Characterization of the Gamboa Virus Serogroup (*Orthobunyavirus* Genus, Peribunyaviridae Family)

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Abstract.

Comprehensive comparative phylogenetic analyses were performed on 17 Gamboa serogroup viruses (GAMSVs) from distinct geographic regions in the Americas and other representative members of the genus *Orthobunyavirus* (Peribunyaviridae), based on small (S), medium (M), and large (L) open reading frame full-length and partial sequences. Genome characterization showed that the GAMSVs divide into four clades or genotypes. The GAMSVs have a genetic organization similar to other orthobunyaviruses, except that they have a larger NSm protein than other orthobunyaviruses. A serosurvey for Gamboa virus antibodies was performed in plasma from birds, other wild animals, and humans living around the Tucuruí hydroelectric dam in Pará state, northern Brazil, a known focus of GAMSV activity. Newborn chicks (*Gallus gallus domesticus*) were experimentally infected with a GAMSV, and the pathogenesis is described. Histopathological changes were primarily in the lungs and liver. Also, a review of the ecology of the GAMSVs in the Americas is included. In sum, this study presents the genomic and evolutionary characterization of the Gamboa group and the potential model of pathogenesis, which would be helpful for diagnostic purposes, epidemiology, and immunopathogenesis studies.

INTRODUCTION

The Gamboa serogroup (*Orthobunyavirus* genus, Peribunyaviridae family) currently consists of five viruses divided into two antigenic complexes: Gamboa and Alajuela. The Gamboa complex includes Gamboa virus (GAMV) and Pueblo Viejo virus (PVV), and the Alajuela complex includes Alajuela (ALJV), San Juan, and Brus Laguna viruses.[1] This

classification was based on the antigenic relationship of the five viruses, as determined by classical serological tests (complement fixation [CF] and neutralization [NT]).[1]

Currently, Gamboa serogroup viruses (GAMSVs) have only been isolated in tropical regions of Central and South America, including Argentina, Brazil, Ecuador, Honduras, Panama, Venezuela, and Suriname.[2–5] Currently, Gamboa serogroup viruses have not been associated with disease in humans, domestic, or wild animals. The principal maintenance cycle of these viruses in nature appears to involve a single mosquito species, *Aedeomyia squamipennis*, as the vector and wild birds as the vertebrate host.[1–5]

As members of the family Peribunyaviridae, the genome of GAMSV consists of a single-stranded negative and/or ambisense RNA comprising three segments, namely, large (L), medium (M), and small (S) segments. The L segment codes for the RNA-dependent RNA polymerase (RdRp); the M segment codes for the two surface glycoproteins (Gn and Gc), in addition to a nonstructural protein (NSm); and the S segment codes a structural nucleoprotein (N) and another nonstructural protein (NSs).[6]

Currently, knowledge about the GAMSVs is very limited, especially about the genetic diversity, ecology, and pathogenesis in potential vertebrate hosts. In this study, we described genome sequences and genetic relationships of 17 GAMSVs. To better understand their pathogenesis in an avian species, newborn chicks (*Gallus gallus domesticus*) were experimentally infected with a Brazilian GAMSV isolate. In addition, a seroepidemiological survey was conducted using plasma from wild birds and animals as well as human residents of the municipality of Tucuruí, Pará state, Brazil, a known focus of GAMSV activity. A brief discussion of the ecology of the GAMSV follows.

METHODS

Virus strains, viral propagation, and RNA extraction.

Virus strains used in this study were obtained from collections of the Department of Arbovirology and Hemorrhagic Fevers of the Evandro Chagas Institute in Belém, Brazil; the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch in Galveston, TX; and the Gorgas Memorial Institute in Panama (Table 1). Most of the viruses used were relatively low passage strains and were propagated in *Chlorocebus aethiops* kidney (Vero) cells. When viral cytopathic effect was apparent, the supernatant was collected and used for the extraction of viral RNA using Trizol LS reagent or QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Nucleotide sequencing and genome assembly.

Total RNA obtained from the supernatant of infected Vero cells was used as a template for the production of cDNA using the Superscript III cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The cDNAs were prepared for high-throughput sequencing using an Ion Torrent device that uses the ion semi conduction method (Thermo Fisher Scientific). The de novo assembling strategy applied to obtain the genomes was the MIRA software v.4.9.2.[7] Contigs were considered if at least five reads were assembled. Quality inspection (base call quality > 20) was set as default to reconstruct the RNA segments. The 3′ and 5′ noncoding sequences (3′ and 5′ noncoding regions [NCRs]) were obtained using both the 3′ and 5′ rapid amplification of cDNA ends method using the specific set of primers (Supplemental Table 1). The genome sequences determined in this study were deposited in GenBank under accession numbers of FX900423 to FX900443 and MG019911 to MG019913.

Genome characterization.

The GAMSV genomes were evaluated regarding size, open reading frame (ORF) descriptions, 5′ and 3′ NCRs, conserved motifs, N-linked glycosylation sites, cysteine residues, and cleavage sites. Analyses were conducted with the Geneious v. 9.1.2 (Biomatters, New Zealand), as well as with InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/), and NetNGlyc v.1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), TMHMM Server v.2.0,[8] and SignalP 4.1 Server.[9] Also, the molecular protein weight for each identified viral protein was predicted using the Protein Molecular Weight Calculator tool (http://www.sciencegateway.org/tools/proteinmw.htm). The RNA secondary fold structure was assessed using the mfold.[10]

Phylogenetic analysis.

Maximum likelihood (ML) phylogenetic trees were reconstructed using 17 strains of GAMSV (Supplemental Table 2) and an additional 89 *Orthobunyavirus* complete coding sequences (S, M, and L) available in the GenBank database (http://www.ncbi.nlm.nih.gov/) until September 21, 2017. The multiple sequence alignments to the nucleotide level were carried out using Muscle v.3.7.[11] The best substitution models were determined by using ModelFinder implemented in IQ-TREE version 1.4.3 software. The phylogenetic reconstructions for all RNA segments in nucleotide level were inferred using the GTR+I+G4 model as the best-fit DNA substitution model, and phylogenetic reconstructions only with Gamboa strains using nucleotide level were inferred using the TIM2+I+G4 to L and M segments, and K2P+I+G4 to S segments as the best-fit DNA substitution model. The phylogenetic trees were performed by using IQ-TREE using 1,000 replicates.[12] Statistical supports for individual nodes were estimated using the bootstrap value. The phylogenetic trees were visualized using the FigTree software v.1.4.2.

Genetic distance identification and prediction of conserved motifs.

The genetic distances among and within clades were calculated based on amino acid alignments from three genes (nucleoprotein, M segment polyprotein, and RdRp) using the *p*-distance values. Standard error estimations were calculated by using the bootstrapping method (1,000 replicates) using the molecular evolutionary genetics analysis v.6 program.[13] The results of the genetic distances among clades are presented in box and whisker plot figures and genetic distances within clades are shown in a table format. The nucleotide and amino acid identity comparisons for GAMSVs and other representative orthobunyaviruses were performed in Geneious 9.1.2 (Biomatters). The presence of potential motifs characteristic for *Orthobunyavirus* were identified using Geneious 9.1.2 (Biomatters).

Reassortment events analysis.

To identify potential reassortment events, the data were analyzed for evidence of distinct phylogenetic topologies based on the depicted trees at the nucleotide level, as described previously. All genes in a single sequence were concatenated, and a multiple alignment was performed using the program MAFFT v7.158b, as described previously.[14] Potential reassortment events among the GAMSVs were then analyzed using the RDP, GENECONV, Bootscan, MaxChi, Chiamera, SiScan, and 3Seq methods implemented in RDP4 program.[15] Common program settings for all methods were used to perceive sequences as linear, to require phylogenetic evidence, to refine breakpoints, and to check alignment consistency. The highest acceptable *P* value was set at 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values.

Prevalence of GAMV antibodies in animals and humans.

A serosurvey was conducted to determine the prevalence of GAMV antibodies in 770 plasma samples from birds of 32 families, 225 serum samples from other wild animals (reptiles, amphibians, rodents, marsupials, edentates, carnivores, Chiroptera, and nonhuman primates) as well as 406 serum samples of humans living in the municipality of Tucuruí, Pará state, Brazil. These samples were collected by the staff of the Evandro Chagas Institute in 1986, during construction of the Tucuruí hydroelectric dam as part of an environmental impact study.[4]

Depending on the sample amount available, we used two different tests for the detection of GAMV antibody: hemagglutination-inhibition (HI)[16,17] tests for samples with small amounts and NT tests in mice[18] for plasma/sera with larger amounts. By using HI test, the samples were initially screened at a 1:20 dilution and positive reactions were titrated at serial 2-fold dilutions from 1:20 to 1:640. The antigen used in the HI tests was a sonicated, sucrose–acetone extracted newborn mouse brain infected with GAMV strain BeAN439546. This virus was isolated from the blood of a bird (*Geotrygon montana*—ruddy quail dove) collected in 1985 during construction of the Tucuruí hydroelectric dam, Pará state, Brazil.[4] The NT test was performed by the intracerebral inoculation of newborn mice, according to the technique reported by Casals.[18] LD_{50} values were calculated as described by Reed and Muench.[19]

Experimental infection of chickens.

To determine the pathogenesis of a representative GAMSV in an avian host, two groups of 12 newborn (2-day-old) chicks (*G. g. domesticus*) were inoculated subcutaneously (sc) or intracerebrally (ic) with approximately $10^{5.5}$ newborn mouse LD_{50} units of GAMV strain BeAn439546. A third group of uninfected chicks served as a control group. After inoculation, one bird in each group was euthanized daily for 11 consecutive days. The 12th and final bird was sampled on day 18 postinfection (p.i.). At the time of euthanasia, blood was collected from each animal for HI antibody determinations,[16,17] using the same GAMV strain (BeAN439546). At necropsy, samples of brain, kidney, spleen, liver, lung, and heart were collected and immediately fixed in 10% buffered formalin for subsequent histopathological and immunohistochemical analyses.[18–20] The animal protocol was approved by the Evandro Chagas Institute Ethics Committee on Animal Research (CEPAN #005/2010).

RESULTS

Nucleotide sequencing, genome organization, and proteins.

Four complete sequences and 13 nearly complete genomes were obtained for the GAMSV group isolates. Three genomic segments, namely, S, M, and L RNAs were assembled with a mean coverage of 87, 124, and 212 times, respectively, with a quality value (base quality) more than 20. The S segment RNA had 1,154 nucleotides (nt), the M segment RNA ranged from 4,919 nt, and the L RNA ranged from 6,933 nt in length. We have identified four distinct ORFs: two in the S RNA (717 amino acids [aa], 393 nt), one in M RNA (4,782 nt), and one in L RNA (6,822 nt). Protein identification with the InterProscan software based on the ORF sequences reveals the presence of the nucleocapsid protein (238 aa, 27 kDa), NSs protein (130 aa, 14.7 kDa); M segment polyprotein (1,591–1,594 aa, 167.6–179.3 kDa); and RdRp (2,273 aa, 264.3 kDa). The analysis of cleavage sites in the glycopolyprotein showed Gn (282 aa, 31.9 kDa), NSm (320 aa, 36.5 kDa), and Gc (869 aa, 97.7 kDa) proteins. Supplemental Table 2 summarizes the genomic characteristics of the 17 GAMV strains. Cleavage sites were identified as shown in Figure 1A as well as the segment

sizes, ORFs and protein products (aa), conserved motifs, and domains and molecular weights (kDa).

3′ and 5′ NCR size heterogeneity, RNA fold structure, cysteine residues, glycosylation sites, and conserved motifs.

Complete 3′ and 5′ NCR sequences were obtained from four GAMSV isolates. Based on the termini sequences, size heterogeneity was observed. RNA fold structures were predicted for all complete sequences, and high conservation among 17 nt of the three RNA segments was observed. Reverse complementarities with A/C mismatch at pairing residues number 9 were identified. Supplemental Figure 1 shows the schematic representation of the termini alignments and the RNA fold structures for the different RNA segments.

A total of 122 cysteine residues were found being conserved for all analyzed GAMSV predicted proteins: *N* = 2; NSs = 5; Gn = 20; NSm = 12; Gc = 39; and RdRp = 44. Also, the potential N-linked glycosylation sites are shown in Figure 1A. Conserved motifs were identified in sequences of GAMSV isolates. For the glycoprotein, we observed the zinc finger and fusion peptide and the RdRp identified region domains named pre-A and A to E, as previously analyzed for other orthobunyaviruses (Supplemental Figures 2–4).

The polyprotein coded by the M segment contains the N-terminal signal peptide that is common to all members of the genus. This polyprotein is cleaved into two different structural proteins, named Gn and Gc, and a nonstructural protein named NSm (Figure 1A). The glycoproteins are predicted to contain transmembrane regions (TMDs): a single TMD in Gn (close to the C-terminus) and a single one close to the C-terminus of Gc, which are responsible for anchoring the glycoproteins in the viral lipid envelope. We observed a distinct organization in the NSm region, with five TMD domains, as opposed to what has been commonly described in other orthobunyaviruses,[6,21] which can indicate that the GAMSVs have a larger NSm protein. Thus, we assume that the NSm protein is larger and not the Gn, based on the topology of the glycopolyprotein during synthesis in the endoplasmic reticulum (Figure 1B). A previous study has shown that the C-terminus of Gn protein in orthobunyaviruses interacts with the N protein during viral assembly.[22] Therefore, we believe that the C-terminus of the Gn protein may have a conserved number of amino acid residues. If the NSm region is the same size as that of other orthobunyaviruses with only three TMDs, we must assume that Gc is larger with two extra TMDs. Thus, the C-terminus is longer than other known orthobunyaviruses, which may compromise the interaction of the proteins with the N protein during viral assembly (Figure 1B). Based on the previous analyses,[6] the amino acid residues present at the N protein level of GAMSV isolates are probably involved in the ribonucleoprotein packaging process, RNA synthesis, and virus RNA ligation to viral proteins.

Phylogenetic analysis of Gamboa serogroup.

Maximum likelihood phylogenetic analysis using 106 complete coding sequences revealed that the GAMSVs are most closely related genetically to Koongol virus and form a monophyletic group in the trees sharing the same common ancestor (bootstrap value = 100) (Supplemental Figure 5A–C). Based on S and L segments (Figure 2A and C), the GAMSVs split into two clades: a basal position clade with Calchaqui virus (designated “Group II”), and an upper clade (Group I) consisting of GAMV, PVV, ALJV, and Soberania virus. On the other hand, the M topology reveals four distinct clades that are highly supported and identified as groups designated as Gamboa, Alajuela, Soberania, and Calchaqui (bootstrap value = 100) (Figure 2B). The Gamboa group comprises PVV and GAMV isolates

BeAN448848, AN439546, BeAR502380, BeAR503385, TR61469, MARU10962, and GAM131. The Alajuela group is formed by ALJV isolates GML382716, GML438524, GAM122, and MARU11079. The Soberania group is composed by strains GAM130, GAM118, GML435718, and GML903023. This latter group was named for the collection place of the mosquitoes in Soberania National Park in central Panama.[5] The Calchaqui group consists of only a single virus, the Calchaqui virus strain AG83-1347 from Argentina. Because the M segment determines the surface glycoproteins, which affect the hemagglutinating and neutralizing antigenic determinants, we divide the GAMSVs into four groups (Figure 2B).

Genetic variability.

We performed the pairwise amino acid sequence distance analysis of the nucleoprotein, M segment polyprotein, and RdRp with GAMSVs and representative members of the *Orthobunyavirus* genus (Supplemental Figure 6). The genetic distances of the GAMSVs to other orthobunyaviruses were estimated to be of ∼63%, ∼61%, and ∼51% for the N protein, glycoprotein, and RdRp, respectively. Interestingly, GAMSVs share the same patterns of amino acid distances with that of the Kongool virus group, whereas the same that is observed in ML trees are noted in the evolutionary distance (Supplemental Figure 5). Lower levels of genetic diversity were depicted within the GAMV serogroup compared with that observed with other *Orthobunyavirus* serogroups. This fact was observed in the *p*-distance intra-clade analysis, based on all three genes (nucleoprotein, glycoprotein, and RdRp), in both nucleotide and amino acid identity comparisons (Supplemental Tables 3 and 4).

Reassortment analyses.

To identify potential reassortment events among GAMSVs, we inspected the nucleotide ML phylogenies for discordances in clade clustering between the S, M, and L trees (Figure 2A–C), combined with RDP4 analyses using concatenated full genomes of all members of Gamboa serogroup sequenced. However, the phylogenetic trees suggests that all Soberania strains could be a result of reassortments; the RDP4 analysis indicated that Soberania strain 118 resulted from a reassortment event, which has S and L from Gamboa strain MARU10962 and a unique M segment (Figure 2D and E).

Prevalence of GAMV antibodies in animals and humans.

Based on both HI and NT tests, we have detected a total of 7.1% (55/770) positive reactions with the GAMV antigen in avian samples tested, distributed in 32 different bird families. However, no pattern of infected was identified among the birds evaluated (Table 2). Also, we have detected 2.67% (6/224) of wild animal sera with HI antibodies to the GAMV antigen (data not shown). Two of the positive sera were from terrestrial rodents, a spiny rat (*Proechimys* sp.), and an agouti (*Dasyprocta agouti*). The other four positive samples were from tortoises (*Chelonoidis carbonaria* and *Chelonoidis denticulata*); but, it should be noted that tortoises were the most common wild animals sampled ($N = 169$); these reptiles represented 75.4% of the nonavian animals sampled. Only 1.5% (6/406) of the human sera had HI antibodies to GAMV antigen; titers in the six positive human samples ranged from 1:20 to 1:80 (data not shown).

Experimental infection of chicks with GAMV.

After infection with GAMV strain BeAN439546, none of the 2-day-old chicks (infected or the controls) developed signs of illness during the 18 days of observation. Their behavior

appeared normal, and animals remained active, including feeding and increase in size. Also, we did not observe gross macroscopic alternations in the organs at necropsy.

However, histopathologic examination of organs of the experimentally infected chicks showed major alterations in the liver and lungs, followed by mild alterations in the kidneys and brain. The tissue changes in the affected organs were observed between the first and seventh day postinoculation (p.i.), with the peak intensity on the fifth and sixth day p.i., especially in the liver and lungs. Also, no pathologic alterations were observed in the organs of birds inoculated sc or intracranially (ic), except that some alterations of neural tissue were found in the ic infected chicks. In the sc and ic infected groups, lung abnormalities were characterized by congestion, edema, trabecular thickening, hemorrhage, and interstitial mononuclear infiltration (lymphocytes and plasma cells) (Figure 3A–C). But, intra-alveolar exudates or intense necrosis were not observed. The degree of tissue injury became more prominent after the third day with peak on the fifth and sixth day p.i. Also, congestion was found in the spleen, in the absence of other abnormalities, but no cardiac alterations were observed.

Alterations of the liver parenchyma were characterized by the presence of hepatocyte ballooning on the second day p.i. in both infected groups (Figure 3D–F). The portal spaces showed mild-to-moderate mononuclear inflammatory infiltrate, accompanied by local congestion, especially during the fifth and sixth day p.i. in both groups. However, signs of hepatocyte regeneration, cholestasis, fibrosis, or remodeling of lobular architecture were not observed.

Abnormalities in the central nervous system were only observed in the group of animals infected by intracranial inoculation. These alterations, which involved the meninges, were characterized by edema and congestion and were mainly found between the fifth and sixth day p.i.; but meningitis was not noted in any of the infected groups. In the kidneys, no significant alterations were observed in the renal parenchyma. On the other hand, congestion and mild lymphocyte infiltration in the interstitium were observed in animals of the control group, but not in glomeruli. Also, the results showed slight swelling of renal tubular cells, in particular on the sixth and seventh day p.i. (Figure 3G and H). However, no significant changes were observed in any of the organs studied between the 8th and 11th days p.i.

Immunohistochemistry revealed visible immunoreactivity in the liver and lungs of newborn chicks infected with GAMV, which included the characteristic brown antigen staining in the cytoplasm of cells (Figure 3D and H). Also, viral antigen was detected between the first and eighth days p.i., with peak intensity on the seventh day p.i. In addition, some immunoreactivity was observed in the kidneys, despite only mild histopathological alterations (Figure 3K). Finally, discrete immunostaining was found in the spleen, heart, and brain, but only detected in a few cells (data not shown).

Serologic response of experimentally infected chicks.

Hemagglutination-inhibition tests performed with the serum samples from the chicks throughout the study period revealed the presence of GAMV antibodies from the 10th day p.i. until the last day of the experiment (18th day) in the two infected groups (ic and sc inoculation). Hemagglutination-inhibition antibody titers were higher in the three birds infected by intracranial inoculation (1:360 on days 10, 11, and 18 p.i.), compared with than titers in the three chicks infected by the subcutaneous route (1:20, 1:20, and 1:80 on days 10, 11, and 18 p.i., respectively). Unfortunately, because of sample limitation, blood was not examined for the presence of viremia; but based on the absence of illness, minimal

histopathological alterations, and the development of antibodies in the infected chicks, we conclude that GAMV infection in birds probably does not cause severe disease.

DISCUSSION

The genomic characterization of 17 GAMSV strains is described. The strains exhibit a similar genomic organization including the presence of the NSs gene, which has been observed in most orthobunyaviruses.[23] Also, we have identified that the RdRp contains the central motifs that are conserved for the polymerase activity (pre-motif A and motifs A through E), which are highly conserved in negative sense RNA viral polymerases and also common to all other orthobunyaviruses.[24] The genomic characterization of GAMSVs revealed one aspect that is highly unusual in orthobunyaviruses. In this study, we observed that GAMSVs possess an NSm protein considerably longer (317 aa) than the typical *Orthobunyavirus* NSm (102–186 aa). Currently, the role of NSm in replication of *Orthobunyavirus* infection is unclear, but previous studies have suggested that protein acts as a polyprotein precursor that is co-translationally cleaved with Gc and Gn. In Bunyamwera virus infection, it has been shown that the NSm protein is critical for assembly and morphogenesis.[22] On the other hand, Maguari virus infection suggests that the NSm protein is not essential for growth in tissue culture.[25] A recent study suggested that a deletion of NSm in Schmallenberg virus also showed reduced virulence in experimental infection.[26] By contrast, the rescue of recombinant Oropouche viruses, with complete deletion of NSm, did not affect viral replication in cultured cells.[27] Further studies using reverse genetics may elucidate the biological importance of the larger NSm proteins found in GAMV.

Phylogenetic analysis of 17 GAMSVs confirms the inclusion of these viruses within the genus *Orthobunyavirus*, Peribunyaviridae family and showed that the GAMSVs are genetically most closely related to the Koongol serogroup. Currently, Koongol and Wongal viruses constitute the Koongol serogroup, but only the complete coding sequence of Koongol virus is available.[28] These viruses were originally isolated from *Culex* mosquitoes collected in Queensland, Australia, in 1960.[29] Also, our phylogenetic tree based on nucleotide sequences of M segment indicates that the GAMSVs can be divided into four clades or complexes, which does not agree with the earlier antigenic classification.[1] However, the previously antigenic classifications were based on the results of CF, HI, and NT tests. Differences observed between the antigenic relationship of orthobunyaviruses species in classical serological tests (CF, HI, and NT tests) and genomic studies have been reported previously.[30,31] These differences are presumed because of the genomic reassortment among the three RNA segments.[31]

The reassortment phenomenon in segmented viruses is a form of genetic exchange that has the potential to provide host/vector range shifts and changes in pathogenicity. Natural reassortment is common among viruses in the *Orthobunyavirus* genus and is an important mechanism for the emergence of new viruses.[31] The first evidence of potential reassortment events among viruses within the GAMV serogroup was based on results of serological assays and the observation that a virus can react with more than one antiserum.[1] Recently, a study has described four isolates of GAMSVs from pools of *Ad. squamipennis* collected in Panama that represented potential reassortment events.[5] Therefore, the potential implications for this phenomenon in the GAMV serogroup need further investigation.

The histopathological alterations observed in the lungs and livers of newborn chicks experimentally infected with GAMV suggest that these tissues are the target organs for replication of the virus. This fact was confirmed by immunohistochemical studies showing that these organs were most affected. The evidence of tropism of GAMSV for the lungs is a characteristic that has not been reported so far for viruses in the *Orthobunyavirus* genus.

Interestingly, the viruses within Bunyavirales order, the lung tropism has most often been associated with orthohantaviruses (Hantaviridae family) that cause hantavirus pulmonary syndrome in humans.[32,33]

Our serological studies showed the highest prevalence (6.2%) of GAMSV HI antibodies in plasma of birds compared with serum samples obtained from other wild animals (2.6%) or humans (1.5%). These findings agree with a previous serosurvey for GAMSV antibodies conducted in the province of Santa Fé, Argentina, which reported a neutralizing antibody rate of 14.5% in birds, but only 3.4% of other wild animals.[1] These results support the hypothesis that birds are the major vertebrate host for GAMSVs because *Ad. squamipennis* is ornithophilic in its feeding preference.[34] The low prevalence of GAMSV antibodies in humans (1.5%) is probably more a reflection of the mosquito vectors' breeding sites and host preference than of human susceptibility to infection. Currently, there have been no reports of human illness or disease associated with GAMSV infection.

The ecology of the GAMSVs is noteworthy because almost all of the arthropod isolates have been obtained from a single mosquito species, *Ad. squamipennis*. This mosquito has a wide geographic distribution within tropical regions of Central and South America.[35] However, it has particular larval breeding sites. *Aedeomyia squamipennis* breeds in and is mostly restricted to permanent bodies of fresh water (lakes, rivers, ponds, and reservoirs), which have a dense growth of aquatic vegetation, such as *Pistia*, *Salvinia*, *Ludwigia*, and *Azolla*.[36–38] The female *Ad. squamipennis* deposit their eggs on the surface of semi-submerged vegetation; when the larvae hatch, the dense vegetation provides refuge for the developing insects.[37] Thus, the available evidence suggests that adult *Ad. squamipennis* feed primarily on birds.[1,4,38]

Environmental impact studies carried out at the Tucuruí dam construction site[4] and the site of another dam on the Bayano River in central Panama[38] illustrate how human-induced ecologic changes in pristine tropical regions can affect the abundance of the *Ad. squamipennis* and GAMSVs. During the preconstruction period, *Ad. squamipennis* mosquitoes were detected in lower numbers at both dam sites; but after the dams were completed and the impoundment areas flooded, the abundance of this mosquito species increased significantly as the impounded water (lake) filled with floating vegetation. The frequency of GAMSV isolations from *Ad. squamipennis* at both sites also increased dramatically in the post-flooding period.[4,38]

A second interesting observation about the ecology of GAMSVs is the apparent frequency of vertical (transovarial) transmission of the virus in its mosquito vector. During extensive field studies in Panama,[3,36] multiple isolations of GAMSVs were obtained from the field-collected *Ad. squamipennis* eggs, larvae, and pupae as well as from adult males throughout the year. Two of the viruses included in this study (Alajuela, GML382716 and Soberania strain, GML435718) were isolated from a second stage *Ad. squamipennis* larva and a pupa, respectively. These findings indicate that vertical transmission of GAMSVs serogroup viruses is relatively common and probably occurs year round in the Neotropics. However, the relative importance of vertical transmission and the mosquito–vertebrate (presumably avian) cycle in the maintenance of this group of viruses is unknown.

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FIGURE 1. Genome organization of Gamboa strain MARU10962 (**A**). The predicted topology of glycoprotein of Gamboa strain MARU10962 (**B**). This figure appears in color at www.ajtmh.org.

FIGURE 2. Maximum likelihood phylogenetic trees of Gamboa serogroup viruses based on nucleotide sequences of small segment (**A**); medium segment (**B**); and large segment (**C**). Reassortment event of Soberania strain 118 (**D.**) and summary of RDP4 analysis to determine potential reassortant (**E**) This figure appears in color at www.ajtmh.org.

FIGURE 3. Bright-field photomicrograph (HE and immunohistochemistry) of tissue samples obtained on postinfection day 7 from chicks (*Gallus gallus domesticus*) subcutaneously infected with Gamboa virus strain BeAN439546. (**A**) Control lung tissue; (**B**) infected lung tissue showing hemorrhage (circle), blood vessel congestion (arrows), and pulmonary trabecular thickening (asterisk); (**C**) lung with, an inflammatory infiltrate (arrows); (**D**) brown cytoplasmic immunostaining of pulmonary cells (arrows); (**E**) control liver; (**F**) infected hepatic tissue showing a perivascular inflammatory infiltrate (arrows); (**G**) hepatocyte edema (arrows); (**H**) hepatic tissue showing immunostained hepatocytes (arrows); (**I**) control renal tissue; (**J**) infected renal tissue showing edema of renal tubular cells; and (**K**) cytoplasmic immunostaining of renal tubular cells (arrows). This figure appears in color at www.ajtmh.org.

TABLE 1

Gamboa serogroup viruses used in this study

Virus name (complex)	Strain number	Source	Locality	Date collected	GenBank accession numbers (RNA segment)
Gamboa	BeAn448848	*Xenops minutus* (bird)	Tucuruí, Pará, Brazil	May 1985	KX900423 (L) KX900424 (M) KX900425 (S)
Gamboa	AN439546	*Columbidae* (bird)	Tucuruí, Pará, Brazil	May 1985	MG019911 (L) MG019912 (M) MG019913 (S)
Gamboa	BeAr502380	*Aedeomyia squamipennis*	Tucuruí, Pará, Brazil	December 1986	KX900426 (L) KX900427 (M) KX900428 (S)
Gamboa	BeAr503385	*Ad. squamipennis*	Tucuruí, Pará, Brazil	N/A	KX900429 (L) KX900430 (M) KX900431 (S)
Gamboa	TRVL-61469	*Aedes* sp.	Paramaribo, Surinam	1964	KX900441 (L) KX900442 (M) KX900443 (S)
Gamboa	MARU10962	*Ad. squamipennis*	Gamboa, Panama	December 1962	KM272180 (L) KM272181 (M) KM272182 (S)
Gamboa	GAM131	*Ad. squamipennis*	Gamboa, Panama	November 2012	KT950266 (L) KT950270 (M) KT950262 (S)
Pueblo Viejo	75V-2621	*Ad. squamipennis*	Vinces, Ecuador	July 1974	KX900438 (L) KX900439 (M) KX900440 (S)
Alajuela	GML382716	*Ad. squamipennis* (larva)	Juan Mina, Panama	September 1985	KX900432(L) KX900433 (M) KX900434(S)
Alajuela	GML438524	*Ad. squamipennis*	Colon, Panama	February 1987	KX900435 (L) KX900436 (M) KX900437 (S)
Alajuela	MARU11079	*Ad. squamipennis*	Gamboa, Panama	January 1963	KM272186 (L) KM272187 (M) KM272188 (S)
Alajuela	GAM122	*Ad.*	Gamboa, Panama	November 2012	KT950264 (L)

		squamipennis			KT950268 (M) KT950260 (S)
Soberania	GAM130	*Ad. squamipennis*	Gamboa, Panama	November 2012	KT950265 (L) KT950269 (M) KT950261 (S)
Soberania	GAM118	*Ad. squamipennis*	Gamboa, Panama	November 2012	KT950263 (L) KT950267 (M) KT950256 (S)
Soberania	GML903023	*Ad. squamipennis*	Bayano, Panama	1977	KM272177 (L) KM272178 (M) KM272179 (S)
Soberania	GML435718	*Ad. squamipennis* (pupa)	Juan Mina, Panama	March 1986	KM272174 (L) KM272175 (M) KM272176 (S)
Calchaqui	AG83-1347	*Ad. squamipennis*	Santa Fe, Argentina	December 1982	KM272183 (L) KM272184 (M) KM272185 (S)

L = large; M = medium; S = small.

TABLE 2

Results of NT and HI tests, using Gamboa virus (BeAN439546) antigen, done on plasma samples from birds collected in Amazon forest

Order	Family	Number of samples tested by*		Number sero-positive		%	
		NT	HI	NT	HI	NT	HI
Caprimulgiformes	Caprimulgidae	1	18	0	4	0.0	22.2
Columbiformes	Columbidae	14	21	2	2	14.3	9.5
Coraciiformes	Alcedinidae	1	1	0	1	0.0	100.0
	Momotidae	0	1	0	0	–	0.0
Cuculiformes	Cuculidae	1	6	0	0	0.0	0.0
Piciformes	Bucconidae	1	2	0	0	0.0	0.0
	Galbulidae	3	8	2	0	66.7	0.0
Galliformes	Cracidae	0	2	0	0	–	0.0
Gruiformes	Rallidae	0	1	0	0	–	0.0
Passeriformes	Conopophagidae	0	2	0	0	–	0.0
	Dendrocolaptidae	9	21	1	4	11.1	19.0
	Formicariidae	12	0	2	0	16.7	–
	Fringiliidae	15	0	3	0	20.0	–
	Furnariidae	18	6	1	4	5.6	66.7
	Grallariidae	0	1	0	0	–	0.0
	Hirundinidae	8	182	1	0	12.5	0.0
	Onychorhynchidae	0	2	0	0	–	0.0
	Passerellidae	0	8	0	0	–	0.0
	Pipridae	4	12	1	1	25.0	8.3
	Rhynchocyclidae	0	1	0	0	–	0.0
	Thamnophilidae	0	31	0	0	–	0.0
	Thraupidae	42	127	2	14	4.8	11.0
	Tityridae	0	7	0	1	–	14.3
	Troglodytidae	1	18	0	2	0.0	11.1
	Turdidae	0	1	0	0	–	0.0
	Tyraniidae	8	17	1	2	12.5	11.8
	Vireonidae	0	3	0	0	–	0.0
	Xenopidae	0	1	0	0	–	0.0
Pelecaniformes	Ardeidae	0	1	0	0	–	0.0
Piciformes	Ramphastidae	0	3	0	2	–	66.7

Psittaciformes	Psittacidae	4	0	0	0	0.0	–
Galliformes	Phasianidae	0	124	0	2	–	1.6
Total by tests		142	628	16	39	11.3	6.2
Total		770		55		7.1	

HI = hemagglutination-inhibition; NT = neutralization.

* Each plasma was tested using just one of the two techniques (NT or HI).

SUPPLEMENTAL FIGURE 1. Schematic representation of the termini alignments and the RNA fold structures for the different RNA segments of Gamboa virus. Segment large (**A**), Segment medium (**B**), Segment small (**C**), and complementary termini of all segments (**D**).

SUPPLEMENTAL FIGURE 2. Multiple sequence alignments showed the predicted N-terminal endonuclease motifs H, PD, and DxK in RNA-dependent RNA polymerase of Gamboa serogroup viruses.

SUPPLEMENTAL FIGURE 3. Multiple sequence alignments showed the predicted Gn zinc finger and peptide fusion motifs in glycoprotein precursor of Gamboa serogroup viruses.

SUPPLEMENTAL FIGURE 4. Multiple sequence alignments showed the predicted conserved polymerase domains (pre-motif A, motif A–E) in the central region in RNA-dependent RNA polymerase of Gamboa serogroup viruses.

SUPPLEMENTAL FIGURE 5. Maximum likelihood phylogenetic trees of Gamboa serogroup viruses with other representative members of *Orthobunyavirus* genus, based on nucleotide sequences of small segment (**A**); medium segment (**B**); and large segment (**C**). Branches are color-coded according to group. The scale bar indicates evolutionary distance in numbers of substitutions per nucleotides substitutions/site, and the principal bootstrap support levels were indicated. Gamboa virus strains sequenced in this study are highlighted with red color.

SUPPLEMENTAL FIGURE 6. Pairwise genetic similarities (amino acid *p*-distance) of Gamboa serogroup viruses compared with other orthobunyaviruses groups, based on the coding sequencing of nucleoprotein (upper), glycopolyprotein (middle), and RNA-dependent RNA polymerase (below).

SUPPLEMENTAL TABLE 1

The specific set of primers used in RACE method to obtain the 3′ and 5′ terminal sequences

Segment	Virus	RACE 5′ primer SP1	RACE 5′ primer SP2	RACE 3′ primer SP1	RACE 3′ primer SP2	RACE 3′ primer
LRNA	MARU10962	CCCTGTCTGACACCATTTTA	GTTCCAATTGAATGTTGGCA	AATGAAGGAACACTGGATGA	CTATGGAGCAGACACAAATT	ACCCCAATCTTCAAAGTCCA
	GML435718					
	GML382716					
	GML438524					
	Alajuela					
	GML903023					
	TR_61469_					
	BeAR503385	CTCCAGTTGAATATTAGCAT	CAACTCTTGATGGTAGGAAG	AAGGCAGTTTAGAGATGC	GATGACTTCGACTCTCCAGA	GAAAATATAGTCTGCATAAT
	BeAR502380					
	BeAR448848					
	Pueblo_Viejo_	GTTCTAATTGAATATTAGCG	TGTGAAGGTTAGTTTTGTTG	AGAAATGCAATAAAGGAATTG	ACAATTCAGGGATGCCTTCA	GATTTTGATTCACCAGAGAA
	Calchaqui_virus	ATTCCAGTTGTATATTTGCA	GTAAATGTTAGCTTTGTTGT	CAAATTGCTGGATTGCAATGGT	TTCAAAGTTCAATATAGCGT	GATTTTGACTCTCCAGAGAA
MRNA	MARU10962	TTCACATGTTTGGTCGAGTGGG	TAAACCACCCAGTTTTAAT	ACCAGAATTGTAATAAAGCCT	GCTTTAAATTTAAAATGTCA	ATGTAATGCTGCATTAAAGA
	GML435718	CACATGTCTGATCTAATGGC	TGAACCAGCCTGTTTTGAT	ATAGAATTGTTATCAAACCT	GAGTTGCCTGGAGATAATAT	TGCATTGAAGACGCAAATTG
	GML382716	CAAGTCTGGTCTAATGGTA	GGTACAGTGGCAGTTGATTT	AGTAGGATTGTAATCAAACCT	GCAATCAACATGAAGTGCCA	ATGTATTAATTAAAGTTTGT

	GML438524	CAAGTCTGGTCTAATGGTA	GGTACAGTGGCAGTTGATTT	AGTAGGATTGTAATCAAACC	TATGCAATCAACATGAAGT	TCTAGAAGGAGACAATGTAT
	Alajuela	CAAGTCTGGTCTAATGGTA	GGTACAGTGGCAGTTGATTT	GAGCAGGATTGTAATCAAAC	TGCAATCAACATGAAGTGCC	ATTAATTAAAGTTTGTAATG
	GML903023	CACATGTCTGATCTAATGGC	TGAACCAGCCTGTCTTGAT	TAGAATTGTTATCAAACCTA	TATGCTATCAATATGAAAT	GAGTTGCCTGGAGACAATAT
	TR_61469_	TCACATGTCTGGTCTAATGGG	TAAACCATCCTGTTTTGATT	ACTAGAATTGTAATCAAACC	ATGCCTTAAATCTAAAATG	TGCAGCATTAAAAACTCAAA
	BeAR503385	TCACATGTCTGGTCTAATGGG	TAAACCATCCTGTTTTGATT	ACTAGAATTGTAATCAAGCC	GCCTACTATAGAAGAATATG	AAATGTCAAAAACCATTAGA
	BeAR502380	CACATGTCTGATCTAACGGA	TAAACCATCCTGTTTTAATT	ACTAGAATTGTAATCAAGCC	AAATGTCAGAAACCATTAGA	AATTAAAGTATGTAATGCAG
	BeAR448848	CACATGTCTGATCTAACGGA	TAAACCATCCTGTTTTAATT	ACTAGAATTGTAATCAAGCC	CGTTAAATTTAAAATGTCAGAA	GATAGTGATACTTTATCAA
	Pueblo_Viejo_	CACATGTTCGGTCCAATGGA	TGAACCATCCTGTTTTAATT	ACTAGAATTGTAATCAAGCC	AATGCCAAAAGCCTTTGGAC	GACTCAGATAGCCGTTTCGA
	Calchaqui_virus	TCACAAGTCTGATCAAGCGGG	CGGGACTGTTGCAGTGGATT	ACCGAATATTGAGGAATATG	GAGTTGCCTGGAGATACAAT	GACTCAAATAGCAGTTTCA
SRNA	MARU10962	TCATTGCTCCATGCTGCCAG	TCAGAGATCGGGTTGATGAC	GTCATCAACCCGATCTCTGA	GGATCAACTGGCAGCATGGA	AGCAG(A/G)AATGTTTC(T/C/A)TACTG
	GML435718					
	GML382716					
	GML438524					
	Alajuela					
	GML903023					
	TR_61469_					
	BeAR503385					
	BeAR502380					
	BeAR44					

	8848 Pueblo_ Viejo_ Calchaq ui_virus					

RACE = rapid amplification of cDNA ends.

SUPPLEMENTAL TABLE 2

Genetic characteristics of the Gamboa virus serogroup genomes according to the RNA segment, 5′–3′ NCR, genes, proteins, and genome sizes

RNA segment	Virus	Genomic region (nt/aa)								Genome size (nt)
		5′-NCR		N	NSs	Polyprotein M	Polyprotein L	3′-NCR		
SRNA				717 nt	393 nt					
	BeAN448848	88	AGTAGTGTACT<u>G</u>CTCAGT…	238 aa	130 aa	NA	NA	428	…GAGGAGCACACTACT	1,233
				717 nt	393 nt					
	BeAR502380	87	AGTAGTGTACTCCTCAGT…	238 aa	130 aa	NA	NA	340	*Not completed*	1,144*
				717 nt	393 nt					
	BeAR503385	62	*Not completed*	238 aa	130 aa	NA	NA		*Not completed*	1,193*
				717 nt	393 nt					
	GML438524	86	AGTAGTGTACTCCTCAGT…	238 aa	130 aa	NA	NA	438	…GAGGAGCACACTACT	1,241
				717 nt	393 nt					
	GML382716	87	AGTAGTGTACTCCTCAGT…	238 aa	130 aa	NA	NA	342	*Not completed*	1,146*
				717 nt	393 nt					
	TR61469	87	AGTAGTGTACTCCTCAGT…	238 aa	130 aa	NA	NA	43	…GAGGAGCACACT	1,235

								0	ACT	
				717 nt	393 nt					
	Pueblo Viejo	87	AGTAGTGTACT CCTCAGT…	238 aa	130 aa	NA	NA	410	…GAGGA GCACACT ACT	1,214
				717 nt	393 nt					
	Alajuela	86	AGTAGTGTACT CCTCAGT…	238 aa	130 aa	NA	NA	350	…GAGGA GCACACT ACT	1,241
				717 nt	393 nt					
	Calchaqui	94	AGTAGTGTACT CCTCAGT…	238 aa	130 aa	NA	NA	440	…GAGGA GCACACT ACT	1,251
				717 nt	393 nt					
	GAM118	0	*Not recovered*	238 aa	130 aa	NA	NA	0	*Not recovered*	717*
				717 nt	393 nt					
	GAM122	0	*Not recovered*	238 aa	130 aa	NA	NA	0	*Not recovered*	717*
				717 nt	393 nt					
	GAM130	0	*Not recovered*	238 aa	130 aa	NA	NA	0	*Not recovered*	717*
				717 nt	393 nt					
	GAM131	0	*Not recovered*	238 aa	130 aa	NA	NA	0	*Not recovered*	717*
	MARU 10962	87	AGTAGTGTACT CCTCAGT…	717 nt	393 nt	NA	NA	350	*Not completed*	1,154*
				238 aa	130 aa					

				717 nt	393 nt					
	GML90 3023	8 6	AGTAGTGTACT CCTCAGT…	238 aa	130 aa	NA	NA	1 0 1	*Not completed*	904*
				717 nt	393 nt					
	GML43 5718	8 6	AGTAGTGTACT CCTCAGT…	238 aa	130 aa	NA	NA		…GAGGA GCACACT ACT	1,240
				717 nt	393 nt					
	BeAn4 39546	8 6	AGTAGTGTACT CCTCAGTA…	238 aa	130 aa	NA	NA	3 9 1	…TTCTTC AATTCAT TTTAGT	1.194*
MR NA						4,782 nt				
	BeAN4 48848	4 2	AGTAGTGTACT GCCGTGTT…	NA	NA	1,594 aa	NA	2 4 1	…ACACG GTAGCA CACTACT	5.065
						4,782 nt				
	BeAR5 02380	4 2	AGTAGTGTACT GCCGTGTT…	NA	NA	1,594 aa	NA	2 4 1	…ACACG GTAGCA CACTACT	5.065
						4,782 nt				
	BeAR5 03385	4 2	AGTAGTGTACT GCCGTGTT…	NA	NA	1,594 aa	NA	2 4 0	...ACACG GTAGCA CACTACT	5.064
						4,779 nt				
	GML43 8524	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,593 aa	NA	2 3 6	…ACACG GTAGCA CACTACT	5.057
						4,785 nt				

	GML38 2716	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,594 aa	NA	2 3 8	…ACACG GTAGCA CACTACT	5.065
						4,782 nt				
	TR_61 469	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,594 aa	NA	2 4 0	…ACACG GTAGCA CACTACT	5.064
						4,782 nt				
	Pueblo Viejo	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,594 aa	NA	2 7 2	…ACACG GTAGCA CACTACT	5.096
						4,779 nt				
	Alajuel a	4 2	AGTAGTGTACT ACCGTGTT…	NA	NA	1,594 aa	NA	2 4 1	…ACACG GTAGCA CACTACT	5.062
						4,773 nt				
	Calcha qui	4 2	AGTAGTGTACT ACCGTGTT…	NA	NA	1,591 aa	NA	1 5 6	…ACACG GTAGCA CACTACT	4.971
						4,782 nt				
	GAM1 18	0	AGTAGTGTACT ACCGTGTT…	NA	NA	1,594 aa	NA	0	…ACACG GTAGCA CACTACT	4.782
						4,782 nt				
	GAM1 22	0	AGTAGTGTACT ACCGTGTT…	NA	NA	1,594 aa	NA	0	…ACACG GTAGCA CACTACT	4.782
						4,782 nt				
	GAM1 30	0	AGTAGTGTACT ACCGTGTT…	NA	NA	1,594 aa	NA	0	…ACACG GTAGCA	4.782

									CACTACT	
						4,782 nt				
	GAM1 31	0	AGTAGTGTACT ACCGTGTT…	NA	NA	1,594 aa	NA	0	…ACACG GTAGCA CACTACT	4.782
	MARU 10962	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	4,782 nt	NA	2 3 4	…ACACG GTAGCA CACTACT	5.058
						1,594 aa				
						4,779 nt				
	GML90 3023	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,593 aa	NA	1 9 0	…ACACG GTAGCA CACTACT	5.011
						4,779 nt				
	GML43 5718	4 2	AGTAGTGTCCT ACCGTGTT…	NA	NA	1,593 aa	NA	1 9 0	...ACACG GTAGCA CACTACT	5.011
						4,782 nt				
	BeAn4 39546	0	*Not recovered*	NA	NA	1,593 aa	NA	2 2 4	…AGAAA ATTTTTG TTTTAAA	5,006*
LR NA							6,882 nt			
	BeAN4 48848	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	6 7	*Not completed*	6,936*
							6,882 nt			
	BeAR5 02380	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	6 6	*Not completed*	6,935*
							6,882 nt			
	BeAR5	4	AGTAGTGTACT	NA	NA	NA	2,293	6	*Not*	6,936*

	03385	7	CCTC…				aa	7	*completed*	
							6,882 nt			
	GML43 8524	4 6	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	7 6	…TATGA GGAGCA CACT	6.944
							6,882 nt			
	GML38 2716	4 7	AGTAGTGTACT CCTC..….	NA	NA	NA	2,293 aa	7 6	…TATGA GGAGCA CACT	6.945
							6,882 nt			
	TR_61 469	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	7 6	…TATGA GGAGCA CACT	6.945
							6,882 nt			
	Pueblo Viejo	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	7 6	…TATGA GGAGCA CACT	6.945
							6,882 nt			
	Alajuel a	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,293 aa	7 6	…TATGA GGAGCA CACT	6.945
							6,760* nt			
	Calcha qui	4 7	AGTAGTGTACT CCTC…	NA	NA	NA	2,253 aa	0	*Not recovered*	6,760*
							6,882 nt			
	GAM1 18	0	*Not recovered*	NA	NA	NA	2,293 aa	0	*Not recovered*	6,882*
							6,882 nt			
	GAM1	0	*Not recovered*	NA	NA	NA	2,293	0	*Not*	6,882*

	22						aa		*recovered*	
							6,882 nt			
	GAM130	0	*Not recovered*	NA	NA	NA	2,293 aa	0	*Not recovered*	6,882*
							6,882 nt			
	GAM131	0	*Not recovered*	NA	NA	NA	2,293 aa	0	*Not recovered*	6,882*
	MARU10962	47	AGTAGTGTAGTCCTC…	NA	NA	NA	6,882 nt	76	…TATGAGGAGCACACT	6.945
							2,293 aa			
							6,882 nt			
	GML903023	46	AGTAGTGTAGTCCTC..	NA	NA	NA	2,293 aa	76	…TATGAGGAGCACACT	6.944
							6,882 nt			
	GML435718	47	AGTAGTGTAGTCCTC…	NA	NA	NA	2,293 aa	76	…TATGAGGAGCACACT	6.945
							6,822 nt			
	BeAn439546	47	AGTAGTGTACTCCTC…	NA	NA	NA	2,273 aa	54	…AAACTTAAAATTAAT	6,923*

aa = amino acids; L = large; M = medium; NA = not applicable; NCR = non coding regions; nt = nucleotides.

* Genomes are incomplete gray shaded isolates: genomes are complete highlighted genomes are complete (including the 5′ and 3′ NCRs).

SUPPLEMENTAL TABLE 3

Estimates of average evolutionary divergence over sequence pairs within groups

Clade	Nucleoprotein	Glycoprotein	RdRp
Gamboa	3.50	10.8	2.1
Koongol	n/c	n/c	n/c
Simbu clade A	20.2	37.5	24.2
Simbu clade B	14.4	47.4	24.7
Simbu Leanyer	n/c	n/c	n/c
Bunyamwera	13.9	27.5	19.3
Guaroa	n/c	n/c	n/c
Wyeomyia	21.7	22.7	16.9
California encephalits	15.7	19.7	12.8
Bwamba	14.5	27.7	23.5
Nyando	30.1	53.6	39.8
Guama	9.8	25.4	07.2
Capim	13.2	24.2	12.1
Enseada	n/c	n/c	n/c
Group C	16.3	16.4	13.7
Mapputta	24.8	40.5	31.7
Tete	34.1	43.3	32.5
Brazoran	n/c	n/c	n/c

RdRp = RNA-dependent RNA polymerase. The number of amino acid differences per site from averaging over all sequence pairs within each clade. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances.

SUPPLEMENTAL TABLE 4

Nucleotide (upper) and amino acid (lower) identity comparisons for Gamboa serogroup viruses and representative orthobunyaviruses

		GAMV	ALJV	PVV	CQIV	BUNV	WYOV	OROV	CARV	LACV	SBV
GAMV	S	–	100.00	92.02	85.29	35.74	35.32	33.76	39.41	35.86	34.19
	M	–	83.18	94.35	77.20	35.32	33.31	27.59	28.47	37.16	28.14
	L	–	99.65	95.38	93.39	49.10	49.32	47.54	47.63	54.62	46.60
ALJV	S	97.21	–	92.02	85.29	35.74	35.32	33.76	39.41	35.86	34.19
	M	74.55	–	82.49	75.88	35.15	34.40	27.30	28.18	37.06	27.91
	L	98.77	–	95.51	93.56	49.01	49.32	47.54	47.63	54.62	46.50
PVV	S	90.79	90.93	–	86.13	35.32	34.89	32.91	40.25	36.29	35.04
	M	83.00	74.74	–	76.88	35.32	33.63	27.71	28.54	37.66	27.71
	L	82.84	82.32	–	93.12	49.10	49.32	47.67	47.50	54.97	46.73
CQIV	S	83.26	82.57	81.87	–	34.89	34.89	36.04	38.98	36.71	34.19
	M	71.33	70.66	71.04	–	35.09	33.90	27.48	28.31	38.48	28.16
	L	79.14	79.20	79.79	–	49.49	49.71	47.85	48.27	54.84	46.75
BUNV	S	46.61	46.47	47.88	47.88	–	63.52	41.63	43.22	43.16	34.19
	M	44.59	44.89	44.49	44.66	–	48.68	32.74	33.15	42.27	31.10
	L	54.91	54.94	55.65	54.79	–	67.29	50.29	51.22	55.83	48.70

WYOV	S	45.34	45.48	45.48	43.93	64.67	–	39.91	41.10	49.57	42.49
	M	45.03	44.83	44.86	44.49	56.31	–	31.38	32.30	41.96	30.70
	L	56.01	55.91	56.24	56.02	67.13	–	47.99	50.78	55.34	49.16
OROV	S	45.11	45.11	45.67	45.96	50.00	48.01	–	41.88	41.88	69.83
	M	40.33	40.09	40.12	39.80	45.92	46.06	–	30.47	31.07	35.17
	L	53.81	53.84	53.68	54.02	55.99	55.43	–	52.77	51.59	57.93
CARV	S	48.75	48.89	49.31	48.61	48.10	49.79	49.37	–	42.86	39.57
	M	40.58	41.60	40.50	40.21	46.13	46.92	46.06	–	32.40	28.36
	L	54.74	54.53	54.28	54.94	57.34	58.07	58.08	–	51.12	49.36
LACV	S	47.06	47.34	47.06	47.90	53.05	56.88	51.63	52.02	–	43.59
	M	45.19	46.16	45.90	45.89	51.80	51.52	44.24	46.09	–	31.57
	L	58.42	58.61	58.97	59.26	58.73	59.41	56.20	56.75	–	50.15
SBV	S	45.54	45.12	45.97	46.11	46.87	50.57	69.53	50.91	53.90	–
	M	39.24	39.31	39.01	38.95	43.91	45.36	46.69	42.86	43.09	–
	L	54.28	54.45	54.03	53.67	55.39	55.66	60.24	55.84	55.40	–

ALJV = Alajuela; BUNV = Bunyamwera; CARV = Caraparu; CQIV = Calchaqui; GAMV = Gamboa; L = large; LACV = La Crosse; M = medium; OROV = Oropouche; PVV = Pueblo Viejo; S = small; WYOV = *Wyeomyia*.

Figure 1

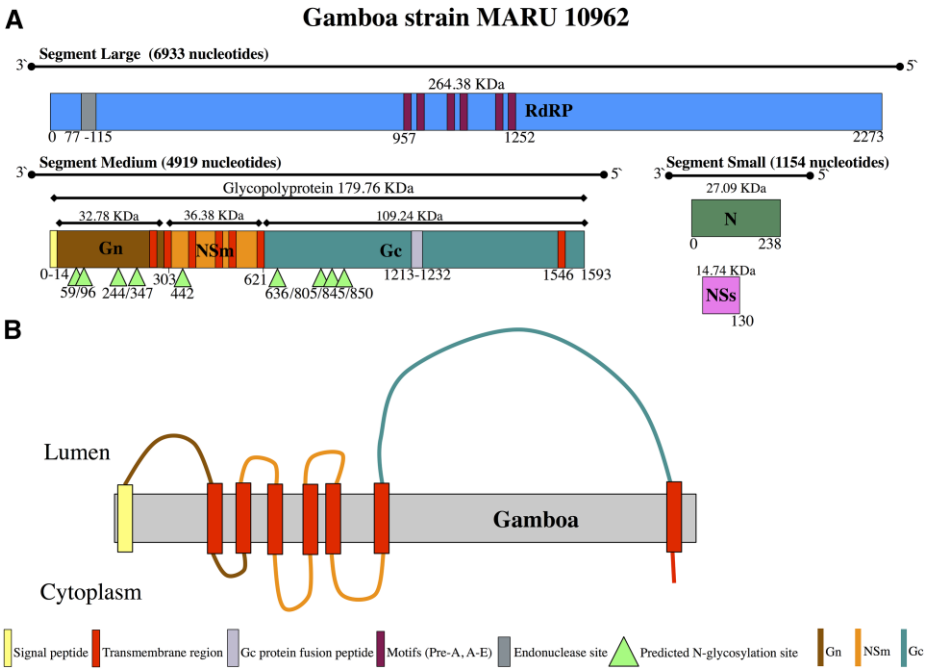
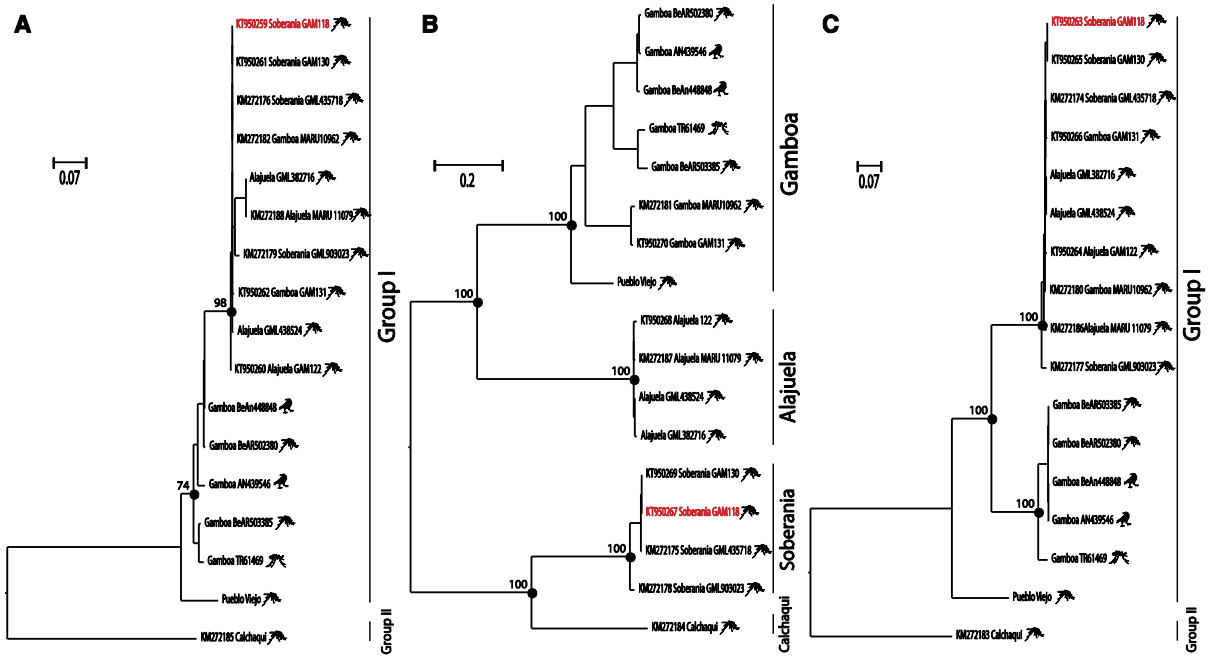
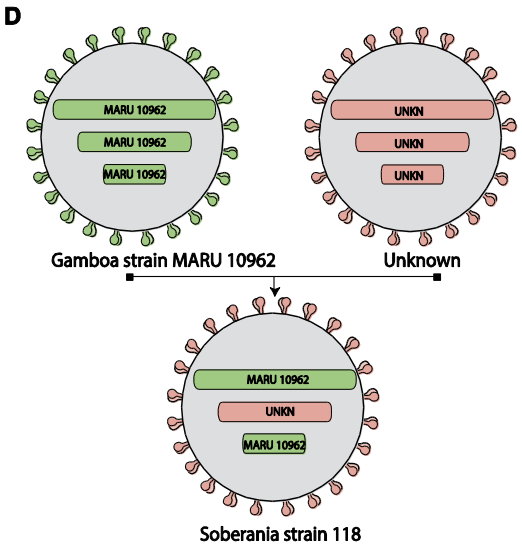

A
Gamboa strain MARU 10962
Segment Large (6933 nucleotides)
3`
5`
264.38 KDa
RdRP
0 77 -115
957
1252
2273
Segment Medium (4919 nucleotides)
Glycopolyprotein 179.76 KDa
32.78 KDa
36.38 KDa
109.24 KDa
Gn
NSm
Gc
0-14
59/96
244/347
303
442
621
636/805/845/850
1213-1232
1546
1593
Segment Small (1154 nucleotides)
27.09 KDa
N
0
238
14.74 KDa
NSs
130
B
Lumen
Gamboa
Cytoplasm
Signal peptide
Transmembrane region
Gc protein fusion peptide
Motifs (Pre-A, A-E)
Endonuclease site
Predicted N-glycosylation site
Gn
NSm
Gc

Figure 2

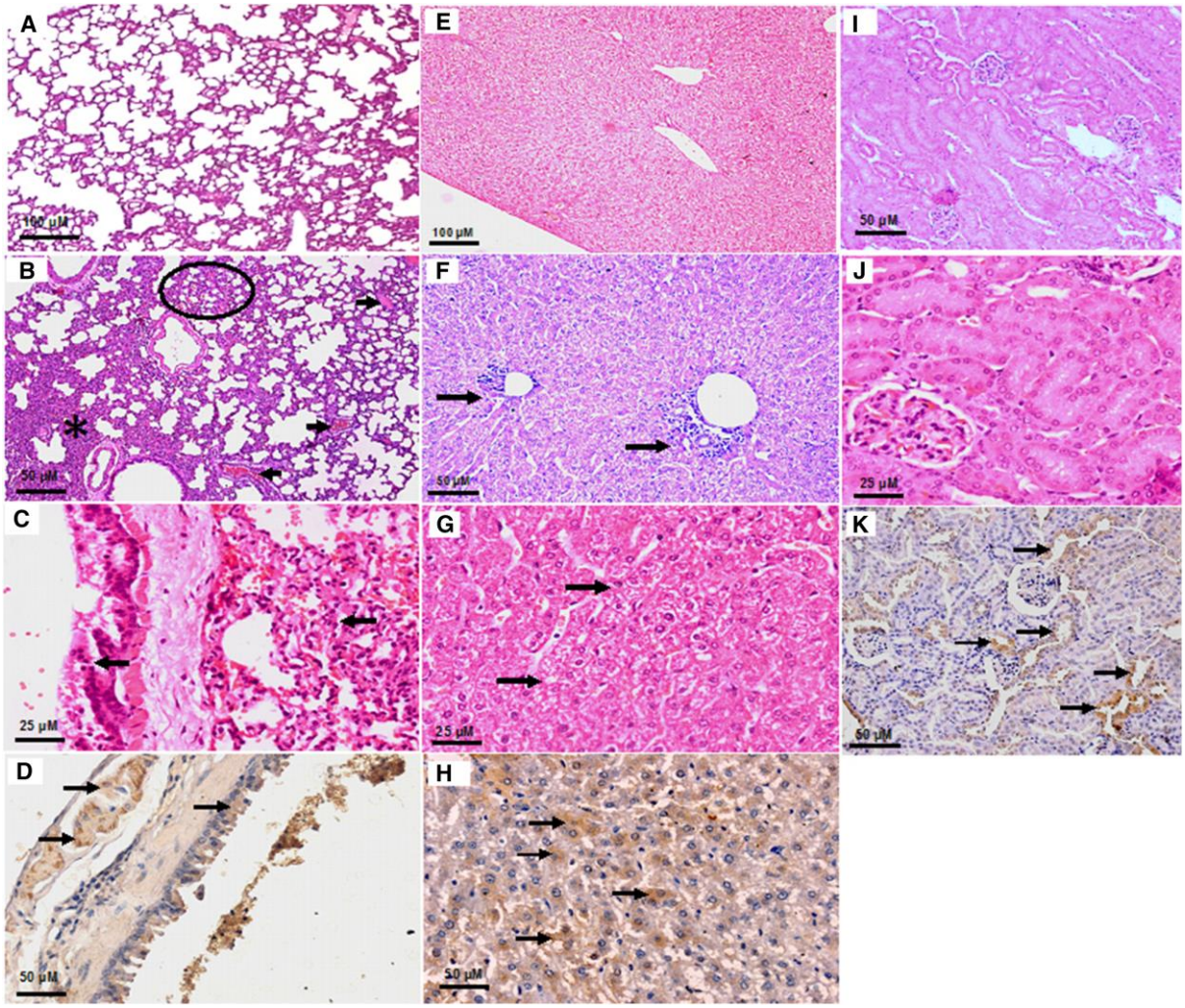




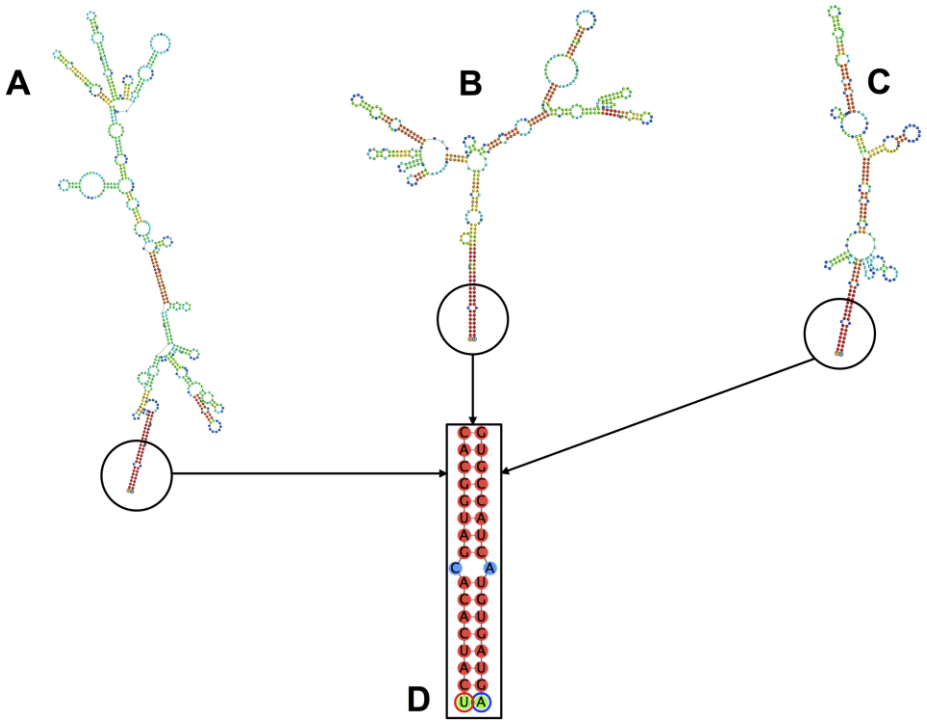
E

Methods	Soberania strain 118
RDP	6.816×10^{-195}
GENECONV	1.454×10^{-227}
BootScan	4.871×10^{-224}
MaxChi	1.288×10^{-42}
Chimaera	2.154×10^{-47}
SiScan	3.042×10^{-100}
3Seq	1.571×10^{-81}

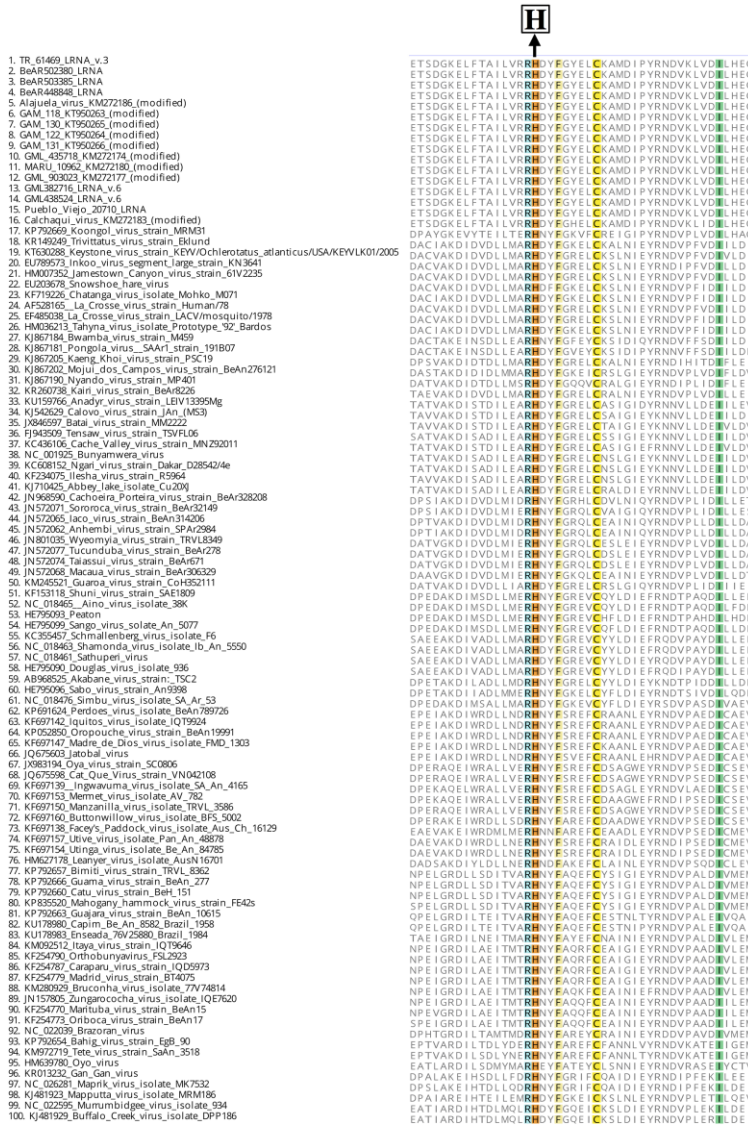
Figure 3


A
100 µM
E
100 µM
I
50 µM
B
50 µM
F
50 µM
J
25 µM
C
25 µM
G
25 µM
K
50 µM
D
50 µM
H
50 µM

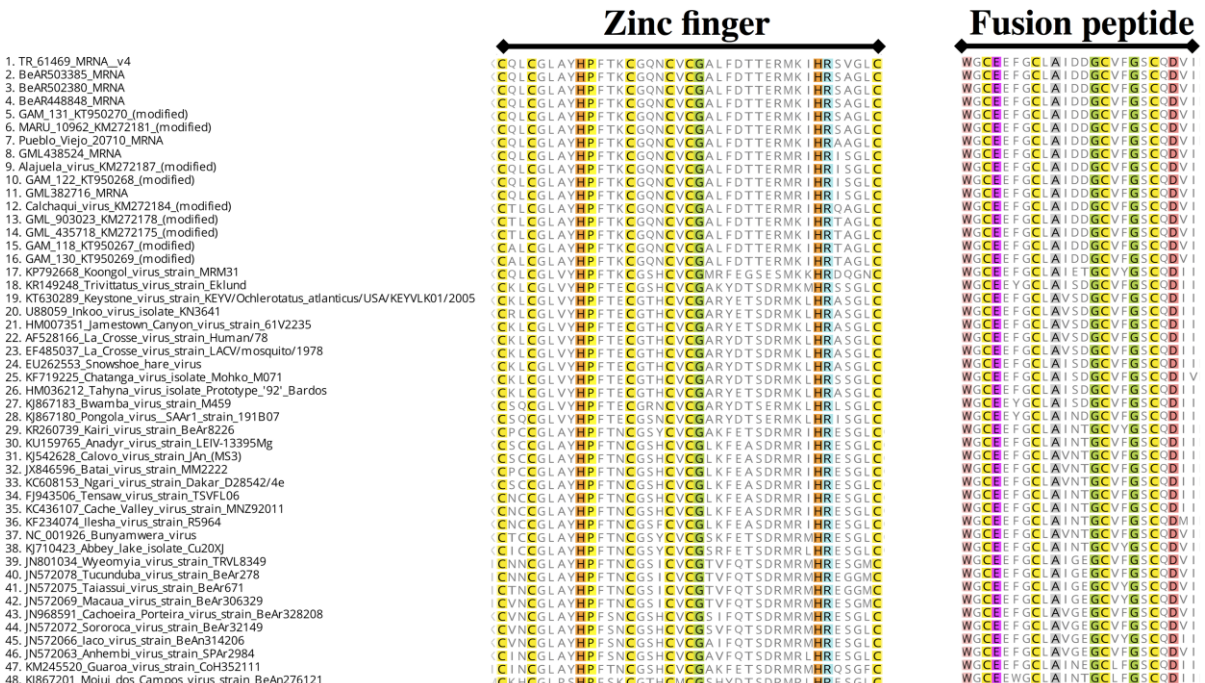
Supplemental Figure 1



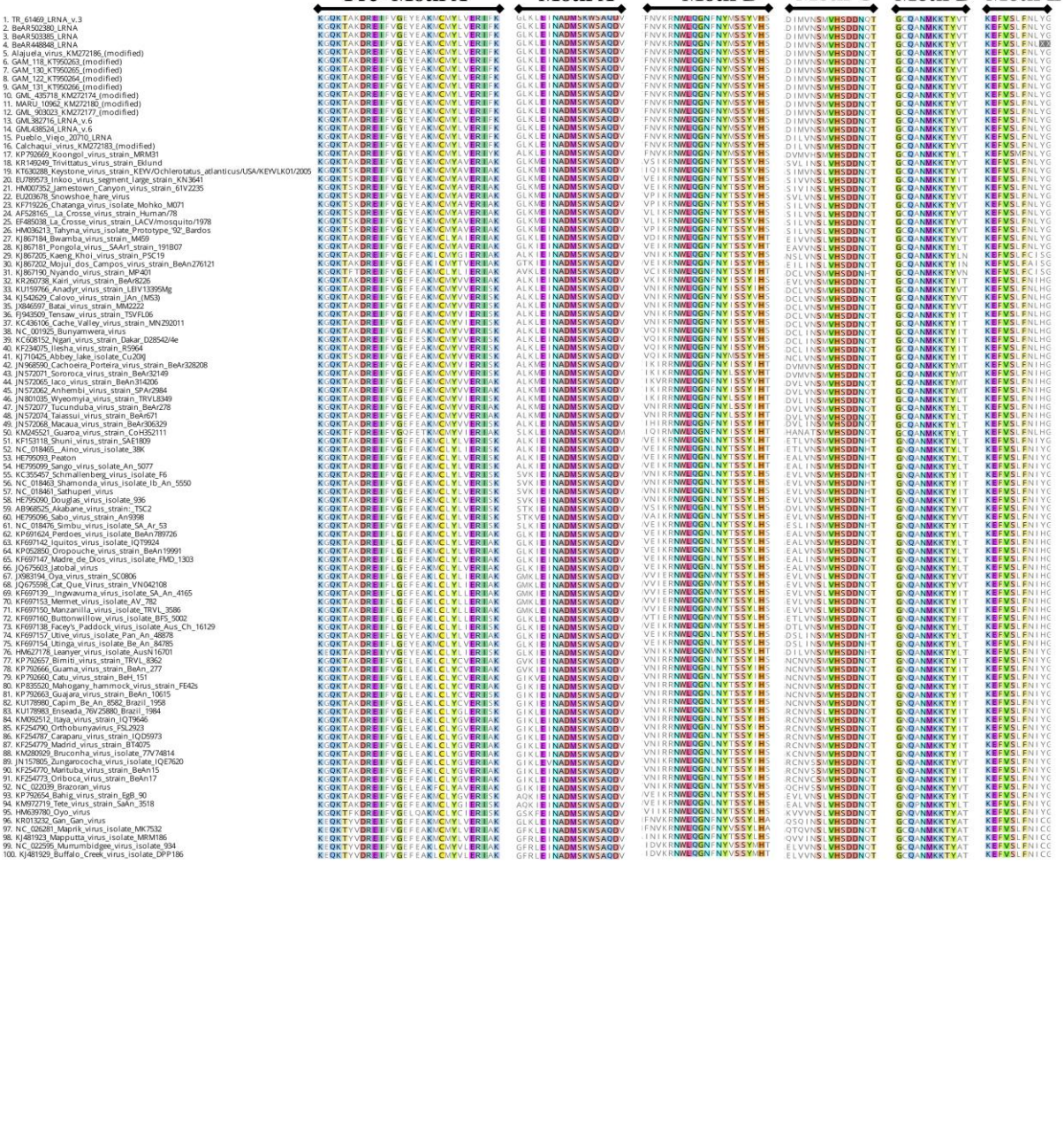
Supplemental Figure 2



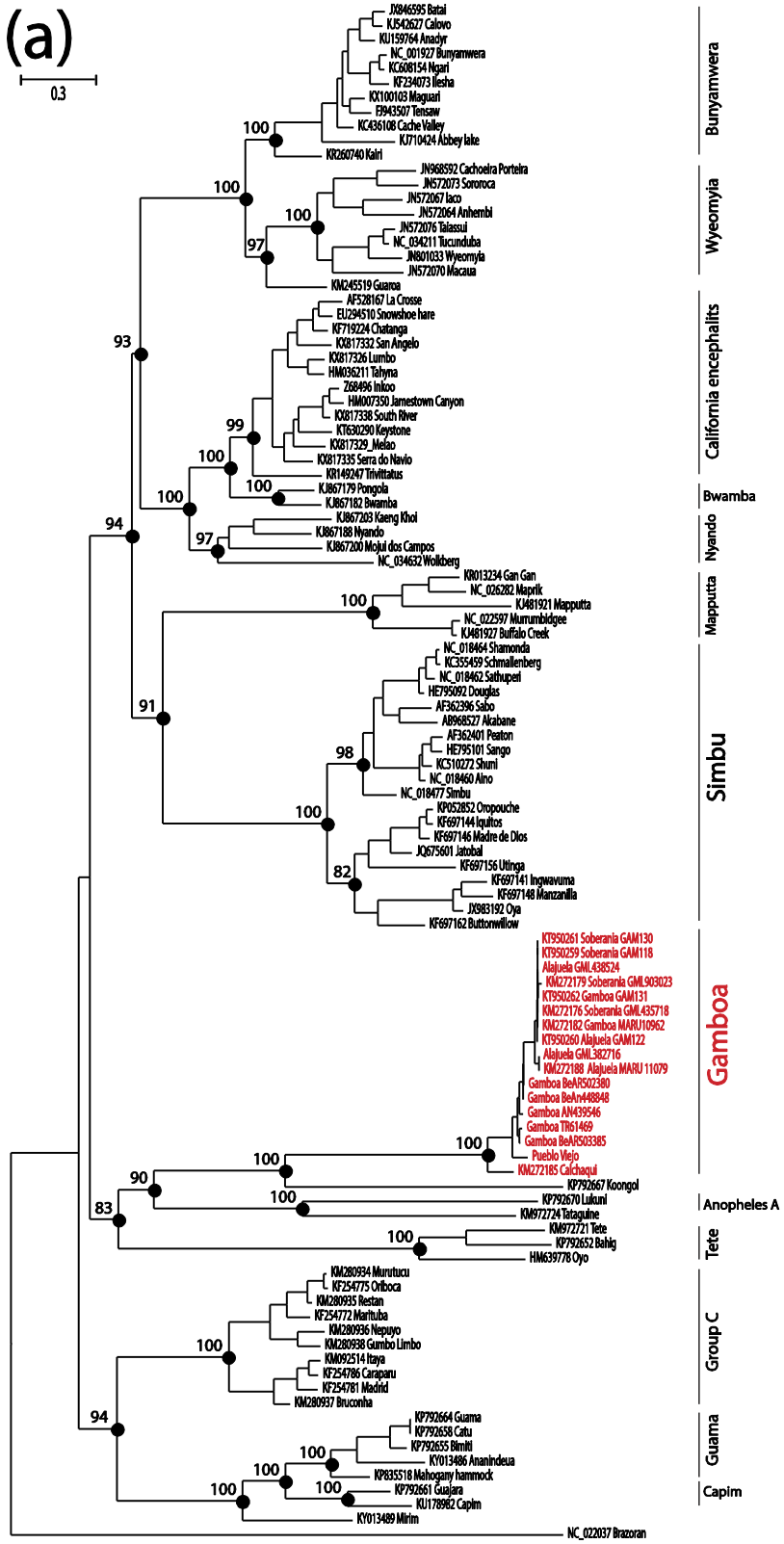
Supplemental Figure 3



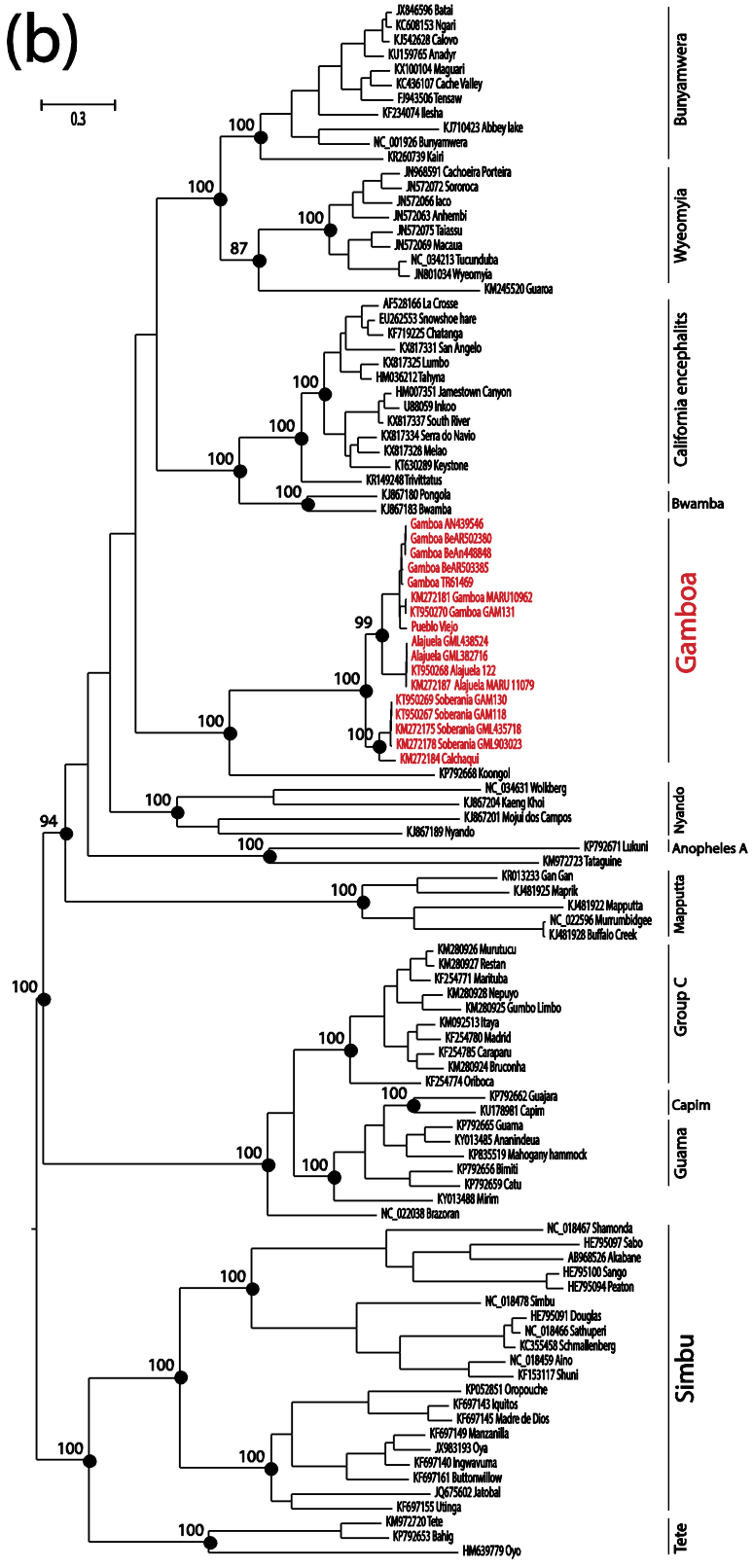
Supplemental Figure 4



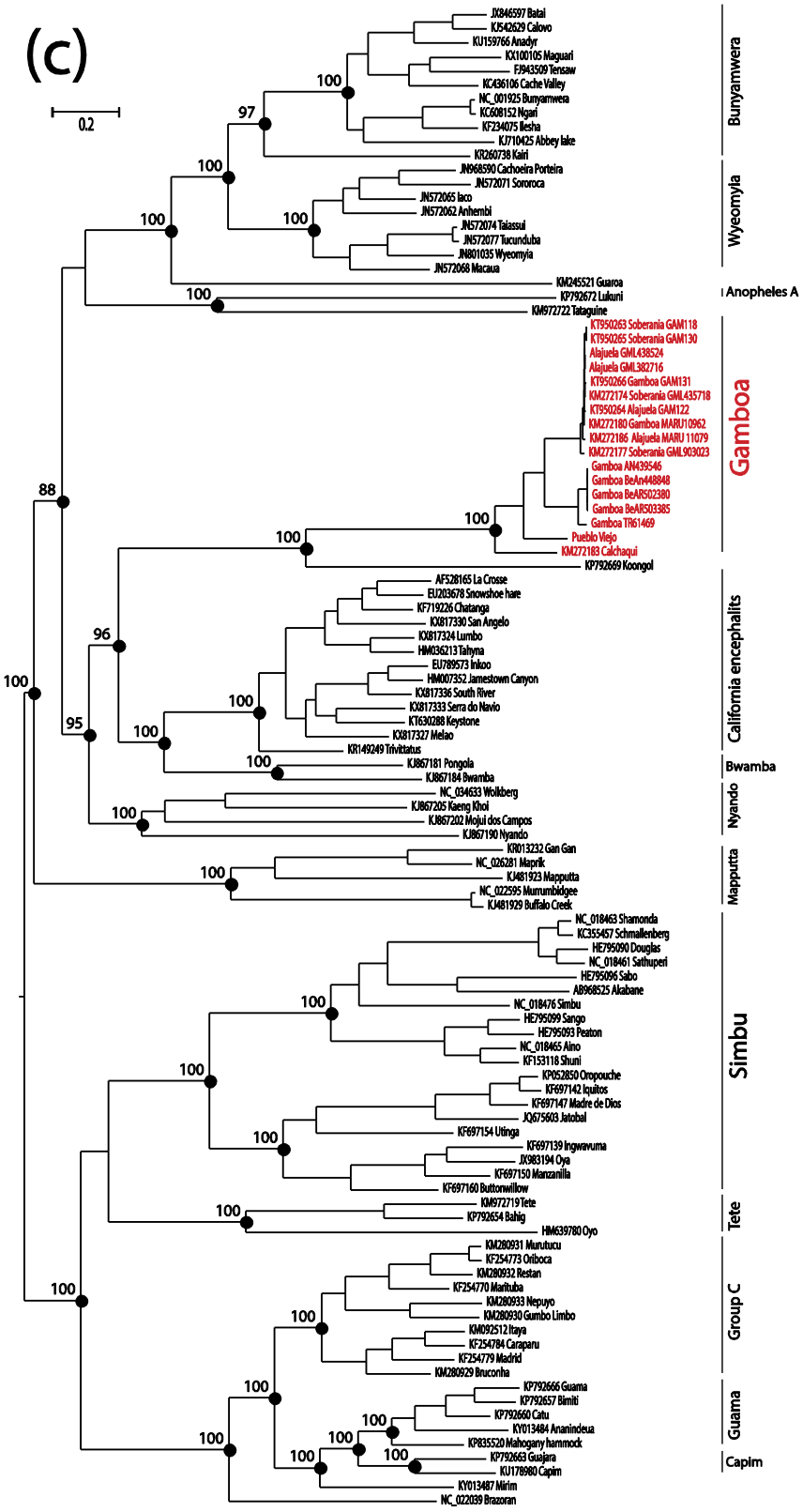
Supplemental Figure 5-1



Supplemental Figure 5-2



Supplemental Figure 5-3



Supplemental Figure 6