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Arteriviruses, Pegiviruses, and Lentiviruses Are Common among Wild African Monkeys

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ABSTRACT

Nonhuman primates (NHPs) are a historically important source of zoonotic viruses and are a gold-standard model for research on many human pathogens. However, with the exception of simian immunodeficiency virus (SIV) (family *Retroviridae*), the blood-borne viruses harbored by these animals in the wild remain incompletely characterized. Here, we report the discovery and characterization of two novel simian pegiviruses (family *Flaviviridae*) and two novel simian arteriviruses (family *Arteriviridae*) in wild African green monkeys from Zambia (malbroucks [*Chlorocebus cynosuros*]) and South Africa (vervet monkeys [*Chlorocebus pygerythrus*]). We examine several aspects of infection, including viral load, genetic diversity, evolution, and geographic distribution, as well as host factors such as age, sex, and plasma cytokines. In combination with previous efforts to characterize blood-borne RNA viruses in wild primates across sub-Saharan Africa, these discoveries demonstrate that in addition to SIV, simian pegiviruses and simian arteriviruses are widespread and prevalent among many African cercopithecoid (i.e., Old World) monkeys.

IMPORTANCE

Primates are an important source of viruses that infect humans and serve as an important laboratory model of human virus infection. Here, we discover two new viruses in African green monkeys from Zambia and South Africa. In combination with previous virus discovery efforts, this finding suggests that these virus types are widespread among African monkeys. Our analysis suggests that one of these virus types, the simian arteriviruses, may have the potential to jump between different primate species and cause disease. In contrast, the other virus type, the pegiviruses, are thought to reduce the disease caused by human immunodeficiency virus (HIV) in humans. However, we did not observe a similar protective effect in SIV-infected African monkeys coinfected with pegiviruses, possibly because SIV causes little to no disease in these hosts.

Viruses that naturally infect wild nonhuman primates (NHPs) are of considerable interest because of their zoonotic potential (1, 2). For example, the study of naturally occurring simian immunodeficiency virus (SIV) infections in African monkeys (i.e., "natural hosts" for SIV) has provided invaluable insight into the origins and pathogenesis of human immunodeficiency virus (HIV) infection in humans and SIV infection in endangered great apes (see references 3–5 for reviews). However, with the exception of SIV, which infects monkeys from over 40 species throughout Africa, the RNA viruses naturally infecting wild NHPs remain largely uncharacterized. This lack of characterization is due in part to limitations in technology and the technical and ethical challenges inherent in invasive sampling of wild NHPs (6–8).

Previously, we used unbiased deep sequencing, a technique that utilizes random hexamers to prime cDNA synthesis from RNA in combination with next-generation sequencing, to discover and characterize blood-borne RNA viruses in wild monkeys from several cercopithecoid (i.e., Old World monkey [OWM]) species in Uganda, Tanzania, and Zambia (9–16). We consistently detected viruses from three genera: lentiviruses (i.e., SIV, *Retroviridae* family), pegiviruses (i.e., simian pegivirus [SPgV], *Flaviviridae* family), and simian arteriviruses (i.e., viruses distantly related to simian hemorrhagic fever virus [SHFV], *Arteriviridae* family). Viruses from these genera are relevant to NHP and human health: human pegivirus (HPgV) is associated with a reduction in pathological immune activation and mortality in

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This work is dedicated to the memory of Austin L. Hughes, who died unexpectedly during the preparation of the manuscript. He was our collaborator, mentor, and friend, and we are deeply indebted to him for the great contributions he made, both to our lives and to this work.

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HIV-infected people (17–19), while simian arteriviruses are prototypical preemergent zoonotic pathogens that have caused numerous outbreaks of viral hemorrhagic fever in captive Asian-origin macaque monkeys (20, 21).

We hypothesized that in addition to lentiviruses, simian pegiviruses and simian arteriviruses are common RNA viruses found in plasma (i.e., the "plasma RNA virome") of monkeys throughout sub-Saharan Africa. To test this hypothesis, we sought to expand our knowledge of the host and geographic ranges of these viruses by applying unbiased deep sequencing to plasma samples collected from wild African green monkeys (AGMs) in Zambia (malbroucks [*Chlorocebus cynosuros*]) and South Africa (vervet monkeys [*Chlorocebus pygerythrus*]). Although AGMs in Gambia (sabaeus monkeys [*Chlorocebus sabaeus*]) are known to harbor simian pegiviruses (22), the AGM species examined in this study are not known to harbor simian pegiviruses or simian arteriviruses and are separated by as much as 2,000 km from the closest known natural host of these viruses (baboons [*Papio* spp.] in Zambia [21]). Additionally, extensive population and cytokine data were already available for a large subset of the South African vervet population included in this study (23). Here, we demonstrate prevalent infection of these AGMs with simian lentiviruses, simian pegiviruses, and simian arteriviruses (but not other RNA viruses), supporting our idea that viruses from these genera are major constituents of the plasma RNA virome in monkeys from a diversity of cercopithecoid species living across a large part of sub-Saharan Africa.

MATERIALS AND METHODS

Ethics statement. All the animals sampled in this study were used according to regulations set forth by the Animal Welfare Act. Sampling of vervet monkeys in South Africa was approved by the Interfaculty Animal Ethics Committee (project no. 13/2010) at the University of Free State. Sampling of vervet monkeys in South Africa and malbrouck monkeys in Zambia was approved by the University of Wisconsin—Milwaukee Animal Care and Use Committee (protocol 07-08 32).

Sample collection. Vervet monkeys were individually trapped by using established methods (24), the details of which were described previously (23). Briefly, each animal was trapped, sedated, and bled via venous puncture. During sampling, a detailed physical examination was performed, clinical signs were assessed, and the approximate age and sex of each individual were noted. Each vervet monkey sampled had a microchip implanted for further identification and for the prevention of duplicate sampling. Upon blood collection, plasma was purified and immediately stored at −80°C.

Unbiased deep sequencing. Samples were processed for sequencing in a biosafety level 3 laboratory as described previously (14), with slight modifications. Briefly, for each sample, RNA was isolated from ~200 μl of plasma by using the Qiagen QIAamp MinElute virus spin kit (Qiagen, Hilden, Germany), omitting carrier RNA. Samples were then treated with DNase, and cDNA synthesis was primed by using random hexamers from a double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Samples were fragmented, and sequencing adaptors were ligated by using the Nextera DNA sample preparation kit (Illumina, San Diego, CA, USA). Deep sequencing was performed on the Illumina MiSeq instrument. Sequence data were processed by using CLC Genomics Workbench 6.5 (CLC Bio, Aarhus, Denmark) and Geneious R5 (Biomatters, Auckland, New Zealand). Low-quality (<Q30, Phred quality score) and short (<100-bp) reads were removed, and coding-complete genome sequences for each virus were acquired by using a combination of mapping and the *de novo* assembly algorithm in CLC Genomics Workbench version 6.5. Viral genomes were annotated in CLC Genomics Workbench version 6.5, and open reading frames (ORFs) were confirmed by querying the NCBI GenBank database (25).

Phylogenetic analysis. Sequences were aligned by using a codon-based version of the open-source software Multiple Alignment Using Fast Fourier Transform (MAFFT), implemented in TranslatorX, without GBLOCKS gene fragment cleaning (26). Phylogenetic history was inferred from aligned nucleotide sequences by using the maximum likelihood method (1,000 bootstrap replicates) via Molecular Evolutionary Genetics Analysis version 6 (MEGA6) open-source software (27). The best nucleotide substitution model, a general time-reversible model coupled with a Γ distribution for rate variation (GTR+Γ), with five rate categories and a +Γ parameter of 1.1092, was estimated by using MEGA6. All positions containing gaps and missing data were eliminated (complete deletion), resulting in a final data set of 4,380 positions. The initial tree for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances, estimated by using the maximum composite likelihood approach.

qRT-PCR. Vervet monkey SIV (SIV_{ver})-specific TaqMan quantitative reverse transcriptase PCR (qRT-PCR) was performed on plasma samples (23). TaqMan qRT-PCR assays were then developed to quantify concentrations of plasma viral RNA for both $SPgV_{ver}$ and DMVV-1 (Drakensberg Mountain vervet virus). First, alignments of the GBV-C_{ver} and DMVV-1 genomes obtained by unbiased deep sequencing were constructed in CLC Genomics Workbench version 6.5 to identify highly conserved regions. Primer3 (28) was then used to design primers and probes specific for these regions [i.e., $SPgV_{ver}$ forward primer 5′-CAGC-(deoxyinosine)-GACATCGGAGAAGC-3′, $SPgV_{ver}$ reverse primer 5′-CTAACA CTTCCCGGCACATT-3′, $SPgV_{ver}$ probe 5′-FAM (6-carboxyfluorescein)-CGGCTGTAACTGGCCTTTAC-BHQ1 (black hole quencher 1)-3′, DMVV-1 forward primer 5′-GTCAGGGCTTCACCCTAGC-3′, DMVV-1 reverse primer 5′-GCCATACCTCCGAAGGGTTA-3′, and DMVV-1 probe 5′-Quasar670-CTTGGTCCCTGACGTGAAAA-BHQ3-3′]. PCR amplicons flanking the primer binding sites for each virus were generated, cloned into the Zero Blunt PCR vector (Invitrogen), and linearized (HindIII; New England BioLabs, Ipswich, MA). Transcription was performed *in vitro* for 6 h (MEGAscript T7 transcription kit; Invitrogen), followed by RNA transcript purification (MEGAclear transcription cleanup kit; Invitrogen), quantification (Qubit RNA high-sensitivity assay kit; Invitrogen), and dilution to a concentration of 1×10^{10} transcript copies/μl. Tenfold dilutions of this transcript were used as a standard curve.

Viral RNA was extracted from 100 μl of plasma with the Viral Total Nucleic Acid Purification kit (Promega, Madison, WI) on the Maxwell 16 MDx instrument (Promega). RNA was reverse transcribed and amplified by using the SuperScript III One-Step qRT-PCR system (Invitrogen) on a LightCycler 480 instrument (Roche, Indianapolis, IN). Reverse transcription was carried out at 37°C for 15 min and then at 50°C for 30 min, followed by 2 min at 95°C. Amplification was accomplished over 50 cycles as follows: 95°C for 15 s and 60°C for 1 min. The reaction mixture contained $MgSO_4$ at a final concentration of 3.0 mM, two amplification primers at a concentration of 500 nM, and a probe at a concentration of 100 nM. The standard curve was linear over 8 orders of magnitude and was sensitive down to 10 copies of RNA transcript per reaction.

Evolutionary analyses. For within-host nucleotide diversity analyses, single-nucleotide polymorphism (SNP) reports were generated by mapping pooled sequencing reads to their corresponding consensus sequence. Next, SNPGenie (29) was used to estimate synonymous nucleotide diversity (π_S) and nonsynonymous nucleotide diversity (π_N) for each ORF by using a new method for pooled-sequencing analyses (30) based on that of Nei and Gojobori (31) (accessible at https://github.com/hugheslab/snpgenie). Briefly, ORF sequences were translated, aligned at the amino acid level, and untranslated by using the ClustalW algorithm in MEGA6 (default settings) (27). These alignments were used to pool nucleotide diversity data for all within-host viral populations. Population-level π_N and π_S values were then calculated in sliding windows of 9 codons. Data were analyzed with R version 3.0.2 (32).

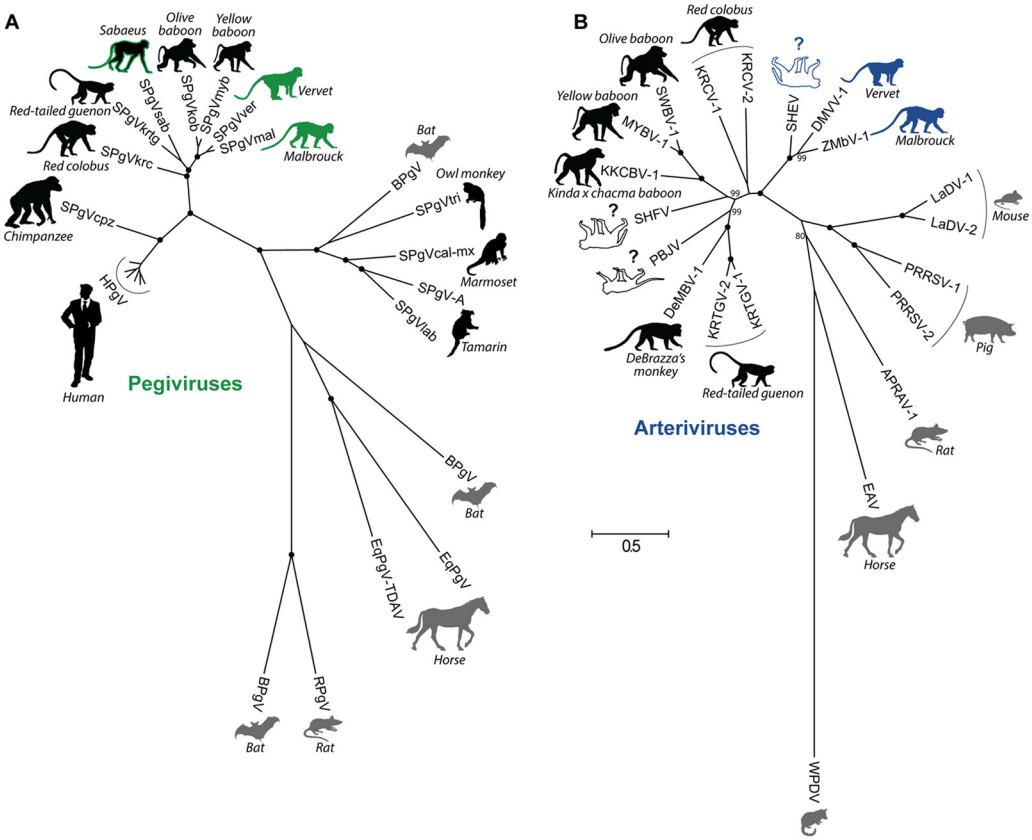

FIG 1 Species-level phylogenetic relationships of all known pegiviruses (A) and arteriviruses (B). Viruses are shown adjacent to the silhouette of their respective host, with host common names in italics. Primate hosts are shown in black, and nonprimate hosts are shown in gray. Viruses discovered in this study are depicted by host silhouettes with solid coloring: green for AGM pegiviruses and blue for AGM arteriviruses. Host silhouettes with a colored outline draw attention to viruses of importance. Sabaeus SPgV ($SPgV_{sab}$) has a green outline because this virus does not group with the AGM pegiviruses presented here. SHEV has a blue outline because of its close relationship to the AGM arteriviruses presented here. White host silhouettes symbolize arteriviruses that have caused outbreaks of viral hemorrhagic fever in captive macaques. Question marks emphasize that the natural host(s) of these viruses remains unknown. Shown is a maximum likelihood tree with 1,000 bootstrap replicates. Black dots indicate splits that are supported by 100% of bootstrap replicates. Bootstrap values below 70 are not shown. The bar shows the calculated genetic distance. SPgVkrc, Kibale red colobus; SPgVkrtg, Kibale red-tailed guenon; SPgVob, SPgV from olive baboon; SPgVmyb, Mikumi yellow baboon; BPgV, bat pegivirus; SPgVtri, owl monkey; SPgVcal-mx, marmoset-mystax; SPgVlab, tamarin; EqPgV, equine pegivirus; TDAV, Theiler's disease-associated virus; RPgV, rat PgV; KRTGV-1, Kibale red-tailed guenon virus; DeMBV-1, Debrazza's monkey virus; PBJV, Peter B. Jahrling virus; KKCBV-1, Kafue kinda-chacma baboon virus; MYBV-1, Mikumi yellow baboon virus; SWBV-1, Southwest baboon virus; KRCV-1, Kibale red colobus virus; LaDV-1, lactate dehydrogenase elevating virus; PRRSV-1, porcine reproductive and respiratory syndrome virus; APRAV-1, African pouched rat virus; EAV, equine arteritis virus; WPDV, wobbly possum disease virus.

Cytokine and chemokine testing. Cytokine testing in plasma was done by using a sandwich immunoassay-based protein array system, the Cytokine Monkey Magnetic 28-Plex panel (Invitrogen), according to the manufacturer's instructions. Results were read by using the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA), which uses Luminex fluorescent-bead-based technology (Luminex Corporation, Austin, TX).

Statistical analyses of host data. All analyses were conducted by using the MASS library (33) with the statistical programming language R (32). For demographic data, linear models were used to determine the relationship between viral infection (response variable) and host age, sex, and viral coinfection (predictor variables). For cytokine data, following outlier removal, the relationship between log cytokine concentration and age, sex, virus binary infection status (infected/uninfected for SIV, simian pegivirus, and simian arterivirus), and coinfection between SIV and simian pegivirus was assessed by using Gaussian-distributed linear models. For each cytokine, final models were selected by using stepwise backward elimination. Mixed-effect models that included sampling location as a random effect did not outperform the null expectation (models were compared using the Akaike information criterion) (results not shown) and were therefore not considered further.

Nucleotide sequence accession numbers. Coding-complete genome sequences for $SPgV_{ver}$, DMVV-1, and SIV_{ver} have been made available in GenBank under accession numbers KR611946 to KR611983 and KR862293 to KR862363. Sequences for malbrouck SPgV ($SPgV_{mal}$) and Zambian malbrouck virus 1 (ZMbV-1) can be found in GenBank under accession numbers KT166442 and KT166441, respectively.

RESULTS

Virus discovery. We performed unbiased deep sequencing of RNA extracted from plasma collected from 12 malbroucks sampled at 3 locations in Zambia and 50 vervets sampled at 9 locations in South Africa. To demonstrate the sensitivity of this method for detecting RNA viruses in these samples, we first assessed our ability to detect SIV_{ver}, an SIV type previously detected in these samples by RT-PCR (23). We found 100% congruence between detection of SIV_{ver} by deep sequencing and detection by PCR (see Table S1 in the supplemental material for sequencing statistics and coverage details).

In addition to SIV_{ver}, deep sequencing revealed two novel pegi-

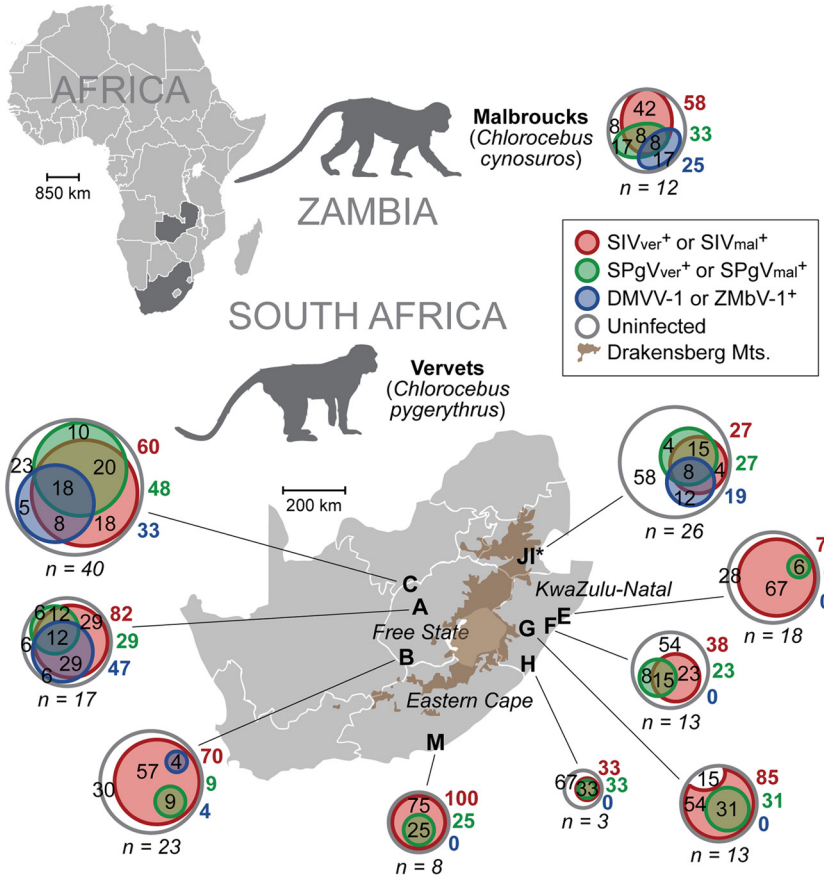

FIG 2 Geographic distribution and prevalence of AGM plasma viruses. Shown are prevalences of SIV, SPgV, and simian arteriviruses in wild AGMs sampled from Zambia (malbrouck monkey [*Chlorocebus cynosuros*]) and South Africa (vervet monkey [*Chlorocebus pygerythrus*]). Venn diagrams of each monkey population show the percentages of uninfected, monoinfected, coinfected, and triple-infected monkeys for each of these three viruses. Gray circles represent the total numbers of monkeys sampled and are proportional to the sample size from each location. Numbers within the gray circles but outside the colored circles are the percentages of each population that are triple negative for virus (white). Colored circles within gray circles show the percentages of each population infected with SIV, SPgV, or DMVV-1/ZMbV-1 that are monoinfected, coinfected, or triple infected. Adjacent colored numbers outside the circles show the percentages of each population infected with the respective virus. Sites I and J are from the Riverside Rehabilitation Center, which houses vervet monkeys from across the region. The prevalence of each virus was determined by using a combination of deep sequencing, qRT-PCR, and RT-PCR.

viruses, $SPgV_{mal}$ and $SPgV_{ver}$, infecting Zambian malbrouck monkeys and South African vervet monkeys, respectively. Compared to other pegiviruses, $SPgV_{mal}$ and $SPgV_{ver}$ were most closely related to one another (Fig. 1A). These viruses were more closely related to simian pegiviruses from baboons (genus *Papio*) sampled in Zambia and Uganda than to simian pegiviruses from the more closely related but geographically distant sabaeus monkeys (*Chlorocebus sabaeus*) sampled in Gambia (9, 16, 22).

We also discovered two novel simian arteriviruses, which we named Drakensberg Mountain vervet virus (DMVV-1) and Zambian malbrouck virus (ZMbV-1). These viruses contained the additional ORFs characteristic of all simian arteriviruses discovered to date (34, 35) and phylogenetically clustered with other simian arteriviruses (Fig. 1B). Of all known simian arteriviruses, DMVV-1 and ZMbV-1 were most closely related to one another. However, DMVV-1 and ZMbV-1 also fell within the same lineage as simian hemorrhagic encephalitis virus (SHEV). SHEV is the simian arterivirus responsible for the first recognized outbreak of simian hemorrhagic fever (SHF) in captive macaques in Sukhumi, former Soviet Union, in 1964 (20, 36), making DMVV-1 and ZMbV-1 the closest relatives of an SHF-causing virus identified to date.

Geographic distribution and prevalence. To examine the prevalence of these viruses in a larger group of AGMs, we tested RNA extracted from the plasma samples of 161 South African vervet monkeys for the presence of $SPgV_{ver}$ and DMVV-1 using highly sensitive qRT-PCR assays designed to amplify conserved regions of the $SPgV_{ver}$ and DMVV-1 genomes. We also used deep-sequencing data, RT-PCR, and serological assays (described previously in reference 23) to screen for SIV_{ver} infection in these animals (see Table S1 in the supplemental material). Taken together with deep-sequencing data from Zambian malbroucks, SIV_{ver} (or SIV_{mal}) and $SPgV_{ver}$ (or $SPgV_{mal}$) were present in monkeys from each sampling site, with 27 to 100% and 6 to 48% of individuals testing positive for lentiviral and pegiviral RNAs, respectively (Fig. 2). In contrast, DMVV-1 was found only in wild AGM populations west of the Drakensberg Mountains in South Africa (sites A, B, and C, i.e., Free State province), with 4 to 47% of individuals from these sites testing positive for viral RNA. ZMbV-1 was found in 25% of malbrouck monkeys in Zambia. All

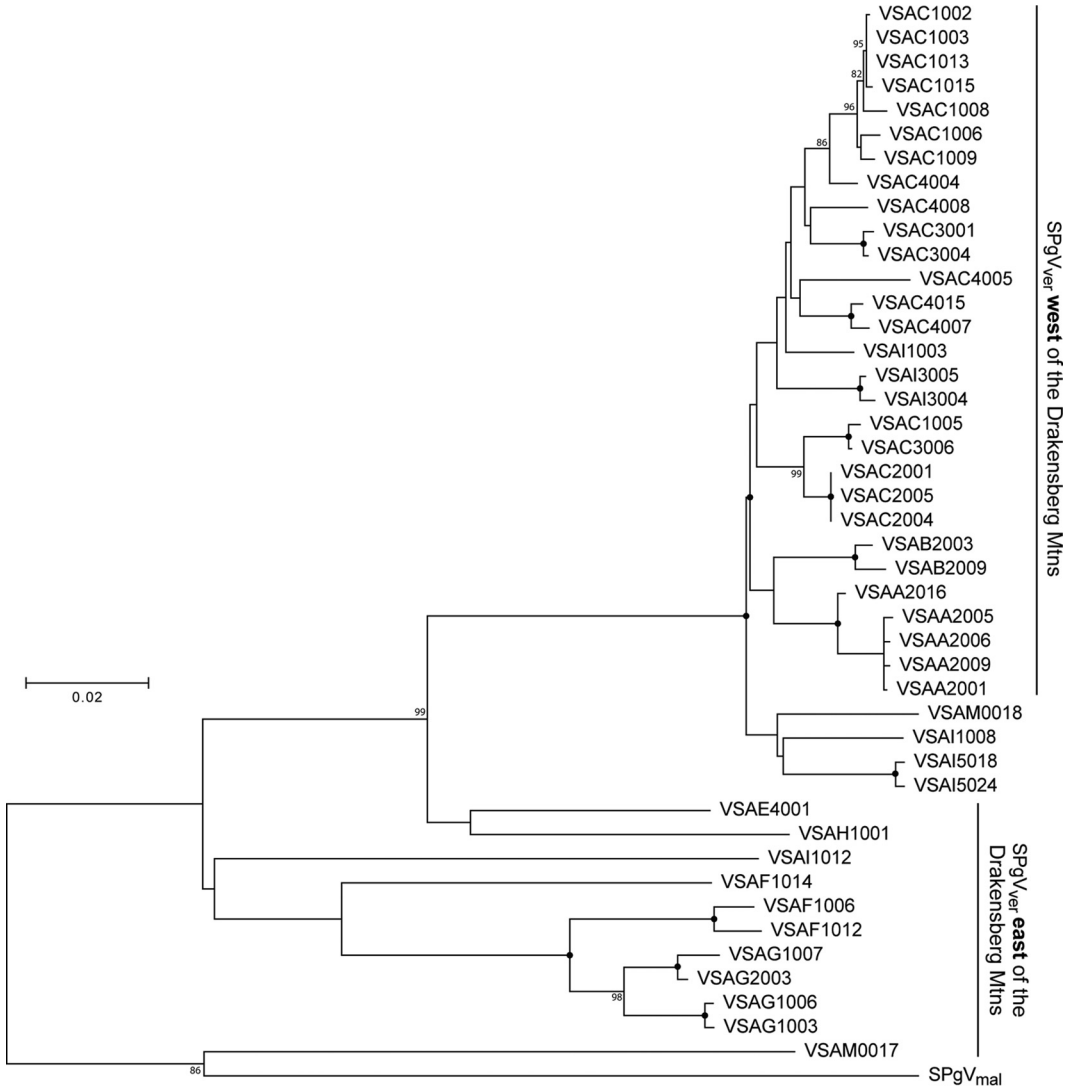

FIG 3 Phylogenetic relationships of all SPgV variants discovered in this study. A PCR amplicon spanning the putative NS3 coding region of the $SPgV_{ver}$ genome was sequenced from samples that tested positive for SPgV RNA by qRT-PCR that were not subjected to unbiased deep sequencing. Shown is a maximum likelihood tree, not rooted, with 1,000 bootstrap replicates. Black dots indicate splits that are supported by 100% of bootstrap replicates. Bootstrap values below 70 are not shown. The bar shows the calculated genetic distance.

three virus types (lentiviruses, pegiviruses, and simian arteriviruses) were detected in the semi-free-living vervet monkeys of the Riverside Wildlife Rehabilitation Center (sites I and J), which serves as a semicaptive sanctuary for AGMs from across South Africa.

Evolutionary relationships among viruses. We amplified regions of the $SPgV_{ver}$ and DMVV-1 genomes corresponding to the highly conserved nonstructural protein 3 (NS3) and ORF1b, respectively, from each monkey that tested positive for viral RNA by qRT-PCR that was not subjected to unbiased deep sequencing. Phylogenetic analysis of $SPgV_{ver}$ and $SPgV_{mal}$ NS3 sequences revealed general clusters of sequence similarity reflective of the various sampling locations, although admixture among locations was observed (Fig. 3). In particular, $SPgV_{ver}$ sequences from monkeys sampled west of the Drakensberg Mountains formed a monophyletic group representing a subclade of viral sequences sampled east of the mountains, which were more diverse and paraphyletic. Two $SPgV_{ver}$ sequences (animals VSAM0017 and VSAM0018) from the southernmost sampling site (site M) were relatively divergent: one grouped with $SPgV_{ver}$ in the western clade, and the other shared the greatest similarity with $SPgV_{mal}$. The phylogeny of DMVV-1 and ZMbV-1 in wild AGMs found to the west of the Drakensberg Mountains revealed a pattern similar to that of $SPgV_{ver}$ in animals west of the Drakensberg Mountains (Fig. 4). In both phylogenies, viruses from the Zambian malbroucks ($SPgV_{mal}$ and ZMbV-1) formed outgroups.

Viremia. To quantify viremia in infected vervet monkeys, we performed virus-specific qRT-PCR on RNA extracted from plasma collected from each vervet (additional analyses were not performed on samples from Zambian malbrouck monkeys due to very limited sample volumes). Virus titers of $SPgV_{ver}$ and DMVV-1, averaging 1.98×10^7 and 1.89×10^7 viral genome copies per ml of plasma, respectively, were significantly higher than those detected for SIV_{ver}, which averaged 1.29×10^6 genome

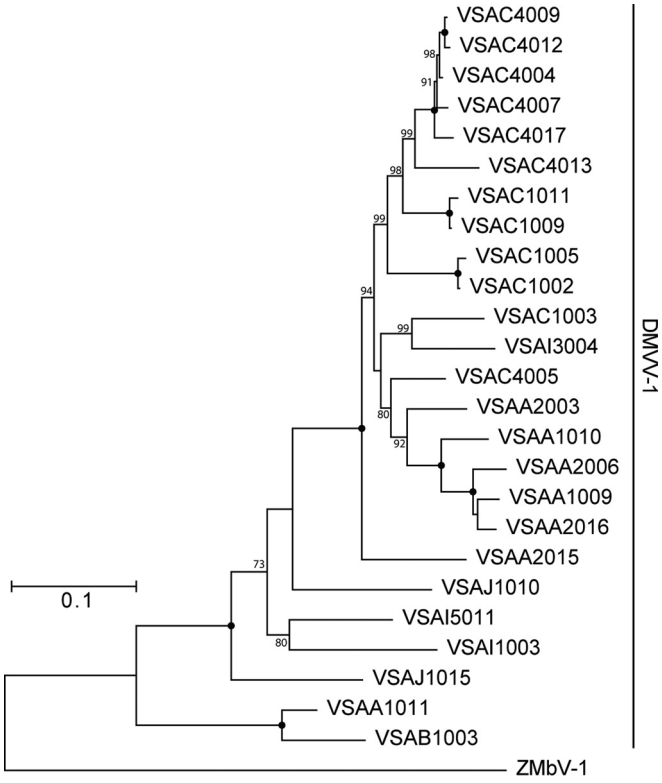

FIG 4 Phylogenetic relationships of all AGM simian arteriviruses discovered in this study. A PCR amplicon spanning ORF1b of the DMVV-1 genome was sequenced from samples that tested positive for DMVV-1 RNA by qRT-PCR that were not subjected to unbiased deep sequencing. Shown is a maximum likelihood tree, not rooted, with 1,000 bootstrap replicates. Black dots indicate splits that are supported by 100% of bootstrap replicates. Bootstrap values below 70 are not shown. The bar shows the calculated genetic distance.

copies per ml of plasma in infected individuals ($P < 0.001$ and $P < 0.001$, respectively, by a two-tailed unpaired t test on log-transformed values) (Fig. 5A). Coinfection with either of the two other viruses did not have a significant effect on plasma titers of SIV_{ver}, $SPgV_{ver}$, or DMVV-1 (Fig. 5B and C).

Viral genetic diversity. Vast genetic diversity is a hallmark of primate lentiviruses and plays an integral role in the immune evasion (i.e., persistence) strategy employed by SIV/HIV (3, 37, 38). The genetic diversity of simian pegiviruses and simian arteriviruses is less well defined. To examine the genetic diversity of these viruses at the population level, we aligned consensus sequences spanning the entire protein-coding region of each viral genome obtained from South African vervet monkeys by deep sequencing. We then created a pairwise matrix to compare each viral sequence to all other sequences in the alignment. SIV_{ver} sequences shared the lowest percent identity on average (81.48% ± 0.23% [mean ± standard error of the mean {SEM}]) (Fig. 6A). However, when SIV_{ver} analysis was restricted to sampling sites A, B, and C, the percent identity among SIV_{ver} sequences was higher (86.35% ± 0.18% [mean ± SEM]) (Fig. 6A) and comparable to the percent identity observed for DMVV-1 sequences from these same locations (85.00% ± 0.38% [mean ± SEM]) (Fig. 6A). Although $SPgV_{ver}$ was found to infect monkeys over a much larger geographical region than DMVV-1, the percent identity among $SPgV_{ver}$ sequences was appreciably higher than that observed for DMVV-1 or SIV_{ver} sequences (95.65% ± 0.20% [mean ± SEM]) (Fig. 6A).

To examine whether this pattern of genetic diversity was also observed at the level of the individual host, we determined the within-host synonymous (π_S) and nonsynonymous (π_N) nucleotide diversities of viral populations for infected vervet monkeys. For this analysis, we considered only samples that yielded sequences with >100× coverage for >99% of the protein-coding regions of the respective viral genome. There were highly significant differences among viruses with respect to both π_S and π_N (determined by a two-tailed unpaired t test) (Fig. 6B). Similar to our population-level analysis, we found that SIV_{ver} had the greatest within-host nucleotide diversity (mean π_S, 1.82×10^{-2} differences/site; mean π_N, 3.69×10^{-3} differences/site) (Fig. 6B). DMVV-1 displayed less within-host diversity than did SIV_{ver} (mean π_S, 1.13×10^{-2}; mean π_N, 1.58×10^{-3}), with an ~2-fold lower π_N value averaged across the genome. However, DMVV-1 diversity was still significantly greater than that of $SPgV_{ver}$ (mean π_S, 2.08×10^{-3}; mean π_N, 2.16×10^{-4}), which had π_S and π_N values that were a full order of magnitude lower than those observed for SIV_{ver} (Fig. 6.B). Viral loads did not correlate significantly with π_S or π_N values obtained for SIV_{ver}, $SPgV_{ver}$, or DMVV-1 (Fig. 6C). For each virus studied, π_S was significantly higher than π_N ($P < 0.001$ for DMVV-1 and $P < 0.01$ for $SPgV_{ver}$ and SIV_{ver}, determined by a paired t test), which is indicative of purifying selection acting across the viral genome (overall π_N/π_S ratios of 0.23 for SIV_{ver}, 0.19 for DMVV-1, and 0.21 for $SPgV_{ver}$).

To examine synonymous and nonsynonymous nucleotide diversities across the genome of each virus, we examined π_S and π_N in a sliding window of 9 codons across each ORF for each virus. For 19 of 23 total ORFs tested, π_S significantly exceeded π_N (determined by a paired t test), which is indicative of the widespread action of purifying selection (Table 1). Of the remaining ORFs, π_N exceeded π_S only in *tat* of SIV, and this difference was not significant. Regions experiencing peaks of nonsynonymous nucleotide diversity ($\pi_N > \pi_S$) indicative of overdominant positive selection were limited to the E1-, E2-, P7-, NS2-, NS5a-, and NS5b-encoding regions of $SPgV_{ver}$; the *env* and *tat* ORFs of SIV_{ver}; and ORFs 1a, TF, 2b, 3, 3′, 4, and 5 of DMVV (Table 2). The proportion of variants with nonsynonymous polymorphisms in these regions ranged from 12 to 47% in $SPgV_{ver}$, 31 to 92% in SIV, and 21 to 100% in DMVV. All 14 variants of DMVV-1 displayed nonsynonymous polymorphisms in a 22-codon region ranging from nucleotides 13922 to 13987 in the viral genome, a region that corresponds to the primary neutralizing antibody epitope that has been mapped in other arteriviruses (39–41), making this an extremely likely target of positive selection in most hosts.

Individual-level correlates of infection. Several host variables associated with natural SIV infection have been described (3, 23). However, host parameters associated with simian pegivirus and simian arterivirus infections have not been evaluated in wild NHPs, including coinfection with other viruses. Consistent with data from previous studies, we found that increased age and female sex were associated with SIV infection in South African vervets (Table 3). In contrast, neither age nor sex was significantly associated with $SPgV_{ver}$ or DMVV-1 infection. We found a strong association between SIV_{ver} and $SPgV_{ver}$ coinfections, similar to the association observed between HIV-1 and HPgV infections in peo-

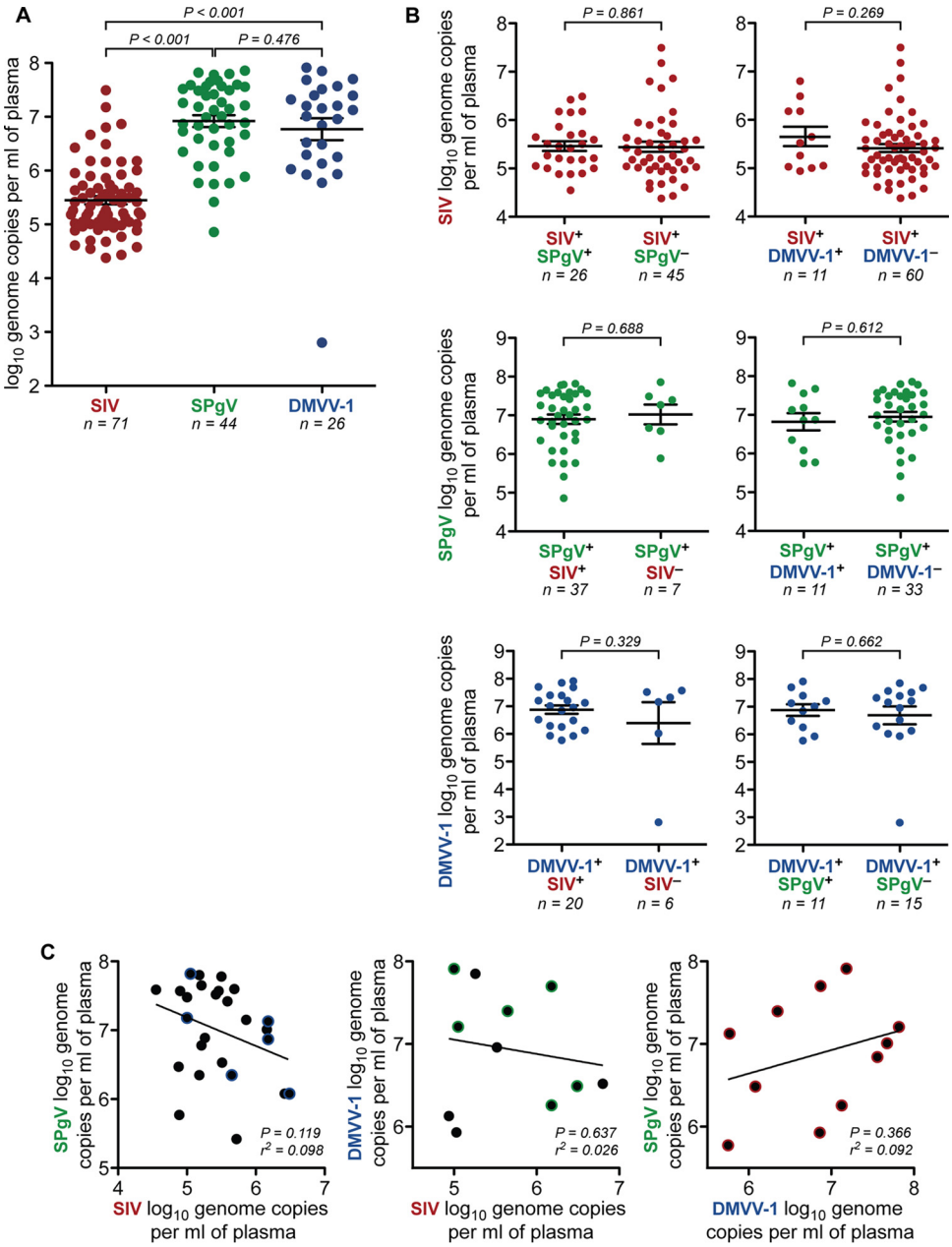

FIG 5 Viremia of the AGM plasma viruses. (A) $SPgV_{ver}$ and DMVV-1 RNA concentrations were quantified from plasma samples of 161 South African vervet monkeys by using highly sensitive virus-specific qRT-PCR assays designed from deep-sequencing data. Only positive results are shown. SIV_{ver} loads were determined previously (23) by the same method. Significance was assessed by using a two-tailed unpaired *t* test on log-transformed values, with error bars showing the standard errors of the means. (B) South African vervets were stratified by coinfection status, and plasma viral load values for the virus in question were plotted. Significance was assessed by using a two-tailed unpaired *t* test on log-transformed values, with error bars showing the standard errors of the means. (C) Linear regression correlating the viral load of each virus in coinfected individuals. Data points with a colored halo indicate triple-infected individuals.

ple (42). However, DMVV-1 infection was not significantly associated with either $SPgV_{ver}$ or SIV_{ver} coinfection.

Cytokines associated with viral infection and coinfection. We obtained data for 28 different plasma cytokines evaluated in 118 vervet monkeys (reported previously in reference 23) and used multivariate linear models to assess the effect of SIV_{ver} infection, $SPgV_{ver}$ infection, SIV_{ver}/$SPgV_{ver}$ coinfection, and DMVV-1 infection on plasma cytokine concentrations. We found that plasma concentrations of several cytokines were negatively correlated with age and female sex (Table 4).

With respect to viral infection, we found a positive association between the presence of SIV_{ver} and plasma concentrations of interleukin-6 (IL-6), as reported previously (23). We also found positive associations between the presence of SIV_{ver} and plasma concentrations of interferon-inducible T cell alpha chemoattractant (I-TAC) (i.e., CXCL11) and macrophage inhibition factor (MIF) and a negative association between interferon gamma (IFN-γ) and the presence of SIV_{ver}. $SPgV_{ver}$ infection was not associated with changes in any plasma cytokine concentrations, with the exception of monokine induced by interferon gamma

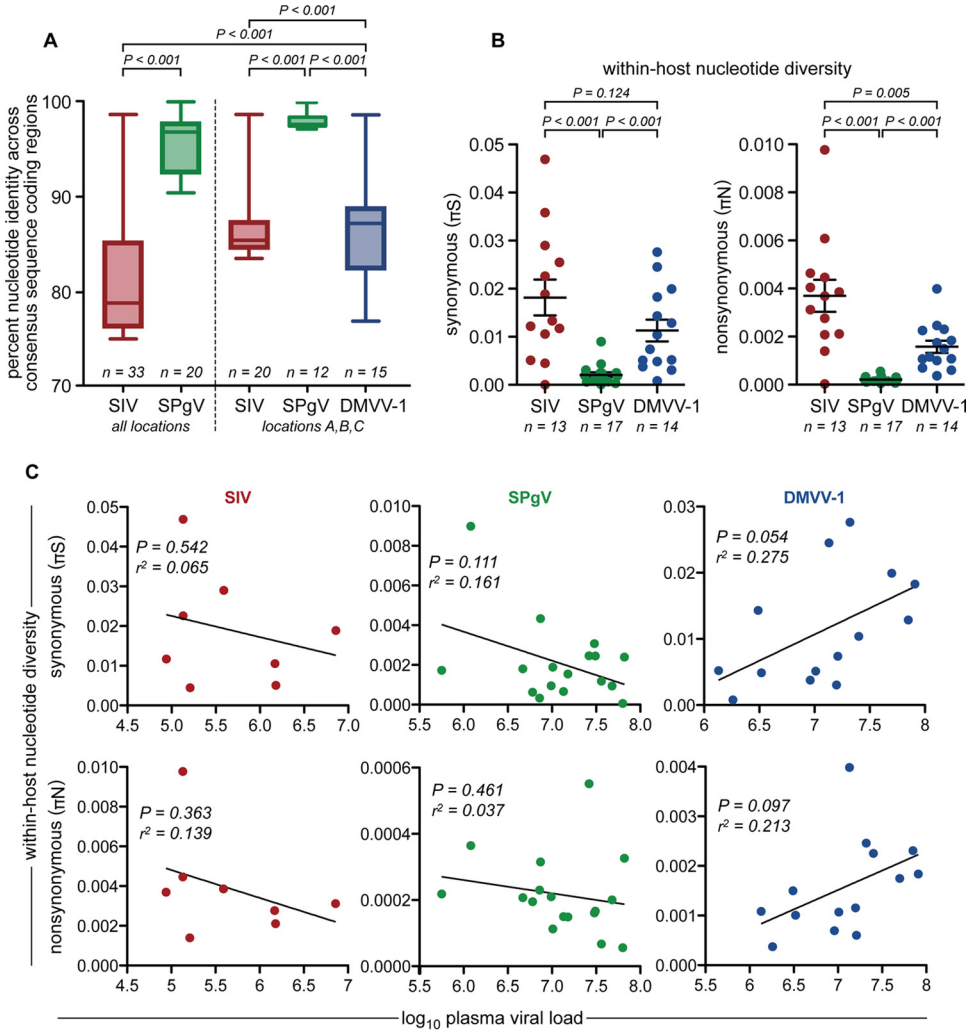

FIG 6 Genetic diversity of AGM plasma viruses. (A) Complete consensus sequences spanning the entire coding region of each virus were aligned, and a pairwise comparison between each aligned sequence was performed. Percent identity values from each comparison were plotted and compared by using a two-tailed unpaired *t* test. Boxes show the middle two quartiles, and whiskers show the minimum and maximum percent identities observed. (B) Synonymous (π_S) and nonsynonymous (π_N) viral nucleotide diversities within each infected monkey, determined by calculating π_S and π_N values across the entire viral genome. Only samples that yielded virus sequences with >100× coverage for >99% of the protein-coding region of the genome were used for this analysis. Significance was assessed by using a two-tailed unpaired *t* test, with error bars showing the standard errors of the means. (C) Linear regression correlating within-host nucleotide diversity and plasma viral load for each virus.

(MIG) (i.e., CXCL9), for which we found a negative association. When we examined cytokine concentrations in SIV_{ver}-infected animals, $SPgV_{ver}$ coinfection did not significantly impact cytokine levels. Additionally, there was a suggestion that the plasma concentrations of several cytokines were increased in association with DMVV-1 infection, including several cytokines and chemokines produced by activated macrophages (IL-1β, granulocyte macrophage colony-stimulating factor [GM-CSF], macrophage inflammatory protein 1β [MIP-1β], epidermal growth factor [EGF], monocyte chemoattractant protein 1[MCP-1], and tumor necrosis factor alpha [TNF-α]) and the anti-inflammatory cytokine IL-10. Importantly, none of the associations that we found between viral infection and plasma cytokine concentrations reached statistical significance after correction for multiple comparisons (Bonferroni correction).

DISCUSSION

Wild AGMs have long been known to harbor species-specific variants of SIV (3) and have played an important role in SIV/HIV pathogenesis research as a "natural host" (5). However, other coinfecting viruses of AGMs have not been similarly well characterized. Here, we searched for blood-borne RNA viruses in AGMs from Zambia and several locations in South Africa using molecular techniques. In addition to SIV_{ver}, we found new viruses from two other genera, pegivirus and simian arterivirus, providing the first comparative analysis of viral infections with these viruses, in their natural state.

Defining the OWM plasma virome: variations on a theme. Our discovery of novel pegiviruses and simian arteriviruses in AGMs in Zambia and South Africa adds to an emerging theme: with few exceptions (43), the plasma RNA virome of African mon-

TABLE 1 Nonsynonymous and synonymous nucleotide diversities for all open reading frames of $SPgV_{ver}$, SIV_{ver}, and DMVV-1 in African green monkeys[a]

Virus	ORF	Mean $\pi_N \pm$ SE	Mean $\pi_S \pm$ SE	*P* value[b]
SIV_{ver}	*gag****	0.002387 ± 0.00029	0.02153 ± 0.00185	1.24×10^{-35}
	*pol****	0.001595 ± 0.00014	0.01808 ± 0.00101	8.59×10^{-70}
	*vif****	0.002705 ± 0.00038	0.00939 ± 0.00216	5.05×10^{-6}
	*vpx****	0.001078 ± 0.00033	0.01158 ± 0.00319	1.62×10^{-6}
	tat	0.006165 ± 0.00118	0.00351 ± 0.00137	0.189
	*rev****	0.005369 ± 0.00117	0.02000 ± 0.00324	9.87×10^{-6}
	*env****	0.006657 ± 0.00057	0.02102 ± 0.00138	1.20×10^{-30}
	*nef****	0.004809 ± 0.00069	0.01738 ± 0.00218	1.19×10^{-10}
$SPgV_{ver}$	SPgV***	0.000214 ± 0.00003	0.00208 ± 0.00019	7.00×10^{-33}
DMVV-1	1a***	0.000954 ± 0.00008	0.01208 ± 0.00078	9.02×10^{-65}
	TF	0.001643 ± 0.00035	0.00287 ± 0.00088	0.0251
	1b***	0.000385 ± 0.00006	0.01289 ± 0.00071	1.70×10^{-82}
	2a′***	0.001221 ± 0.00027	0.01175 ± 0.00158	5.73×10^{-13}
	3′***	0.002715 ± 0.00052	0.01108 ± 0.00203	1.96×10^{-6}
	4′***	0.001358 ± 0.00036	0.01023 ± 0.00195	1.96×10^{-8}
	2a***	0.001255 ± 0.00044	0.01212 ± 0.00262	5.41×10^{-5}
	2b**	0.001889 ± 0.00032	0.00551 ± 0.00109	0.000391
	3***	0.003706 ± 0.00064	0.01117 ± 0.00179	3.34×10^{-5}
	4	0.005481 ± 0.00082	0.00730 ± 0.00124	0.0433
	5a*	0.007072 ± 0.00153	0.02214 ± 0.00511	0.000655
	5	0.009080 ± 0.00181	0.01100 ± 0.00195	0.156
	6***	0.000371 ± 0.00014	0.00705 ± 0.00180	4.45×10^{-6}
	7***	0.001382 ± 0.00033	0.00887 ± 0.00197	2.68×10^{-5}

[a] Bonferroni significance levels were used to account for the use of 23 tests, one for each open reading frame, as follows: *, α value of <0.05 if the *P* value was <0.00217; **, α value of <0.01 if the *P* value was <0.000435; ***, α value of <0.001 if the *P* value was $<4.35 \times 10^{-5}$. Significance levels refer to a paired *t* test where π_N equals π_S.
[b] Determined by a paired *t* test.

TABLE 2 Peaks of nonsynonymous nucleotide diversity for all open reading frames of $SPgV_{ver}$, SIV_{ver}, and DMVV-1 in South African vervet monkeys[a]

Virus	ORF	Start position	Stop position	Length (no. of codons)	% of variants with nonsynonymous polymorphism
SIV_{ver} ($n = 13$)	*gag*	—	—	—	—
	pol	—	—	—	—
	vif	—	—	—	—
	vpx	—	—	—	—
	tat	5685	5735	17	31
		7980	8120	47	85
	rev	—	—	—	—
	env	6107	6226	40	92
		6335	6376	14	85
		7013	7078	22	62
	nef	—	—	—	—
$SPgV_{ver}$ ($n = 17$)		1053	1082	10	24
		1326	1376	17	29
		2244	2282	13	24
		2403	2438	12	12
		2604	2630	9	29
		6696	6746	17	12
		8922	8966	15	47
DMVV-1 ($n = 14$)	1a	733	768	12	21
	TF	2924	3064	47	71
		3152	3232	27	50
		3512	3541	10	21
	1b	—	—	—	—
	2a′	—	—	—	—
	3′	11389	11442	18	64
		11527	11565	13	57
	4′	—	—	—	—
	2a	—	—	—	—
	2b	12470	12526	19	79
	3	13201	13230	10	64
	4	13227	13307	27	86
		13512	13571	20	71
	5a	—	—	—	—
	5	13748	13849	34	79
		13922	13987	22	100
	6	—	—	—	—
	7	—	—	—	—

[a] Peaks were identified conservatively as 9-codon sliding windows in which π_N exceeded both the respective window's π_S and the mean value of π_S for the ORF. Start and stop sites refer to approximate nucleotide coordinates in the genome sequence. Dashes indicate the absence of a nonsynonymous peak in that ORF.

keys is a distinct and definable entity consisting of various combinations of simian immunodeficiency viruses, simian pegiviruses, and simian arteriviruses. To date, species-specific simian pegiviruses and simian arteriviruses have been found in cercopithecoid monkeys belonging to both major African subfamilies (i.e., Cercopithecinae and Colobinae) and both cercopithecine tribes (i.e., Cercopithecini and Papionini). This finding suggests that like SIV, simian pegiviruses and simian arteriviruses are widely distributed among African monkeys of different species, with many more variants likely remaining to be discovered. Our discovery of these viruses in monkeys from South Africa also greatly expands the known geographic range of these virus groups in African monkeys (Fig. 7).

The widespread distribution of pegiviruses in African monkeys is not entirely surprising because pegiviruses are already known to infect a diversity of primates, including humans (HPgV), chimpanzees ($SPgV_{cpz}$), and New World monkeys (SPgV-A). This wide species range suggests that pegiviruses may have infected an ancient ancestor common to Old World and New World monkeys and cospeciated with these hosts, a hypothesis generally supported by the phylogenetic relationships among pegiviruses from these different hosts. However, a more detailed examination of the simian pegivirus phylogeny suggests a more nuanced history, with evidence for cross-species transmission of simian pegiviruses among OWMs and a viral phylogeny seemingly better predicted by host geographical location than by host phylogeny. More extensive sampling and more detailed analyses would be required to differentiate cospeciation from geographic spread of simian pegiviruses, as has been done for SIV (44).

Locally within South Africa, geography seems to be important for the distribution of simian arteriviruses, as evidenced by the lack of detection of simian arteriviruses in AGMs east of the Drakensberg Mountains. The absence of simian arteriviruses in AGMs from Gambia (*Chlorocebus sabaeus*) despite the detection of simian pegiviruses and SIV in these monkeys by unbiased deep sequencing suggests that simian arteriviruses generally have a patchy distribution across African OWM populations (22). One possible explanation for this observation is that simian arteriviruses are susceptible to "bottleneck" effects and local extinction, as was recently shown in the case of SIV and SPgV in the AGMs that now populate the Caribbean island of St. Kitts (22). Alternatively, it is possible that simian arteriviruses entered African OWMs relatively recently and have not yet spread beyond certain geographical confines. Sampling of wild primates from additional geographic locations, including locations that allow geography-based dating of simian pegiviruses and simian arteriviruses, similar to what has been done for SIV (45), will hopefully provide additional data points and add clarity to the natural histories of these viruses.

TABLE 3 Relationship between viral infection status and demographic features of South African vervet monkeys

Response variable	Predictor variable[a]	df	*t*	*P*
SIV_{ver}	Age*	112	6.208	<0.001
	Male sex*		−2.287	0.02
	SPgV infection*		4.402	<0.001
	DMVV-1 infection		0.585	0.559
$SPgV_{ver}$	Age	112	−0.938	0.350
	Male sex		1.241	0.217
	SIV infection*		4.402	<0.001
	DMVV-1 infection		1.420	0.158
DMVV-1	Age	112	−0.361	0.719
	Male sex		−0.675	0.501
	SIV infection		0.585	0.560
	SPgV infection		1.420	0.158

[a] Asterisks signify predictor variables with a statistically significant relationship.

Genetic and genomic features of RNA plasma viruses in African monkeys. Lifelong viremia is a hallmark of SIV infection and explains why the prevalence of SIV infection in AGMs increases with age. Persistent/prolonged infection also appears to be a feature of both simian pegivirus and simian arterivirus infections, which may explain the relatively high prevalence of these viruses in this study (9, 11). However, we did not find any correlation between the prevalence of simian pegivirus or simian arterivirus and age in AGMs, suggesting that these infections are not lifelong but are cleared after some undetermined period of time.

A key component of SIV's persistence strategy is the integration of proviral DNA into the host's genome, a persistence mechanism that, to our knowledge, is not utilized by simian pegiviruses or simian arteriviruses. However, SIV also relies upon several additional methods to maintain high levels of viral replication. The rapid accumulation of mutations that alter the amino acid sequence of viral proteins targeted by host antibodies and T cells decreases the effectiveness of these responses and enables viral persistence, a mechanism known as "immune escape," which leaves a signature of nonsynonymous nucleotide diversity in the viral genome (37, 38). To gain insight into the persistence mechanisms used by simian pegiviruses and simian arteriviruses, we searched for signatures of nonsynonymous nucleotide diversity in the genomes of SIV_{ver}, $SPgV_{ver}$, and DMVV-1. As expected, we found hotspots of nonsynonymous variation in the regions of the SIV_{ver} envelope gene that correspond to the "highly variable loops" that are preferentially targeted by host antibodies (23, 46). We found a similar pattern of adaptive changes in the DMVV-1 genome, with signatures of nonsynonymous diversity found primarily in genes that code for envelope glycoproteins and/or regions corresponding to antibody epitopes that have been extensively mapped in other arteriviruses (47–51). This observation suggests that vervet monkeys infected with DMVV-1 produce antibodies that target DMVV-1 glycoproteins but that these antibodies may not be effective in clearing DMVV-1 infection, a scenario that may be akin to immune escape observed during SIV infection. Longitudinal studies of monkeys infected with simian arteriviruses in captivity would be required to more thoroughly evaluate the relationship between host immune responses and viral sequence changes.

Low levels of nonsynonymous diversity were found in the $SPgV_{ver}$ genome despite the high levels of viremia found within infected individuals. This finding is consistent with data from previous studies of pegiviruses in captive NHPs (9, 52, 53) and suggests that the persistence mechanism(s) used by pegiviruses is fundamentally different from that of DMVV-1 or SIV, that pegivirus

TABLE 4 Relationship between viral infection status and plasma cytokine concentrations in South African vervet monkeys[a]

Virus	Cytokine	*t* value for cytokine	*P* value (uncorrected) for cytokine	*P* (corrected) for cytokine	Model covariate	*t* value for model covariate	*P* value (uncorrected) for model covariate	*P* (corrected) for model covariate
DMVV-1	EGF	2.27	0.040	>1.0	Age	−2.07	0.040	>1.0
	GM-CSF	3.19	0.002	0.05	Age	−0.27	0.788	>1.0
					Sex (male)	2.22	0.029	0.81
	IL-1β	2.29	0.024	0.67	Age	−2.42	0.017	0.48
					Sex (male)	3.19	0.002	0.05
	IL-10	2.36	0.020	0.56	Sex (male)	1.71	0.089	>1.0
	MCP-1	1.95	0.049	>1.0	Age	−2.05	0.043	>1.0
					Sex (male)	3.83	<0.01	<0.01
	MIP-1β	3.09	0.003	0.07	Age	−2.53	0.012	0.36
	TNF-α	2.10	0.038	>1.0	Sex (male)	2.10	0.038	>1.0
$SPgV_{ver}$	MIG	−2.24	0.027	0.76	Sex (male)	4.06	<0.01	<0.01
					SIV	−0.84	0.404	>1.0
					SIV*SPgV	2.46	0.016	0.43
SIV_{ver}	IFN-γ	−2.79	0.006	0.18				
	IL-6	1.79	0.043	>1.0	SPgV	−1.19	0.236	>1.0
					SIV*SPgV	1.52	0.131	>1.0
	I-TAC	2.20	0.030	0.84				
	MIF	2.15	0.034	0.95	Age	−2.14	0.034	0.95

[a] A multivariate linear model was developed, and model covariates were identified by using stepwise backward elimination. Uncorrected *P* values are those inferred by the model. Corrected *P* values represent Bonferroni-corrected *P* values for a total of 28 hypotheses tested (one for each cytokine). For a full list of cytokines tested, see Table S1 in the supplemental material.

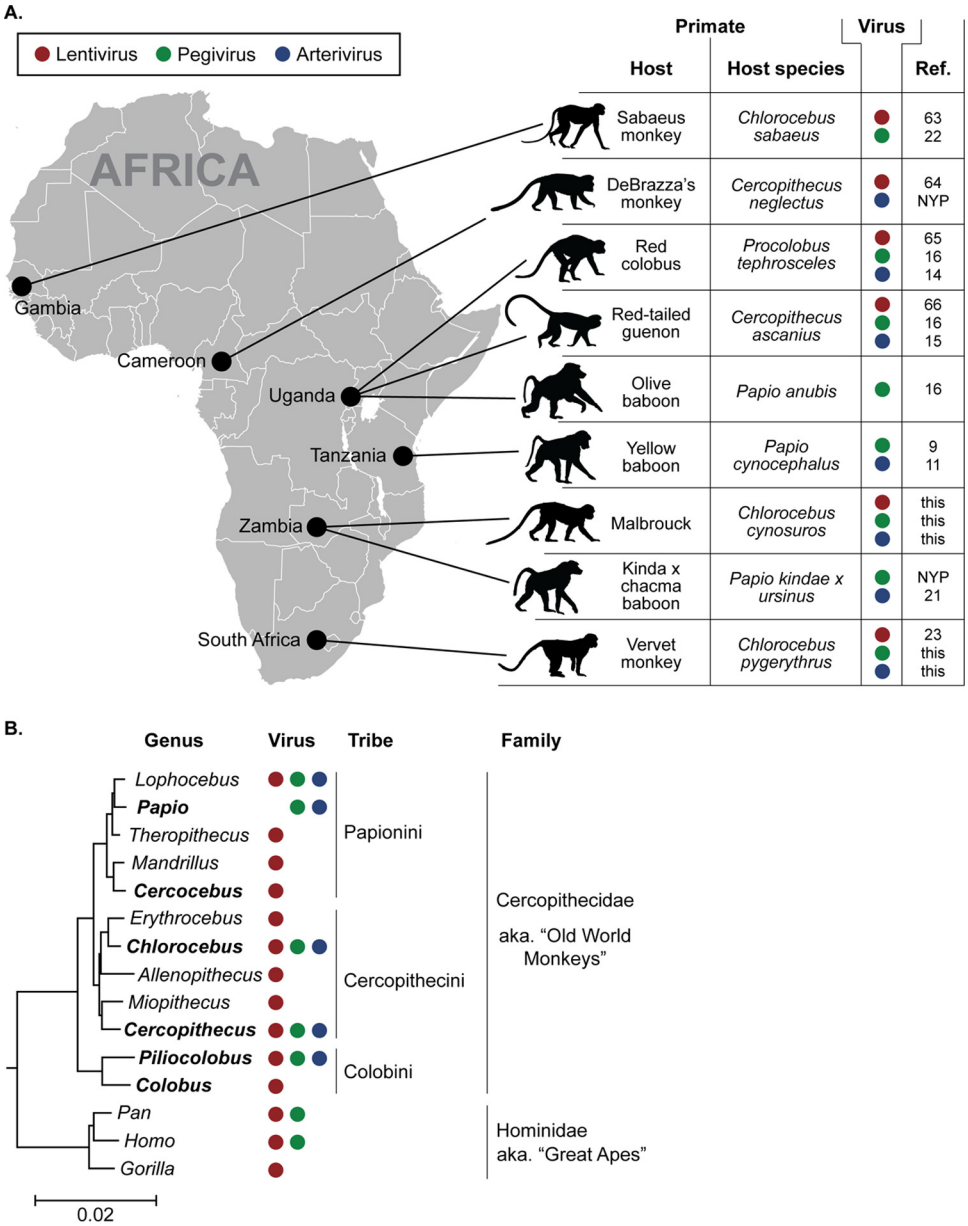

FIG 7 Known geographic and host ranges of African monkey plasma viruses. (A) Map of Africa showing the sampling locations of monkeys in which simian pegivirus or simian arterivirus infection was identified. In the table, a colored dot indicates that SIV (red), SPgV (green), or a simian arterivirus (blue) infection was detected in that particular primate from that particular location (61–64). NYP, not yet published. (B) Genus-level phylogenetic tree of African OWMs and great apes. Colored dots indicate that SIV (red), GBV-C (green), or SHFV (blue) infection was detected in a primate from that genus. Names in boldface type indicate genera from which we have sampled more than 10 wild primates by unbiased deep sequencing (for a comprehensive list of species naturally infected with SIV, see reference 65). (Adapted from *PLoS Genetics* [66].)

genomes are relatively constrained by purifying selection, or that pegivirus genomes are subject to a much lower mutation rate, as also suggested by their low levels of synonymous diversity (54). Whether the low level of nonsynonymous genetic diversity observed in simian pegiviruses is due to an increase in selective constraint, an inherently low mutation rate, or both remains an important question that future *in vivo* and *in vitro* studies need to address.

Pathogenesis and coinfection. The study of SIV infection in wild and captive African monkeys has been invaluable for understanding the pathogenesis of HIV, because unlike HIV in humans, SIV infection in these natural hosts does not lead to the development of AIDS (5). The leading explanation for the apparently low pathogenicity of SIV in African monkeys is that SIV has coevolved with these hosts over thousands of years, and this host-virus relationship has now reached a state of near commensalism. However, the impact of simian pegivirus infection on SIV pathogenesis in African monkeys has never been assessed, as pegiviruses in African monkeys have been described only recently. Given that HPgV infection attenuates HIV pathogenesis and improves mortality in HIV-infected humans (17–19, 55), we examined the impact of $SPgV_{ver}$ coinfec-

tion on cytokines influenced by SIV_{ver} using a multivariate linear model. We did not find a significant difference in cytokine concentrations in SIV_{ver}/$SPgV_{ver}$-coinfected monkeys compared to SIV_{ver}-monoinfected monkeys, nor did we find a difference in SIV loads between $SPgV_{ver}$-positive and $SPgV_{ver}$-negative AGMs. These findings suggest that simian pegiviruses do not significantly impact SIV pathogenesis (as measured by cytokine levels) in African OWMs, possibly because SIV in these natural hosts (unlike SIV in macaque monkeys and HIV in humans) causes little to no change in these parameters.

Similarly, we used a multivariate linear model to identify cytokines associated with DMVV-1 infection in vervet monkeys. In DMVV-1-infected animals, we found mildly elevated concentrations of several proinflammatory cytokines that are produced by activated macrophages, including IL-1β, GM-CSF, MIP-1β, EGF, MCP-1, and TNF-α. Although these associations did not reach statistical significance, this pattern of cytokine associations is biologically intriguing: macrophages support the replication of simian hemorrhagic fever virus (SHFV) (the simian arterivirus type strain) *in vitro* and are thought to be the primary cell type infected by simian arteriviruses *in vivo*, as has been shown for other nonsimian arteriviruses (34, 56). Additionally, increased expression of IL-10, an anti-inflammatory cytokine which we also found to have elevated levels in DMVV-1-infected vervets (albeit to nonsignificant levels), is associated with a reduction in virulence for both simian and nonsimian arteriviruses (56–58). Macaques infected with SHFV have increased plasma concentrations of proinflammatory cytokines but not increased levels of IL-10, possibly suggesting that IL-10 may be a host factor that protects African monkeys from simian arterivirus pathogenesis and SHF (59, 60).

Taken together, these data suggest that the simian lentiviruses, simian pegiviruses, and simian arteriviruses have a small to negligible effect on blood cytokine profiles in South African vervet monkeys. Extrapolating from these findings, we predict that these viral infections result in little to no overt disease in African OWMs, although the examination of other disease parameters, with greater statistical power, is needed to further clarify the long-term effect of these viral infections on NHP health.

Conclusion. It is reasonable to expect that viruses from additional families will be discovered in the coming years as the sensitivity and throughput of virus discovery technology improve. Indeed, the prevalence of simian immunodeficiency viruses, simian pegiviruses, and simian arteriviruses in many OWM populations and the high levels of viremia that these viruses cause in infected individuals have simplified their discovery and characterization. Viruses that cause acute infections, replicate to low titers, or are primarily cell associated are undoubtedly more difficult to identify with current sequencing-based methods. While efforts to discover novel animal viruses will continue to be of high yield, further characterization of newly discovered viruses in controlled laboratory settings will also be important, especially for select viruses with the potential to impact human and wildlife health. Such studies are already under way for simian pegiviruses and simian arteriviruses (9, 21). Considering that NHP models faithfully recapitulate many important features of viral diseases in humans, future research to discover novel primate viruses has the potential to be particularly fruitful.

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