

## Genetic Characterization of the Patois Serogroup (Genus *Orthobunyavirus*; Family *Peribunyaviridae*) and Evidence That Estero Real Virus is a Member of the Genus *Orthonairovirus*

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**Abstract.** Estero Real virus (ERV) was isolated in 1980 from *Ornithodoros tadaridae* ticks collected in El Estero Real, Sancti Spiritus, Cuba. Antigenic characterization of the isolate based on serological methods found a relationship with Abras and Zegla viruses and, consequently, the virus was classified taxonomically within the Patois serogroup. Given the fact that genetic characterization of Patois serogroup viruses has not yet been reported and that ERV is the only virus within the Patois serogroup isolated from ticks, we recently conducted nearly complete genome sequencing in an attempt to gain further insight into the genetic relationship of ERV with other Patois serogroup viruses and members of *Peribunyaviridae* family (*Bunyavirales* order). With the exception of ERV, our sequencing and phylogenetic studies revealed the close relationship of the Patois serogroup viruses to each other, forming a clear divergent clade from other members of the *Orthobunyavirus* genus (*Peribunyaviridae* family). Notably, our analysis also revealed that ERV forms a monophyletic clade that is closely related to species of the *Orthonairovirus* genus (*Nairoviridae* family) in all the genome segments. In light of these findings, we believe that the taxonomic classification of ERV should be revised.

### INTRODUCTION

The *Bunyavirales* order contains more than 300 viruses that have been reclassified by the International Committee on Taxonomy of Viruses (ICTV) in nine families, which includes *Hantaviridae*, *Feraviridae*, *Fimoviridae*, *Jonviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phasmaviridae*, *Phenuiviridae*, and *Tospoviridae*.<sup>1</sup> Based on this new classification, four of the nine virus families, including *Hantaviridae*, *Nairoviridae*, *Peribunyaviridae*, and *Phenuiviridae*, contain viruses that are pathogenic in humans. The family *Peribunyaviridae* is the largest with more than 170 named viruses subdivided into 18 serogroups on the basis of serological reactivity.<sup>2</sup> Among them, the Patois serogroup is one of the least studied group of viruses despite their frequent isolation during ecological investigations. Viruses in this serogroup have been isolated from mosquitoes in South, Central, and North America, and rodents have been incriminated as the main reservoir host.<sup>3,4</sup> Currently, at least seven viruses have been grouped within the Patois serogroup: Pahayokee virus (PAHV), Shark River virus (SRV), Patois virus (PATV), Zegla virus (ZEGV), Abras virus (ABRV), Babahoyo virus (BABV), and Estero Real virus (ERV). The PATV and ZEGV were originally isolated from mosquitoes collected in Panama in 1961,<sup>5</sup> and initial antigenic characterization of these viruses identified them as members of the Group C viruses; however, subsequent studies reclassified them as members of the Patois serogroup.<sup>6</sup> Both PATV and ZEGV were frequently isolated along the tropical Gulf coast of southeastern Mexico as part of ecological investigations conducted during 1963–1968.<sup>7,8</sup> These studies also revealed the presence of hemagglutination inhibition and neutralizing

antibodies to PATV in sera of people residing at the study site and in terrestrial wild mammals, including cotton rats, opossums, and raccoons.<sup>8</sup> The PAHV and SRV were isolated in 1963 and 1964, respectively, from *Culex (Melanoconion)* species captured in the Everglades National Park in Florida<sup>6</sup>; however, SRV-like viruses have also been isolated from sentinel hamsters in Mexico and Guatemala providing evidence about their vast geographic distribution.<sup>8</sup> ABRV and BABV were isolated from *Culex* spp. mosquitoes and from the blood of sentinel hamsters collected in the coastal areas of Ecuador and found to be closely related by complement fixation but distinct to other described members of the Patois serogroup.<sup>9</sup> Last, ERV was isolated from argasid *Ornithodoros tadaridae* ticks collected from leaves of a palm tree colonized by bats in El Estero Real in Cuba in April 1980 and, to this date, it constitutes the only Patois serogroup virus isolated from ticks.<sup>10</sup> Because of the unusual association of a Patois serogroup virus with ticks and the limited information available about the genetic relationship of this group of viruses with other members of the *Bunyavirales* order, we initiated a coordinated effort to determine the complete genome nucleotide (nt) sequences of six of the Patois serogroup viruses described to date. We report here our phylogenetic analyses which confirm that the Patois serogroup viruses formed a divergent clade from other members of the *Orthobunyavirus* genus (*Peribunyaviridae* family) and, more importantly, we provide evidence that ERV is an orthonairovirus.

### MATERIALS AND METHODS

**Virus strains and RNA extraction.** Prototype Patois serogroup viruses were obtained from the World Reference Center for Arboviruses and Emerging Viruses and propagated in African green monkey kidney (Vero) cells and maintained at 37°C. The cell cultures were examined daily for evidence of cytopathic effect (CPE). On the appearance of CPE or 10 days after virus inoculation, cell culture supernatants were harvested and clarified by centrifugation, and viral RNA was

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TABLE 1  
Viruses included in this study

| Virus name        | Abbreviation | Strain  | Collection date | Source of isolate              | Location                              | Accession numbers            |
|-------------------|--------------|---------|-----------------|--------------------------------|---------------------------------------|------------------------------|
| Abras virus       | ABRV         | 75V1183 | 8/21/1974       | <i>Culex (Mel) paracrybda.</i> | Naranjal, Ecuador                     | MH017269, MH017275, MH017287 |
| Babahoyo virus    | BABV         | 75V2858 | 2/28/1975       | <i>Culex (Mel) ocosa</i>       | Abras, Ecuador                        | MH017270, MH017276, MH017282 |
| Patois virus      | PATV         | 63A49   | 1963            | <i>Culex</i> spp               | Veracruz, Mexico                      | MH017273, MH017277, MH017283 |
| Shark River virus | SRV          | 64U80   | 1964            | <i>sentinel hamster</i>        | Mexico                                | MH017271, MH017278, MH017284 |
| Zegla virus       | ZEGV         | BT5012  | 6/23/1961       | <i>Sigmodon hispidus</i>       | Almirante, Panama                     | MH017272, MH017279, MH017285 |
| Estero Real       | ERV          | K329    | 04/26/1980      | <i>Ornithodoros tadaridae</i>  | El Estero real, Sancti Spiritus, Cuba | MH017274, MH017280, MH017286 |

extracted as previously described<sup>11</sup> using Trizol reagent (Invitrogen, Carlsbad, CA).

**Next generation sequencing.** Following RNA extraction, viral RNA was fragmented and a sequence library was prepared and sequenced on a HiSeq 1000 using the 2 × 50 paired-end protocol. Reads were quality-filtered and assembled using the de novo assembly program ABySS.<sup>12</sup> Host reads were filtered out before the de novo assembly. The longest contigs were selected and reads were mapped back to the contigs using bowtie<sup>2</sup><sup>13</sup> and then visualized with the Integrated Genomics Viewer<sup>14</sup> to verify that the assembled contigs were correct. Total reads ranged from 1.5 to 12 million and the percentage of reads mapping to the virus genome in each sample ranged from 12% to 33%. Additional details are available on request. The complete genome sequences of Patois serogroup viruses obtained in this study were deposited in the GenBank. Accession numbers are listed in Table 1.

**Genome characterization.** Virus genomes were evaluated regarding size, open reading frames (ORF) descriptions,

5' and 3' noncoding regions, and conserved motifs with Geneious 9.1.2 (Biomatters, Auckland, New Zealand). We submitted data to the TOPCONS webserver for identification of transmembrane regions and signal peptide, and we submitted data to the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) for identification of glycosylation sites. The annotations of protein domains in the M segment were performed with InterProScan in Geneious 9.1.2 (Biomatters) and Conserved Domain Database.<sup>15</sup>

**Phylogenetic analysis.** Maximum likelihood (ML) phylogenetic trees were reconstructed using alignments of complete coding sequences of all RNA segments of the Patois serogroup viruses and representative orthobunyaviruses (S, M, and L) available in the GenBank database (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments were carried out using the combinations of PROMALS3D and Gblocks method.<sup>16,17</sup> The phylogenies were inferred using IQ-TREE version 1.4.3 software under the best-fit model based on Bayesian Information Criterion determined by Modelfinder with 1,000 replicates.<sup>18,19</sup>

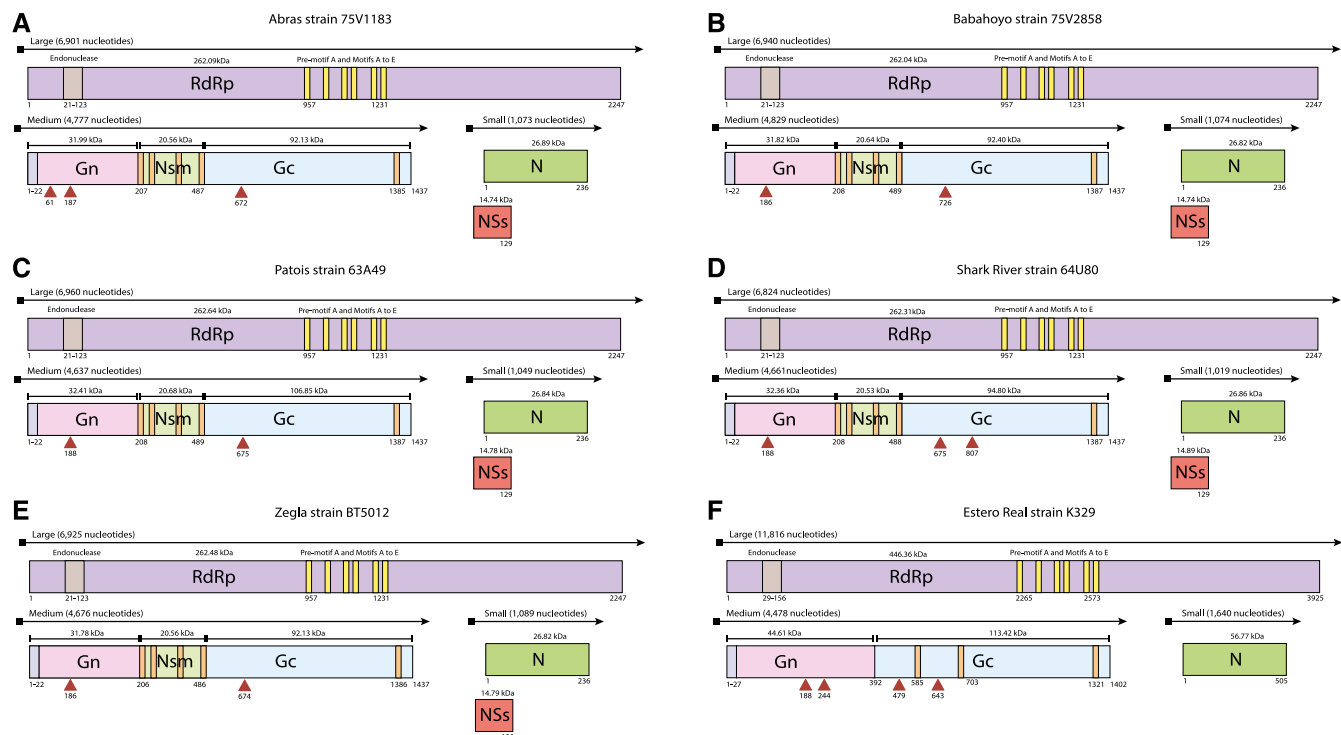


FIGURE 1. Genome organization of Patois serogroup. (A) Abras virus, (B) Babahoyo virus, (C) Patois virus, (D) Shark River virus, (E) Zegla virus, and (F) Estero Real virus. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).



between amino acid positions 389 to 392 (Figure 1F). In addition, we identified the Zinc finger region, which is important to the viral RNA binding site and probably is involved in viral assembly.<sup>23</sup> Therefore, the GPC shows a topology similar to members of the *Hughes* serogroup (*Hughes orthonairovirus* species group).<sup>22</sup>

Analyses of the L segment sequences revealed that viruses in the Patois serogroup possess an L segment ranging from 6,824 to 6,960 nt in length that codes for an RNA dependent RNA polymerase (RdRp) of 2,247 amino acids, with a predicted molecular weight of ~262 kDa. Surprisingly, the L segment of ERV is 11,816 nt in length and encodes an RdRp of 3,925 aa, with a predicted molecular weight of ~446.36 kDa. However, all the proteins contain conserved polymerase activity domains consisting of pre-motif A and motifs A through E, which are highly conserved in negative sense RNA viral polymerases and common to all other *Bunyvirales* characterized so far<sup>24</sup> (Figure 1). In addition, the ERV RdRp contains a characteristic catalytic ovarian tumor-like cysteine protease motif that is common to all orthonairoviruses.<sup>22</sup>

**Phylogenetic relationship of Patois serogroup viruses with other orthobunyaviruses.** The phylogenetic trees that were generated based on amino acid sequences of 72 orthobunyaviruses produced 14 monophyletic groups, but with different topologies in each segment. Thirteen of the well-supported clades corresponded to already established serogroups: Bunyamwera, Wyeomyia, California encephalitis, Bwamba, Nyando, Simbu, Mappoutta, Gambia, Koongol, Tete, Group C, Guama, and Capim (Figure 2). Furthermore, we assigned a divergent clade as a proposed new “group,” containing the six viruses of the Patois serogroup (Figure 2). The phylogenetic analysis based on all segment sequences reveals that the members of the Patois serogroup form a monophyletic clade with Bellavista virus. This observation suggests that these viruses might have the same evolutionary origin. In addition, the Patois serogroup viruses were also closely related to Guama, Capim, and Group C groups.

We next conducted pairwise amino acid sequence distance analysis based on the N, GPC, and RdRp sequences of Patois serogroup and representative orthobunyaviruses, and results of an inter-clade analysis are shown in Figure 3. The genetic distances of the Patois serogroup viruses to other orthobunyaviruses was estimated to be ~57%, ~60%, and ~48% for the N, GPC, and RdRp, respectively. Lower levels of genetic diversity were observed among the members of Patois serogroup viruses when compared with those observed with other orthobunyavirus clades. This fact was observed in the *p*-distance intra-clade analysis, based on all three genes (*N*, *GPC*, and *RdRp*), in both nt and amino acid identity comparisons (Table 2).

**Phylogenetic relationship of ERV with other orthonairoviruses.** Because blast search analysis revealed the close association of ERV to other orthonairoviruses, we conducted phylogenetic analyses based on amino acid sequences of ERV and 37 complete coding sequences of orthonairoviruses. The ML trees showed 13 well-supported monophyletic groups, but with different topologies in each segment. Twelve of the well-supported clades corresponded to already established orthonairoviruses species.<sup>22</sup> The phylogenetic analysis reveals that ERV forms a monophyletic clade and is genetically more closely related

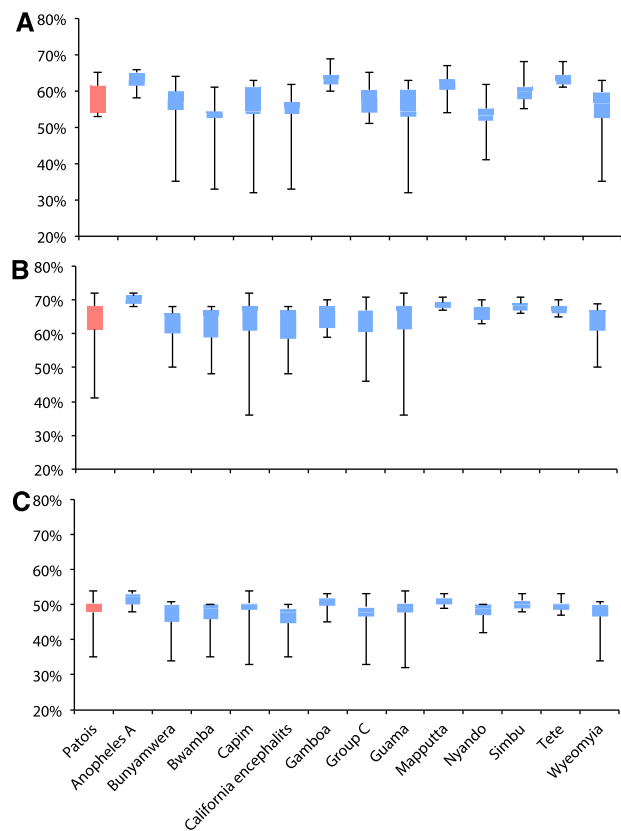


FIGURE 3. Pairwise genetic similarities (amino acid *p*-distance) of Patois serogroup compared with other orthobunyavirus groups based on the coding sequencing of N protein (A), glycopolyprotein (B), and RdRp (C). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

to the species of *Hughes* serogroup (*H. orthonairovirus* species group) in all segments (Figure 4). In addition, the *p*-distance intra-clade analysis, based on the N, GPC, and RdRp sequences of ERV and representative orthonairoviruses, indicates that ERV is a distinct species within the genus (Figure 5). The findings further confirm the close association of ERV with orthonairovirus but not with the Patois serogroup viruses.

TABLE 2

Nucleotide and amino acid identity comparisons for members of Patois serogroup

|      |   | PATV  | PATV-like | ABRV  | BABV  | SRV   | ZEGV  |
|------|---|-------|-----------|-------|-------|-------|-------|
| PATV | S | –     | 94.49     | 76.59 | 76.59 | 95.76 | 98.31 |
|      | M | –     | 81.75     | 70.49 | 70.65 | 89.42 | 80.71 |
|      | L | –     | 82.65     | 76.54 | 76.17 | 82.53 | 82.40 |
| ABRV | S | 75.81 | 77.36     | –     | 98.58 | 77.97 | 77.12 |
|      | M | 68.42 | 69.35     | –     | 84.02 | 70.28 | 70.29 |
|      | L | 76.54 | 76.57     | –     | 92.41 | 76.96 | 76.50 |
| BABV | S | 75.95 | 77.22     | 99.02 | –     | 77.97 | 77.12 |
|      | M | 69.19 | 69.40     | 74.38 | –     | 70.93 | 70.63 |
|      | L | 76.17 | 74.73     | 92.41 | –     | 77.28 | 76.57 |
| SRV  | S | 89.87 | 86.22     | 76.93 | 76.23 | –     | 94.92 |
|      | M | 80.30 | 74.82     | 69.44 | 70.12 | –     | 79.39 |
|      | L | 82.53 | 83.66     | 76.96 | 77.28 | –     | 83.36 |
| ZEGV | S | 97.05 | 89.59     | 77.07 | 76.93 | 88.89 | –     |
|      | M | 74.59 | 80.11     | 69.01 | 69.08 | 74.13 | –     |
|      | L | 82.40 | 94.37     | 76.50 | 76.57 | 83.36 | –     |

ABRV = Abras virus; BABV = Babahoyo virus; PATV = Patois virus; SRV = Shark River virus; ZEGV = Zegla virus.

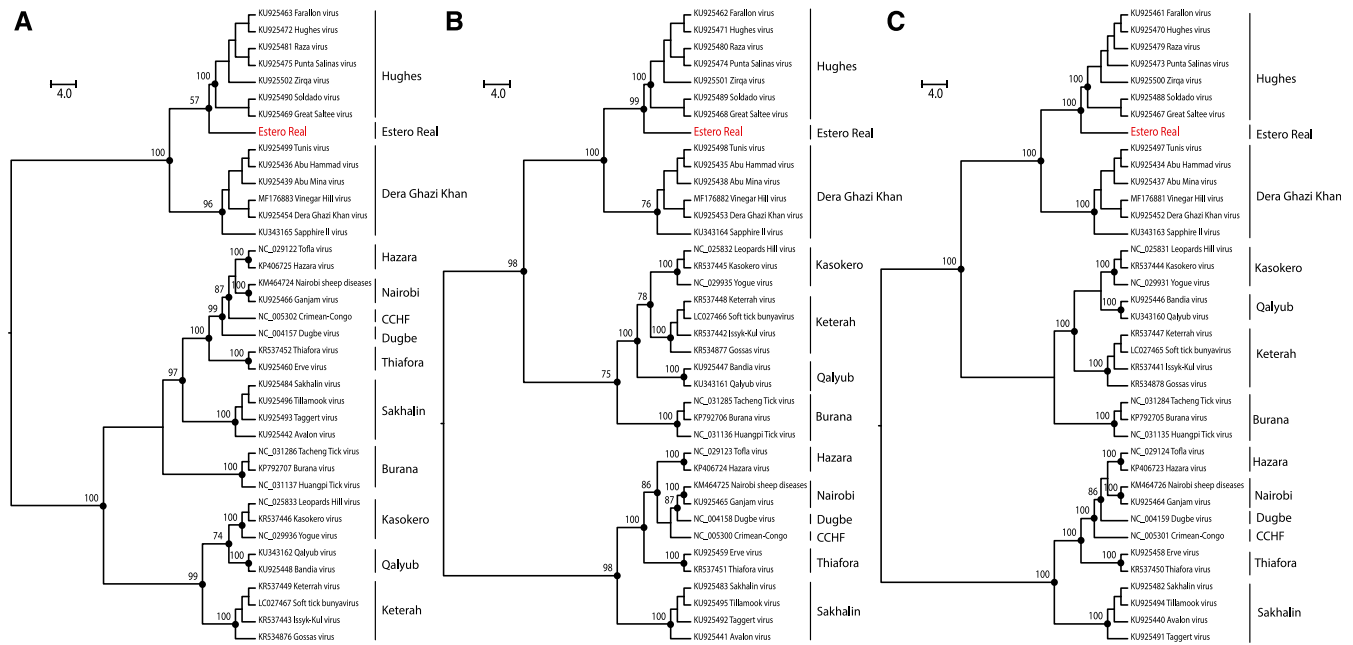


FIGURE 4. Maximum likelihood phylogenetic trees of ERV within *Nairoviridae* family. The nucleoprotein (A) and glycoprotein (B) based on LG + I + G4 model amino acids substitution model and RdRp (C) based on LG + F + I + G4 model amino acids substitution model. Phylogenies are midpoint rooted and with proportional branches for clarity of presentation. The scale bar indicates evolutionary distance in numbers of substitutions per amino acids substitutions/site, and the principal bootstrap support levels were indicated. Estero Real virus sequenced in this study is highlighted in light grey and in red (online). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

**Reassortment analyses.** To identify the potential reassortment events among viruses of the Patois serogroup, we inspected the nt ML phylogenies for discordances in clade clustering between the S, M, and L trees (Figure 2), together with Sliding-window RDP4 analyses using concatenated full genomes of 72 orthobunyaviruses available in GenBank. These analyses indicate that the ZEGV BT5012 strain is a potential reassortant that contains the S and L segment from PATV, and an M segment of unknown origin (Supplemental Figure 1). Based on tree topologies and additional analysis to identify potential reassortment events using the RDP4 program,<sup>25</sup> we did not observe evidence of reassortment with ERV.

## DISCUSSION

In this report, we present the complete coding sequences and genomic characteristics of six of the seven members of the Patois serogroup within the family *Peribunyaviridae*. All strains exhibit similar genomic characteristics as other orthobunyaviruses. We found that the RdRp contains the endonuclease and main motifs that are conserved for the polymerase activity (pre-motif A and motifs A through E), and are highly conserved in negative sense RNA viral polymerases.<sup>24</sup> Our analysis also revealed that, with the exception of ERV, all members of the Patois serogroup viruses sequenced in this study contain putative ORFs for the NSs protein, which has a start codon before the pre-N start codon, as previously described for Enseada and Brazoran viruses.<sup>26,27</sup> The NSs protein has been detected in most orthobunyaviruses that infect vertebrates and is known to be an important virulence factor.<sup>28–31</sup> Because infection with Patois serogroup viruses has not yet been associated with human illness, it would be interesting to determine whether the NSs of these viruses have

similar virulence properties as those described for other orthobunyaviruses.

Our phylogenetic analysis revealed that, with the exception of ERV, the Patois serogroup viruses are most closely genetically related to Bellavista virus, which has not been associated with human illness. Although the Patois serogroup viruses have not been confirmed to cause human disease, they share a common ancestor with Capim, Guama, and Group C serogroup viruses. Both Guama and Group C viruses have been associated with human disease and are a significant cause of febrile illness in Latin America.

We also conducted pairwise amino acid sequence distance analysis of the Patois serogroup viruses and observed a divergence of ~57%, ~60%, and ~48% for the N, GPC, and RdRp, respectively. When these viruses were compared with the amino acid distance from other serogroups, we noted that the degree of evolutionary divergence is equivalent to those observed with other orthobunyavirus serogroups. The Patois serogroup viruses exhibit a higher amino acid distance within the *Orthobunyavirus* genus for the N and GPC, whereas the distance rates for the RdRp were lower. This means that these viruses are under the same selective pressure, with a more conserved polymerase protein and more plastic N and GPC, as previously reported for other orthobunyaviruses.<sup>26</sup>

The natural reassortment process in segmented viruses is a form of genetic exchange that has the potential to provide host/vector range shifts and changes in pathogenicity and/or virulence, as well as an important mechanism for the emergence of new viral species. Reassortment events have been suggested as the driving force in the evolution of bunyaviruses because they have been detected among members of the Group C, Simbu, and Bunyamwera serogroups.<sup>32</sup> Within the Patois serogroup, the first evidence of potential reassortment



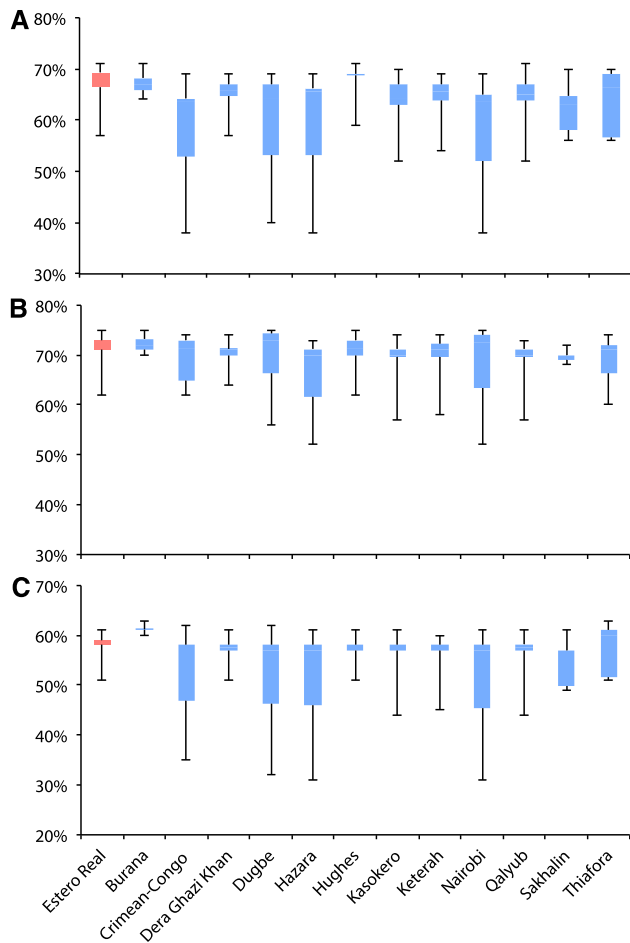


FIGURE 5. Pairwise genetic similarities (amino acid  $p$ -distance) of ERV compared with other orthonairovirus groups based on the coding sequencing of N protein (A), glycopolyprotein (B), and RdRp (C). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

events was based on the results of serological assays and the observation that SRV and PAHV have almost identical L and S RNA oligonucleotide fingerprints, but different M RNA fingerprints.<sup>33</sup> Here, based on the topology of the phylogenetic trees obtained from complete genome sequences of each segment, combined with a RDP4 analysis, we have confirmed that the ZEGV strain BT5012 is a natural reassortant of PATV. The S and L segments of this virus were provided by PATV, whereas the M segment is of unknown origin (Figure 2). This finding challenges the current ICTV classification which designates ZEGV as a distinct species (*Zegla orthobunyaviruses*)<sup>1</sup> and suggests that the virus should be considered instead as a member of the *Patois* serogroup (*Patois orthobunyavirus* species group). Interestingly, ZEGV and PATV were isolated from the hispid cotton rat (*Sigmodon hispidus*) captured near Almirante city in Panama in June, 1961.<sup>5</sup> The potential implications for this reassortment phenomenon in the *Patois* serogroup need further investigation.

The most important finding from this study is the observation that ERV is closely related genetically to members of the *Orthonairovirus* genus and not to the *Patois* serogroup viruses. The genome organization of ERV shares the same characteristics of viruses within the *Orthonairovirus* genus and phylogenetic analyses clearly corroborated these findings by placing ERV in a distinct clade but closely related to species of *H. orthonairovirus* in all segments analyzed, suggesting

that its current placement with other members of the *Patois* serogroup viruses should be revised.

In summary, our study provides new insights into the genetic diversity and evolution of the *Patois* serogroup viruses described to date (with the exception of PAHV) and provides genetic evidence that the ERV taxonomic status does not belong with this group of viruses. It also highlights the need for future studies to determine the epidemiology, geographic distribution, and public health impact of ERV. Furthermore, the sequences generated in this study could be used to develop diagnostic tests and evaluate the potential public health impact of viruses included in the *Patois* serogroup.

Received March 7, 2018. Accepted for publication April 19, 2018.

Published online June 11, 2018.

Note: Supplemental figure appears at [www.ajtmh.org](http://www.ajtmh.org).

Acknowledgments: This study was supported by the National Institutes of Health (NIH) grants number F31 AI124662-01 (JAS) and R24 AI120942 (PVA), WMS was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP grants no. 12/24150-9 and 17/13981-0; MJF was supported by the FAPESP grant no. 16/01414-1; MRTN was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico grants number 302032/2011-8 and 200024/2015-9).

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