both recombinant proteins. The concordance between antibody detection using recombinant proteins and that using peptides was high for P16 (NS4AB; 93.75%) and P9 (NS5A; 56.25%) but much lower for P4 (21.43%) (Table 5). Thus, antibody detection with P4 had the lowest positive predictive value for RNA and the weakest concordance with antibody detection using recombinant proteins. With the elimination of samples reactive only for P4, the results for 16/20 peptide-positive samples (80.00%) were concordant with results obtained using recombinant proteins.

**DISCUSSION**

Here we developed antibody tests for detection of HPgV-2 employing two recombinant proteins (NS4AB and E2), and we used the tests to obtain updated seroprevalence estimates of HPgV-2 infections. More-sensitive recombinant antibody tests detected 13 (92.86%) of 14 HPgV-2-viremic samples from 13 individuals, compared to only 10 (71.43%) of 14 samples using tests based on synthetic peptides (Table 2). Notably, antibodies were detected in the presence of HPgV-2 RNA (Table 5). The positive predictive values of peptide reactivity for the presence of HPgV-2 RNA were greatest for P16 (64.28%) and P9 (50.00%) but much lower for P4 (21.43%) (Table 5). Thus, antibody detection with P4 had the lowest positive predictive value for RNA and the weakest concordance with antibody detection using recombinant proteins.

### TABLE 2 Antibody responses of HPgV-2-viremic samples to recombinant antigens

| HPgV-2 isolate | Antibody/RNA reactivity | Antibody reactivity to peptides<sup>a</sup> | S/CO<sup>b</sup> for antibody reactivity to recombinant protein | HPgV-2 viral load (log RNA copies/ml) |
|----------------|------------------------|-------------------------------------------|---------------------------------------------------------------|--------------------------------------|
|                | HCV | HPgV-1 | HIV |                            | HCV-2 NS4AB | HCV-2 E2 |                           |
| UC0125.US<sup>c</sup> | ++ | 1/2+ | –/- |                          | 9.0 (+)     | 4.6 (+) | 6.2                          |
| ABT0096P.US    | ++ | –/+ | –/- |                          | 7.46 (+)    | 26.25 (+) | 3.5                          |
| ABT0070P.US    | ++ | –/+ | –/- |                          | 12.68 (+)   | 23.60 (+) | 5.2                          |
| ABT0188P.US    | ++ | –/+ | –/- |                          | 1.08 (+)    | 43.40 (+) | 2.5                          |
| ABT0055A.US    | ++ | –/+ | –/- |                          | 0.23        | 16.04 (+) | 3.8                          |
| ABT0029A.US    | ++ | –/+ | –/- |                          | 1.33 (+)    | 1.74 (+)  | 4.6                          |
| ABT0128A.US    | ++ | –/+ | –/- |                          | 0.61        | 2.15 (+)  | 4.5                          |
| ABT2039AN.US   | –/- | –/- | –/- |                          | 0.05        | 0.14      | 5.8                          |
| ABT0030P.US    | ++ | –/+ | –/- |                          | 0.14        | 4.24 (+)  | 6.6                          |
| ABT0035P.US<sup>d</sup> | ++ | –/+ | –/- |                          | 0.33        | 8.46 (+)  | 5.9                          |
| ABT0041P.US<sup>d</sup> | ++ | –/+ | –/- |                          | 0.62        | 11.42 (+) | 6.2                          |
| ABT0116A.US    | ++ | –/+ | –/- |                          | 5.45 (+)    | 30.14 (+) | 5.2                          |
| ABT0118A.US    | ++ | –/+ | –/- |                          | 7.06 (+)    | 10.97 (+) | 6.2                          |
| ABT0130A.US    | ++ | –/+ | –/- |                          | 12.04 (+)   | 16.03 (+) | 3.2                          |

<sup>a</sup>Peptide reactivity testing was performed as described previously (2).
<sup>b</sup>The cutoff value was based on the population median plus 7 SDs.
<sup>c</sup>Limited volume was available for testing of the index case, UC0125.US; the sample was diluted 1:1 in normal human serum that had been prescreened for HPgV-1 and HPgV-2 RNA and antibodies. The sample was not screened for antibodies to HPgV-1 E2 due to insufficient testing volume.
<sup>d</sup>Samples were obtained from the same donor, with bleed dates 7 weeks apart.

### TABLE 3 Prevalence of antibodies to HPgV-2 in HCV-infected, HIV-infected, HBV-infected, and volunteer control samples

| Reference group | No. HPgV-2 antibody positive | Antibody prevalence (% [95% CI]) | P value<sup>a</sup> | S/CO<sup>b</sup> | NS4AB (RNA) | Peptide (RNA) | NS4AB and peptide (RNA) | NS4AB, peptide, or RNA |
|----------------|-------------------------------|----------------------------------|--------------------|----------------|-------------|----------------|------------------------|------------------------|
| HCV infected<sup>d</sup> (n = 726) | 24 | 3.11 (2.13–4.88) | NA | 11 | 11 (7) | 14 (7) | 11 (7) | 18/24 (75.00) |
| HCV infected (RNA negative/antibody positive) (n = 299) | 3 | 1.00 (0.21–2.90) | 0.0502 | 1 | 1 (1) | 2 (1) | 1 (1) | 2/3 (66.67) |
| HIV (n = 434) | 0 | 0.00 (0.00-0.85) | 0.0001 | 0 | 0 (0) | 0 (0) | 0 (0) | 0/0 (0.00) |
| HBV (n = 456) | 3 | 0.66 14-1.91 | 0.0023 | 2 | 2 (0) | 0 (0) | 0 (0) | 2/3 (66.67) |
| Volunteer donors (n = 416) | 1 | 0.24 (<0.01-1.33) | 0.0002 | 0 | 0 (0) | 1 (0) | 0 (0) | 0/0 (0.00) |
| Total non-HCV (n = 1,306) | 4 | 0.31 (0.08-0.78) | 0.0001 | 2 | 2 (0) | 1 (0) | 0 (0) | 2/4 (50.00) |
| Total (n = 2,331) | 31 | 1.33 (0.91-1.88) | NA | 12 | 14 (8) | 17 (7) | 12 (8) | 22/31 (70.97) |

<sup>a</sup>P values were calculated using Fisher’s exact test, comparing the HCV group with the HIV, HBV, and volunteer control groups. NA, not applicable.
<sup>b</sup>Supplemental testing was performed for HPgV-2 RNA, anti-NS4AB, or peptide reactivity.
<sup>c</sup>The sample set includes only HCV RNA-positive/antibody-positive samples; it does not include the previously identified samples UC0125.US and ABT0239AN.US (HCV RNA positive only). The longitudinal bleed samples ABT0035P.US and ABT0041P.US were counted as 2 samples in the analysis.
TABLE 4 Comparison of HPgV-1 and HPgV-2 prevalence rates using molecular and serological tools

| Group                      | Total no. (%) positive (RNA or antibody) | No. (%) RNA positive | No. (%) RNA and anti-E2 positive/total no. RNA positive | No. (%) RNA and anti-E2 positive/total no. RNA positive | P value<sup>a</sup> |
|----------------------------|------------------------------------------|----------------------|--------------------------------------------------------|--------------------------------------------------------|------------------|
|                            | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 | HPgV-1 | HPgV-2 |
| HCV infected<sup>b</sup>   | (n = 726) | 354 (48.76) | 24 (3.31) | 0.0001 | 67 (9.23) | 11 (1.51) | 3/287 (1.04) | 11/24 (45.89) | 3/67 (4.47) | 11/11 (100.00) | 0.0001 |
| HCV antibody positive/RNA  | 173 (57.86) | 3 (1.00) | 0.0001 | 15 (5.02) | 1 (0.33) | 1/158 (0.63) | 1/3 (33.33) | 1/15 (7.86) | 1/1 (100.00) | 0.1250 |
| negative (n = 299)        |                                        |                      |                        |                        |                      |                    |                        |                    |                      |
| HIV infected (n = 434)    | 181 (41.71) | 0 (0.00) | 0.0001 | 51 (11.75) | 0 (0.00) | 2/130 (1.54) | 0/0 (0.00) | 2/51 (3.92) | 0/0 (0.00) | NA |
| HBV infected (n = 456)    | 53 (11.62) | 3 (0.66) | 0.0001 | 8 (1.75) | 0 (0.00) | 2/45 (4.44) | 0/3 (0.00) | 2/8 (25.00) | 0/0 (0.00) | NA |
| Volunteer control (n = 416) | 55 (13.22) | 1 (0.24) | 0.0001 | 17 (4.09) | 0 (0.00) | 0/38 (0.00) | 0/1 (0.00) | 0/17 (0.00) | 0/0 (0.00) | NA |
| Total (n = 2,331)         | 816 (35.00) | 31 (1.33) | 0.0001 | 158 (6.78) | 12 (0.51) | 8/658 (1.21) | 12/31 (38.71) | 8/158 (5.06) | 12/12 (100.00) | 0.0001 |

<sup>a</sup>P values were determined using Fisher’s exact test, comparing HPgV-1 and HPgV-2. NA, not applicable.

<sup>b</sup>The group does not include the previously identified samples UCO125.US and ABT0239AN.US (HCV RNA positive only). Overall, of the 14 known HPgV-2 RNA-positive samples, 13 (92.86%) had detectable anti-E2 responses.
The serological analysis presented here reveals the stark differences in the prevalence of HPgV-1 and HPgV-2 in donor populations. The total prevalence rates (detection of RNA and/or E2 antibodies) among the populations tested (n = 2,331) were 35.00% and 1.33% for HPgV-1 and HPgV-2, respectively (Table 4). The prevalence rates for HPgV-1 (48.76%) and HPgV-2 (3.31%) among actively HCV-infected individuals were higher than those observed among volunteer donors or those infected with HIV or HBV (Table 4). HPgV-1 RNA was detected in all groups tested (6.78%), whereas HPgV-2 RNA was restricted to the HCV-infected group (1.51%). Similarly, the presumably nonpathogenic and transfusion-transmitted anelloviruses have been detected in all of these groups, including healthy donors, and have distributions comparable to that of HPgV-1 (26–28). Thus, there is a notable dichotomy in which commensal viruses (anelloviruses and HPgV-1) are more prevalent across all populations, with HPgV-2 showing clear enrichment in populations coinfected with the pathogenic virus HCV. Although evidence of any clinical association with HPgV-2 is currently not known, the specific association with pathogenic HCV may be relevant to HPgV-2 infections.

![FIG 3](http://jcm.asm.org/)

Distribution of S/CO values for anti-HPgV-2 E2 reactivity. Numbers of samples are plotted on the y axes, and the corresponding S/CO values are plotted on the x axes. Dashed vertical lines, cutoff values of the population-based median plus 7 SDs; values greater than 1 were considered positive. *, positive samples (S/CO of >1); accompanying numbers indicate the numbers of samples. The x axis for the HCV data set was modified to show samples with greater S/CO values.

![TABLE 5](http://jcm.asm.org/)

**TABLE 5** Correlation between HPgV-2 peptide and recombinant protein results

| Peptide      | No. (%) of samples positive for recombinant antigen and peptide/total no. | No. (%) of HPgV-2 RNA-positive samples with peptide reactivity/total no.* |
|--------------|---------------------------------------------------------------------------|---------------------------------------------------------------------|
| P4 (NS3)     | 1/16 (6.25)                                                               | 3/14 (21.43)                                                          |
| P9 (NS5A)    | 9/16 (56.25)                                                              | 7/14 (50.00)                                                          |
| P16 (NS4AB)  | 15/16 (93.75)                                                             | 9/14 (64.28)                                                          |

* Reactivity was based on an S/CO value of >1, from the study by Berg et al. (2).
With these new tools, we now possess the ability to detect both active and resolved HPgV-2 infections. In screening populations that share blood-to-blood contact as a route of transmission (HCV, HIV, and HBV), we have noted active infections only in the HCV-infected group, but the low antibody prevalence in all groups tested suggests that HPgV-2 infections are not restricted to HCV-infected individuals. It is thus expected that over time, as larger populations are screened, we may find additional HPgV-2 cases without HCV coinfection. In particular, screening for HPgV-2 in populations at risk of parenteral transmission but without HCV coinfection (e.g., patients who have received multiple transfusions and intravenous drug users who test negative for HCV) will be of interest in finding HPgV-2 cases. As more strains of HPgV-2 from different geographic regions are revealed, molecular and serological assays will likely be refined to detect diversity and to provide a complete epidemiological picture. Continued efforts to identify more cases of HPgV-2 infection are under way, for better understanding of the clinical relevance, epidemiology, and pathogenesis of HPgV-2.

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