Human Pegivirus Molecular Epidemiology in Argentina: Potential Contribution of Latin American Migration to Genotype 3 Circulation

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In order to determine the human pegivirus (HPgV) genotypic diversity in Argentina taking into account the potential contribution of human migration from neighboring countries, samples from 130 Argentine injecting drug users, 116 Argentine- and 50 immigrant-pregnant women were analyzed. HPgV RNA prevalence among human immunodeficiency virus (HIV)-positive injecting drug users was similar to HIV-positive pregnant women, as was the case when comparing HIV-negative injecting drug users and HIV-negative pregnant women ($P > 0.05$). HPgV genotype 2 (HPgV/2) was prevalent among both Argentine injecting drug users and pregnant women, in contrast to HPgV/3 observed among pregnant women from Latin American countries with predominant indigenous populations and who had experienced their initial sexual intercourses—and possibly their source of infection—in those countries ($P < 0.01$). In addition, HPgV vertical and horizontal transmission was proven by molecular analysis of E2 gene and construction of identity matrixes with epidemiologically non-related isolates. This study shows that human migration from neighboring Latin American countries with predominant indigenous populations might contribute to HPgV/3 circulation in Argentina. ***J. Med. Virol.***

KEY WORDS: human pegivirus (HPgV); Argentina; viral genotypes; injecting drug users; pregnant women

INTRODUCTION

Human pegivirus (HPgV), formerly GB virus C or GBV-C, is a positive-strand member of the fourth genus of the *Flaviviridae* family, named *Pegivirus* (pe, persistent; g, GB or G) [Stapleton et al., 2011].

Although high prevalence of HPgV infection is recorded among subjects with the risk of parenteral exposures, sexual contact, and vertical route has also been reported [Oubiña et al., 1999; Mathet et al., 2003; Shankar et al., 2008]. Due to shared transmission modes, co-infection with HPgV is common among people infected with human immunodeficiency virus 1 (HIV-1) and/or hepatitis C virus (HCV). Approximately, 10–25% of chronic HCV patients and 14–36% of injecting drug users seropositive for HIV-1 show evidence of HPgV co-infection. The much higher HPgV triple infection rate of 30–36% among individuals with HIV/HCV co-infection has been reported [Schwarze-Zander et al., 2006].

HPgV has been classified into seven genotypes and many subtypes based on their sequence diversity of full-length genomes. Geographically, HPgV genotypes show distinct distribution patterns related to the co-evolution of the viruses with human beings during migrations along the history [Simmonds, 2001]. In general, genotype 1 is predominant in Africa; genotype 2 is detected in the European and American continents; genotype 3 is common in Asia, including

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Japan and China, but can also be found in indigenous populations from Latin America; in contrast, genotype 4 is predominant in Southeastern Asia; genotype 5 in South Africa; and genotype 6 in Indonesia. Recently, a seventh genotype was reported among injecting drug users from Yunnan, China [Feng et al., 2011].

Although no association with any known disease has been demonstrated, there is a provocative evidence of certain degree of protective effect of HPgV among some subjects infected with HIV. Its co-infection with HIV-1 may produce some favorable outcomes, associated with a lower mortality rate, slower disease progression, and longer survival term. Furthermore, HPgV genotypes 2 and 5 have been related specifically with a more delayed progression to AIDS [Shankar et al., 2008].

Although Argentine people is considered usually as a population with some degree of European ancestry, people of several ancestries from neighboring South American countries are still moving to Argentina, where HPgV genotypes 1, 2, and 3 have been reported [Oubiña et al., 1999; Mathet et al., 2003]. In order to determine the HPgV genotypic diversity in Argentina taking into account the potential contribution of such human migration, in this study: (a) the HPgV RNA and genotypic prevalence was assessed retrospectively among injecting drug users as well as local and immigrant pregnant women residing in Buenos Aires city and its suburbs; and (b) putative chains of viral transmission were investigated among all pregnant women infected with HIV who tested positive for HPgV serum RNA.

METHODS

Patients' Samples

Serum samples and epidemiological data from 166 pregnant women were collected by the "Ramón Sardá" Maternity Hospital in Buenos Aires during the period 2008–2010. Fifty of them were immigrants from Latin American countries (30.1%). Mean age ± SD was 27.2 ± 7.3 years for the whole group (n = 166; median = 26), while it was 25.8 ± 6.9 years for HIV-negative women (n = 100; median = 25) and 29.3 ± 7.2 years for HIV-positive women (n = 66; median = 30). Such values passed the D'Agostino & Pearson omnibus normality test. This population was not randomly selected. One hundred HIV-negative pregnant women were recruited after arrival to the Maternity Hospital within 2 months of the beginning of the study. The remaining 66 HIV-positive pregnants were included in this study throughout a 3-year elapsed time.

In addition, 130 samples obtained from Argentine injecting drug users recruited throughout a previous "snowball" sampling study during the period 1998–2008 [Trinks et al., 2008] were included, as well. Mean age ± SD was 33.3 ± 8.3 years for the whole group (n = 130; median = 32), while it was 30.8 ± 9 years for HIV-negative injecting drug users (n = 51; median = 29) and 34.9 ± 7.4 years for HIV-positive injecting drug users (n = 79; median = 33). Only the HIV-negative injecting drug users passed the D'Agostino & Pearson omnibus normality test.

Serological Tests, HPgV RT-PCR, and Genotype Assignment

All serum samples were kept at −70°C until use. Serological tests for HBsAg, anti-HBcAg (AxSYM, Abbott, Chicago, IL), anti-HIV (Bio-Rad, Fujirebio, Tokyo, Japan), and anti-HCV (AxSYM) antibodies were carried out in all collected samples.

Serological HPgV studies were precluded due to the commercial unavailability of an antibody specific detection kit. Hence, HPgV prevalence was assessed by RT-PCR.

Viral RNA was extracted and reverse-transcribed. The 5′ UTR of HPgV was amplified by nested-PCR, as described previously [Quarleri et al., 1999] and PCR products were sequenced bi-directionally using Big-Dye Termination chemistry system (Applied Biosystems, Life Technologies, Foster City, CA). Kwok and Higuchi rules were followed strictly [Kwok and Higuchi, 1989].

The HPgV nucleotide sequences obtained in this study were aligned with sequences downloaded from GenBank database and ascribed to all reported HPgV genotypes by using ClustalX version 2.0.12 software. Genotype assignment was done by means of neighbor-joining (NJ) analysis with Kimura's 2-parameter distance model, as well as maximum likelihood (ML)—a more robust phylogenetic inference method-included in the PHYLIP package (version 3.5c).

Vertical/Horizontal Transmission Assessment

The possibility to include their children and sexual partner(s) in the study was offered to all pregnant women infected with HIV who tested positive for HPgV serum RNA.

Viral RNA was extracted and reverse-transcribed to cDNA. First, in order to detect the presence of HPgV RNA in all newly obtained samples, the 5′ UTR was amplified by nested-PCR [Quarleri et al., 1999]. The PCR products were sequenced bi-directionally and the viral genotype was determined by phylogenetic analysis.

Although the 5′ UTR is used widely for HPgV detection due to the high degree of sequence conservation among isolates, this fact limits its potential to trace chains of transmission, despite the presence of scattered sub-regions with higher heterogeneity [Mathet et al., 2003]. Therefore, in all HPgV RNA positive samples, a second genomic region, which shows a higher diversity among isolates—the E2 gene—was amplified partially by nested-PCR [Mathet et al., 2003]. The PCR products were sequenced bi-directionally and the viral genotype was determined by phylogenetic analysis. To facilitate the proper comparison between epidemiologically related

and unrelated sequences, five further serum samples obtained from three pregnant women and two injecting drug users without epidemiological links were also included.

To rule out the possibility of cross-contamination, samples obtained from women and their sons and/or current sexual partner were processed independently from the RNA extraction step (each sample of a given pair was processed in 2-week separated experiments).

Identity matrixes for the 5′ UTR and E2 gene sequences of HPgV were constructed by using BioEdit Sequence Alignment Editor, version 7.0.1.

Statistical Analyses

EPIDAT 3.1 (Dirección Xeral de Innovación e Xestión da Saúde Pública, Xunta de Galicia, Galicia, Spain, and Pan American Health Organization) and Tadpole program (University of Cambridge, UK) were used for statistical analysis. Student's *t*-test was used to compare the means and standard deviations between any pair of samples. A P value <0.05 was considered statistically significant.

Ethical Considerations

All participants provided their informed written consent to perform this study, which was approved by two Ethics Committees on Research from: (a) "Ramón Sardá" Maternity Hospital for the pregnant women samples, their children, and sexual partner(s) who agreed to take part in this study; and (b) CIEI-FM-UBA for the injecting drug users samples.

RESULTS

Serological Tests

Among the pregnant women, 66 out of 166 samples (39.7%) showed seropositivity for HIV, five (3%) for HBV, and six (3.6%) for HCV. All the HCV and three out of the five HBV infected women were also co-infected with HIV (Fig. 1A). Fifteen percent of the HIV-positive and 39% of the HIV-negative pregnant women were immigrants from Latin American countries ($P < 0.01$).

Among the injecting drug users, 79 out of 130 samples (60.8%) were seropositive for HIV, 100 (76.9%) for HBV, and 90 (69.2%) for HCV. While 70 injecting drug users (53.8%) were co-infected with HIV, HBV, and HCV; 13 (10%) were seropositive for HCV and HBV and 9 (6.9%) for HBV and HIV (Fig. 1B).

Detection of HPgV RNA

Among the pregnant women, HPgV RNA was amplified in 18 out of 166 samples (10.8%). Thirteen out of these 18 HPgV-positive samples (72.2%) were co-infected with HIV; therefore, the relative frequency of HPgV RNA among HIV-positive and -negative pregnant women was 19.7% and 5%, respectively ($P < 0.01$). In contrast, the relative frequency of HPgV RNA among HCV-positive and -negative pregnant women was 33.3% (2/6) and 10% (16/160), respectively ($P > 0.05$). The triple HPgV/HCV/HIV infection was observed in 33.3% (2/6) of the HCV/HIV co-infected women. None of the HPgV RNA-positive samples were co-infected with HBV (Fig. 1A).

HPgV RNA was detected in 16 out of 130 injecting drug users (12.3%). Fourteen of these HPgV RNA-positive samples (87.5%) were co-infected with HIV; therefore, the relative frequency of HPgV RNA among HIV-positive and -negative injecting drug users was 17.7% (14/79) and 3.9% (2/51), respectively ($P < 0.05$). In contrast, the relative frequency of HPgV RNA among HCV-positive and -negative injecting drug users was 17.8% (16/90) and 0% (0/40), respectively ($P < 0.01$). Moreover, the relative frequency of HPgV RNA among HBV-positive and -negative injecting drug users was 16% (16/100) and 0% (0/30), respectively ($P < 0.01$). The quadruple HPgV/HBV/HCV/HIV infection was recorded in 20% (14/70) of the injecting drug users co-infected with HBV/HCV/HIV (Fig. 1B).

HPgV Genotypic Prevalence

Among pregnant women, 14 of the HPgV RNA-positive samples (77.8%) were ascribed to HPgV genotype 2 and the remaining 4 (22.2%) to genotype 3 (Fig. 2 and Supplementary Fig. 1).

While HPgV genotype 2 prevailed among the HIV-positive pregnants (92%), 60% of the HIV-negative pregnant serum samples were assigned to genotype 3 ($P < 0.01$) (Fig. 2 and Supplementary Fig. 1). Interestingly, all sequences ascribed to genotype 3 ($n = 4$) belonged to immigrant women who had experienced their initial sexual intercourses in their birth countries, for example, Bolivia or Peru (HPgV genotype 3 from non-Argentine pregnants vs. HPgV genotype 3 from Argentine pregnants: $P < 0.01$).

Among the injecting drug users, 13 of the HPgV-positive samples (81.2%) were ascribed to HPgV genotype 2 and the remaining 3 (18.8%) to genotype 3 (Fig. 2 and Supplementary Fig. 1).

The only two HPgV samples detected among the group of the HIV-negative injecting drug users were ascribed to genotype 2. In comparison, 11 samples from the group of the HIV-positive injecting drug users belonged to such genotype (78.6%) and the remaining three to genotype 3 (21.4%). These values did not reach statistically significant differences.

Vertical/Horizontal Transmission Assessment

After offering all pregnant women infected with HIV who tested positive for HPgV RNA the possibility to include their children and their respective sexual partner in this part of the study, two mothers accepted the proposal.

A mother named "M1" was a 30-year-old woman from Buenos Aires city who exhibited seropositivity for HIV during the pregnancy of her son "S1." M1 received the complete antiretroviral treatment to

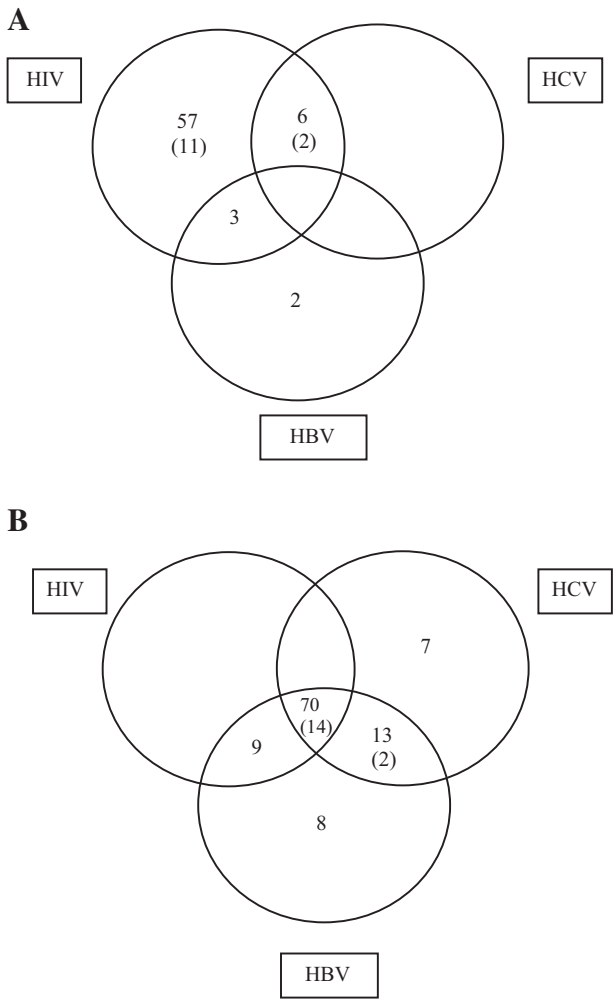

Fig. 1. HPgV RNA frequency (between brackets) in HIV, HBV, and/or HCV infected pregnant women (**A**) and injecting drug users (**B**).

avoid HIV vertical transmission and her viral load was under the detection limit (<50 RNA copies/ml). S1 was born prematurely by cesarean section and included in this study at the age of 9 months. He was tested three times by means of HIV DNA PBMC PCR detection, rendering negative results.

HPgV RNA was detected in M1 and her son. Both 5′ UTR sequences were ascribed to genotype 2 showing a 100% identity between them (Fig. 2 and Supplementary Fig. 1). To confirm HPgV vertical transmission, E2 gene was also amplified partially. Phylogenetic analysis revealed that sequences obtained from M1 and S1 belonged to genotype 2 showing a 100% identity between them (Fig. 3 and Supplementary Fig. 2).

Another mother—"M2"—was a 29-year-old woman born in Lima, Peru, who exhibited seropositivity for HIV during the pregnancy of her second son "S2.2." M2 received the complete antiretroviral treatment to avoid HIV vertical transmission and her viral load was under the detection limit (<50 RNA copies/ml). S2.2 was born in term by a natural birth and included in this study at the age of 2 months. He was tested once by means of HIV DNA PBMC PCR detection rendering negative results.

S2.2's father—"F2"—a 29-year-old man born in Buenos Aires who was seropositive for HIV, and S2.2's stepbrother—"S2.1"—, a 11-year-old Peruvian boy who was serologically negative for HIV and was born as a result of a previous sexual intercourse of M2 in Peru, were also included in this part of the study.

HPgV RNA was detected in M2 and her current sexual partner F2, but not in her sons S2.1 or S2.2. Both 5′ UTR sequences were ascribed to genotype 3, showing a 100% identity between them (Fig. 2 and Supplementary Fig. 1). E2 gene was also amplified partially from both sera. Phylogenetic analysis revealed that sequences obtained from M2 and F2 belonged to genotype 3, showing a 99.6% identity between them (Fig. 3 and Supplementary Fig. 2), a significantly higher value than the one observed when comparing other HPgV genotype 3 sequences obtained from non-epidemiologically related isolates from Bolivia and Argentina (n = 5; 93.7% identity among them), and from Asia (n = 7; 89%). After a thorough chromatograms' examination, both E2 nucleotide sequences revealed the presence of C and T in HPgV nucleotide position 1283 from M2 sequence, but only a C in the same position from F2 isolate (figure available upon request).

DISCUSSION

HPgV Prevalence Among Injecting Drug Users and Pregnant Women: Association With Viral Co-infections

While a previous study showed that the HPgV RNA relative frequency among HIV co-infected injecting drug users from Buenos Aires was 30% (n = 70) [Oubiña et al., 1999], such value reached 17.7% in this study, which analyzed an almost identical number of serum samples (n = 79; $P > 0.05$). Mean age ± SD were similar between both groups. No statistically significant difference was neither observed when the HPgV RNA rate was compared between the group of HIV-positive pregnant women studied by Mathet et al. [2003] (n = 34; 19%), and the one herein reported (n = 66; 19%), nor between the blood donors from Buenos Aires (n = 200; 5.5%) [Oubiña et al., 1999], and the HIV-negative pregnant women recruited in this study (n = 100; 5%). Although these two latter groups differ in their composition, the similarity of the relative frequency rates here compared suggests that they represent genuinely what it might be observed in the general population from Buenos Aires city, where HIV-positive prevalence reaches up to ≈0.2–0.36% [Gendler and Pascuccio, 2007]. Nevertheless, comparison between HPgV RNA prevalence among blood donors and the whole pregnant women population studied (HIV-negative plus HIV-positive: 18/166:

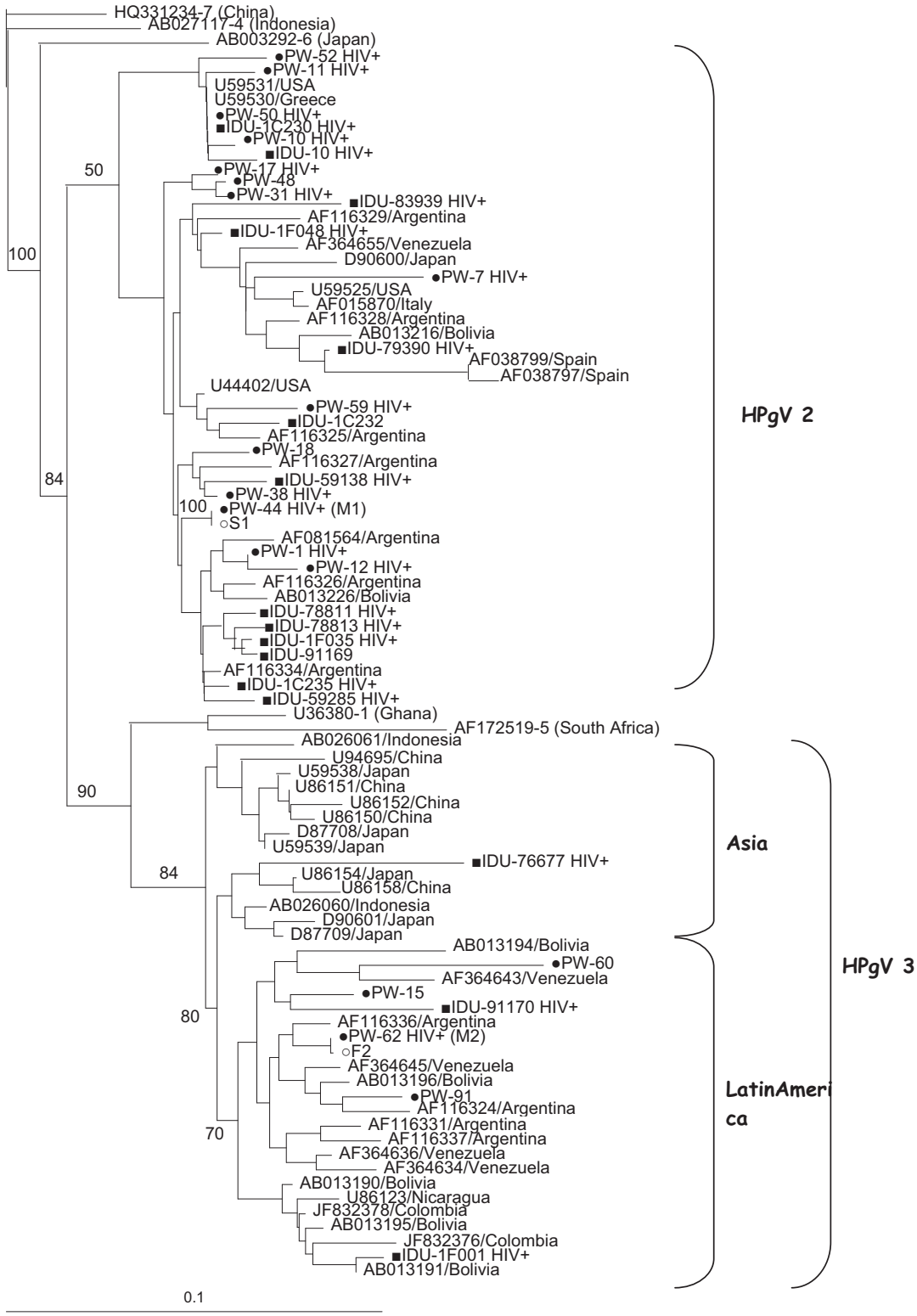

Fig. 2. A phylogenetic neighbor-joining tree with Kimura's 2–1 parameter distance model constructed by using partial HPgV 5′ UTR sequences (encompassing nt 41–367) from HPgV genotypes 1 to 7, used as reference isolates. While the black colored squares indicate the strains reported in this study from injecting drug users (IDUs), the black colored and open circles represent the strains from pregnant women (PW) and those obtained to evaluate HPgV putative horizontal (F2), and vertical transmission (S1), respectively. The GenBank/EMBL/DDBJ accession numbers of the 5′ UTR sequences reported in this study are: JQ928647–JQ928682. Bootstrap values of 100 replications are shown above the main branches. A 70 bootstrap value was considered as a limit of significance. The scale bar represents the number of nucleotide substitutions per site.

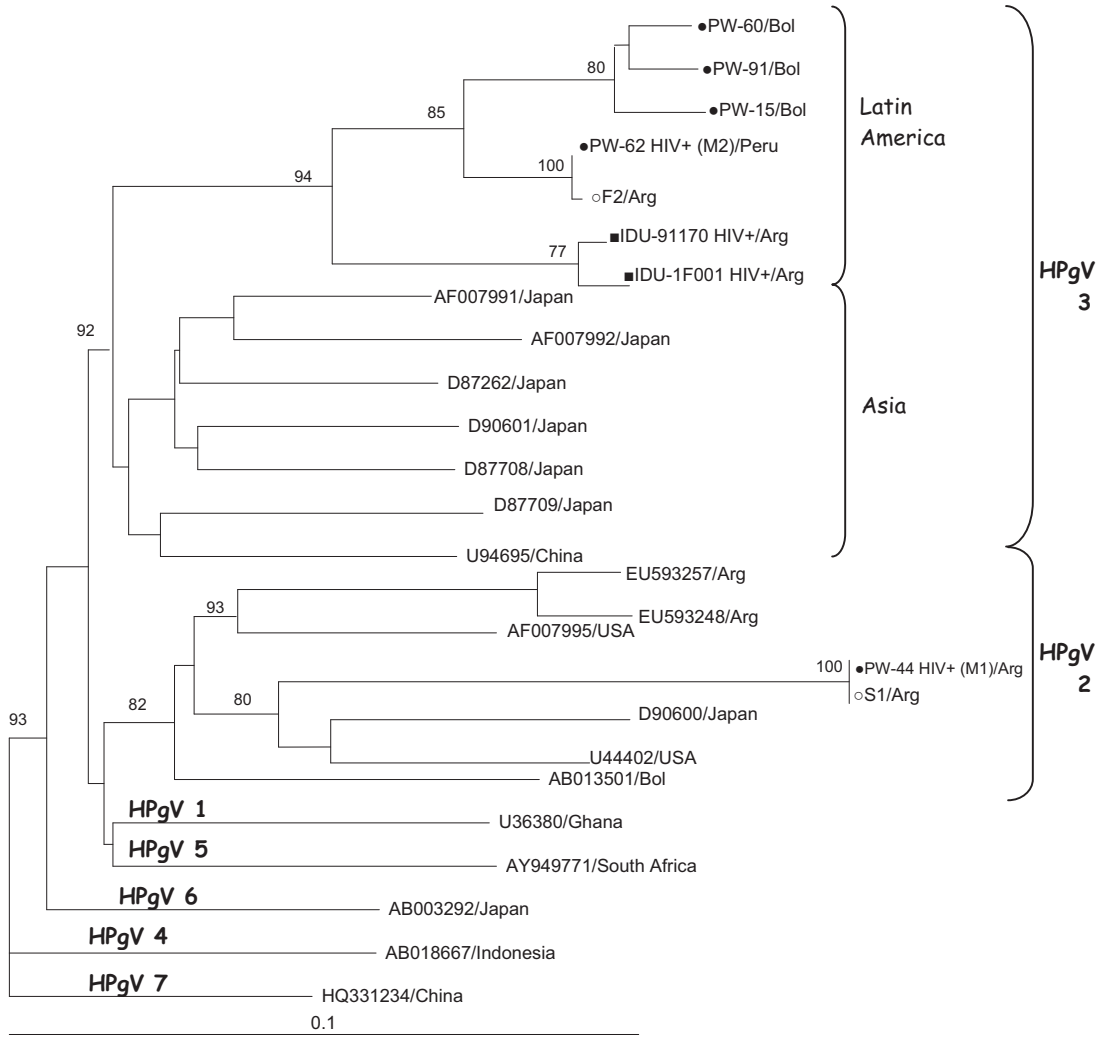

Fig. 3. A phylogenetic neighbor-joining tree with Kimura's 2-parameter distance model constructed by using partial HPgV E2 gene sequences (encompassing nt 1149–1396) from HPgV genotypes 1–7, used as reference isolates. While the black colored squares indicate the strains reported in this study from injecting drug users (IDUs), the black colored and open circles represent the strains from pregnant women (PW) and those obtained to evaluate HPgV putative horizontal (F2), and vertical transmission (S1), respectively. The nationality of those subjects studied herein born in Bolivia (Bol), Peru, and Argentina (Arg) are indicated. The GenBank/EMBL/DDBJ accession numbers of the E2 sequences reported in this study are: JQ928643–JQ928646 and JX520636–JX520640. Bootstrap values of 100 replications are shown above the main branches. A 70 bootstrap value was considered as a limit of significance. The scale bar represents the number of nucleotide substitutions per site.

10.8%) also failed to reach statistical significance ($P > 0.05$). In spite of such similarity, it must be taken into account that the pregnant women population in this study had not been randomly recruited, thus precluding a proper comparison with values obtained from blood donors.

The HPgV RNA rate was similar among HIV-positive pregnant women and HIV-positive injecting drug users (19.7% vs. 17.7%; $P > 0.05$), as was the case when comparing such rate in both HIV-negative injecting drug users and HIV non-infected pregnant women (5% vs. 3.9%; $P > 0.05$). This would suggest that the presence of HPgV genome is associated with HIV co-infection and not merely with another vulnerability factor, such as the history of injecting drug abuse. No statistical differences were recorded when the triple co-infection HIV/HCV/HPgV was analyzed in comparison with HIV/HPgV co-infection: 33.3% (2/6) versus 16.7% (11/66) in HIV-positive pregnant women, respectively, and 20% (14/70) versus 17.7% (14/79) in HIV-positive injecting drug users, respectively.

Although the sample size encompassing both populations was limited and no proper conclusions may be drawn, it is noteworthy that mono-infections either with HBV or HCV were not associated with the presence of HPgV RNA when compared with HBV/HCV co-infections or even HIV mono- or co-infections (Fig. 1).

HPgV Genotypes Among Injecting Drug Users and Pregnant Women: Influence of Human Migrations into Argentina

Regarding the HPgV genotypic prevalence, genotype 2 was the most prevalent among both injecting drug users from Buenos Aires city and pregnant women born in the same place (Fig. 2 and Supplementary Fig. 1). In contrast, an absolute predominance of genotype 3 was observed among pregnant women coming from other Latin American countries with predominant indigenous populations (e.g., Bolivia or Peru) and who had experienced their first sexual intercourse—and possibly their source of infection—in those countries. Moreover, four pregnants born in Peru, Bolivia, or Paraguay who had their first sexual intercourses after arriving to Argentina, were infected with HPgV genotype 2.

These results might be a reflection of different human migrations, which took place throughout centuries and are still in progress. The presence of HPgV genotype 2 in the American continent is thought to be a result of the strong influence of migrations from Europe. Moreover, the Asian genotype 3 is also found in South American aboriginal communities, as well as in countries with predominant indigenous populations, as a consequence of the movements of Mongoloids who migrated from Asia to America through the Bering Strait a long time ago [Simmonds, 2001].

In this study, it was possible to evaluate the phylogenetic relationship among the HPgV genotype 3 isolates from Buenos Aires and other Latin American countries. While HPgV genotype 2 sequences obtained in this study were interspersed among other worldwide isolates, the sequences ascribed to genotype 3 herein were grouped into two defined clusters: (i) Asian sequences (a single one isolate from an injecting drug user), and (ii) Latin American sequences (the remaining seven isolates—two from injecting drug users, four from immigrant pregnant women, and the sexual partner of one of them; Fig. 2 and Supplementary Fig. 1).

The HIV/HPgV co-infection is associated frequently with a lower HIV viral load, to a higher number of $CD4^+$ T cells and to a higher survival rate as compared to those observed among HIV mono-infected patients; thus, it can be inferred that this co-infection would be relevant to these patients [Shankar et al., 2008]. Moreover, HPgV genotyping is also important to them, as genotype 3 is related to lower levels of $CD4^+$ T cells when compared to genotype 2 [Shankar et al., 2008].

Vertical/Horizontal Transmission Assessment

In this study, all HIV-positive pregnant women who tested positive for HPgV RNA were offered the possibility to include their children and their respective sexual partner in the study. Only two mothers accepted such proposal. In order to confirm HPgV vertical transmission, a fragment of the 5′-end of E2 gene—which presents a higher diversity degree among the isolates as compared with the 5′ UTR—was amplified.

In the first studied case, HPgV vertical transmission was confirmed, as the analysis of the nucleotide sequences of E2 obtained from mother M1 and her son S1 showed an identity of 100%, which proves the maternal origin of the son's infection (Fig. 3 and Supplementary Fig. 2).

In the second studied case, HPgV RNA could only be detected in HIV/HPgV co-infected mother M2's current sexual partner (F2) and father of her second son (S2.2), but not in any of her two sons (S2.1 or S2.2). Both M2 and F2 were infected with HPgV genotype 3. As mentioned above, the presence of genotype 3 in Buenos Aires might be the result of human immigration from Latin American countries, with predominant indigenous populations; for example, Peru where M2 was born. Therefore, a working hypothesis of an eventual horizontal transmission was proposed. In order to prove it, a fragment of the 5′-end of E2 gene was amplified and sequenced. The E2 nucleotide sequences' analysis obtained from M2 and her sexual partner F2 showed an identity of 99.6% (Fig. 3 and Supplementary Fig. 2). Such identity rate is significantly higher than that observed when non-epidemiologically related isolates from Latin America and Asia were compared (93.7 ± 1.4 and $89\% \pm 2.2$, respectively), thus suggesting compellingly a horizontal transmission between M2 and her sexual partner F2.

While the presence of two viral populations was detected from M2 sample—being the population with C predominant over the one with T at HPgV nucleotide position 1283 [C>T]—, in F2 it was only observed the population with C at the same nucleotide position of HPgV genome, which might suggest the HPgV transmission from M2 (showing a more heterogeneous viral population) to F2 (figure available upon request). However, the inability to detect the population T1283 in the F2 sample does not exclude its presence in circulation, taking into account that it is reportedly known that Sanger's method for nucleotide sequencing requires roughly a minimum of 30% of the total of template to produce a peak in any specific nucleotide position. On the other hand, differences in the HPgV quasispecies between individuals M2 and F2 are likely due to different host pressures exerted by each individual's immune system. Thus, the inability to detect the single nucleotide polymorphism (SNP) T1283 in individual F2 could also represent quasispecies diversity in different hosts, especially since it is not known when the virus was transmitted from one individual to the other and how much time it had to diversify. Nevertheless, since this study provides the first HPgV E2 genotype 3 sequences from Argentina [Ruiz et al., 2010], Bolivia and Peru, the authors are precluded to evaluate them further together with

other sequences from such geographical area and thus, to be able to draw a conclusive statement.

Although both horizontal and vertical routes of HPgV transmission have already been documented [Semprini et al., 1998; Mathet et al., 2003; Björkman and Widell, 2008; Shankar et al., 2008; Stapleton et al., 2011], this study strengthens previous reports and undoubtedly shows the contribution of non-parenteral routes to the dissemination of HPgV in HIV-positive subjects residing in Argentina. It seems worth to analyze the genotype-specific impact on the clinical outcome of HIV-positive Argentine patients, bearing particularly in mind the presence of a separate HPgV genotype 3 cluster currently including Latin American isolates.

CONCLUSION

As a whole, this study shows that HPgV genotype 2 is the most prevalent in Argentina and that the occurrence of genotype 3 is most likely due to immigration from Latin American countries with predominant indigenous populations, in which genotype 3 prevails. Moreover, the detection of HPgV RNA is associated with HIV co-infection, independently of other vulnerability factors, such as the history of injecting drug abuse, as documented when comparing HIV-positive (non-drug addict) pregnant women and HIV-positive injecting drug users.

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SUPPORTING INFORMATION

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