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Broad diversity of simian immunodeficiency virus infecting *Chlorocebus* species (African green monkey) and evidence of cross-species infection in *Papio anubis* (olive baboon) in Kenya

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Abstract

Background: Simian immunodeficiency virus (SIV) naturally infects African non-human primates (NHPs) and poses a threat of transmission to humans through hunting and consumption of monkeys as bushmeat. This study investigated the as of yet unknown molecular diversity of SIV in free-ranging *Chlorocebus* species (African green monkeys—AGMs) and *Papio anubis* (olive baboons) within Mombasa, Kisumu and Naivasha urban centres in Kenya.

Methods: We collected blood samples from 124 AGMs and 65 olive baboons in situ, and detected SIV by high-resolution melting analysis and sequencing of PCR products.

Results: Simian immunodeficiency virus prevalence was 32% in AGMs and 3% in baboons. High-resolution melting (HRM) analysis demonstrated distinct melt profiles illustrating virus diversity confirmed by phylogenetic analysis.

Conclusions: There is persistent evolutionary diversification of SIVagm strains in its natural host, AGMs and cross-species infection to olive baboons is occurring. Further study is required to establish pathogenesis of the diverse SIVagm variants and baboon immunological responses.

KEYWORDS

African green monkeys, evolution, high-resolution melting analysis, olive baboon, SIVagm, strain diversity

1 | INTRODUCTION

African non-human primates (NHPs) are reservoirs for diverse zoonotic retroviruses of human health importance including simian immunodeficiency virus (SIV), genus *Lentivirus* and simian foamy virus (SFV)[1] besides being incidental hosts for Ebola[2] and Marburg virus.[3] Diverse strains of SIV infect over 40 species of African NHPs.[4] Although not pathogenic to its natural host, the NHPs, the virus continuously poses a threat of transmission to humans because of hunting and consuming NHPs as bushmeat.[5,6] It has been shown that human immunodeficiency virus (HIV) originated from cross-species transmission of SIVcpz strain from chimpanzees (*Pan troglodytes*) and SIVsmm from sooty mangabeys (*Cercocebus atys*), resulting in zoonotic infections that respectively produced human immunodeficiency virus type 1 (HIV-1) and HIV-2 pandemic.[7,8] Despite the high prevalence of natural SIV infections in African NHPs species,[6,9,10] comprehensive surveys and molecular characterisation of lentiviruses infecting East African monkeys remain limited.

Chlorocebus spp. (African green monkeys—AGMs or vervet monkey), *Cercopithecus* spp. (Sykes' monkey and blue monkey) and *Papio* spp. (baboon) belong to the family *Cercopithecidea* and are common NHPs found within most urban and peri-urban environments in East Africa. AGMs are a super-species comprising several phenotypically and geographically distinct subspecies of monkeys.[11] The AGMs subspecies in Kenya include *Chlorocebus aethiops* mostly found in rift valley and western regions of the country and *Chlorocebus pygerythrus* species commonly found along the coastal forests and urban centres. Two species of baboons that occur in Kenya include *Papio anubis* (olive baboon), widely distributed in central and western parts of the country and *Papio cynocephalus* (yellow baboon) in south eastern regions.[12] Common *Cercopithecus* spp. include *Cercopithecus mitis albogularis* (Sykes monkey) found in south eastern and *Cercopithecus mitis stuhlmanni* (blue monkey) found in western regions.[13] As common monkeys widely distributed in Kenya, these NHPs are regarded as pests in most towns and villages.[14]

Viral diseases associated with African NHPs as reservoirs or incidental hosts have emerged as major source of disease outbreaks in humans.[15] Some of these diseases are acquired through bushmeat handling, especially involving monkeys,[16] which increases the risk of contracting zoonotic simian retroviruses such as SIV, simian t-lymphotropic virus (STLV) and SFV by humans.[17-19] Further, aggressive encounters with NHPs resulting in bites[20] and high incidence of zoonotic retroviruses in NHPs[17] increase the risks of infection. Hence, proximity to urban-restricted NHPs such as AGMs and olive baboons necessitate surveillance for potentially high-risk viruses. Although most SIV surveillance studies have focused on African NHPs inhabiting rural and forest habitats,[6,21] urban-restricted NHPs can also be reservoirs of these viruses. Notably, synanthropic or urban-restricted free-ranging monkeys are potential sources of zoonotic viral diseases of human health importance such as chikungunya,[22] dengue, yellow fever and Zika viruses.[23]

The AGMs subspecies harbour distinct but related SIV strains considered as subtypes that cluster according to respective hosts within the SIVagm lineage.[24-26] Unlike AGMs, baboons are non-receptive to SIV infection. However, previous serosurvey of lentiviruses in *Papio cynocephalus* (yellow baboons) in Tanzania has shown exposure to SIV,[27] and follow-up molecular tests confirmed infection by SIVagm. Another study recovered SIVagm strain from a healthy *Papio ursinus* (chacma baboon) sampled in South Africa.[28] Both studies suggest that exposure to infected AGMs during predation can result in SIVagm transmission into sympatric baboon populations.

Despite their importance as reservoir hosts of zoonotic viral diseases, surveys for SIV diversity in free-ranging Kenyan NHPs have been opportunistic, and mostly on captive AGMs[29] and Sykes' monkeys.[30] Understanding the extent of viral diversity in natural hosts and determinants of successful cross-species transmission or spillover is important for zoonotic diseases surveillance programs especially in urban and peri-urban centres where human interactions with NHPs are potentially high, and risk of sharing pathogens remains unknown.

Serological techniques including antigen-based enzyme-linked immunosorbent assay (ELISA) and Western blot assays are routinely used for serosurvey of SIV exposure in NHPs populations.[6,9,10,30] However, real-time PCR high-resolution melting (HRM) analysis has emerged as a valuable molecular tool for rapid identification of diverse microorganism in biological specimens[31] and has been utilised to identify HIV infections.[32] Therefore, we undertook a comprehensive molecular survey of SIV strains infecting urban-restricted NHPs in Kenya, using PCR-HRM analysis to determine infections and virus diversity.

2 | MATERIALS AND METHODS

2.1 | Ethics statement and permits

The Institutional Scientific Review Committee (ISERC) at the Institute of Primate Research (IPR), Kenya, reviewed this projects' experimental design (ISERC/04/18). The institutional review informed the Kenya Wildlife Service (KWS) on the authorisation for in situ sampling of free-ranging non-human primates within urban and peri-urban centres.

2.2 | Sampling sites

We sampled NHPs found in urban and peri-urban centres of Mombasa, Kisumu and Naivasha in Kenya (Figure 1) after complaints of rampant property destruction and crop raiding and the need to translocate troops of monkeys to nearby animal reserves. The sampling sites in Mombasa Island (4°3′S, 39°39′E) were Tudor water sports facility located northwest of the Island, Mbaraki (Mbk) in the south of the island, and government administration compounds (GAC) along the eastern parts of the Island. Baboons were trapped and sampled in Mazeras (3°58′S, 39°33′E), on the mainland and within the outskirts of Mombasa town. In Kisumu (0°6′S, 34°45′E), a city located along the shore of Lake Victoria in Western Kenya three sites were targeted, the Kenya Wildlife Impala Park (and zoo) within the town centre, a residential golf course (GC) area near the Kisumu International Airport and Kajulu (KAJ), a peri-urban centre located 5 km from the city. In Naivasha (0°43′S, 36°25′E), located in the Great Rift Valley, we sampled animals in Kimana area, an ecotourism destination at the shores of Lake Naivasha.

2.3 | Sampling of NHPs

Free-ranging NHPs were baited with fresh carrots, bananas and maize cobs and captured using wire mesh cages fitted with self-triggering slide doors as previously described.[33] Individual animals were anaesthetised by administration of xylazine (2%): ketamine hydrochloride (10%) (Sigma Aldrich) in the ratio of 1:3. Age was determined using dental eruption before blood was collected once in situ. Whole blood

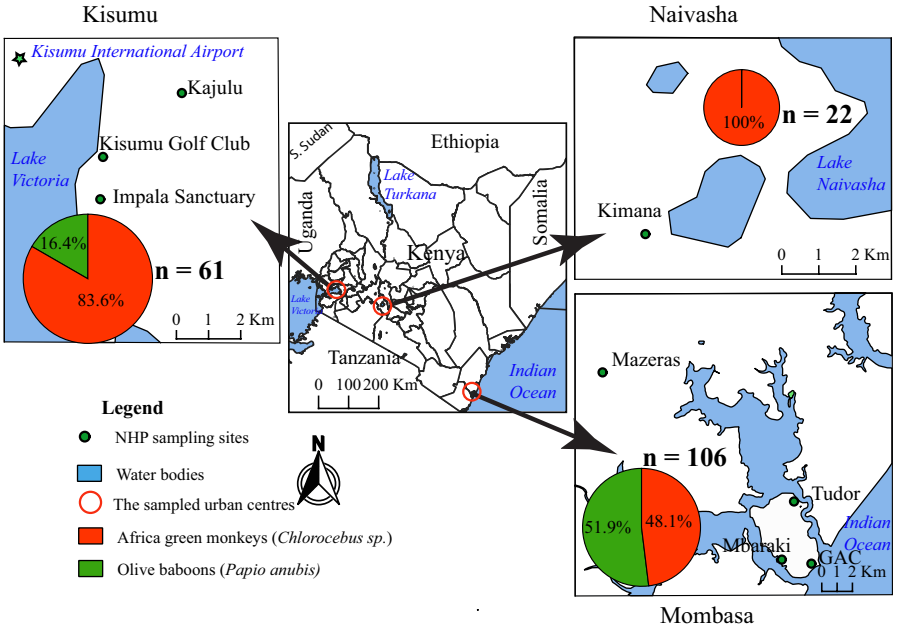

FIGURE 1 Urban and peri-urban sampling sites. Map of Kenya (centre) showing the geographical locations of the three study urban and peri-urban centres (red circles) namely Kisumu, Mombasa and Naivasha where the non-human primates were trapped. The three sampling centres are shown offset with sampling sites represented by the green circles. The proportions of each NHP species captured for each sampling centres is shown on the pie charts as percentages. The proportion of the AGMs are shown in red while the baboons are shown in green for each representative pie chart

(10 mL) was drawn from the femoral vein by venipuncture using 21-gauge needle and 10-mL syringe. Four milliliter of each blood sample was preserved in sterile vacutainer tubes with EDTA as anticoagulant (10 mL BD vacutainer© K2E [EDTA, BD]), 4 mL was aliquoted into serum tubes (6.0 mL BD vacutainer©, BD), transported in dry ice and stored at −80°C. Two drops of blood were spotted onto Whatman FTA cards (Sigma Aldrich), air-dried and stored at room temperature. For infants, drops of blood were sampled from sterilised fingertips to alleviate stressful venipuncture sampling. Sampled animals were monitored until they regained full consciousness before being released.

2.4 | DNA extraction and screening for SIVs

Total genomic DNA was extracted from 200 µL of each blood sample using the MagNA 96 Pure DNA and Viral NA Small Volume Kit (Roche Applied Science) in a MagNA Pure 96 (Roche Applied Science) automated extractor as previously described.[34] We confirmed the NHP species by amplifying mitochondrial cytochrome oxidase 1 (CO1) gene.[35] Screening for SIV was done by nested PCR-HRM targeting the proviral DNA.[36] In each PCR amplification, we included a positive SIV control isolated from a captive Sykes monkey and routinely used as a positive control for SIV cell culture at the Institute of Primate Research. Primary amplification was done in Veriti™ thermo cycler (Life technologies holdings PTE LTD) using primers (NDR1, 5′-TRGAYACAGGRGCWGAYGA-3′; POLOR1, 5′-ACB ACY GCN CCT TCH CCT TTC-3′) targeting 2.7 kb *pol* gene fragment.[36] The amplification reaction contained 2.5 µL of 10× standard buffer (New England Biolabs Inc), 0.5 µL of 10 mmol/L dNTPs, 0.5 µL of 10 µmol/L each primer, 0.125 µL of 5 U Taq polymerase (New England Biolabs Inc) and 2 µL of DNA template. Secondary PCRs were performed in a RotorGene Q thermocycler (QIAGEN) using primers (Polis4, 5′-CCA GCN CAC AAA GGN ATA GGA GG-3′; Uni2, 5′-CCC CTA TTC CTC CCC TTC TTT TAA AA-3′) targeting a 650-nucleotide (nt) fragment *pol* gene broadly conserved in lentiviruses.[37] Prepared reaction mix contained 2 µL of 5× HOT FIREPol® EvaGreen® HRM Mix (Solis BioDyne), 0.5 µL of each 10 µmol/L primer and 1 µL of primary PCR products as DNA template. Amplification conditions for primary PCR included an initial denaturation at 95°C for 15 minutes followed by 35 cycles at 95°C for 30 seconds, 49°C for 30 seconds, and 72°C for 2 minutes and a final extension at 72°C for 5 minutes. Secondary PCR conditions included denaturation at 95°C for 15 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing was done at 55°C for 30 seconds, extension at 72°C for 30 seconds and a final extension was done at 72°C for 5 minutes. Subsequent HRM analysis was conducted as previously described.[34] Briefly, PCR products were denatured at 95°C for 1 minute, annealing at 40°C for 1 minute and equilibrating at 75°C for 90 seconds, and then increasing the temperature in 0.1°C increments up to 90°C, with fluorescence acquisition after 2 seconds incremental holding periods. Distinct HRM profiles, normalised in the range of 80-90°C, were visually determined for each reaction after completion of HRM data acquisition.

2.5 | Molecular identification of SIVs

Simian immunodeficiency virus strains were characterised by sequencing the partial *pol* gene fragment amplified by PCR-HRM and a 900-nt envelope (*env*) gp120 fragment encompassing the V3-V5 regions. The partial *env* gene fragment was amplified by nested PCR using primers (ENVA, 5′-GAA GCT TGT GAT AAA ACA TAT TGG AT-3′; ENVB, 5′-AGA GCT GTG ACG CGG GCA TTG AGG-3′) for primary amplification. Primers sets (ENVC, 5′-GTG CAT TGA CAG GGT TAA TGA ATA CAA CAG-3′; ENVD, 5′-TTC TTC TGC TGC AGT ATC CCA GCA AG-3′) were used for secondary amplification.[27] Each 20 µL PCR mixture for primary amplification contained; 10 µL 2× Multiplex PCR Plus Kit

Mastermix (QIAGEN), 2 μL Q-Solution and 0.5 μL of 10 μmol/L each forward and reverse primers, 5 μL of nuclease free PCR water and 2 μL of template DNA. Nested amplifications also contained the same PCR components as described above but 1 μL of the primary amplicon was used as template. Amplification reactions were done in a SimpliAmp™ thermocycler (Applied Biosystems). The cycling conditions included an initial incubation at 95°C for 5 minutes, 35 cycles of extension at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and final extension at 68°C for 10 minutes for both primer sets. Nested PCR products were visualised under UV after electrophoresis on a 1.5% agarose gel containing 0.5 μmol/L ethidium bromide. Amplicons with the expected fragment size of 650- and 900-nt were purified using ExoSAP-IT protocol (USB Corporation) following the manufacturer's instructions and sequenced at Macrogen.

2.6 | Phylogenetic and evolutionary selection analyses

Consensus sequences generated using Geneious v10[38] were aligned using MUSCLE.[39] Maximum-likelihood phylogenetic reconstruction was performed using the Akaike information criterion[40] implemented in PhyML[41] with 1000 bootstrap replicates used to ascertain the phylogenetic tree's robustness. Further, a tip-date calibrated, relaxed (uncorrelated lognormal) molecular clock phylogeny of *pol* gene sequences was calculated using BEAST v1.10.1,[42] implementing a constant population size coalescent prior and an HKY model of nucleotide substitution. Adequate effective sample size (ESS) values (>200) were obtained by running the Markov chain Monte Carlo analysis for 100 million generations.[43] The log files generated by BEAST were examined using tracer version 1.7 to assess ESS values and determine the burn-in depth.[44] Maximum clade credibility (MCC) trees were summarised using TreeAnnotator v1.10.1.[42]

The alignment of SIV *env* gene sequences was screened for evolutionary selection signatures using methods implemented on the adaptive evolution server (www.datamonkey.org).[45] These methods included single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL),[46] Fast Unconstrained Bayesian Approximation (FUBAR),[47] mixed effects model of evolution (MEME)[48] and internal fixed effects likelihood (IFEL).[49] Obtained results were considered significant at a 0.05 level of significance for SLAC, MEME, IFEL and FEL, while a posterior probability >0.9 was considered significant for FUBAR.

3 | RESULTS

3.1 | Species of NHPs and distribution

We trapped 124 urban-restricted free-ranging AGMs (53 females, 71 males) and 65 (21 females, 44 males) peri-urban olive baboons of different age groups alive with minimal bruises (Table S1), sampled them under anaesthesia and released back to their habitat. Based on dental eruption pattern, animals estimated to be below 11 months old were classified as infants, those 12-37 months old were considered subadults, while all animals whose teeth were fully erupted (>38 months) were classified as adults.[50] PCR amplification and sequencing of a 650-nt mitochondrial DNA fragment coding for CO1 identified the AGM species sampled in Kisumu and Naivasha as *Chlorocebus aethiops* based on 99% (629/633) nucleotide identity for the topmost blastn search (accession number AF312703.1). The AGMs sampled in Mombasa were identified as *C pygerythrus*, based on 99% (661/666) nucleotide identity to the topmost hit from blastn search (accession number EF597501.1). DNA samples from baboon were identified as *Papio anubis* based on 98% (659/668) nucleotide sequence identity to the topmost blastn search hit (accession number KC757406.1).

3.2 | Detection and prevalence of SIV in AGMs and baboons

Simian immunodeficiency virus prevalence was determined by a combination of PCR-HRM of the partial *pol* gene and nested PCR amplification of partial *env* gene. Representative PCR-HRM amplification profiles of the *pol* gene were used for non-subjective molecular characterisation (Figure 2). Molecular prevalence of SIV was 32% (39/124) in AGMs and 3% (2/65) in baboons. SIV infection in male AGMs was 34% (24/71) and females 28% (15/53). Two male baboons, one from Kajulu in Kisumu and the other from Mazeras, in Mombasa were positive for SIV. Amongst NHPs' age groups, infection was higher in adults (27/72, 38%) followed by subadults (9/36, 25%) and infants (3/16, 19%). The distribution of SIV infections in different sampling sites was uneven and ranged from 8% to 48% in AGMs (Table 1). Infections by sampling sites were highest in Mbaraki, Mombasa (10/22, 48%) followed by Kimana in Naivasha (17/51, 33%) and Impala park in Kisumu (7/25, 28%). The AGMs sampled in Tudor, Mombasa had the lowest SIV prevalence of 8% (1/12). In some cases, SIV amplification was successful for *pol* region but not in the *env* region and vice versa as previously observed for SIVagm.[21] More SIV positive samples (22/39, 56%) were amplified using the *env* primer sets indicating it is more robust in SIV detection compared to the primers used for *pol* region (19/39, 49%). This could be attributed to the nature of primers sets used which included both universal primers for *pol* region and AGM lineage specific primers used for *env* region.

3.3 | Analysis of HRM amplicons

High-resolution melting analysis of SIV *pol* gene PCR amplicons demonstrated distinct melt profiles (Figure 2), suggesting detection of diverse viruses. Sequencing confirmed the PCR-HRM amplicons as SIV genomic fragments. Positive virus amplification from specimens AGM675-NVS, AGM699-NVS and AGM682-NVS sampled from the same location resulted in distinct melt peaks and profiles indicating existence of sequence differences in terms of nucleotide base composition between the genomic amplicons. Distinct melt

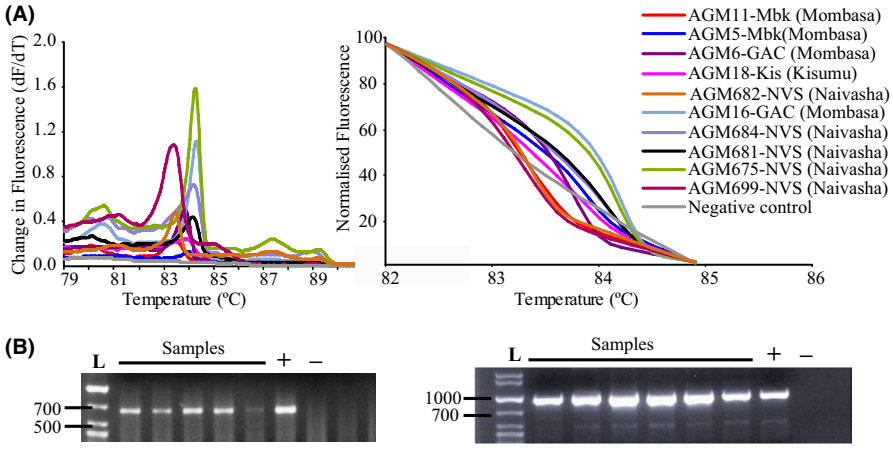

FIGURE 2 Detection of SIV in NHPs by a combination of PCR-HRM and gel electrophoresis. A, Representative melt peaks (left panel) and HRM melt profiles (right panel) generated by PCR-HRM of the partial *pol gene* (650 bp) from SIV positive samples. B, The amplified and electrophoresis size separated SIV partial pol gene (left panel) and *env* gene (right panel) fragments of 650 and 900 bp, respectively. L represents the molecular weight ladder; + and − represent SIV positive and negative controls, respectively

TABLE 1 Prevalence of SIV infection in AGMs and olive baboons according to species and sampling sites from Mombasa, Naivasha and Kisumu as detected by combination of PCR-HRM and nested PCR of both partial *pol* and *env* gene fragments

		Infected% (SIV positive/Total sampled NHPs)	
Sampling town	**Sampling sites**	**AGMs**	**Baboons**
Mombasa	Mbaraki	47.82% (11/23)	
	Tudor	8.33% (1/12)	-
	GAC	31.25% (5/16)	-
	Mazeras		1.82% (1/55)
	Subtotal	33.33% (17/51)	1.82% (1/55)
Kisumu	Kajulu	19.05% (4/21)	10.00% (1/10)
	Golf Course	20.00% (1/5)	-
	Impala	28.00% (7/25)	-
	Subtotal	23.53% (12/51)	10.00%(1/10)
Naivasha	Kimana	45.45% (10/22)	
Total		31.45% (39/124)	3.08%(2/65)

profiles were also generated for each SIV amplicon from AGM18-Kis (Kisumu) and AGM6-GAC (Mombasa), AGM675-NVS (Naivasha), and AGM11-Mbk from Mombasa. In contrast, similar melt profiles were exhibited for SIV amplicons from AGM675-NVS, AGM684-NVS sampled in Naivasha and AGM16-GAC sampled in Mombasa suggesting their low divergence (Figure 2). The HRM melt profiles for the representative positive samples shown in Figure 2 ranged from 83.33 to 84.30°C (Table 2) illustrating SIV sequence diversity.

3.4 | Phylogenetic analyses of SIVagm infecting AGMs and baboons in Kenya

Samples that were SIV positive by PCR-HRM were amplified for sequencing using primers targeting *pol* (19 samples) or *env* (22 samples) genes. Out of these, 14 samples generated unambiguous sequences for *pol* and 17 for *env* gene fragments, respectively. These sequences have been deposited in GenBank under accession numbers MG590108-MG590139 and MG757169-MG757171. A BLAST search of NCBI database using amplified partial *pol* and *env* revealed 81%-93% and 79%-85% sequence identity, respectively, to published SIVagm from Africa. Upon phylogenetic analysis, partial *pol* sequences from this study segregated in a monophyletic cluster with SIVagm sequences previously isolated from Kenya (Figure 3). There was no clear phylogeographic clustering according to sampling towns amongst sequences from Kenya. Close phylogenetic relationship was inferred between the sequence from AGM18-Kis sampled in Kisumu and SIVagmTAN-1 (Accession number U58991) from an AGM in Eastern Uganda (Figure 3).

Phylogenetic analysis of the partial *env* gene (Figure 4) revealed similar topology to the tree from the *pol* gene where sequences from this study clustered with published SIVagm sequences from Kenya. The SIV sequence amplified from an olive baboon in Kisumu clustered, with high bootstrap support, with those from sympatric AGMs. Epidemiologically linked SIVs were identified in Naivasha (AGM682-NVS and AGM4-NVS, AGM681-NVS and AGM21-NVS, Figure 3) and Kisumu (AGM5-GC and baboon 5, Figure 4).

The molecular clock analysis (Figure 5) was consistent with the maximum-likelihood phylogenetic analysis where SIVagm derived from Kenyan AGMs segregated in a monophyletic cluster distinct from SIVagm sequences from southern and western Africa. Based on the bayesian tree topology, the SIVagmVer clade from Kenya that included sequences from this study shared a common ancestor with the South African SIVagmVer clade. While all except one SIVagm

TABLE 2 Summary of the high-resolution melting scores for each representative melt peak shown in Figure 2A

Sample ID	Sampling town	HRM score (°C)
AGM11-Mbk	Mombasa	83.33
AGM5-Mbk	Mombasa	83.97
AGM6-GAC	Mombasa	83.85
AGM18-Kis	Kisumu	83.92
AGM682-NVS	Naivasha	83.45
AGM16-GAC	Mombasa	84.30
AGM684-NVS	Naivasha	84.20
AGM681-NVS	Naivasha	84.17
AGM675-NVS	Naivasha	84.27
AGM699-NVS	Naivasha	83.40
Negative control	-	-

sequences from this study clustered within the SIVagmVer lineage specific to *C aethiops*, there were three subclusters; two from AGMs whose subspecies was identified as *C aethiops* and one subcluster mainly composed of sequences from Mombasa whose AGM hosts were identified as *C pygerythrus*. The one exception SIVagm sequence from AGM18-Kis (Kisumu) isolated from *C aethiops* clustered with the SIVagmTan variant isolated from *C tantalus*. All the SIVagmVer sequences from AGMs sampled in southern Africa formed a monophyletic clade alongside SIVagmMal isolated from *C cynosurous* from Zambia.[24] In general, the SIVagmSab clade appeared to be ancestral to the SIVagmVer and SIVagmTan clades.

Overall, amino acid conservation was higher in *pol* protein compared to the *env* protein as previously reported.[51] The *pol* sequence identities ranged between 91%-99% for Naivasha samples, whereas the samples from Mombasa had 88% amino acid identity. The SIV amino acid sequences from AGM18-Kis sampled in Kisumu had 79%-84% identity with other sequences from Kenya. The diversity results from the protein sequence analyses agree with HRM and phylogenetic analysis where samples from Naivasha, AGM682-NVS and AGM675-NVS with similar melt profiles (Figure 2) had 99% identity. SIVs infecting AGM11-Mbk from Mombasa and AGM699-NVS from Naivasha had 90% identity, a trend illustrated by their melt profile differences, whereas SIV variants from AGM699-NVS and AGM675-NVS with different melt profiles (Figure 2) had 97% identity for *pol* amino acids sequences.

Conversely, the amino acids conservation was lower in the *env* protein compared to the *pol* protein. The *env* protein sequences from Naivasha had 77%-98% amino acid identity. These sequences from Naivasha had 77%-89% identity to sequences from Mombasa and 80%-91% identity to sequences from Kisumu. The *env* amino acid sequence from SIV infecting Baboon5 had 96% identity with SIV from AGM5-GC sampled from the same habitat in Kisumu despite maintaining 80%-89% identity to the rest of the sequences. This supports epidemiological linkage of the two isolates as implied by phylogenetic analysis (Figure 4). Despite the phylogenetic diversity, well-conserved domains and hypervariable domains (V3-V5) were identified from the *env* amino acid sequence alignment (Figure 6). The identified V3 domain had 34 amino acids residues comparable to previous reports while maintaining the well-conserved MAG motif that largely determines cellular tropism in SIV.[24] All eight cysteines, major viral functional domains including the CD4 binding domain, N-linked glycosylation sites and viral fusion peptide were conserved as expected within the sequenced *env* region.[21,52]

Analysis of evolutionary selection pressure revealed evidence of episodic and pervasive diversification on the *env* gene. Codons 18 and 58 (Table 3) were under purifying/negative selection by IFEL and FEL approaches (0.05 significance level). The FUBAR method revealed 14 sites under pervasive diversifying selection at posterior probability of >0.9, while MEME revealed 21 sites to be under episodic diversifying selection at a 0.05 level of significance. Overall, using FUBAR and MEME, eight sites were observed to be under diversifying selection (Table 3).

4 | DISCUSSION

This study reports on the first molecular survey of SIV variants circulating in common free-ranging monkeys, AGMs and olive baboons, inhabiting selected urban and peri-urban centres of Kenya. We also present evidence of extensive SIV diversification in the natural host, AGMs and partial characterisation of a strain capable of infecting olive baboon via cross-primate species transmission. These NHP populations can be potential reservoir hosts maintaining zoonotic lentiviruses in urban ecologies, with a risk of transmission to naïve human population through frequent contacts and butchering of monkeys as previously observed in West and Central African countries.[6,17] Our results also reveal modestly high overall SIV prevalence of 32% amongst AGMs in the sampled urban centres compared to previously documented 26%-28% seroprevalence in captive and semi-captive AGMs in Kenya.[29,53]

Despite the 32% SIV prevalence amongst AGMs in Kenya being lower than the overall 46% recorded in AGMs from South Africa[21] and 44% in Gambia,[54] it is still within the reported range of 30%-50% infection rates in wild AGMs.[25,52,53] The SIV prevalence in baboons (3%) was lower compared to AGMs. This could be attributed to non-productive infection in baboons after cross-species transmission resulting in low quantity of virus particles therefore limiting our molecular assay whose sensitivity relies on viral load. Serological assays including antigen-based ELISAs and Western blot assays could have ascertained the real SIV exposure in the sympatric baboon populations and the natural host, AGM. However, these diagnostic approaches are also limited by narrow virus specificity while also being subject to non-specific cross-reactivity.[30]

We detected higher SIV prevalence in adults (37.5%) than subadult (25%) and infant (18.8%) AGMs. The low prevalence amongst infants could be attributed to the rare vertical transmission of SIV in AGMs as previously reported.[29,55] Variance of SIV prevalence was also apparent in NHPs sampled from different urban centres with Naivasha having the highest prevalence (46%) followed by

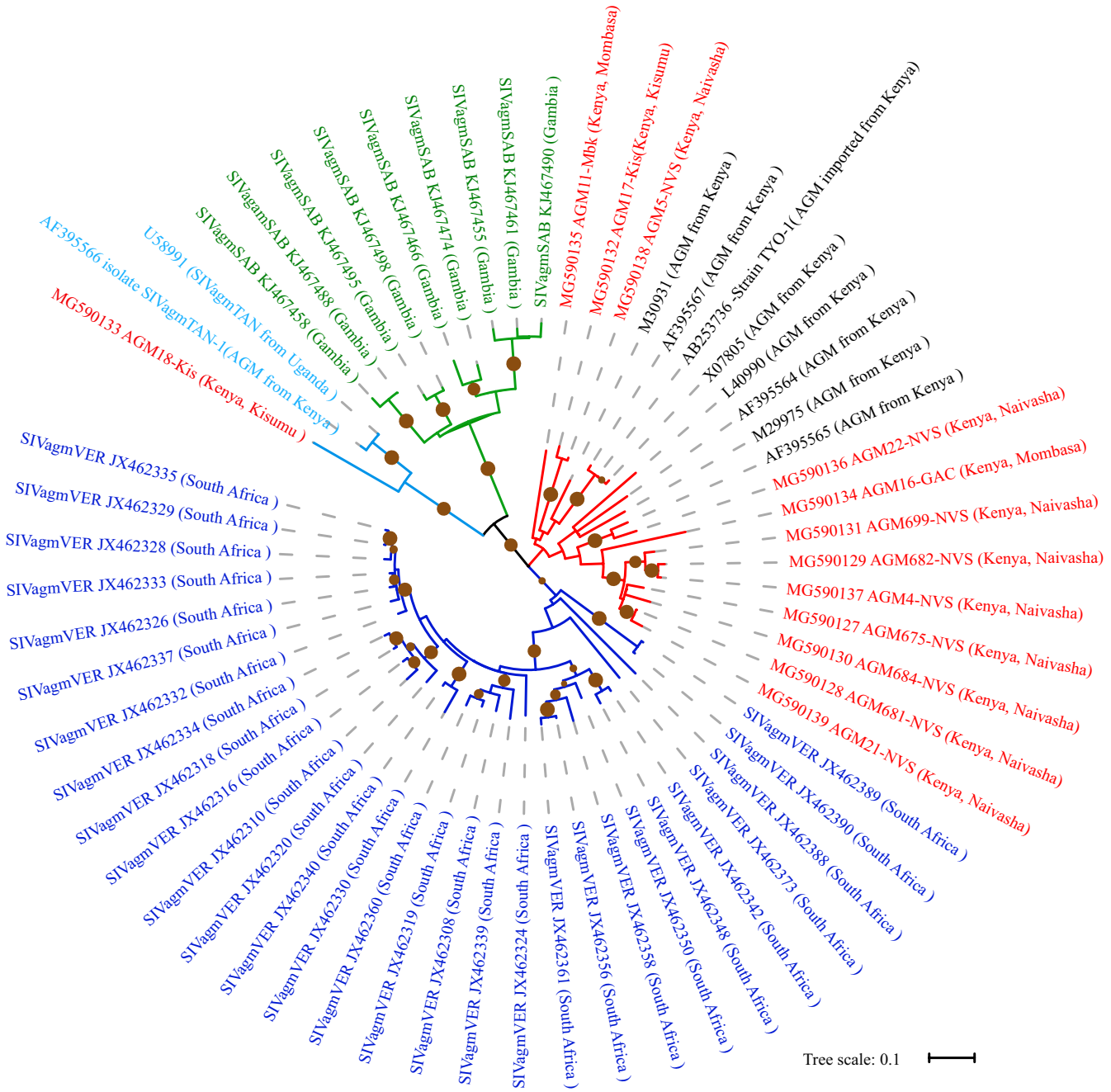

FIGURE 3 A maximum likelihood tree generated from partial *pol* gene (650 bp) illustrating genetic diversity of SIV from synanthropic (urban dwelling) AGMs from three urban centres in Kenya. Sequences from Kenya are highlighted in red, South Africa in blue and West Africa in green. Sequences obtained from GenBank with the host species originating from Kenya are shown in black. The sequence from Kisumu is closely related to sequences from tantalus subspecies of AGMs from Uganda is highlighted in light blue. Solid brown circles represent bootstrap support values of >70 while the bar represents the number of nucleotide substitutions (0.1) per site. Sequences generated in this study are named according to the monkey species (AGM), the animal number and sampling site

Mombasa (33%) and Kisumu (23%). The variance was comparable to SIV findings from wild NHPs in South Africa[21] and Cameroon[10] that attributed this difference to environmental and viral factors such as inefficient adaptation for replication in new host species.

For the first time, SIVagm was detected in a free-ranging olive baboon sampled alongside AGMs population in Kenya. The detection of this AGM specific strain from olive baboon sampled in western region of Kenya corroborates previous reports of SIVagm from yellow baboon in Tanzania[27,56] and chacma baboons in Southern Africa.[24,28] Phylogenetic analyses placed the baboon-derived SIV sequence in a strongly supported cluster (100% bootstrap) with the virus isolated from AGM5-GC sampled in Kisumu (Figure 4). High amino acid identity (96%) between these two isolates suggests epidemiological linkage to a common ancestor. Given that a baboon-specific SIV has not been identified this far, detection of SIVagm in olive baboon confirms the possibility of SIVagm establishing in new hosts as previously

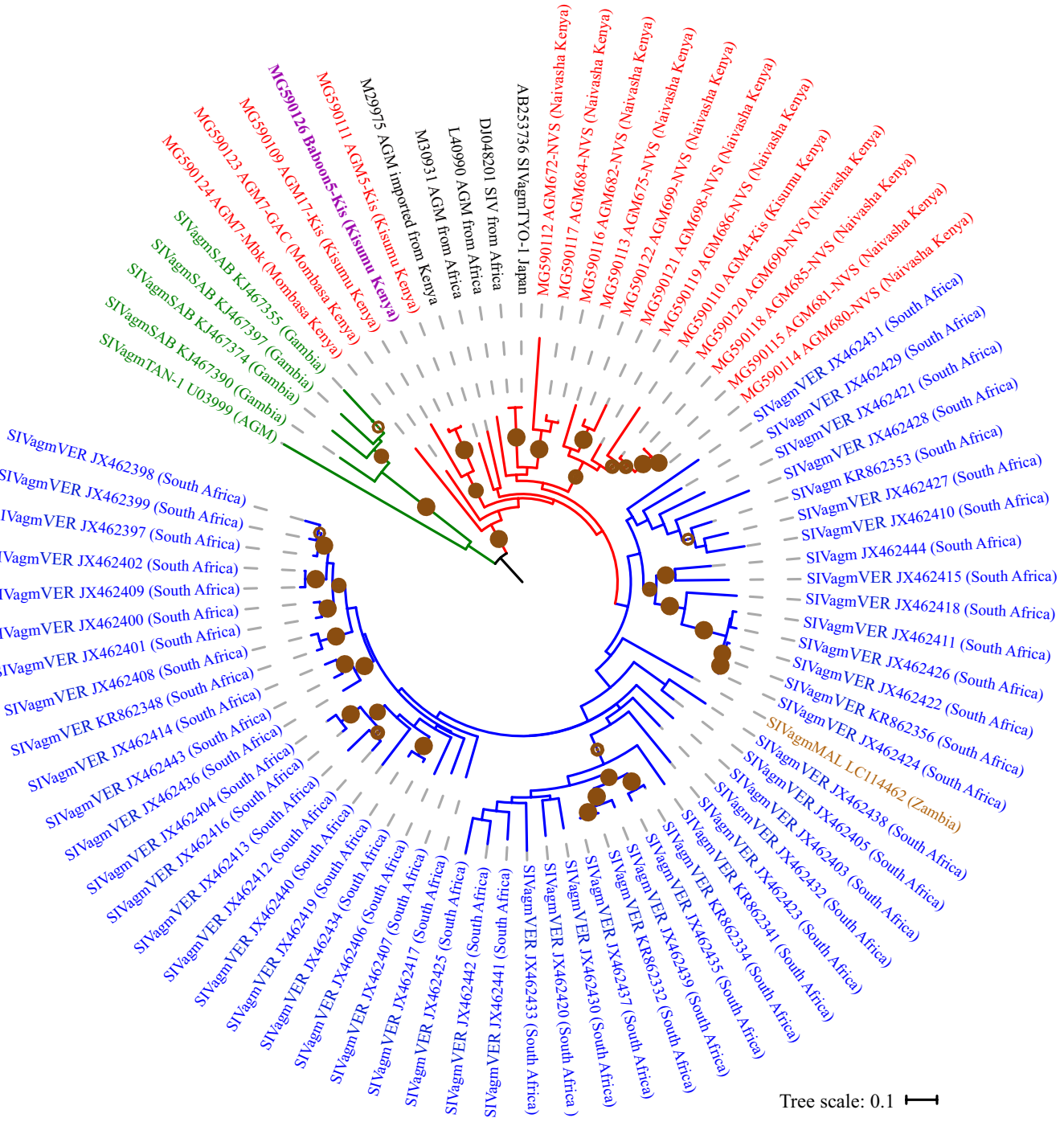

FIGURE 4 A maximum likelihood tree generated from partial *env* gene (900 bp) illustrating genetic diversity of SIV infecting synanthropic AGMs from several urban centres in Kenya. Sequences from Kenya are highlighted in red, South Africa in blue and West Africa in green. The SIVagm sequences obtained from a baboon is shown in purple while the SIVagmMAL sequences from Malrouck subspecies in Zambia is shown in brown. Sequences obtained from GenBank with the host species originating from Kenya are shown in black. Solid brown circles represent bootstrap support values of >70 while the bar represents the number of nucleotide substitutions (0.1) per site. Sequences generated in this study are named according to the monkey species (AGM—African green monkey), the animal number and sampling site

suggested.[27] The characterisation of SIVagm infecting baboons will not only be important in elucidating the determinants of cross-species transmission but also host adaptation to the virus. Of medical interest, understanding the infectious outcomes of SIVagm in baboons could offer insights into the development of a baboon model for HIV therapeutic research.

Analysis of HRM amplicons as complimented by phylogenetic analysis suggests existence of SIV intra-lineage diversity as supported by maximum-likelihood tree (Figure 3). However, PCR-HRM analysis could not explicitly characterise SIV strains based on their melt profiles in that one of the sequence (AGM18-Kis) that appeared in a different phylogenetic cluster (Figure 3) did not demonstrate a distinct

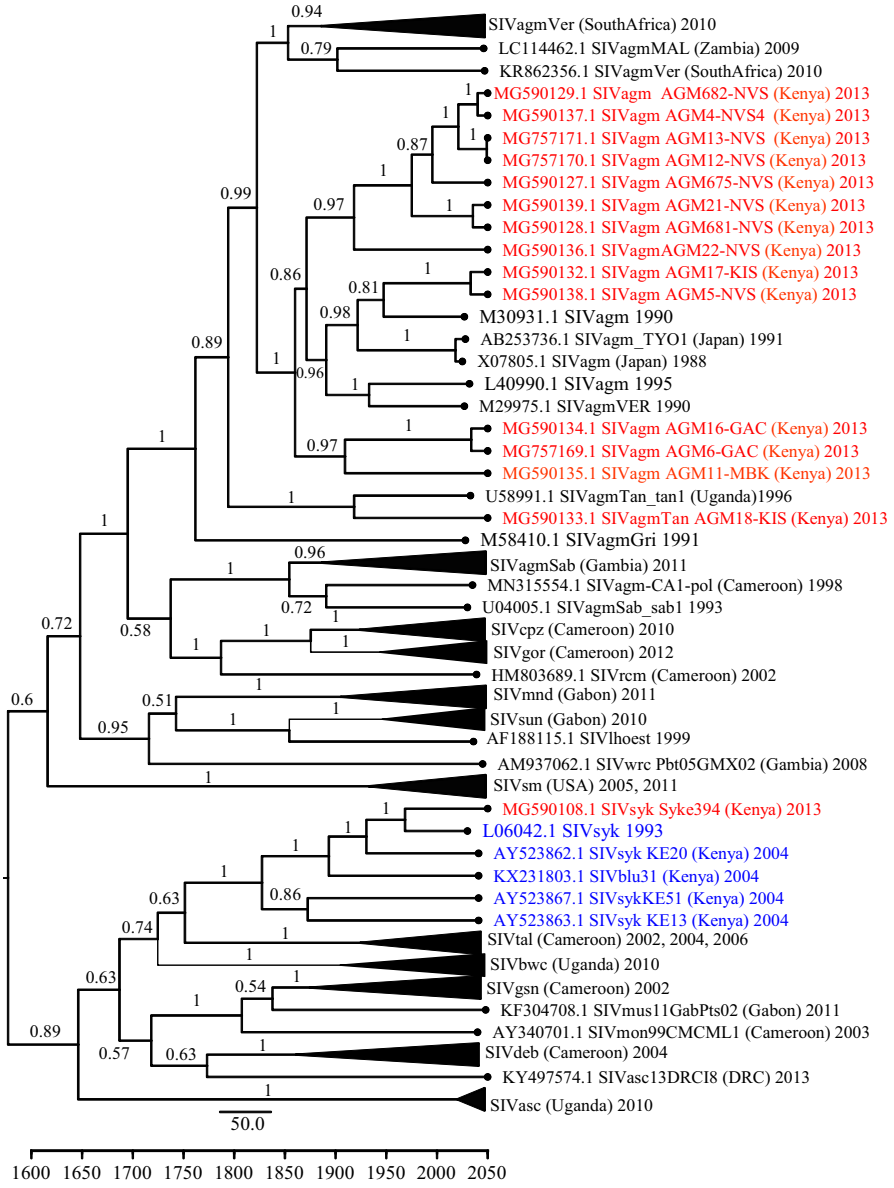

FIGURE 5 Time scaled Bayesian phylogenetic tree for *pol* gene from the newly derived SIVagm sequences from vervets in Kenya. The tip-date calibrated tree inferred from a lognormal distribution using the uncorrelated relaxed clock model shows the ultrametric divergence pattern between different SIV lineages. Internal nodes show divergence date estimates where all nodes had a posterior support >0.5. Branch lengths are expressed in substitutions per site as indicated by the scale bar

profile by HRM analysis. The use of a 650-nt PCR amplicon for HRM analysis in this study could have compromised HRM's melt profile resolution since it is recommended to use primers targeting smaller amplicon sizes (<300-nt) for better analysis.[34] In studies where single nucleotide polymorphisms are critical, the robustness of HRM in analysing miniature differences between PCR generated DNA fragments has proved to be reliable.[57] As reviewed by Tong and Giffard,[58] an increase in length of the amplicon size beyond 100-nt becomes difficult to resolve by HRM especially for sequences with nucleotide plasticity such as SIV and HIV. Despite the shortfall in SIV characterisation, the utility of HRM as a sensitive and robust probe free technique for detecting infectious pathogens and differentiate genetic variants of viruses can be improved by further implementing statistical analyses as illustrated in characterisation of HIV diversity.[32,59]

The inferred SIVagm phylogeny for partial *env* and *pol* gene fragments placed all the sequences amplified in this study except one within SIVagmVer cluster that included previous isolates from Kenya.[26] With SIV variants being host species specific, we confirmed the species of AGMs sampled (Figure 7) to support the phylogenetic placement of SIVagm from these animals within the SIVagmVer lineage. The two distinct sub-clusters formed by SIVagm sequences from *C pygerythrus* sampled in Mombasa and those from *C aethiops* sampled in Naivasha and Kisumu (Figure 5) suggest SIV co-divergence with the hosts but not phylogeographic clustering. High bootstrap support values amongst SIV sequences from AGMs sampled in Naivasha imply epidemiologic linkage suggesting continuous local transmission. The phylogenetic interspersing of sequences from Kisumu and Mombasa indicates that divergent strains can also occur in one location as previously reported in mustached monkeys[19] and chimpanzees.[60]

Despite the molecular identification of AGM18-Kis as *C aethiops*, its SIV isolate clustered in a distinct clade alongside SIVagmTan from a tantalus monkey whose habitat range include eastern Uganda and western Kenyan border. This agrees with the west-to-east SIV transmission model across existing AGM geographic ranges given the

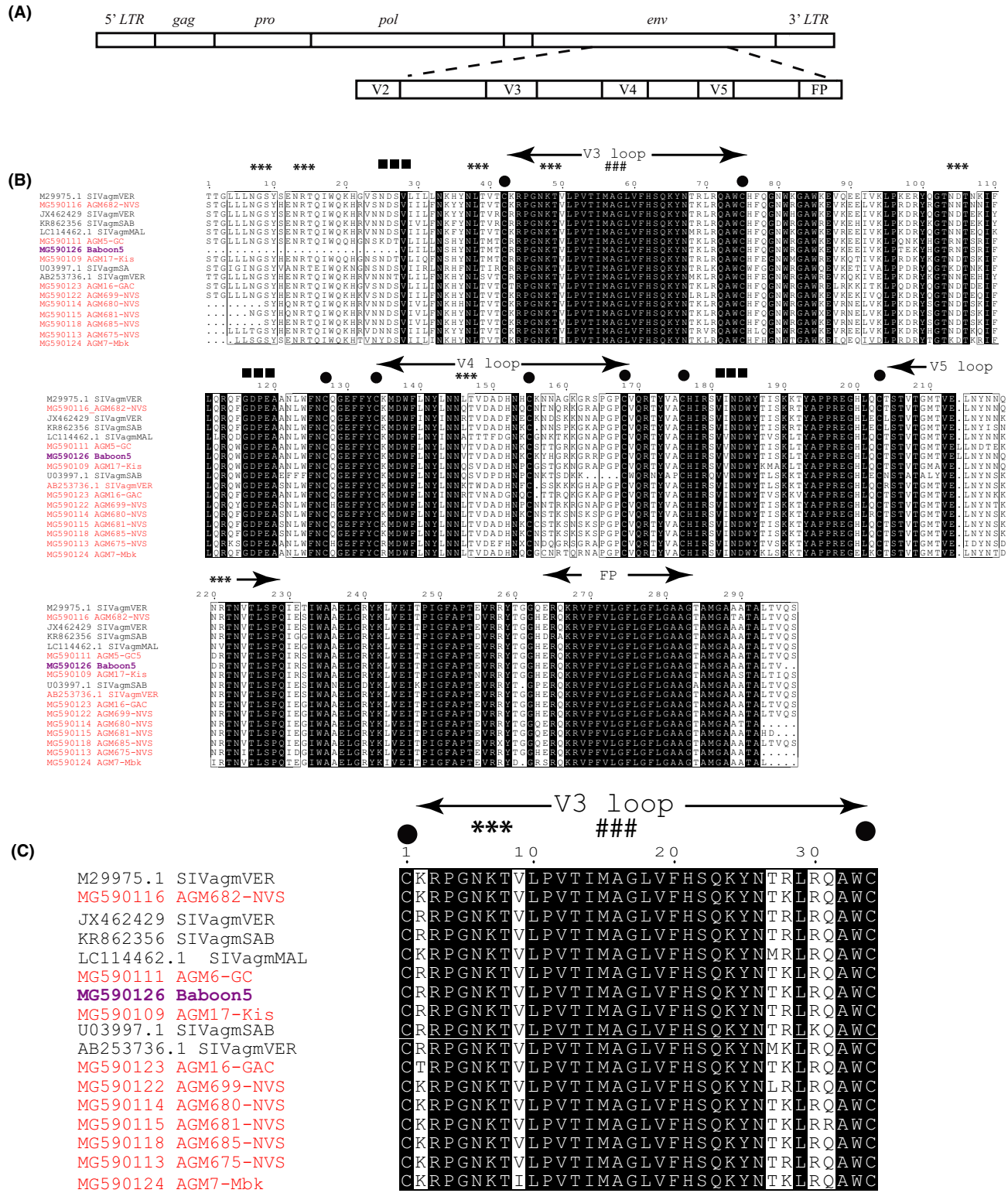

FIGURE 6 SIV genome architecture and partial env protein domains. A, A cartoon representing SIV genome architecture showing respective genes and offset is a representation of the V3-V5 and the fusion protein ([FP] domain) domains of the partial env glycoprotein. B, Env protein amino acids alignment showing the V3 to V5 domains, the fusion protein, the potential N-linked glycosylation sites highlighted with asterisks (***). The conserved cysteine residues are highlighted with black dots while potential CD4 binding sites are indicated as black squares, the MAG motif is highlighted hash signs (###). C, A trimmed alignment V3 hypervariable domain showing the MAG motif, the domain was comprises of 34 residues as reported from previous studies and expected for this region. Sequences obtained from AGMs in this study are shown in red, the sequence obtained from a baboon is shown in purple while reference SIVagm sequences retrieved from Genbank are shown in black

TABLE 3 SIV *env* gene codons under evolutionary selection pressure. This analysis was performed using the molecular evolution tools in the Datamonkey Web servers (Delport et al[45]) with default settings

	Diversifying selection		Negative selection	
	FUBAR ($(\beta > \alpha) \geq 0.9$)	MEME ($P < .05$)	IFEL ($P < .05$)	FEL ($P < .05$)
Codon	Pervasive	Episodic	Negative	Negative
2	0.930754	0.006223	-	-
18	-	-	0.010375	0.03724
58	-	-	0.020702	0.046966
70	0.926138	0.001725	-	-
124	0.939274	0.002007	-	-
161	0.926871	0.019448	-	-
170	0.965838	0.01065	-	-
296	0.927782	0.01812	-	-
297	0.960401	0.00871	-	-
301	0.927481	0.012432	-	-

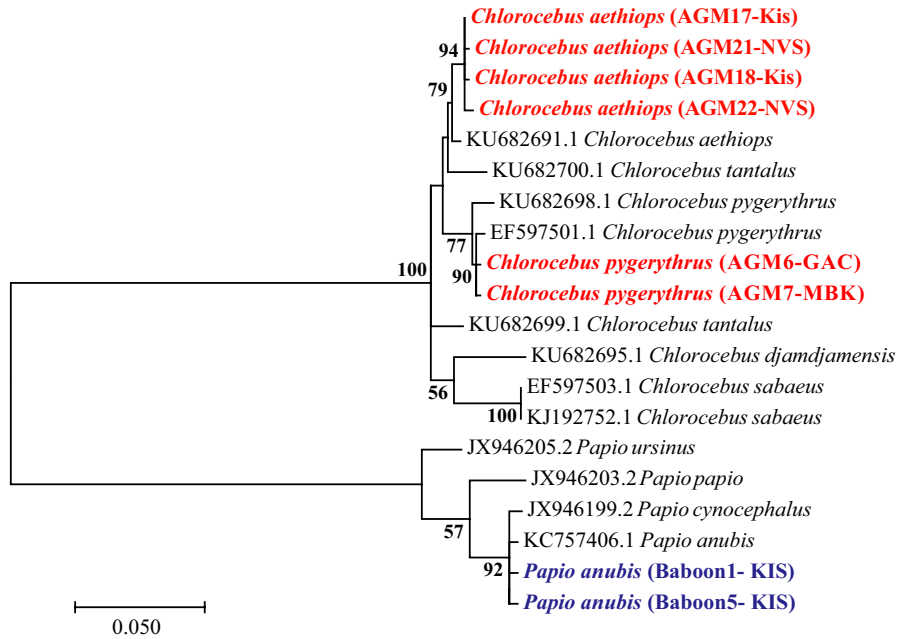

FIGURE 7 Maximum likelihood phylogenetic relationships among AGM and baboon mitochondrial cytochrome oxidase 1 (CO1) gene (650 nt). The evolution history inferred by maximum likelihood tree was midpoint rooted. AGM sequences from this study are highlighed in red while the baboon sequences are highlighted in blue. The AGM sequences from Kisumu and Naivasha segregated with *C aethiops* subspecies while the AGM sequences from Mombasa segregated with the *C pygerythrus* subspecies. The CO1 sequence for AGMKis18 from Kisumu clustered with *C aethiops* subspecies despite being infected with SIVagm specific to *C tantalus* subspecies. Maximum likelihood nonparametric bootstrap support values (>50%) based on 1000 replicates are shown on the nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. There were a total of 632 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

location of Kisumu relative to Uganda boarder.[61] The geographic proximities of their hosts' ranges suggest a common evolutionary history (Figure 3) as previously reported for STLV infecting mustached monkeys.[19] Attempts to amplify the *env* gene fragment for this sample were not successful. Thus, we could not ascertain the probability of this isolate being a recombinant or a case of cross-species transmission. Of note, in depth phylogenetic analysis of SIVagmTan from a tantalus monkey sampled in Cameroon has provided significant evidence suggesting this strain to be a recombinant.[62] However, the identification of SIVagmTan in a *C aethiops* from Kenya illustrates unresolved complexity of SIVagm strain diversity in African green monkeys especially in East Africa. Given the wide geographic range of *Chlorocebus* species in Kenya and a nexus where cases of hybridisation are inevitable,[13] the complexity of SIVagm diversity might be greater than estimated in this study. Polyspecific association amongst sympatric *C aethiops* and *C pygerythrus* in Kenya, as previously reported for mustached monkeys in Cameroon,[63] is one of the plausible explanations for the extensive SIVagmVer diversity documented in this study.

Contrary to the reports of ancient SIV infection in AGMs,[21] our tip-date calibrated phylogenetic tree corroborates the findings from Wertheim and Worobey that estimated SIV infection in AGMs to be more recent than previously thought.[61] The tree topology places SIVagmSab basal to both SIVagmTan and SIVagmVer in line with the postulated idea of west-east SIV infection dynamic in AGMs by Wertheim and Worobey.[61] Nonetheless, our tip-dated phylogenetic tree should be interpreted cautiously since the tree was inferred using small genomic region derived from *pol* region and could not represent the cumulative signal for the full-length genome. Further, the tree's molecular clock calibration was based on sequence tip-dates, which might suffer from inaccuracy in their estimated sampling dates owing to reporting biasness.

Nucleotides of SIVs from sampled AGMs demonstrated extensive genetic diversity indicating a suitable niche for viral evolutionary changes within the natural host.[64] The codon-oriented FUBAR and MEME methods also revealed evidence of diversifying (positive) selection within the SIV *env* sequences. This sequence diversification and evolutionary selection in a natural host is a typical characteristic of potentially zoonotic viruses as opposed to viruses that diversify in a dead-end host thus limiting onward transmission.[65] When selection favours mutations within amino acids critical for host-virus interactions, the virus fitness in a new host is favoured.[66] This phenomenon underscores the significance of SIV diversification as a potential precursor for pathogen spill over.

Results from this study distinctively demonstrate, although from a small population of sampled animals from few selected urban centres, that there is persistent evolutionary diversification of SIV strains in its natural host, AGMs, with the potential to cross-infect other primate species including olive baboons. Partial genetic characterisation of SIVagm provides pertinent information on the changes involved in SIV natural evolution and epidemiology and it is important for designing reliable urban health policies for zoonoses control.

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AUTHORS' CONTRIBUTIONS

MJ, JV, DM, RN and VO conceived the study and designed the experiments. IL, MJ, JT and MO collected the samples. RN, MJ, JV and VO conducted the laboratory analysis, EKM, RN, MJ, JV and VO analysed the data. RN, JV, MJ, MO, EKM and VO drafted the manuscript. All the authors revised the manuscript and approved the final manuscript draft.

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