Original Article

Prevalence and potential impact of human pegivirus-1 on HIV-1 disease progression among Indian PLHIV

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Abstract

Introduction: Human pegivirus-1 (HPgV-1) influences the pathogenesis and outcome of viral infections. We investigated the prevalence and impact of HPgV-1 due to the paucity of studies on Indian people living with HIV (PLHIV).

Methodology: Samples were collected from 347 treatment-naïve PLHIV; and 100 blood donors negative for HIV, HBV, and HCV. CD4+ T-cell and HIV-1 viral load were measured using flow-cytometry and quantitative polymerase chain reaction (qPCR), respectively. HPgV-1 was quantified and genotyped by qPCR and Sanger sequencing, respectively.

Results: HPgV-1 viremia in PLHIV and controls was 11% (38/347) and 1% (1/100), respectively. We found HPgV-1 genotype-2a in PLHIV and genotype-2b in controls. Male preponderance was seen in HIV-1 mono-infection and co-infection groups (166 vs. 143 and 33 vs. 5; $p < 0.0001$). The peak prevalence of HPgV-1 was at 31–50 years ($p = 0.02$). CD4+ T-cell count (245.5 vs. 240; $p = 0.59$) and HIV-1 log viral load (4.7 vs. 4.9; $p = 0.50$) were not significantly different between the HIV-1 mono-infected and coinfected individuals. However, a direct correlation existed between HpgV-1 viral load and CD4+ T-cell count ($r = 0.27$, $p = 0.05$) and an inverse correlation with HIV-1 viral load ($r = -0.21$, $p = 0.10$).

Conclusions: This is the first study in India to estimate the HPgV-1 prevalence in PLHIV with the predominance of genotype-2a. HPgV-1 viremia had a moderate impact on CD4+ T-cells and HIV-1 viral load, which requires a longitudinal study to identify the beneficial influence on HIV-1 disease progression and outcome.

Key words: Human pegivirus; HIV-1; HPgV-1; genotype; India.

Introduction

Human pegivirus-1 (HPgV-1) belongs to the family Flaviviridae, genus *Pegivirus*, and species *hominis* [1]. It is a positive-sense single-stranded RNA with a genomic size of approximately 9.3 kb, comprising two structural and six non-structural proteins [2,3]. HPgV-1 is classified into seven genotypes with distinctive geographical distributions [4–6].

The global prevalence of HPgV-1 in the general population ranged from 0.8% to 44.6%, whereas the prevalence in the risk group, including intravenous drug users, commercial sex workers, and men who have sex with men, varied from 1.8% to 75.3% [7]. The prevalence of HPgV-1 in developing countries was higher than in developed countries [7]. Given the similar transmission routes (parenteral, sexual, and mother to child), the risk of co-infection of human immunodeficiency virus-1 (HIV-1) and HPgV-1 is high [1]. The HIV-1/HPgV-1 co-infection prevalence varies between 5% to 47.9%, globally [7]. In Asia, the prevalence of HIV-1/HPgV-1 co-infection varied among different countries: Indonesia (88%), Lebanon (30%), Russia (26.2%), China (25.6%–38.8%), Japan (20.3%), Iran (19.2%), Cambodia (10.8%), and Singapore (10%) [8–15].

Several studies have reported a direct correlation between CD4+ T cell count and HPgV-1 viral load, and an inverse correlation between HIV-1 and HPgV-1 viral load. Laboratory findings in HIV-1/HPgV-1 coinfection show an elevated CD4+ T cell count,

decreased HIV-1 viral load, and an association with clinically slower HIV progression. However, HIV-1/HPgV-1 co-infection studies in the Indian population are rare. The aim of this study was to determine the prevalence of HPgV-1 in the antiretroviral treatment (ART) naïve HIV-1-infected population and HIV-1 negative healthy controls from India, to identify the HPgV-1 genotypic distribution, and to ascertain its influence on HIV-1 viral load and CD4+ T cell count.

Methodology

This cross-sectional study was done in the Department of Clinical Virology of a tertiary care centre in India, with samples collected between 2009 and 2019. The study was approved by the Institutional Review Board (minutes No. 11055, dated 20 December 2017). Archived plasma samples from patients who had given informed consent for the use of samples for further testing were stored at -80 °C. The study group included antiretroviral treatment (ART)-naïve HIV-1-infected individuals above the age of 18 years. HIV-2-positive individuals were excluded. The minimum sample size calculated for the study was 323, and we included 347 samples for the study. The control group included 100 healthy blood donors who were HIV, and hepatitis B and C negative.

Estimation of CD4+ T cells and HIV-1 viral load

The CD4+ T cell estimations were carried out at the time of sample collection using flow cytometry (BD FACS Count system, San Jose, U.S.A., and Sysmex CyFlow Counter, Sysmex, Goerlitz, Germany). HIV-1 viral load testing in these samples was done using artus® HI Virus-1 RG RT-PCR Kit (Qiagen GmbH, Hilden, Germany) and Abbott RealTime HIV-1 assay (Abbott, Abbott Park, Illinois, USA).

Extraction of nucleic acids for HPgV-1 RNA

RNA extraction was done using QIAamp® viral RNA manual extraction reagents (Qiagen GmbH, Hilden, Germany) and an automated Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega Corporation, Madison, USA) using 140 µL plasma samples and 50 µL of elution. The extracts were stored at 4 ºC for immediate use or -80 ºC for later use. Nuclease-free water and RNA constructs were used as negative and positive controls, respectively.

Qualitative real-time HPgV-1 PCR

The qualitative real-time reverse transcriptase polymerase chain reaction (RT PCR) was done using previously published primers and probes [16].

Preparation of RNA constructs and HPgV-1 standards

The segment of the hepatitis G virus (GenBank: U44402.1; 9392 bp) that was compatible with the already-published primers [16] was used for creating the template for the construct. The selected segment was 140 bp, and the BLAST in the NCBI database showed a 100% match (E value: 9e-65) with pegivirus-1 (Accession No. MK684252.1) [17]. A spacer and the T7 promoter (TAATACGACTCACTATAGGG) sequence were included at the 5' end of the desired sequence, and gBlocks® gene fragments were synthesized with the desired sequence by IDT (Integrated DNA Technologies, Coralville, Iowa, United States). gBlocks® are custom, double-stranded, sequence-verified fragments of DNA up to 500 bp developed by IDT. Transcription-mediated amplification was done on the desired gBlocks® as a template using RiboMAX™ Large Scale RNA Production System — T7 (# P1300; Promega, Madison, USA). All the manufacturer's instructions were followed. The concentration of RNA transcripts was measured, and multiple aliquots of the desired concentration were stored at -80 °C until use. These were used as a positive control for the real-time PCR until a positive sample was obtained from the tested study samples.

A 356 bp fragment of the 5' UTR was amplified from the RNA of the suspected HPgV-1 infected individual using conventional PCR (discussed below). The sample was confirmed as HPgV-1 by Sanger sequencing. The HPgV-1 amplicon was cloned into an expression vector using the TOPO TA Cloning Kit for subcloning, with One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, USA) as per the manufacturer's instructions. The DNA concentration was checked using a spectrophotometer (EPOCH, Agilent, Santa Clara, USA). The copy number of the plasmids was calculated, and different plasmid dilutions were made for use as standards.

Quantitative real-time HPgV-1 PCR (qPCR)

The extracted RNA was amplified with already-published primers and probes targeting the 5' UTR region, and the target size was 86 bp [16]. The PCR was performed using QuantiFast pathogen + IC kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions on Rotor gene 6000 (Qiagen, Hilden, Germany). The standards ranged from 10^7 to 10^3 copies/mL and were included in every PCR run. The PCR reaction consisted of 5 µL of 5X QuantiFast pathogen MM (Qiagen, Hilden, Germany), 0.25 µL of 100X QuantiFast pathogen RT Mix (Qiagen, Hilden,

Germany), 1 µL of 10X pathogen-specific mix, 2.5 µL of 10X IC assay, 2.5 µL of 10X IC RNA, 8.75 µL of nuclease-free water, and 5 µL of RNA extract. The cycling conditions were 50 ºC for 20 minutes, initial denaturation at 95 ºC for 5 minutes, followed by 50 cycles of 95 ºC for 15 seconds, and 60 ºC for 30 seconds.

Conventional PCR of HPgV-1 for genotyping

Conventional PCR was performed with the HPgV-1 positive samples. The forward primer 5' - CCA GAA ACC GAC GCC TAT CT -3' (nucleotide position 45 to 64 according to GenBank accession number U44402) and reverse primer 5'- CCC ACT GGT CCT TGT CAA CT -3' (nucleotide position 400 to 381) targeting the 5' UTR region were used. The PCR reaction comprised 10 mL of 5X PCR buffer, 2 µL of dNTPs, 1 µL each of forward and reverse primers (40 picomoles/µL), 2 µL of *Taq* polymerase, 14 µL of nuclease-free water, and 20 µL of the RNA extract. The cycling conditions were 50 °C for 30 minutes, initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 45 seconds, 55 °C for 1 minute, 72 °C for 2 minutes, and final extension at 72 °C for 7 minutes. The PCR products were detected using 2% agarose gel electrophoresis. For this, 5 µL of each PCR product (size: 356 bp) was mixed with 3 µL of tracking dye (bromophenol blue with sucrose). The gels were visualized on a gel documentation system (Gel Doc, Bio-Rad, Hercules, USA), and images were analyzed using the Quantity One software version 4.1.1 (Bio-Rad, California, USA).

HPgV-1 sequencing and phylogenetics

Sequencing was performed for all positive samples for which extracts were available. The sequencing was done using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequenced samples were aligned using MUSCLE, and a maximum-likelihood phylogenetic tree was created using PhyML 3.0 in Geneious Prime®

Table 1. Characteristics of the study population based on HPgV-1 RNA status.

Characteristics	HIV-1 mono infected	HIV-1/HPgV-1 co-infected	*p* value
Study subjects (n = 347)	309 (89%)	38 (11%)	Not applicable
Gender			< 0.0001
Male (n = 199)	166 (83.4%)	33 (16.6%)	
Female (n = 148)	143 (96.6%)	5 (3.4%)	
Age (Median, IQR)	38 (32-45)	40 (37-44)	0.49
Age groups (n%)			0.02
18–30 years (n = 61)	58 (95.1%)	3 (4.9%)	
31–50 years (n = 244)	210 (86.1%)	34 (13.9%)	
51–70 years (n = 42)	41 (97.6%)	1 (2.4%)	
State-wise distribution (n)			0.04
Andhra Pradesh (n = 55)	45	10	
Assam (n = 6)	5	1	
Bihar (n = 3)	2	1	
Chhattisgarh (n = 4)	4	0	
Jharkhand (n = 10)	10	0	
Karnataka (n = 7)	3	4	
Kerala (n = 7)	6	1	
Maharashtra (n = 2)	2	0	
Manipur (n = 2)	2	0	
Mizoram (n = 2)	2	0	
Nagaland (n = 3)	3	0	
Odisha (n = 3)	3	0	
Tamil Nadu (n = 212)	192	20	
Telangana (n = 3)	3	0	
West Bengal (n = 25)	24	1	
Unknown (n = 3)	3	0	
CD4+ T cells (Median (IQR))	248.5 (155.3–417.3)	240 (180–423.5)	0.67
CD4+ T cells (cells/µL) stratified, n (%)			0.43
< 200 cells/µL (n = 123)	110 (38.2%)	13 (35.1%)	
200–499 cells/µL (n = 142)	124 (43.0%)	18 (48.7%)	
≥ 500 cells/µL (n = 60)	54 (18.8%)	6 (16.2%)	
Total	288 (100%)	37 (100%)	
Not available (n = 22)	21	1	
HIV-1 VL (copies/mL), (median (IQR))	4.7 (3.8-5.3)	4.9 (3.9-5.4)	0.48
HIV-1 VL (copies/mL) stratified; n (%)			0.94
< 1,000 copies/mL (n = 42)	38 (90.5%)	4 (9.5%)	
1,000–9,999 (n = 57)	51 (89.5%)	6 (10.5%)	
≥ 10,000 copies/mL (n = 248)	220 (88.7%)	28 (11.3%)	

HPgV-1: human pegivirus-1; HIV: human immunodeficiency virus.

2023.2.1 [18]. A heat map was created to compare the identity percentage of sequences from the current study and sequences from an earlier study [19].
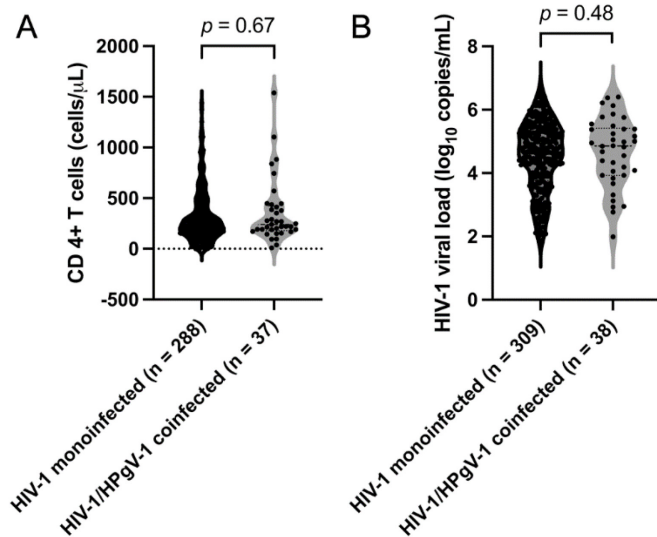
Statistical analysis

The demographic data and laboratory data were collected and analyzed using Microsoft Excel 365. Fisher's exact test was used for gender data, Chi square test was used for comparison of age and CD4+ T cell counts, and Mann Whitney test for comparison of median CD4+ T cells and HIV-1 viral load between HIV-1/HPgV-1 co-infection vs. HIV-1 mono-infection. The data generated from CD4+ T cells and HIV-1 viral load between HPgV-1 positive and HPgV-1 negative were plotted using a violin plot. Pearson correlation tests and linear regression analyses were used for HPgV-1 viral load vs. CD4+ T cells and HIV-1 viral load. All statistics were performed using GraphPad Prism version 10.0.2 for macOS (GraphPad Software, San Diego, California, USA, www.graphpad.com). A *p* value of ≤ 0.05 was considered significant.

Results

The HPgV-1 qPCR was standardized with a limit of quantification of 7.5 copies/reaction, and a dynamic range of 10^3 to 10^8 copies/mL. HPgV-1 RNAemia was significantly higher in the ART-naïve HIV-1-infected individuals (11%, 38/347) than the healthy controls (1%, 1/100); *p* = 0.002. A male preponderance was seen in both HIV-1 mono-infection (male: 166 vs. female: 143) and co-infection (male: 33 vs. female: 5); *p* <

0.0001. There was no significant difference in the median age between HIV-1 mono-infected and HIV-1/HPgV-1 coinfected individuals (38 vs. 40, *p* = 0.49). However, peak HPgV-1 prevalence was observed between the ages of 31 and 50 years (*p* = 0.02) when compared to other age groups. Additional characteristics of HIV-1 mono-infected and HIV-1/HPgV-1 coinfected groups are presented in Table 1.

The baseline median absolute counts throughout the cross-sectional estimation of CD4+ T cells did not show a significant difference between HIV-1 mono-infected and HIV-1/HPgV-1 coinfected individuals (248.5 vs. 240, *p* = 0.67) (Figure 1A). Stratification based on the baseline CD4+ T cell counts (< 200 cells/µL, 200–499 cells/µL, $\geq$ 500 cells/µL) also showed no significant difference between HIV-1 mono-infected and HIV-1/HPgV-1 coinfected individuals. A significant, weak direct correlation was observed between HPgV-1 viral load and nadir CD4+ T cell count (r = 0.27, *p* = 0.05)

Figure 2. Pearson correlation of HPgV-1 viral load with (A) CD4+ T cell count and (B) HIV-1 viral load.

HPgV-1: human pegivirus-1; HIV: human immunodeficiency virus.

Figure 1. Violin plots of the distribution of (A) CD4+ T cells and (B) HIV-1 viral load in HIV-1 mono-infected and HIV-1/HPgV-1 coinfected individuals.
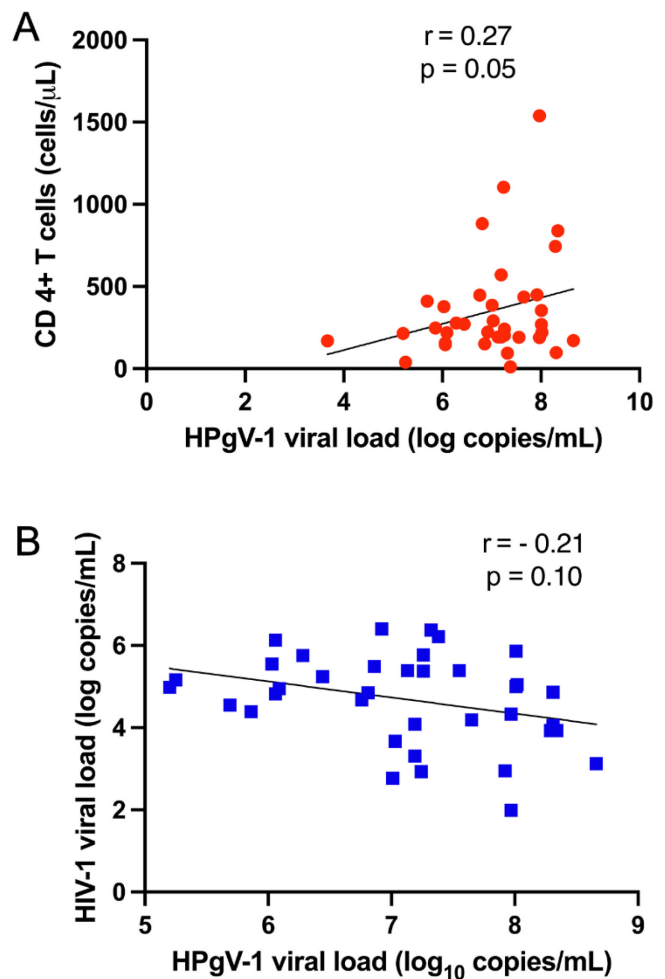

HIV: human immunodeficiency virus; HPgV-1: human pegivirus-1.

(Figure 2A). Similarly, the baseline HIV-1 viral load between the HIV-1 mono-infected and HIV-1/HPgV-1 coinfected groups was not significantly different (4.7 vs. 4.9 log copies/mL, $p = 0.48$) (Figure 1B). A weak inverse but insignificant correlation was observed between the HPgV-1 viral load and HIV-1 viral load (r = –0.21, $p = 0.10$) (Figure 2B).

Out of the 38 HIV-1/HPgV-1 positive samples, 32 were successfully genotyped (Figure 3) and identified as HPgV-1 genotype 2a. All 32 HPgV-1 sequences have been submitted to GenBank (accession nos. ON584189, ON637706-ON637728, and OR231960-OR231967).

Figure 3. Maximum likelihood tree of HPgV-1 genotypes using the 5' UTR region.
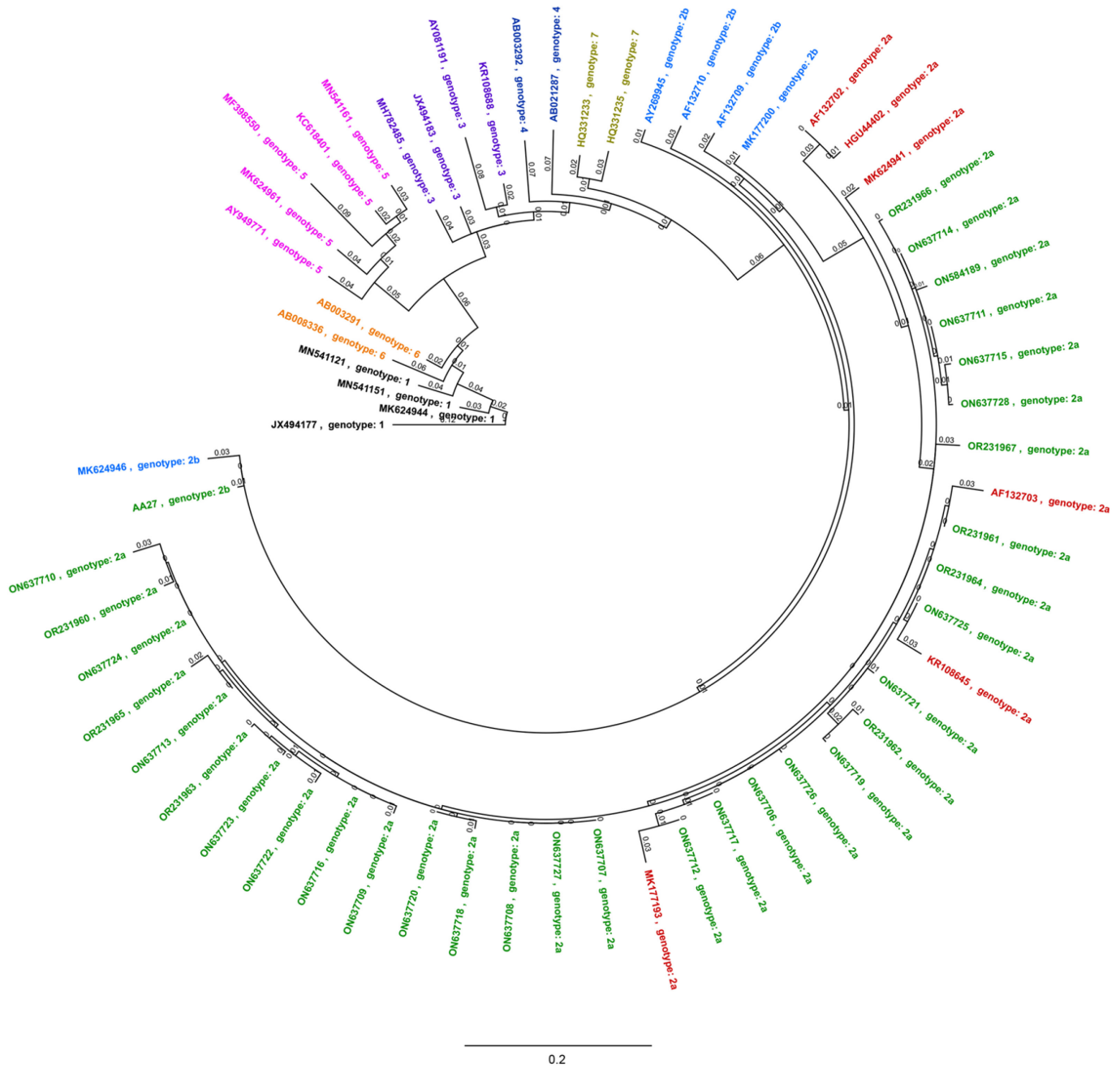
HPgV-1 sequences from 32 HIV-1-infected individuals and 1 healthy blood donor were compared with the known genotypes of HPgV-1. The scale bar represents substitutions per site and is shown on the branch labels. The reference genotype sequences are denoted by various colors with their GenBank accession numbers. The study samples are denoted by green color. HPgV-1: human pegivirus-1.

Figure 4. Maximum likelihood tree of HPgV-1 genotype 2 country-wise using the 5' UTR region.
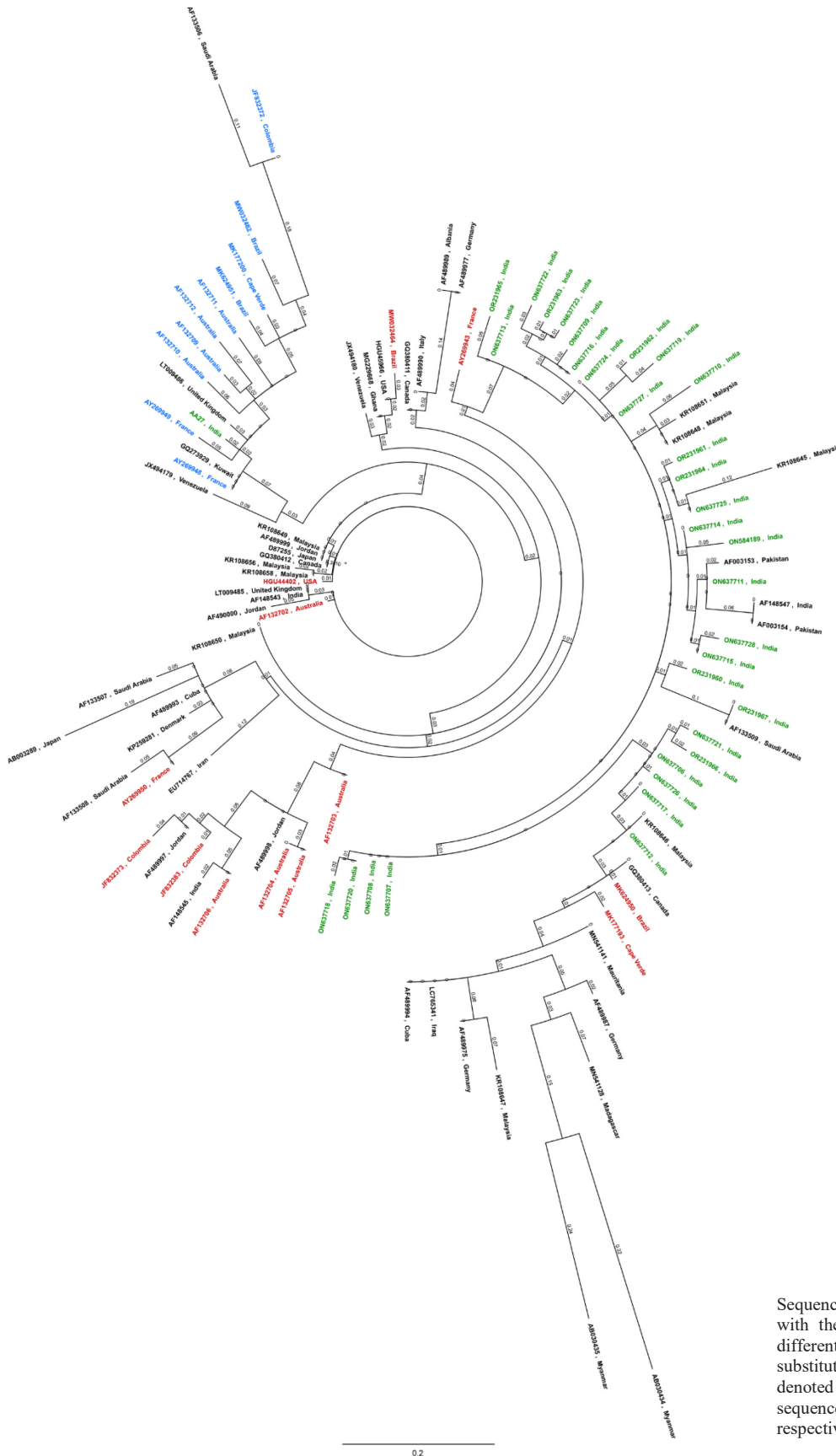
Sequences from 33 study samples were compared with the known genotypes 2, 2a, and 2b from different countries. The scale bar represents substitutions per site. The study samples are denoted in green. The genotypes 2, 2a, and 2b sequences are denoted in black, maroon, and blue, respectively. HPgV-1: human pegivirus-1.

During the phylogenetic analysis, all 32 sequences clustered with sequences retrieved from Malaysia, Pakistan, and Saudi Arabia (Figure 4). However, the healthy blood donor HPgV-1 sequence clustered with HPgV-1 genotype 2b sequences from Europe (Figure 4). HPgV-1 genotype 2a isolates were from the states of Tamil Nadu (56.3%), Andhra Pradesh (21.9%), Karnataka (12.5%), Assam (3.1%), Bihar (3.1%), and Kerala (3.1%). The HPgV-1 genotype 2b isolated was from Tamil Nadu. Additionally, HPgV-1 genotype 2a was found in non-HIV disease groups in a previously published study conducted at this centre [19]. The nucleotide similarity between the HPgV-1 sequences from current and earlier studies was 97.54%, and ranged from 87.5% to 100% (Figure 5) [19].

Discussion

The prevalence of HIV in India is 0.22%, which is equivalent to approximately 2.3 million people living with HIV [20]. With the availability of highly effective ART, the risk of non-communicable diseases is high among HIV-infected individuals. HPgV-1 infection is known to have beneficial effects on infectious and non-infectious diseases [21]. However, the prevalence of HPgV-1 in HIV-1-infected individuals in India is unavailable, and its influence on the HIV disease is not well studied.

In the present study, the prevalence of HPgV-1 among the ART-naïve HIV-1 cohort in a hospital-based setting was 11%, which is comparatively lower than in China, with a prevalence of 25.6–38.8%. On the contrary, the prevalence of HPgV-1 was significantly lower in healthy controls (1%) reflecting the possible spread in HIV-infected individuals. Furthermore, we have also reported that HPgV-1 RNA positivity in South Indian blood donors was 4.5% consistently lower

in the healthy population [22]. However, in North India, the prevalence of HPgV-1 among the general population and commercial blood donors varied between 4% and 46% [23]. In this study, HPgV-1 virus positivity was higher among the South Indian population when compared to North Indians.

There was a significant male preponderance of HPgV-1 RNA positivity in this study, which differed from a recent study done in Cabo Verde [24]. The reason for the male preponderance needs to be investigated further in detail. Compared to other age groups, the peak prevalence of HPgV-1 RNA in HIV-1-infected individuals was at 31–50 years ($p = 0.02$).

Studies have shown that there is a slower AIDS progression and longer survival in HIV-1/HPgV-1 co-infected individuals [16,25–27]. The prolonged survival of individuals with HPgV-1 viremia was demonstrable only 5–6 years post-HIV seroconversion, and the loss of HPgV-1 RNA by 5–6 years was associated with a poor prognosis [26]. A meta-analysis showed that HPgV-1 infection later in HIV disease reduced mortality in HIV-1/HPgV-1 co-infected individuals [28]. One of the limitations of the study was the cross-sectional design, where we were unable to ascertain the time of HIV-1/HpgV-1 acquisition, the effect of seroconversion, and the duration of HPgV-1 viremia.

Given the increased prevalence of HPgV-1 in HIV-1-infected individuals and the male preponderance, we compared the impact of HPgV-1 co-infection on CD4+ levels and HIV-1 log viral loads. Although CD4+ T cell count and HIV-1 log viral load were not significantly different between mono and co-infected groups, HPgV-1 viral load directly correlated with CD4+ T cell counts, suggesting the beneficial influence of HPgV-1 viremia on the levels of CD4+ T cell count. This observation

Figure 5. Heat map showing nucleotide identity percent between HPgV-1 sequences in the current and earlier study (Abraham *et al.*, 2002) from the same centre.
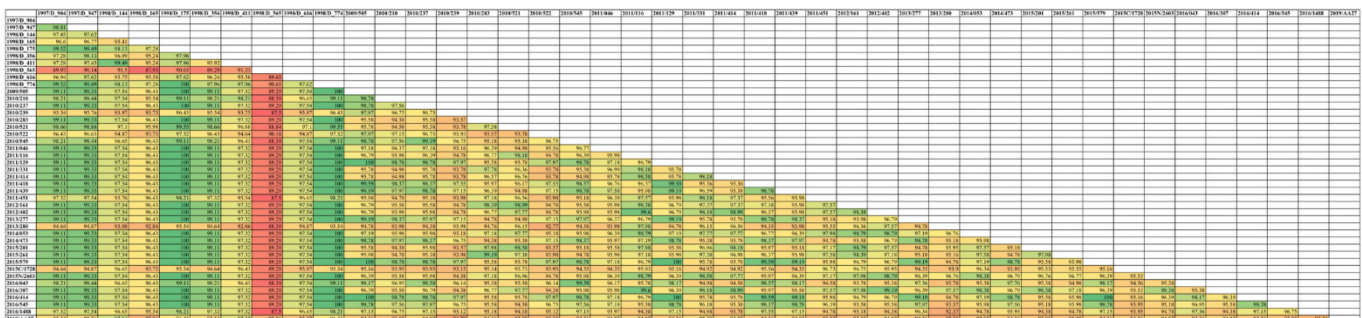
The current study includes 33 samples from 2009 to 2019. The previous study included 10 samples from 1998 and 1999. The nucleotide similarity between the current and earlier studies ranged from 87.5% to 100%, with a median of 97.54%. HPgV-1: human pegivirus-1.

was further validated by the finding that HPgV-1 viremia correlated inversely with HIV-1 viral load, emphasizing the possible involvement of cytokine-mediated control [29]. These findings concurred strongly with the global data, which showed a significant positive correlation between HPgV-1 viral load and CD4+ T cell count, and an insignificant inverse correlation between HPgV-1 viral load and HIV-1 viral load [27,30]. This could be attributed to HPgV-1 non-structural protein 5A, which is known to decrease the expression of surface CXCR4, exerting a direct control on HIV-1 progression late in the seroconversion period [31,32].

HPgV-1 genotype distribution varies globally and is known to confer variable influence on HIV-1 disease progression [4,33–35]. In the present study, the phylogenetic analysis of 5' UTR revealed HPgV-1 genotype 2a to be prevalent in ART-naïve HIV-1-infected individuals from India. Compared to previous studies from the same centre, the genotype predominance has been stable for over a decade, with HPgV-1 genotype 2a being prevalent among renal transplant recipients, individuals with hematological disorders, and chronic liver disease [19]. Phylogenetic analysis showed that all HPgV-1 2a strains from HIV-1-infected individuals clustered with Asian sequences, probably matching population migration, and HPgV-1 2b in the control group clustered with European sequences. CD4+ T cell levels were lower in HIV-1-infected individuals infected with HPgV-1 genotype 2a when compared to HIV-1-infected individuals infected with genotype 2b; but in the current study, there was a lack of HPgV-1 2b genotype among HIV-1-infected individuals [35]. A detailed, large-scale study is needed to delineate the importance of the HPgV-1 geographical distribution in India. Furthermore, when the current HPgV-1 sequences were compared with the collections of decade-old strains from this centre, it revealed high (97.54%) nucleotide similarity ranging from 87.5% to 100% [19]. This suggests that the HPgV-1 genome is stable, and there is low selective pressure, which conforms to the global data [36].

Conclusions

The prevalence of HPgV-1 in the ART-naïve HIV-1-infected Indian population was lower than in neighboring countries, with an exclusive predominance of HPgV-1 genotype 2a clustering with Asian sequences and exhibiting high genome stability. In addition to a strong male preponderance, a positive correlation of HPgV-1 viral load with CD4+ T cells and an inverse correlation with HIV-1 viral load suggest some beneficial effects on HIV disease progression and outcome. However, this requires a detailed longitudinal study to further elucidate the precise role of HPgV-1 in HIV infection.

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