We used portable genome sequencing to investigate reported dengue virus transmission in Angola. Our results show that autochthonous transmission of dengue serotype 2 (cosmopolitan genotype) occurred in January 2018.

In Africa, the prevalence of disease caused by Aedes mosquito–borne viruses might be similar to that in the Americas (1,2). However, the transmission and genetic diversity of arthropodborne viruses (arboviruses) in Africa remains poorly understood because of a paucity of systematic surveillance. Moreover, syndromic surveillance might confound symptomatically similar illnesses, and serologic diagnostic tests can be misleading because of cross-reactivity between related circulating flaviviruses (3). Improved viral genomic surveillance can assist in better understanding viral transmission dynamics in Africa.

During 2013, Angola experienced a large dengue outbreak that was concentrated in Luanda Province (4). Cases detected in travelers returning from Angola to other parts of the world showed that the virus rapidly disseminated from Angola to Europe, Asia, and the Americas (5). Although infections were predominantly caused by dengue virus (DENV) serotype 1 (6), all DENV serotypes were reported in returning travelers from Angola (7).

The Study
To investigate the timing and frequency of dengue occurrence in Angola, we conducted rapid diagnostic tests by using the SD Bioline Dengue Duo kit (Alere, https://www.alere.com) to detect the presence of dengue-specific IgM, IgG, and, since January 2017, nonstructural protein 1 (NS1) (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/4/18-0958-App1.pdf). During January 1, 2016–May 15, 2018, we collected samples from 6,839 patients (3,276 male, 3,563 female) in central Luanda for whom a physician suspected dengue as the cause of an illness with symptoms consistent with DENV infection. Samples were originally obtained for routine diagnostic purposes from persons visiting local clinics. Thus, we used residual samples without informed consent, with ethics approval from the National Ethical Committee of the Angola Ministry of Health.

We identified 80 DENV NS1–positive cases among the tested samples (Appendix Figure). The first confirmed infections were detected in May 2017, and the number of cases appeared to peak around May 2018 (Figure, panel A).

We tested 153 randomly selected serum samples (IgG, IgM, or NS1-positive) collected during July 21, 2017–January 31, 2018, for DENV RNA by using real-time quantitative reverse transcription PCR (qRT-PCR) at the Instituto Nacional de Investigação em Saúde (INIS) in Luanda, Angola. We used the US Centers for Disease Control and
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Prevention’s DENV1–4 Real-Time RT-PCR assay kit on an Applied Biosystems 7500 Fast Real-Time PCR System (https://www.thermofisher.com), according to the manufacturer’s instructions (7). Of these 153 samples, 1 (isolate AO-1) yielded a qRT-PCR cycle threshold of 22.5 for DENV serotype 2 (DENV-2). This sample was obtained from a 48-year-old man living in Luanda who visited a clinic on January 18, 2018. The patient reported traveling to Mussulo Island, a resort 30 km from Luanda, during December 24, 2017–January 2, 2018.

We subjected the qRT-PCR–positive sample to viral genomic amplification and sequencing by using a multiplex PCR primer scheme designed to amplify the entire coding region of DENV-2. We aligned published genomes of non-sylvatic DENV-2 and used them to generate a 90% consensus sequence that formed the target for primer design.

Figure. Investigation of DENV infections in Luanda, Angola, January 1, 2016–May 15, 2018. A) Number of DENV infections (i.e., cases positive for DENV NS1), Luanda, Angola, May 1, 2017–May 15, 2018. B) Midpoint rooted maximum-likelihood phylogeny of DENV-2 whole genomes. Support for branching structure is shown by bootstrap values at nodes. On the right side, the cosmopolitan genotype clade containing the Angola DENV-2 sequence is expanded. Colors indicate geographic location of sampling. The Angola DENV-2 is shown in bold. Support for branching structure is shown by bootstrap values at nodes (bootstrap scores >70 shown). C) Geographic distribution of available DENV-2 sequence data (>100 bp). Pie chart radii are log-proportional to the number of sequences available in each country and are colored according to genotype DENV, dengue virus; DENV-2, dengue virus serotype 2; NS1, nonstructural protein 1; SE, Southeast; seq, sequences; und, undefined.

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designed primers that generated overlapping amplicons 980 bp in length with an overlap of 20 bp as previously described (9; Appendix Table). Details of cDNA synthesis, multiplex PCRs, library preparation, sequencing, and generation of consensus sequences are described in the Appendix.

We originally used the 90% consensus sequence as a reference genome for mapping sequencing reads, but we later refined this reference to a more appropriate reference genome (GenBank accession no. LC121816) by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to identify DENV sequences with high identity to the provisionally mapped data. The median sequencing depth was 11,448 reads, and 75% of the genome had a depth of at least 2,419 reads. In total, we sequenced 96% of the coding region of DENV-2 (isolate AO-1; GenBank accession no. MH460898). Raw and processed data are available on GitHub (Appendix).

We constructed phylogenetic trees to explore the relationship of the sequenced AO-1 genome to those of other isolates. We retrieved 1,395 DENV-2 genome sequences with associated date and country of collection from GenBank. From this dataset, we generated a subset that included all 35 identified sequences from Africa, 200 globally sampled sequences (randomly sampled from the remaining 1,360 sequences), and the novel AO-1 sequence. We aligned these sequences by using MUSCLE, as implemented in Geneious 9.0.5 (10). We constructed a maximum-likelihood phylogenetic tree by using a general time-reversible model with gamma distributed among site rate heterogeneity (4 categories) in RAXML version 8.2.10 (11). We performed 500 nonparametric bootstrap replicates to evaluate statistical support for phylogenetic nodes. We also constructed a phylogeny that includes additional partial gene sequences (Appendix).

Phylogenetic estimation strongly supports placement of the isolate from Angola in the cosmopolitan genotype of DENV-2 (Figure, panel B). The Angola strain forms part of a well-supported monophyletic clade that comprises genomes sampled in East Africa and is most closely related to DENV isolated from a returning traveler from this region. Viruses from this clade have been present in East Africa since at least 2013 (Figure, panel B).

To explore the global distribution of the DENV-2 cosmopolitan genotype and identify geographic gaps in DENV genomic surveillance that might bias phylogenetic interpretation, we generated maps of the distribution of currently available DENV sequence data. We downloaded from GenBank all DENV sequences >100 bp of any serotype with known location of sampling (including those from returning travelers). We genotyped DENV-2 sequences by using the Genome Detective online classification tool (http://www.genomedetective.com). Most sequenced DENVs in Africa belong to DENV-2 (49%), of which 70% belong to the cosmopolitan genotype (Figure, panel C). We found that although 16% of all global clinically apparent dengue infections have been estimated to occur in Africa (2), DENV serotype 1–4 sequences from Africa currently represent <1% of the available global DENV sequence data. No data exist from the Democratic Republic of the Congo, which has been epidemiologically linked with Angola during past arbovirus outbreaks (12). Additional data will help to address transmission dynamics of DENV-2 in Angola and identify common routes of virus importation into the country.

Conclusions
The DENV-2 portable sequencing approach we describe represents a useful tool for genomic characterization and molecular epidemiology of outbreaks in Africa and elsewhere. On the basis of phylogenetic evidence and the geographic distribution of detected genotypes, the DENV-2 cosmopolitan genotype detected in Angola is probably endemic in Africa. The AO-1 genome we analyzed probably represents an early transmission event from an ongoing DENV-2 epidemic in Luanda. Further sequencing of DENV in the region is required to determine whether the cosmopolitan genotype is endemic to Angola or if it represents a more recent introduction from elsewhere (e.g., East Africa or other unsampled locations).

Acknowledgments
We would like to thank all the patients involved and the staff who assisted with sample collection.

This study was made possible by funding from the Wellcome Trust and Royal Society Sir Henry Dale Fellowship (grant no. 204311/Z/16/Z), the Higher Education Funding Council for England’s Global Challenges Research Fund (grant no. 005073), and the John Fell Research Fund (grant no. 005166). Travel to Angola by S.C.H. and N.R.F. was supported by Africa–Oxford Travel Grants (grant nos. AfiOx-48 and AfiOx-60).

This work forms part of the ArboSPREAD project.

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To revisit the August 2017 issue, go to: https://wwwnc.cdc.gov/eid/articles/issue/23/8/table-of-contents
Early Genomic Detection of Cosmopolitan Genotype of Dengue Virus Serotype 2, Angola, 2018

Appendix

DENV2 Complete Genome MinION Nanopore Sequencing

Between the 15 and 23rd February 2018, we attempted sequencing using the Oxford Nanopore MinION device at Instituto Nacional Investigação em Saúde (INIS), Ministry of Health of Angola, Luanda, on the AO-1 isolate, as part of the ArboSPREAD project focused on genomic surveillance of arthropod-borne viruses. Diagnostic, sequencing and genetic analysis results were presented at the INIS to local public health authorities on the 23rd February 2018. The detailed sequencing protocol has been previously described in (1). Following cDNA synthesis using random primers, multiplex PCR is conducted to generate overlapping amplicons of the whole genome of the targeted viral strain (1). Extracted RNA was reverse-transcribed to cDNA using the Protoscript II First Strand cDNA synthesis Kit (New England Biolabs, Hitchin, UK) and random hexamer priming. DENV2 genome amplification was attempted using 35 cycles of PCR according to the reaction mix and thermocycling conditions given in Quick et al (1), and with the primers shown in Appendix Table. PCR products were cleaned up using AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and quantified with the Qubit dsDNA High Sensitivity assay on a Qubit 3.0 instrument (Life Technologies). Presence of correctly-sized bands were checked on an E-Gel electrophoresis machine. PCR products for the AO-1 sample were barcoded using the Native Barcoding Kit (NBD103, Oxford Nanopore Technologies, Oxford, UK) and pooled in an equimolar fashion. Sequencing libraries were generated from the barcoded products using the Genomic DNA Sequencing Kit SQK-LSK108 (Oxford Nanopore Technologies). We used 250 ng of total DNA input in the library preparation. The library was loaded onto a flow cell (FLO-MIN106) and sequencing data were collected for 24 hours.
Processing of Sequencing Data and Generation of Consensus Sequences

Consensus sequences were generated from raw data reads using a pipeline that has been extensively described previously (1), with only minor modifications. First, raw data were basecalled using Albacore Sequencing Pipeline Software version 2.1.10 (Oxford Nanopore Technologies). Basecalled data were demultiplexed into separate barcodes using Porechop version 0.2.3 (https://github.com/rrwick/Porechop), with reads being assigned to barcodes only when barcodes were present at both ends of the sequence. Demultiplexed data were subject to consensus calling using python scripts (the main script ‘zibra.py’ and all other scripts called by this code are available at https://github.com/zibraproject/zika-pipeline, and have been previously detailed in the scientific literature [1]). Briefly, each basecalled FASTA file is mapped to the reference genome using bwa v 0.7.16a-r1181 (http://bio-bwa.sourceforge.net/bwa.shtml#13) and a BAM file is produced. Primers are trimmed based on positions relative to the reference genome given in input BED files and coverage is normalized to improve computational speed. Nanopolish (version 0.8.4) (https://github.com/jts/nanopolish) is used to call variants. Variants with ≥20 depth are used to generate consensus sequences, and regions with lower coverage, and those in primer binding regions were masked with N characters. To prevent our consensus sequence being affected by the reference sequence chosen, we first mapped basecalled reads to a 90% consensus sequence of a representative non-sylvatic DENV2 alignment and extracted the consensus of this. The generated consensus was then used as input to a BLAST query to find the closest identity genome available in GenBank. The closest identity sequence (GenBank accession number: LC121816) was then reused as the reference genome and the consensus generation pipeline was rerun. Key data files produced by the pipeline, input BED and reference files, and raw FAST5 files linked to the barcodes associated with dengue that were included in this library are available on GitHub (https://github.com/arbospread/DENV2-Angola-2018_01).

Partial Gene Phylogeny

Most DENV sequences available in GenBank are partial gene sequences. We therefore supplemented the whole genome alignment with all DENV2 sequences >1000 bp from Africa that belonged to the same genotype as the novel sequence from Angola. Sequences were aligned to the envelope region of DENV2, and we estimated a separate phylogeny from this alignment.
by using the models we have described. The closest relative to AO-1 was not affected by 
inclusion of these partial sequences, and no additional diversity (i.e., DENV sampled from other 
years and/or countries) was included within the monophyletic African clade containing AO-1, so 
this tree is not shown.

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Appendix Table. Sequencing primers used for MinION sequencing of DENV2.

| Primer Name  | Primer sequence (5'-3') |
|--------------|-------------------------|
| DENV2_1_LEFT | AGCAGATCTCTGTAGAATAACCAACG |
| DENV2_1_RIGHT| TTTTTGCGATCGTGCACACA |
| DENV2_2_LEFT | TCGCTCCATTCAATGACAATGC |
| DENV2_2_RIGHT| CCATTTCAGGCTGACCTTGAG |
| DENV2_3_LEFT | AGATTGGTAGCTTTGAAAAATCCC |
| DENV2_3_RIGHT| TGAAAGGGATTGCTGTGAGAAG |
| DENV2_4_LEFT | AGATTGGTAGCTTTGAGCATG |
| DENV2_4_RIGHT| CCGGACGACATTCGTGATGA |
| DENV2_5_LEFT | TCATGCAGGAGAGAAGATC |
| DENV2_5_RIGHT| TCTGAAGAGTAGTCAGGTCA |
| DENV2_6_LEFT | TGGAAATCAGACCATTGGAAGAGAAGAAG |
| DENV2_6_RIGHT| TCTCACTGTGGATTCCTTTC |
| DENV2_7_LEFT | CCAATCTGCTAATAACATGACAGAT |
| DENV2_7_RIGHT| TATGCGTGGGTTGGATATC |
| DENV2_8_LEFT | AGATGAAGATGACATTTCGAGAAGA |
| DENV2_8_RIGHT| CCATGTATATGACTGTCATTTCATT |
| DENV2_9_LEFT | ATGCGAGTAGCCACTCTAGTG |
| DENV2_9_RIGHT| CACCCACTGTGGAGATGGCAT |
| DENV2_10_LEFT| ACCAGAAAAACAGAGAACACCCC |
| DENV2_10_RIGHT| CACCACCGTTCCATTCTTTT |
| DENV2_11_LEFT| GGAGCTGGACTTCTTTTCAT |
| DENV2_11_RIGHT| GACGTCCCAAGGTTTTGTCAGC |
| DENV2_12_LEFT| AGAGCATGAAACATCATGGCACT |
| DENV2_12_RIGHT| GTGCCCTCTTGGGTTGGTCTTT |
| DENV2_13_LEFT| TGGGACACAAGATGCATTAGCAG |
| DENV2_13_RIGHT| CGGCACCATTGCTCTTCTT |
| DENV2_14_LEFT| TGGGACACAAGATGCATTAGCAG |
| DENV2_14_RIGHT| CGGCACCATTGCTCTTCTT |
Appendix Figure. Summary of the serologic results obtained from 6,839 tests performed in Luanda between Jan 2016 and 15th May 2018. Panels A, C and E show positive results; panels B, D and F indicate number of negative results through time. Note that while IgM (panels C and D) and IgG (panels E and F) screening started in Jan 2016, NS1 screening (panels A and B) started only in Jan 2017. The IgM and IgG positive cases throughout 2016 are possibly due to the presence of antibodies against yellow fever virus.