Flaviviruses have been major emerging zoonotic pathogens in Africa within the past decade (1). In South Africa, West Nile virus (WNV) is the main flavivirus detected in animals and humans (2,3). Several other lesser-known flaviviruses were first described in South Africa but are understudied and potentially underreported, including Wesselsbron, Usutu, and Banzi (BANV) viruses (4). In South Africa, mosquito surveillance is not routinely performed and studies on flavivirus ecology are outdated (5). In this study, we aimed to update flavivirus vector epidemiology in northeastern provinces of South Africa through a large-scale ecologic survey.

The Study
We selected 15 sites (4 sentinel sites, 11 ad hoc sites) across 5 provinces in South Africa for mosquito collection according to recent cases of arboviral disease in humans and animals (2,3) (Figure 1). We established sentinel sites in Boschkop and Kyalami, both located in the Gauteng province (periurban sites), and Lapalala and Marakele, both located in the Limpopo province (conservation sites); collections were performed during 2011–2018 (Table 1). Opportunistic supplementary collections occurred at 10 ad hoc sites during 2015–2018 spanning 3 additional provinces that included urban, periurban, and conservation sites. We performed additional ad hoc collections during March–April 2017 in and around Kruger National Park (KNP) located within Limpopo and Mpumalanga provinces (periurban/conservation sites) (6). The methodologies for mosquito collection, identification, pooling, processing, flavivirus screening, and COX1 gene sequencing have been previously described (7). To focus on months from midsummer to autumn in South Africa, when availability of mosquito breeding sites and vectorial capacity increases because of warmer and wetter weather conditions (8), we only screened mosquitoes collected during January–June. To submit sequences to GenBank, we generated NS5 gene fragments >200 bp by using heminested PCR with 0.4 µmol/L each of forward primer (FU1, 5′-TACAACATGGGAAAGAGAA-3′) and reverse primer (CFD2, 5′-GTGTCCCAGCCGGCTGTCATCAGC-3′) and Platinum Taq DNA Polymerase (Thermo Fisher Scientific, https://www.thermofisher.com). For WNV positive pools, we performed reverse transcription PCR to amplify a 1,525 bp fragment of the WNV envelope protein gene for phylogenetic analysis (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/1/22-0036-App1.pdf). We calculated the mosquito minimum infection rate (MIR) per site by using a standard formula: (number of positive pools/total number of individual mosquitoes tested) × 1000.

We collected >40,000 mosquitoes from 5 provinces in South Africa during 2011–2018 and screened for zoonotic flaviviruses. We detected West Nile virus in mosquitoes from conservation and periurban sites and potential new mosquito vectors; Banzi virus was rare. Our results suggest flavivirus transmission risks are increasing in South Africa.
West Nile and Banzi Viruses, South Africa

(Culex, Mansonia, Anopheles, and Aedes) and screened for flaviviruses. We detected WNV in 16 (1.09%) and BANV in 2 (0.14%) pools. We did not detect other zoonotic flaviviruses; however, insect-specific flaviviruses were detected and described elsewhere (7). WNV outbreaks can be expected once the MIR rises above 1 (9). We observed the highest MIRs in Kyalami (periurban site, MIR = 2.53), KNP (periurban/conservation site, MIR = 1.22), and Lapalala (conservation site, MIR = 1.01) (Table 2). Therefore, we identified those areas as higher risk sites for WNV outbreaks in humans and animals. These results correlated with areas where Culex spp. mosquitoes were the most abundant and where WNV cases were previously reported in humans and animals in South Africa (3,10).

Only 11 of 16 WNV-positive pools had partial NS5 gene sequences of sufficient quality to perform maximum-likelihood analysis; we confirmed all 11 pools were WNV and also confirmed 2 BANV-positive pools (Figure 2; bootstrap value = 100 for both viruses). We observed high nucleotide similarity (94.62%–100.00%) between the identified WNV NS5 gene sequences and those from previously identified, highly neuroinvasive strains from South Africa isolated from either equines or humans. We successfully amplified the 1,525 nt region of the envelope protein gene for 5 of 16 WNV-positive pools (Appendix Figure 2).

We performed COX1 gene sequencing for 9 of 16 WNV-positive pools and confirmed morphologic identification of those mosquitoes as Cx. univittatus except for 1 pool collected in KNP that was identified by sequencing as Cx. perexiguus (Table 2; Appendix Figure 3), a mosquito species not known to be present in South Africa (11). This 1 pool might have contained a mix of both species, but Sanger sequencing was unable to distinguish between the 2 species. In addition, the COX1 reference sequences for Cx. perexiguus mosquitoes obtained from online databases may not be accurate because this species has not been identified in South Africa. Recently, COX1 gene amplification using universal primers followed by next-generation sequencing was shown to distinguish between species in mixed pools (12) and might be useful in future studies to resolve this ambiguity. Further studies are necessary to clarify the status of the Cx. perexiguus mosquitoes in South Africa. Most of the WNV-positive pools consisted of Cx. univittatus, Cx. pipiens s.l., and Cx. theileri mosquitoes, which we collected in high abundance. This result reiterates the importance of these species as WNV vectors in South Africa (5). We identified Cx. simpsoni, Cx. bitaeniorhynchus, An. gambiae sensu lato, and Cx. poicilipes mosquitoes, none of which have been previously associated with WNV in South Africa, as new potential vectors for WNV by using COX1 gene sequencing (4). Globally, from this list of species, only Cx. poicilipes mosquitoes from Senegal were found to be infected with WNV (13). Experimental studies have shown that the Cx. bitaeniorhynchus mosquito is a likely vector...
for WNV because this species was able to successfully transmit WNV (14). We were unable to genetically characterize the remaining 7 of 16 pools because of insufficient material for DNA extraction; we identified mosquitoes in those pools by morphologic characteristics.

Table 2. Detection of flaviviruses and their associated potential mosquito vectors in survey of West Nile and Banzi viruses in mosquitoes, South Africa, 2011–2018

<table>
<thead>
<tr>
<th>Site</th>
<th>Virus</th>
<th>Positive pool ID</th>
<th>No. mosquitoes†</th>
<th>Morphologic ID‡</th>
<th>Molecular ID§</th>
<th>MIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boschkop</td>
<td>WNV</td>
<td>GAU11MP26</td>
<td>2</td>
<td>Culex p. sensu lato</td>
<td>Cx. univittatus</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>GAU17MP72</td>
<td>33</td>
<td></td>
<td>Cx. univittatus</td>
<td></td>
</tr>
<tr>
<td>Kruger National</td>
<td>WNV</td>
<td>KNP17MP714</td>
<td>4</td>
<td>Cx. simpsoni</td>
<td>Cx. simpsoni</td>
<td>1.23</td>
</tr>
<tr>
<td>Park</td>
<td>WNV</td>
<td>KNP17MP718</td>
<td>36</td>
<td>Cx. univittatus</td>
<td>Cx p. perexiguus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>KNP17MP720</td>
<td>1</td>
<td>Cx. bitaeniorhynchus</td>
<td>Cx. bitaeniorhynchus</td>
<td></td>
</tr>
<tr>
<td>Kyalami</td>
<td>WNV</td>
<td>KYA11MP11</td>
<td>14</td>
<td>Cx. univittatus</td>
<td>Not done</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>KYA11MP13</td>
<td>10</td>
<td>Cx. p. s.l.</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>KYA14MP133</td>
<td>10</td>
<td>Cx. univittatus</td>
<td>Cx. univittatus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>KYA14MP134</td>
<td>19</td>
<td>Cx. univittatus</td>
<td>Cx. univittatus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>KYA14MP115</td>
<td>5</td>
<td>Cx. theleri</td>
<td>Cx. theleri</td>
<td></td>
</tr>
<tr>
<td>Lapalala</td>
<td>WNV</td>
<td>LAP13LP71</td>
<td>44</td>
<td>Anopheles spp.</td>
<td>Not done</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>LAP13LP28</td>
<td>50</td>
<td>Anopheles spp.</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>LAP13LP22</td>
<td>50</td>
<td>Aedes spp.</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>LAP14MP394</td>
<td>2</td>
<td>Cx. univittatus</td>
<td>Cx. univittatus</td>
<td></td>
</tr>
<tr>
<td>Marakele</td>
<td>WNV</td>
<td>MAR13MP77</td>
<td>50</td>
<td>Cx. poicilipes</td>
<td>Not done</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>MAR15MP18</td>
<td>1</td>
<td>An. gambiae s.l.</td>
<td>An. gambiae s.l.</td>
<td></td>
</tr>
<tr>
<td>Lapalala</td>
<td>BANV</td>
<td>LAP13MP25</td>
<td>49</td>
<td>Cx. spp.</td>
<td>Cx. rubinotus</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>BANV</td>
<td>LAP13MP26</td>
<td>50</td>
<td>Cx. spp.</td>
<td>Cx. annuloris</td>
<td></td>
</tr>
</tbody>
</table>

*BANV, Banzi virus; ID, identification; MIR, minimum infection rate; WNV, West Nile virus.
†Number of mosquitoes in each positive pool.
‡Species of mosquito identified by morphologic characteristics.
§Species of mosquito identified by sequencing the COX1 gene.
Figure 2. Phylogenetic analysis of flaviviruses using NS5 gene sequences in survey of West Nile and Banzi viruses in mosquitoes, South Africa, 2011–2018. Maximum likelihood analysis was used to identify flaviviruses found in mosquitoes after partial sequencing of the flavivirus NS5 gene region (226 nt, Kimura 2-parameter model plus gamma distribution plus proportion of invariable sites). Sequence data were edited by using CLC Main Workbench version 8.0.1 (QIAGEN, https://www.qiagen.com). Reference genomes were downloaded from GenBank. Multiple sequence alignments were created by using MAFFT (http://mafft.cbrc.jp/alignment/server/index.html) with default parameters. Phylogenetic analysis was performed by using MEGA X software (MEGA, https://www.megasoftware.net) with bootstrap support for network groupings calculated from 1,000 replicates. Bootstrap values (>70%) are displayed on branches. GenBank accession numbers for newly sequenced virus strains: OL411950 (KYA11MP13 isolate), OL411951 (GAU11MP26 isolate), OL411952 (KYA14MP13 isolate), OL411953 (KYA14MP13 isolate), OL411954 (LAP13MP26 isolate), OL411955 (MAR15MP18 isolate), OL411956 (LAP13MP22 isolate), OL411957 (KNP17MP714 isolate), OL411958 (KNP17MP720 isolate), OL411959 (KNP17MP18 isolate), OL411960 (KYA11MP11 isolate), OL411961 (LAP13MP25 isolate), and OL411962 (LAP13MP26 isolate). Solid black triangles are new viral sequences that were detected in mosquitoes in this study. Scale bar indicates nucleotide substitutions per site.
Detection of BANV in mosquito pools in South Africa has not been described since the late 1970s (15), and a lack of surveillance raises the question regarding the true incidence of this virus. Only the Cx. rubinotus mosquito is recognized as a vector for BANV (15). Despite unclear morphologic identification, we identified Cx. rubinotus and Cx. annulitoris mosquitoes in the 2 BANV-positive pools through COX1 gene sequencing (Table 2; Appendix Figure 3), which should be investigated further to confirm vector status.

Conclusions
The first limitation of our study is that we did not separate voucher specimens for mosquito species from the pools before homogenization. A voucher specimen is a mosquito species that is preserved and serves as a reference used to document identity. Second, identifications of mosquito species not previously associated with WNV infection are preliminary findings, and further investigation of vector competency is required.

Mosquito surveillance is not routinely performed for arboviruses in South Africa, and most studies were performed 40 years ago (5). In this study, 5 provinces were targeted for mosquito surveillance over a 7-year period. These investigations revealed a wide range of new potential vectors that require further investigation. Both WNV and BANV were identified in mosquitoes in periurban and conservation areas at the animal/human interface in South Africa, suggesting increasing circulation potential for those viruses between humans, wildlife, domestic animals, and avian species that are common in those areas.

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References


Address for correspondence: Marietjie Venter, Zoonotic Arbo- and Respiratory Virus Program, Centre for Viral Zoonosis, Department of Medical Virology, Pathology Building, Prinshof Campus South, University of Pretoria, Private Bag X323, Gezina 0031, South Africa; email: marietjie.venter@up.ac.za

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Survey of West Nile and Banzi Viruses in Mosquitoes, South Africa, 2011–2018

Appendix

Appendix Table. PCR primers used to amplify a 1,525-bp fragment of the West Nile virus envelope protein gene for phylogenetic analysis in a survey of West Nile and Banzi viruses in mosquitoes, South Africa, 2011–2018*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer Name</th>
<th>Orientation</th>
<th>Primer sequence, 5′–3′</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR, 1st round</td>
<td>WNV1EF</td>
<td>Sense</td>
<td>AAGACAGAATCATGGATACTG</td>
<td>1,853</td>
</tr>
<tr>
<td></td>
<td>WNV1ER</td>
<td>Anti-sense</td>
<td>GCCATTAAGGATGACGCTGAAC</td>
<td></td>
</tr>
<tr>
<td>RT-PCR, 2nd round</td>
<td>WNV2EF</td>
<td>Sense</td>
<td>CATTGGATGGATGCTAGGAAG</td>
<td>1,525</td>
</tr>
<tr>
<td></td>
<td>WNV2ER</td>
<td>Anti-sense</td>
<td>CACATGCAGAAGGAGTCTCGG</td>
<td></td>
</tr>
</tbody>
</table>

*RT-PCR, reverse transcription PCR.

Appendix Figure 1. Distribution of mosquito genera collected annually at sentinel and ad hoc sites in survey of West Nile and Banzi viruses in mosquitoes, South Africa, 2011–2018. The number of mosquitoes collected per site per year is displayed, and the annual average rainfall is for each region is indicated. Annual average rainfall data were obtained from the weather service in South Africa (https://www.weathersa.co.za/home/historicalrain). Culex mosquitoes were the most abundantly collected genera. A total of 25,397 (38.31%) Culex mosquitoes were collected followed by Anopheles (32.52%, n = 21,560), Aedes (20.47%, n = 13,573), Mansonia (6.79%, n = 4,500), and other genera combined (1.91%, n = 1,269); other genera combined consisted of Uranotaenia, Aedeomyia, Ficalbia, Coquillettida, Mimomyia, Culiseta and Eretmapodites. BEN, Benoni; COET, Pretoria North; GAU, Boschkop; KNP,
Appendix Figure 2. Phylogenetic analysis of flaviviruses using envelope protein gene sequences in survey of West Nile and Banzi viruses in mosquitoes, South Africa, 2011–2018. Maximum likelihood analysis was used to identify flaviviruses found in mosquitoes after partial sequencing of the flavivirus envelope protein gene region (1,440 nt, GTR + G model [general time-reversible model + gamma distribution]). Bootstrap values (>70%) are displayed on the branches. Reference genomes were downloaded from GenBank. GenBank accession numbers for the newly sequenced strains are: OL411963 (MAR15MP18 isolate), OL411964 (KNP17MP718 isolate), OL411965 (LAP13MP394 isolate), OL411966 (KYA14MP134 isolate), and OL411967 (KYA14MP133 isolate). Solid black triangles are new viral sequences detected in mosquitoes in this study. Scale bar indicates nucleotide substitutions per site.
Appendix Figure 3. Phylogenetic analysis of mosquitoes using COX1 gene sequences in survey of West Nile and Banzí viruses in mosquitoes, South Africa, 2011–2018. Maximum likelihood analysis was used to identify mosquitoes after partial sequencing of the COX1 gene region (657 nt, GTR + I model [general time-reversible model + gamma distribution]). Reference genomes were downloaded from GenBank or the Barcode of Life Data System (Boldsystems, https://www.boldsystems.org). GenBank accession numbers are: OL457134 (GAU17MP72 isolate), OL457138 (KNP17MP714 isolate), OL457137 (KNP17MP718 isolate), OL457140 (KNP17MP720 isolate), OL457144 (KYA14MP133 isolate), OL457135 (KYA14MP134 isolate), OL457139 (KYA14MP115 isolate), OL457136 (LAP14MP394 isolate), OL457143 (MAR15MP18 isolate), OL457142 (LAP13MP25 isolate), and OL457141 (LAP13MP26 isolate). COX1 gene sequencing was not performed for GAU11MP26, KYA11MP11, KYA11MP13, LAP13LP71, LAP13LP28, LAP13LP22, or MAR13MP77. Open triangles are COX1 sequences from mosquitoes collected in this study. Scale bar indicates nucleotide substitutions per site.