

Deep sequencing identifies two genotypes and high viral genetic diversity of human pegivirus (GB virus C) in rural Ugandan patients

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Human pegivirus (HPgV), formerly ‘GB virus C’ or ‘hepatitis G virus’, is a member of the genus *Flavivirus* (Flaviviridae) that has garnered significant attention due to its inhibition of HIV, including slowing disease progression and prolonging survival in HIV-infected patients. Currently, there are six proposed HPgV genotypes that have roughly distinct geographical distributions. Genotypes 2 and 3 are the most comprehensively characterized, whereas those genotypes occurring on the African continent, where HPgV prevalence is highest, are less well studied. Using deep sequencing methods, we identified complete coding HPgV sequences in four of 28 patients (14.3%) in rural Uganda, east Africa. One of these sequences corresponds to genotype 1 and is the first complete genome of this genotype from east Africa. The remaining three sequences correspond to genotype 5, a genotype that was previously considered exclusively South African. All four positive samples were collected within a geographical area of less than 25 km², showing that multiple HPgV genotypes co-circulate in this area. Analysis of intra-host viral genetic diversity revealed that total single-nucleotide polymorphism frequency was approximately tenfold lower in HPgV than in hepatitis C virus. Finally, one patient was co-infected with HPgV and HIV, which, in combination with the high prevalence of HIV, suggests that this region would be a useful locale to study the interactions and co-evolution of these viruses.

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INTRODUCTION

Human pegivirus (HPgV), formerly ‘GB virus C’ or ‘hepatitis G virus’, was discovered in 1995 and assigned to the *Flaviviridae*, a family containing several important human disease agents, including yellow fever virus, West Nile virus, Dengue fever virus, and hepatitis C virus (HCV; ICTV, 2012; Leary *et al.*, 1996; Simons *et al.*, 1995; Stapleton *et al.*, 2011). Within the flaviviridae, HPgV has recently been classified as a member of the newly proposed

genus *Pegivirus*, which infects non-human primates (Birkenmeyer *et al.*, 1998), rodents (Drexler *et al.*, 2013; Kapoor *et al.*, 2013b), bats (Quan *et al.*, 2013) and horses (Chandriani *et al.*, 2013; Kapoor *et al.*, 2013a). Like all flaviviruses, HPgV has a single stranded, positive sense RNA genome (Leary *et al.*, 1996; Linnen *et al.*, 1996b). The HPgV genome is 9.4 kb in length and contains a single long ORF flanked by 5′ and 3′ untranslated regions (Mohr & Stapleton, 2009). The closest relative of HPgV known to infect humans is HCV; however, unlike HCV, HPgV is lymphotropic, establishes subclinical infection, and is not associated with hepatitis (George *et al.*, 2006; reviewed by Stapleton, 2003).

The nucleotide sequences reported in this study were deposited in GenBank with accession numbers KC618398 – KC618401.

HPgV has a worldwide distribution, with studies in developed countries showing that between 1% and 5% of healthy blood donors are viraemic (Moaven *et al.*, 1996; Mohr & Stapleton, 2009; Stapleton, 2003). Prevalence is higher in developing countries, with the highest blood donor infection rates (between 11.1 and 18.9%) on the African continent (Mohr & Stapleton, 2009). HPgV is transmitted sexually, vertically, and through exposure to infected blood (Bhattarai & Stapleton, 2012). It is therefore highly prevalent in populations with sexually transmitted or blood borne infections. For example, previous studies have reported HPgV viraemia in 17–40% of HIV-positive individuals (Heringlake *et al.*, 1998b; Mohr & Stapleton, 2009; Stapleton *et al.*, 2004).

Despite being non-pathogenic (Berzsenyi *et al.*, 2005; Polgreen *et al.*, 2003), HPgV has garnered significant research attention because of its interactions with HIV. Several clinical studies have shown that HPgV infection slows disease progression in individuals infected with HIV. Specifically, HPgV infection can prolong survival and reduce mortality in HIV-infected patients through reduction in HIV viral load, increased CD4+ T-cell counts, and improved response to antiretroviral therapy (Heringlake *et al.*, 1998a; Tillmann *et al.*, 2001; Williams *et al.*, 2004; Xiang *et al.*, 2001). The mechanisms by which HPgV modulates HIV infection may include direct interference with HIV entry and replication and indirect regulation of host factors that prevent or reduce disease progression (reviewed by Bhattarai & Stapleton, 2012).

Given the proposed use of HPgV as a bio-therapeutic agent in the treatment of AIDS (Gretch, 2012), significant attention has been placed on understanding the epidemiology, evolution and genetic diversity of this virus (Bagasra *et al.*, 2012; Wu *et al.*, 2012). HPgV sorts into six genotypes with roughly distinct geographical origins (Muerhoff *et al.*, 1996, 2005; Saito *et al.*, 1998; Smith *et al.*, 2000). Initially, three genotypes were described as originating in Africa (genotype 1), Europe (genotype 2), and Japan (genotype 3). Later, viruses from south-east Asia (genotype 4), South Africa (genotype 5) and Indonesia (genotype 6) were described (Muerhoff *et al.*, 2005, 2006; Naito *et al.*, 1999; Sathar *et al.*, 1999; Smith *et al.*, 2000; Tucker *et al.*, 1999). Most recently, three novel full-length genomes were sequenced from injecting drug users in south-western China; these have been proposed to represent a seventh genotype (Feng *et al.*, 2011). Of the 46 full-length coding genomes now available in public databases, the majority are genotypes 2 and 3, likely representing focused sampling from the geographical areas corresponding to these genotypes. Only two full coding genomes exist from genotype 1, despite evidence from partial-genome sequencing suggesting that genotype 1 may be the most genetically diverse genotype (Parreira *et al.*, 2012). Full coding genomes from the African continent include two from west Africa (Leary *et al.*, 1996; Saito *et al.*, 1999), one from east Africa (Erker *et al.*, 1996), and one from South Africa (Muerhoff *et al.*, 2005).

Here, we describe four new HPgV genome sequences from western Uganda. These sequences were collected from a region of east Africa that is heavily burdened by infectious diseases, including a high prevalence of HIV, reported at 16.1% in individuals age 15–49 in 2008 (Rubaihayo *et al.*, 2010). The sequences correspond to genotypes 1 and 5, the most poorly characterized groups in terms of availability of full-length viral genome sequences (Parreira *et al.*, 2012). Our results expand the known diversity and geographical range of these genotypes and demonstrate natural co-circulation of HPgV and HIV in rural east Africa.

RESULTS

As part of an effort to identify the causative agents of febrile illness, blood samples from a total of 28 subjects presenting with fevers of unknown origin at one of two health clinics in rural Uganda were deep sequenced to identify RNA viruses. From this cohort, four patients showed evidence of HPgV viraemia. *De novo* assembly of HPgV deep sequencing reads (total of 13 880 to 148 032) from these patients yielded near-complete HPgV genomes (9 281–9 339 bp), with an average coverage depth ranging from $686 \times$ to $6326 \times$. Two of these patients were female (Hu5 and Hu6), two were male (Hu20 and Hu21), and each resided in one of three villages located within 25 km² of each other. HPgV titres, assayed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and expressed as HPgV genome copies ml⁻¹ serum, were high in all four infected individuals: Hu5, 8.0×10^7 ; Hu6, 5.1×10^7 ; Hu20, 1.4×10^6 ; and Hu21, 3.1×10^6 .

HPgV polyprotein cleavage sites were predicted based on an alignment with members of the *Pegivirus* and *Hepacivirus* genera and through manual (Nielsen *et al.*, 1997) and *in silico* (Petersen *et al.*, 2011) signalase and NS3-NS4A protease cleavage site prediction. The genomic architecture of the four viral sequences was consistent with that of known HPgV sequences, containing a single ORF of 8 528 bp that putatively encodes two envelope proteins (E1 and E2), a 6.4 kDa ion channel protein (P7-like protein; as identified using the ExPASy server; Gasteiger *et al.*, 2005) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B; reviewed by Stapleton *et al.*, 2011). For each of the new HPgVs, the first Met in-frame with the coding sequence aligned well with the experimentally determined translation initiation codon for HPgV (Simons *et al.*, 1996). As observed previously in other pegiviruses, the sequence encoding a putative core (i.e. nucleocapsid) protein was absent or truncated in all four new genomes. The four viral nucleotide sequences were annotated and deposited in GenBank with accession numbers KC618398–KC698401.

Partial 5'UTR sequences of 532 to 534 nt were recovered by deep sequencing, representing $\geq 96\%$ of the 554 nt HPgV 5'UTR (Linnen *et al.*, 1996a; Xiang *et al.*, 2000). These sequences were 88 to 89% similar to the 5'UTRs of HPgV reference strains (AF121950 and NC_001710) and

90% to 99% similar to each other. Partial 3'UTR sequences of 219 to 278 nt were recovered for the new HPgVs, representing 70% to 89% of the 312 nt HPgV 3'UTR (Linnen *et al.*, 1996b; Xiang *et al.*, 2000). As observed for the 5'UTR, the 3'UTRs of the novel HPgVs and HPgV reference strains (AF121950 and NC_001710) were well conserved; 3'UTR sequences acquired by deep sequencing were $\geq 97\%$ similar to aligned regions of the reference strains and $\geq 98\%$ similar to each other.

Deep sequencing also identified five HIV-positive individuals within the study cohort, containing 40 to 180 reads per sample. Four individuals were infected with HIV-1 subtype A1/D and one was infected with HIV-1 subtype A1. This infection frequency (17.9%) is consistent with a previously reported HIV prevalence for this region of 16.1% (Rubaihayo *et al.*, 2010). One of the above patients, Hu20, was co-infected with HIV-1 subtype A1/D and HPgV. HPgV motifs previously proposed to be important for HIV antagonism were conserved in all new sequences described herein, including the co-infected sample (George *et al.*, 2012; Jung *et al.*, 2007; Koedel *et al.*, 2011; Timmons *et al.*, 2013; Xiang *et al.*, 2006, 2008).

To compare our newly described sequences to published HPgV sequences, we reconstructed a Bayesian phylogenetic tree that included our sequences as well as publicly available full-length HPgV sequences. Our Bayesian phylogeny is largely consistent with recently published HPgV phylogenies (Feng *et al.*, 2011; Parreira *et al.*, 2012), showing 100% posterior clade support for proposed genotypes 1–5 (Fig. 1). The phylogeny includes sequences identified as recombinant, as assessed using the recombination analysis software RDP4 (Martin *et al.*, 2010): AB021287 (genotype 4; recombination event spanning E2, P7 and NS2), U75356 (genotype 3; recombination events in NS4B and NS5B), and D87715 (genotype 3; recombination event in NS5B). These sequences were retained because removal of these putative recombinants did not change the topology of the tree.

Sequence Hu6 (KC618399) fell within the genotype 1 clade (100% posterior probability), sharing 89.6% and 88.8% nucleotide identity with the other complete genotype 1 sequences, U36380 and AB013500, respectively (Fig. 1). The three remaining sequences, Hu5 (KC618398), Hu20 (KC618400) and Hu21 (KC618401) sorted with genotype 5 (Muerhoff *et al.*, 2005) with 100% posterior clade probability. Hu20 and Hu21 are sister taxa (92% similarity), and are 88.8% similar to Hu5 and published sequence AY949771. Hu5 and AY949771 share 89.2% similarity with each other. Of note, our phylogeny supports the presence of a sixth genotype, represented by two complete sequences from Japan (AB008336 and AB003291). These sequences share 97.7% nucleotide identity with each other and are 87.7% and 87.8% similar to genotype 1 and 5, respectively (Table 1). Simian pegivirus from chimpanzees (SPgV_{tro}) was approximately 71% similar to all human genotypes (Table 1).

The new genotype 1 and 5 sequences made possible the calculation of within-genotype average pairwise nucleotide similarity. For genotype 1, this value was 88.8% (standard error, 0.003), and for genotype 5 this value was 89.3% (standard error, 0.002). Among putative proteins, nucleotide sequences were most conserved in the truncated core (94.0%) and NS5A (92.2%) regions. The least conserved areas were located in the P7-like region (89.8%) and in NS2 (89.7%; Table 2).

To characterize HPgV diversity within infected hosts, we quantified single-nucleotide polymorphisms (SNP) within each individual, which reflects the diversity of the viral 'quasispecies' (Lauring & Andino, 2010). SNP frequencies in HPgV were lower compared to HCV for both synonymous (silent) and non-synonymous (amino acid changing) mutations (Lauck *et al.*, 2012a). HPgV from patient Hu5 contained the highest total number of SNP (123 synonymous and six non-synonymous), although Hu20 had a higher total number of non-synonymous SNP, at seven (Table 3). The majority of SNP occurred at frequencies of 5–10%, suggesting that these SNP may reflect random nucleotide misincorporations. We also estimated nucleotide diversity as the average pairwise difference between sequences for all sites (π), synonymous sites (π_S) and non-synonymous sites (π_N). Hu5 displayed the highest overall nucleotide diversity throughout the genome (π of 0.00921 substitutions per site, Table 4). Comparing values of π_S and π_N for each protein-coding region, π_S was consistently higher than π_N , with the exception of the core protein region from patient Hu6 (Table 4). While no HPgV protein-coding gene was consistently more diverse than the other protein-coding genes, NS5B had the highest average π_N value in three out of the four patients: Hu5, Hu20 and Hu21. Intra-host diversity estimates for patient Hu20, co-infected with HIV, did not differ appreciably from estimates for other patients infected with HPgV alone (Table 4).

DISCUSSION

This study examined HPgV infection in western Uganda, where 4 out of 28 (14.3%) of patients screened were identified as HPgV-positive. This result is consistent with previous findings of high HPgV prevalence in developing countries, ranging from 5% to 18.9% (Mohr & Stapleton, 2009). This study identified two genotypes, 1 and 5, co-circulating in the area. To our knowledge, the only complete genome previously reported from east Africa sorted with genotype 2, a lineage containing a number of European sequences, which is suggested to reflect early human migrations from Africa to Europe (Smith *et al.*, 2000). Genotype 1 has previously been identified as an Africa-wide clade, originating from west and central Africa, (Leary *et al.*, 1996; Smith *et al.*, 1997; Tanaka *et al.*, 1998). Our discovery of a genotype 1 sequence in east Africa confirms that this genotype is widespread throughout the African continent. Genotype 5 has previously been

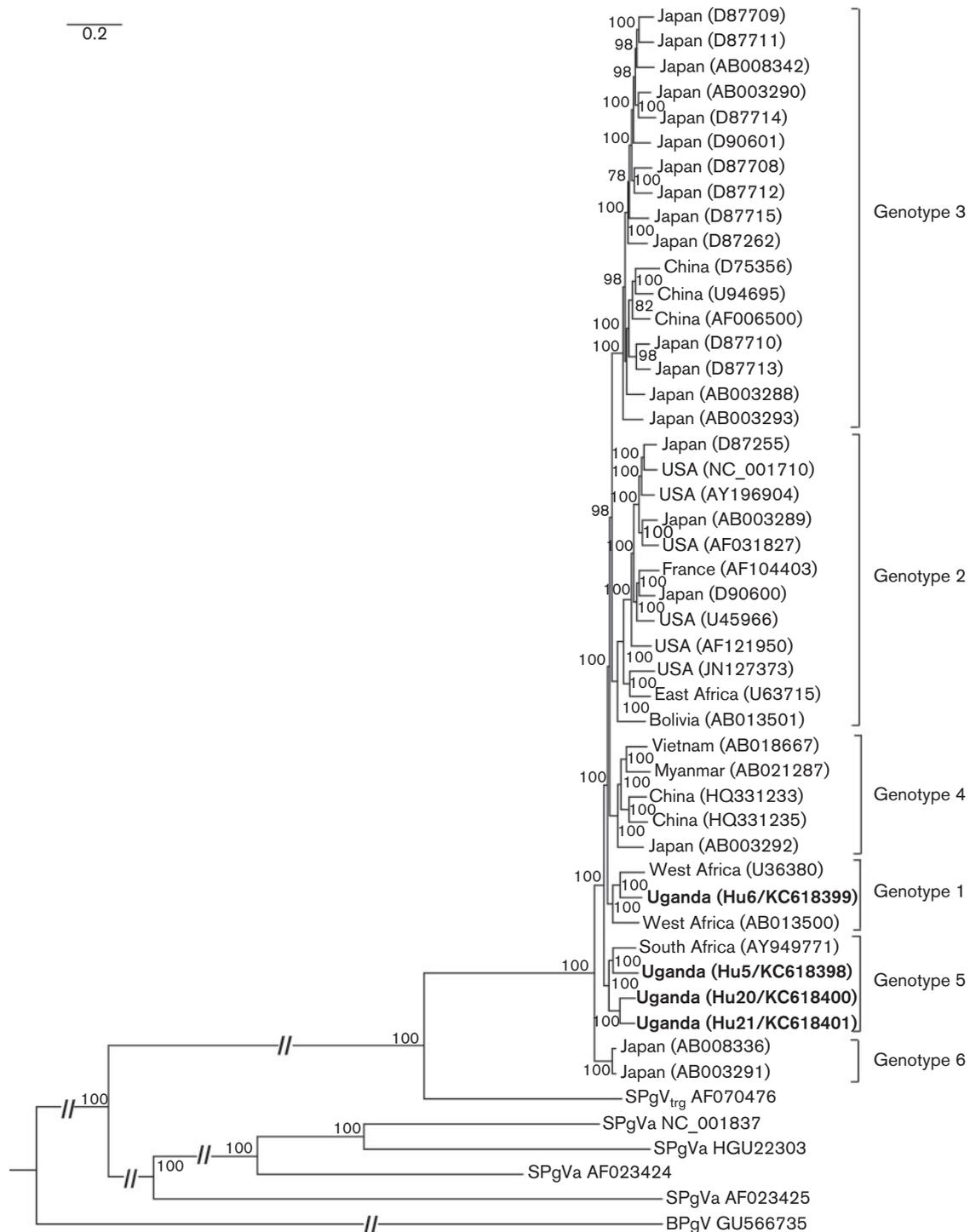


Fig. 1. HPgV Bayesian phylogenetic tree produced from an alignment of 38 full coding genomes including the four new full coding sequences described herein. Putative recombinants AB021287, U75356, D87715 were included because their inclusion did not alter the topology of the tree. More distantly related pegiviruses, including simian pegivirus from chimpanzees (SPgV_{trg}; AF070476) and New World monkeys (AF023424, AF023425, HGU22303 and NC_001837), as well as bat pegivirus (BPgV; GU566735) were included as outgroups. Newly discovered sequences are in bold with sample identification numbers and GenBank accession numbers in parentheses. Posterior clade probability values are shown as percentages at nodes; scale bar indicates nucleotide substitutions per site.

Table 1. Percentage nucleotide identity between HPgV genotypes

Percentage nucleotide identities below the diagonal and standard errors above the diagonal are shown. Simian pegivirus from chimpanzees (SPgV_{tro}) was included for comparison.

	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5	Genotype 6	SPgV _{tro}
Genotype 1	.	0.003	0.003	0.003	0.003	0.003	0.005
Genotype 2	86.7	.	0.003	0.003	0.003	0.004	0.004
Genotype 3	87.3	86.8	.	0.003	0.003	0.003	0.004
Genotype 4	87.0	86.5	87.1	.	0.003	0.003	0.004
Genotype 5	87.6	86.7	87.3	87.0	.	0.003	0.005
Genotype 6	87.7	86.5	87.1	86.6	87.9	.	0.005
SPgV _{tro}	70.7	70.5	70.7	70.8	70.9	70.7	.

characterized as a South African clade, with a single complete coding sequence and several partial sequences reported from this country (Muerhoff *et al.*, 2005; Sathar & York, 2001; Tucker *et al.*, 1999; Tucker & Smuts, 2000). Our discovery of three genotype 5 sequences in Uganda demonstrates that the geographical range of this viral genotype is more extensive than previously documented.

The four complete HPgV coding sequences described herein provide new support for the phylogenetic positions of previously undersampled genotypes. While genotype 2 and 3 have been well characterized phylogenetically, previous studies have had limited success resolving HPgV evolutionary history because of few genotype 1, 4 and 5 complete coding sequences (Parreira *et al.*, 2012). The additional complete coding sequences described here demonstrate consistency in the phylogenetic relationships previously established for genotypes 1 and 5 despite expansion of their known geographical range.

Our sequence analyses and Bayesian phylogeny suggest the existence of six HPgV genotypes based on six strongly supported clades. The proposed sixth genotype comprises two Japanese sequences (Suzuki *et al.*, 1999; Tanaka *et al.*, 1998) previously considered members of genotypes 1 and 4, respectively (Feng *et al.*, 2011; Muerhoff *et al.*, 2005), but also shown to be genetically distinct from other genotypes by principal coordinate analysis (Parreira *et al.*, 2012). Our results also suggest that another previously proposed genotype, represented by a single sequence (Muerhoff *et al.*, 2006), as well as other sequences proposed to form a

seventh genotype (Feng *et al.*, 2011), should be considered part of genotype 4, with which they cluster with 100% posterior clade support according to our analyses. Interestingly, one of our putative genotype 6 sequences, (GenBank accession number AB003291), clusters with 15 partial African NS3 and NS5a 'indel types' from central Africa (Tanaka *et al.*, 1998). The 12 amino acid indel that typifies this group may be an ancestral trait that was lost in other HPgV lineages, perhaps indicating that this is an ancestral viral lineage with a geographical origin in Africa (Pavesi, 2001; Tanaka *et al.*, 1998). Our results demonstrating multiple, diverse HPgV genotypes co-circulating within a very small geographical area (<25 km², with genotype 1 from Hu6 and genotype 5 from Hu20 originating from the same village) further supports the idea of a centre of HPgV endemism and diversity in sub-Saharan Africa.

Our study also confirms that HIV-1 and HPgV co-circulate in sub-Saharan Africa on a local scale. Previous studies have identified motifs within the HPgV genome that are associated with the inhibition of HIV replication. Particularly, the HPgV non-structural phosphoprotein NS5A inhibits replication by reducing surface expression of the HIV co-receptor CXCR4 and increasing the release of its ligand, SDF-1 into supernatant (Xiang *et al.*, 2006, 2008). The NS5A motif (position 152–165) responsible for this activity is conserved in all known sequences, including the four new sequences identified here. Similarly, HPgV E2 envelope protein has been identified as preventing HIV

Table 2. Average percentage nucleotide identity among sequences within HPgV by gene

Genotype	Overall	Core	E1	E2	P7-like	NS2	NS3	NS4A	NS4B	NS5A	NS5B
1	88.8	95.2	88.4	88.3	89.9	87.3	87.7	88.8	87.7	90.6	89.9
2	89.7	94.3	89.1	89.0	87.2	88.9	89.0	90.0	89.0	90.6	91.0
3	90.5	92.5	89.7	91.0	91.3	89.7	89.5	92.5	89.5	91.9	91.2
4	89.0	96.2	89.7	88.3	86.5	87.7	87.7	91.8	88.5	91.5	89.4
5	89.3	92.9	89.6	88.3	86.5	87.5	88.1	91.0	89.2	81.5	90.6
6	97.7	92.9	97.5	97.6	98.1	87.0	98.7	96.4	97.2	97.3	97.5
Average	90.8	94.0	90.7	90.4	89.8	89.7	90.1	91.8	90.2	92.2	91.6

Table 3. Intra-host diversity of HPgV

Assessed as the number of synonymous (S) and non-synonymous (NS) mutations and dN/dS ratios (for whole coding genomes only) across the full coding viral genomes from four patients in western Uganda. –, not calculated.

Frequency (%)	Hu5			Hu6			Hu20			Hu21		
	S	NS	dN/dS	S	NS	dN/dS	S	NS	dN/dS	S	NS	dN/dS
5–10	66	4	–	17	2	–	36	5	–	14	1	–
10–30	31	1	–	24	2	–	16	2	–	11	1	–
30–50	26	1	–	1	0	–	6	0	–	5	0	–
Total	123	6	0.049	42	4	0.095	58	7	0.121	30	2	0.0667

viral entry by modifying HIV gp41 fusion peptide (Jung *et al.*, 2007; Koedel *et al.*, 2011; Timmons *et al.*, 2013). E2 (269–286) is also conserved among the new sequences, suggesting that these new sequences also have HIV-inhibitory properties.

Finally, this study expands our understanding of HPgV intra-host viral genetic diversity. It has been suggested that HPgV can exist as a ‘quasispecies’ with different tissue tropisms in a single individual (Fogeda *et al.*, 2000; Ruiz *et al.*, 2010). However, intra-host genetic diversity of HPgV has, to our knowledge, remained previously undescribed. We found that the total number of SNPs detected in HPgV was approximately tenfold lower than in HCV, measured using nearly identical methods (Lauck *et al.*, 2012a). Although the reasons for this difference are unclear, we speculate that the non-pathogenic nature of HPgV reflects its stable co-evolution with human hosts and immunological tolerance, while pathogenic viruses like HCV must diversify to evade host immunity. Additionally, the majority of HPgV intra-host mutations occurred at a frequency of less than 10% but were not randomly distributed across the genome. Of particular interest, we found the greatest number of synonymous and non-synonymous intra-host mutations in NS5B. This result was

surprising, given that NS5B contains conserved stem-loop structures that may constrain its evolution (Cuceanu *et al.*, 2001). Both our intra-host diversity data and nucleotide diversity estimates demonstrated a tendency toward synonymous mutations over non-synonymous mutation, which indicates purifying selection (i.e. selection against potentially deleterious non-synonymous mutations). These results corroborate previous findings of low rates of non-synonymous-to-synonymous substitutions, averaging 0.04 across all coding regions (Romano *et al.*, 2008).

Overall, our results expand the known geographical range of HPgV genotypes 1 and 5 and demonstrate that multiple HPgV lineages can co-circulate in the same area. We also show that levels and patterns of intra-host HPgV diversity do not mirror levels or patterns of inter-host HPgV variability. Furthermore, the presence of diverse HPgV lineages in an HIV-endemic region, and documented co-infection with both viruses, suggests that this region of sub-Saharan Africa may be a useful locale for investigating immunological and evolutionary interactions between HPgV and HIV, including their clinical effects in co-infected patients. As more data are generated on the distribution and prevalence of the HPgV genotypes and co-infection with HIV, it should be possible to investigate

Table 4. Nucleotide diversity

Estimated as average pairwise distances between sequences for non-synonymous sites (π_N), synonymous sites (π_S), and all sites (π) by gene and for the entire coding region (ORF).

		Core	E1	E2	P7-like	NS2	NS3	NS4A	NS4B	NS5A	NS5B	ORF
Hu5	Mean π_S	0.0151	0.0189	0.0239	0.0430	0.0296	0.0277	0.0151	0.0268	0.0216	0.0256	0.0253
	Mean π_N	0.0022	0.0024	0.0021	0.0013	0.0025	0.0016	0.0016	0.0018	0.0017	0.0031	0.0021
	Mean π	0.0067	0.0073	0.0088	0.0144	0.0109	0.0096	0.0057	0.0097	0.0075	0.0099	0.0092
Hu6	Mean π_S	0.0072	0.0077	0.0092	0.0093	0.0076	0.0069	0.0042	0.0099	0.0064	0.0081	0.0078
	Mean π_N	0.0075	0.0014	0.0012	0.0010	0.0017	0.0014	0.0010	0.0014	0.0017	0.0024	0.0017
	Mean π	0.0074	0.0034	0.0036	0.0038	0.0035	0.0031	0.0020	0.0040	0.0031	0.0041	0.0035
Hu20	Mean π_S	0.0277	0.0200	0.0172	0.0183	0.0131	0.0168	0.0189	0.0155	0.0129	0.0200	0.0168
	Mean π_N	0.0012	0.0019	0.0018	0.0024	0.0027	0.0033	0.0024	0.0015	0.0018	0.0039	0.0026
	Mean π	0.0132	0.0075	0.0065	0.0073	0.0059	0.0075	0.0074	0.0060	0.0051	0.0087	0.0069
Hu21	Mean π_S	0.0034	0.0090	0.0144	0.0144	0.0103	0.0118	0.0130	0.0121	0.0102	0.0135	0.0119
	Mean π_N	0.0027	0.0016	0.0014	0.0014	0.0024	0.0014	0.0017	0.0017	0.0015	0.0032	0.0019
	Mean π	0.0029	0.0039	0.0054	0.0054	0.0048	0.0046	0.0051	0.0049	0.0041	0.0063	0.0050

lineage-specific HPgV–HIV interactions. Understanding these natural dynamics of infection and co-infection may, in turn, lead to novel therapeutic approaches for patients infected with HIV.

METHODS

Ethics. All samples and data were collected with informed consent in accordance with World Health Organization guidelines. Permission to conduct this research was granted by the Uganda National Council of Science and Technology, McGill University, and the University of Wisconsin–Madison. Samples were shipped internationally following IATA guidelines and regulations.

Patients and sample collection. Individuals visiting one of two health clinics outside of Fort Portal (one of the largest towns in western Uganda) who presented with a fever over 100.4 °F were asked to participate in a larger study whose aim was to identify aetiologies of fever. Demographic information including gender, age, and village were recorded, and blood was drawn from the median cubital vein into an evacuated plasma collection tube (Becton Dickinson) by staff nurses at the clinic. Blood was kept cool until processing (<4 h), then was separated using centrifugation in a field laboratory and frozen immediately in liquid nitrogen for storage and transport to the USA. Samples were shipped to North America in an IATA-approved dry shipper.

Amplification of viral RNA. RNA was prepared from blood plasma and directly sequenced on an Illumina MiSeq instrument as previously described (Lauck *et al.*, 2011; Lauck *et al.*, 2013). Sequence data were processed using CLC Genomics Workbench (version 5.5; CLC Bio); sequences were stripped of adaptor sequences, quality/length trimmed (minimum of q30, ≥ 100 bp) and assembled *de novo*. Contiguous sequences were characterized using BLASTN (Altschul *et al.*, 1990); all sequences were mapped to a reference viral database acquired from GenBank. Novel and reference HPgV ORF sequences were aligned using TranslatorX (Abascal *et al.*, 2010). HPgV protein products were identified by comparison to published sequences and annotated using CLC Genomics Workbench. The four complete sequences generated were deposited in GenBank under accession numbers KC618398–KC618401.

Analysis. Phylogenetic relationships within the *Pegivirus* genus were estimated from nucleotide sequences of full-length published coding genome sequences. If two or more genomes were greater than 99% identical, one sequence was chosen to represent both. All genomes with minimum sequence ambiguity were selected (AB003288, AB003289, AB003290, AB003291, AB003292, AB003293, AB008336, AB008342, AB013500, AB013501, AB018667, AB021287, AF006500, AF031827, AF121950, AY196904, AY949771, D87255, D87262, D87708, D87709, D87710, D87711, D87712, D87713, D87714, D87715, D90600, D90601, HQ331233, HQ331235, JN127373, NC_001710, U36380, U45966, U63715, U75356, U94695). Putatively recombinant forms were identified using RDP4, version 4.16 (Martin *et al.*, 2010). Sequences were aligned using the MAFFT algorithm in TranslatorX (Abascal *et al.*, 2010), and regions of ambiguous alignment were removed using the embedded Gblocks algorithm under default settings (Castresana, 2000). More distantly related pegiviruses, including simian pegivirus (SPgV) from chimpanzees (SPgV_{tro}; AF070476), New World monkeys (AF023425, AF023424, NC_001837, HGU22303), and bat pegivirus (BPgV; GU566735) were used as outgroups. Bayesian trees were reconstructed using MrBayes, version 3.2.1 (Ronquist & Huelsenbeck, 2003) with a substitution model of the form GTR+ Γ , selected using jModelTest and the Akaike information criterion (AIC), with Δ AIC to second-best

model GTR+I+ Γ =70.1 (Posada, 2008), model parameters estimated from the data under default priors, and Markov chains run for 2.5 million generations, with the first 25% of sampled trees discarded as burn-in.

To estimate inter-host viral genetic diversity, nucleotide-level percentage sequence identity among sequences was calculated as the pairwise proportion of nucleotide differences (p-distance) in MEGA5.05 (Tamura *et al.*, 2011). To estimate intra-host viral genetic diversity, single-nucleotide polymorphism (SNP) analysis was performed using CLC's SNP analysis tool, with the following parameters: window length, 7; maximum gap and mismatch count, 2; minimum central quality base, 30; minimum average quality for window bases, 25; minimum coverage, 100 \times ; and minimum variant frequency, 5%. This method has been used previously for intra-host viral genetic diversity analysis of HCV to ensure that only high quality and high coverage areas are considered in SNP calling (Lauck *et al.*, 2012a). To achieve fine genetic resolution of each viral population, estimates of nucleotide diversity were calculated using all available deep sequences, including those sequencing with SNPs below our highly conservative threshold. Nucleotide diversity estimates (π) were calculated using the PoPoolation software version 1.2.2 (Kofler *et al.*, 2011) with disabled corrections and a transversion penalty of six.

Titres of HPgV were determined using TaqMan RT-qPCR. RNA was extracted from 200 μ l of blood plasma using a Qiagen QIAamp MinElute virus spin kit (Qiagen). HPgV quantification was performed on a Lightcycler 480 real-time instrument (Roche) using a Superscript III platinum one-step RT-qPCR kit (Invitrogen) and the following original primers and probe: forward 5'-TACGACGACT-GCCCTACAC-3'; reverse 5'-TTTGCCAGCTIACATCAGG-3'; probe 5'-FAM-CGCAGCCGTCGCTGCTGACAT-BHQ-3'. Standard Lightcycler RT-qPCR conditions were used, incorporating primers at 500 nM and the TaqMan probe at 100 nM. A standard curve was prepared from viral RNA (from patient Hu5) amplified using RT-qPCR with the following primers: forward: 5'-GCACAGGAGAGGAA-GGTC-3' and reverse: 5'-CCCAGTCTGTCACCACCAC-3'. The resulting 588 bp amplicon was (i) cloned (Zero Blunt[®] PCR Cloning kit; Life Technologies), (ii) linearized (*Hind*III digestion; NEB), (iii) transcribed (MEGAscript T7 kit; Ambion) and (iv) quantified fluorometrically (Qubit 2.0, Invitrogen). The fidelity of the RNA standard was verified by Sanger sequencing.

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