BRIEF REPORT

Genomic Analysis of Lassa Virus during an Increase in Cases in Nigeria in 2018

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SUMMARY

During 2018, an unusual increase in Lassa fever cases occurred in Nigeria, raising concern among national and international public health agencies. We analyzed 220 Lassa virus genomes from infected patients, including 129 from the 2017–2018 transmission season, to understand the viral populations underpinning the increase. A total of 14 initial genomes from 2018 samples were generated at Redeemer's University in Nigeria, and the findings were shared with the Nigerian Center for Disease Control in real time. We found that the increase in cases was not attributable to a particular Lassa virus strain or sustained by human-to-human transmission. Instead, the data were consistent with ongoing cross-species transmission from local rodent populations. Phylogenetic analysis also revealed extensive viral diversity that was structured according to geography, with major rivers appearing to act as barriers to migration of the rodent reservoir.

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Lassa fever is a viral hemorrhagic illness that is endemic to parts of West Africa and causes more than 300,000 cases of illness and 3000 deaths each year.[1] Despite the burden of disease, there is currently no approved vaccine, and the only available pharmacologic therapy is early intravenous administration of the antiviral agent ribavirin.[2-4] For these reasons, Lassa fever has been recognized by the World Health Organization (WHO) and the Coalition for Epidemic Preparedness Innovations as an important threat to global health and as

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a disease that is in urgent need of attention in the area of research and development.[5-7]

During the 2018 transmission season (approximately December 2017 through May 2018), an unusually large increase in Lassa fever cases occurred in Nigeria, which led the WHO to declare it an outbreak, classified as a grade 2 public health emergency.[8] By May 27, the Nigerian Center for Disease Control (NCDC) had reported 431 laboratory-confirmed cases in patients from 21 states in 2018, including 37 health care workers, with an estimated case fatality rate of 25%.[9] Confirmed cases were concentrated in the southwestern states of Edo (42%) and Ondo (24%) and the southeastern state of Ebonyi (15%). The factors underlying the increase in cases were not known, which raised concern among public health officials that something about this endemic disease had fundamentally changed.

In infectious disease emergencies, genomic analysis of contemporaneous samples collected from infected patients can complement conventional epidemiologic data and provide important information about the nature of the emergency (see, e.g., studies of influenza virus,[10] Zika virus,[11,12] and Ebola virus[13-15]). Of particular relevance to the situation in Nigeria, genomic data can rapidly reveal relationships between cases, indicating changes in the infectivity of particular strains or in patterns of transmission. In this case, the dominance of a single strain would contrast with previous phylogenetic investigations of Lassa virus in Nigeria, which showed extensive, ancient viral genetic diversity, including at least three distinct, coexisting viral lineages[16-19]. Similarly, an increase in human-to-human transmission would leave a clear genetic signature in a disease that typically results from contact with infected rodents (*Mastomys natalensis*, the major natural reservoir[20]) or their excreta. Instances of human-to-human transmission have been documented, particularly in hospital settings, and are a focus of public health concern,[21,22] but they are exceptions to the usual transmission pattern.

We analyzed genomes of Lassa virus from patient samples from the 2017–2018 transmission season, as well as from the two transmission seasons preceding it, to assess whether genetic changes in the viral populations circulating in Nigeria might have contributed to the increase in cases. This work was done in partnership with the Irrua Specialist Teaching Hospital (ISTH), which has the largest Lassa fever facility in Nigeria and is a major diagnostic referral center, receiving samples from patients with suspected Lassa fever from across the country. Initial sequencing was undertaken locally by the African Center of Excellence for Genomics of Infectious Diseases (ACEGID) at Redeemer's University in Ede, Nigeria, and key findings were reported to the NCDC in real time to aid in their understanding of factors contributing to the unusual increase in cases in 2018. This data set of Lassa virus samples from 2015 through 2018 provides genomic context for the recent increase in Lassa fever cases and further resolves the geographic structure of the endemic Lassa virus population across Nigeria.

METHODS

SAMPLES FROM PATIENTS

We obtained samples through a study that was evaluated and approved by the institutional review boards at the ISTH (Irrua, Nigeria), Redeemer's University, and Harvard University (Cambridge, Massachusetts). Study staff obtained written informed consent from participants who were enrolled in the research study at the ISTH. In addition, excess clinical samples were obtained under a waiver of consent granted by the ISTH Research Ethics Committee. Samples from suspected cases of Lassa fever were tested for Lassa virus by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). Samples were deidentified, and demographic and clinical data were obtained in accordance with ethics approval.

VIRAL SEQUENCING

All the samples were inactivated in buffer AVL (Qiagen) at the clinical laboratory at the ISTH before being sent to either Redeemer's University or the Broad Institute for subsequent processing. Institutional biosafety committees approved this work before it began, where appropriate. We extracted RNA from patient plasma using the QiAmp viral RNA mini kit (Qiagen) or Pathogen RNA/DNA kit (MagMax) according to the manufacturer's instructions. We removed contaminating DNA by means of DNase treatment, synthesized complementary DNA, and prepared sequencing libraries with the Nextera XT kit

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(Illumina), as previously described.[23] We constructed sequencing libraries directly from clinical samples without culture adaptation or any other intervention that might alter the genetic sequence of the virus. We extracted, prepared, and sequenced samples at the ACEGID (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org) and the Broad Institute (Table S2 in the Supplementary Appendix). The initial 14 genomes from 2018 were obtained from 26 samples chosen arbitrarily from the RT-qPCR–positive patient samples we had access to at that time. Subsequently, 115 genomes from the 2017–2018 season were obtained from 369 samples. This includes more than 90% of the RT-qPCR–positive patient samples received at the ISTH from January through April 2018, during the height of the emergency. In addition, we prepared and sequenced a similar number of samples from across the two previous transmission seasons (i.e., 2015–2016 and 2016–2017), from which we obtained 91 Lassa virus genomes. Failure to produce a Lassa virus genome from a RT-qPCR–positive sample may have been due to low viral titer, degradation due to lack of sufficient cold chain, or technical sample-handling issues. Samples that did not produce a Lassa virus genome in the first attempt were not investigated further. We performed replicate sequencing from independent plasma aliquots of 14 samples at both sites, which showed high concordance (Fig. S1 in the Supplementary Appendix). We sequenced all the samples with an Illumina MiSeq, HiSeq 2500, or NovaSeq machine with 100 nucleotide paired-end reads.

GENOMIC DATA ANALYSIS

We analyzed the sequencing data using our publicly available software (viral-ngs, version 1.19.2[24,25]) implemented on the DNAnexus cloud-based platform. In brief, we demultiplexed individual libraries, removed reads mapping to the human genome or to other known technical contaminants (e.g., sequencing adapters), and filtered the remaining reads against previously published Lassa virus genomes. We performed de novo assembly using Trinity software[26] and scaffolded contigs against one of three Lassa virus reference genomes (GenBank accession number KM821997-8, GU481072-3, or KM821772-3), representing the major viral lineages (II, III, and IV, respectively). We used KrakenHLL software, version 0.4.8,[27] to identify other viral taxa present in the samples. To do so, we first built a database that encompassed the known diversity of all viruses that infect humans, along with the genomes of several other species (similar to a database described elsewhere,[28] but without insect species). We searched for viral species in the samples by classifying all unique 31-bp subsequences (k-mers) in the sequencing reads. Species for which the number of unique k-mers was at least 10 times as high as the number of reads classified to it were manually investigated for potential hits. We did not find evidence of other pathogenic viral infections in any of the samples.

To construct the phylogenetic tree of Lassa virus, we performed a multiple sequence alignment of the 220 genomes reported here with a set of 193 previously published Lassa virus genomes from Nigeria, Sierra Leone, Liberia, and Côte d'Ivoire.[17] We used MAFFT software, version 7.402,[29] to perform codon-based multiple sequence alignments of the genes encoding nucleoprotein (NP), glycoprotein precursor (GPC), and polymerase (L). To estimate maximum-likelihood phylogenies of L and of concatenated alignments of NP and GPC with IQ-TREE software, version 1.5.5,[30,31] we used a general time reversible nucleotide-substitution model with a gamma distribution of rate variation among sites and ultrafast bootstrapping. To create time-aware phylogenies for the Nigerian lineage II Lassa virus genomes, we then performed Bayesian phylogenetic analyses with BEAST software, version 1.8.4,[32] incorporating the collection date for each sequence. We included GPC and NP lineage II alignments as separate partitions. We used a model that incorporated a Hasegawa–Kishino–Yano nucleotide-substitution model with "1+2+3" codon partitioning,[33] an uncorrelated relaxed clock with a log-normal distribution, and a Bayesian SkyGrid coalescent tree prior distribution. All the Bayesian analyses were run for 200 million Markov chain Monte Carlo steps, with parameters and trees sampled every 5000 generations. Maximum clade credibility trees summarizing all Markov chain Monte Carlo samples were generated with the use of TreeAnnotator software, version 1.8.4 (BEAST), with a burn-in rate of 10%.

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RESULTS

REAL-TIME GENOMIC ANALYSIS

The number of suspected and confirmed Lassa fever cases tested at the ISTH in early 2018 was notably higher than in previous years. From November 2017 through May 2018, the ISTH tested specimens from more than 2700 clinically suspected cases of Lassa fever, of which 436 were RT-qPCR–positive for Lassa virus (Fig. 1). These 436 cases represent more than 90% of all laboratory-confirmed cases reported by the NCDC over this period during the 2017–2018 transmission season.[9] To characterize the Lassa virus population underpinning the increase in cases, we initially performed unbiased whole-genome sequencing with a subset of samples from patients with confirmed Lassa virus infection. This initial data set comprised 14 complete or partial Lassa virus genomes (Table 1). The mean unambiguous assembly length of these genomes was 10,258 bases (range, 4450 to 10,610), and the mean coverage depth was 207× (range, 1 to 1834) (Table 1). The per-sample quality metrics are summarized in Table S1 in the Supplementary Appendix. All the Lassa virus genome data have been publicly released at the National Center for Biotechnology Information under BioProject number PRJNA436552.

The 14 patients from whom these genomes were obtained were representative of the demographic characteristics of patients with contemporaneous cases that were confirmed at the ISTH (Fig. S2 and Table S3 in the Supplementary Appendix). In our initial data set, 13 of the 14 patients came from southwestern Nigeria, which was consistent with the overall geographic distribution of the 2018 cases[9] and the distribution seen during previous transmission seasons. The samples were obtained from January through March 2018, during the peak of the increase in cases. Hemorrhage was documented in 2 of the

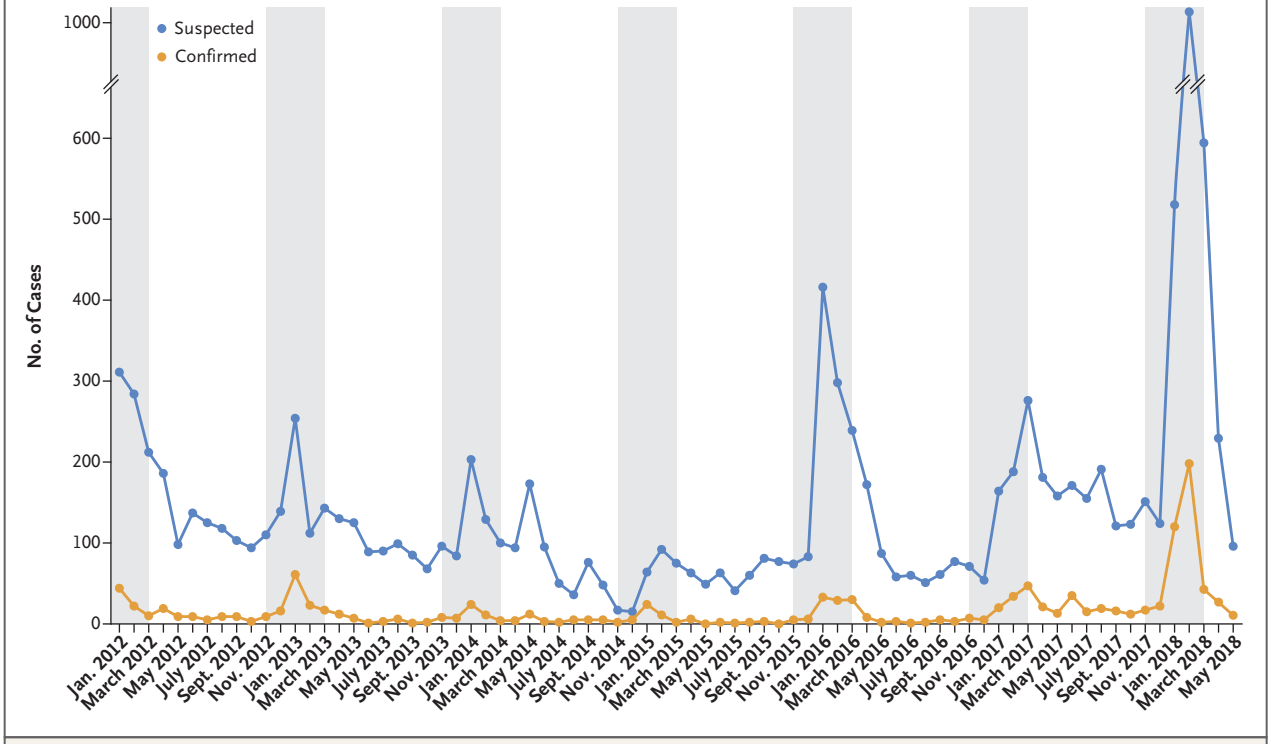

Figure 1. Lassa Fever Cases over Time.

The numbers of clinically suspected and confirmed (reverse-transcriptase quantitative polymerase-chain-reaction–positive) cases of Lassa fever identified at the Irrua Specialist Teaching Hospital are shown for each month from January 2012 through May 2018. Gray shading indicates the typical dry season in Edo State, Nigeria, which overlaps with the period of peak Lassa fever incidence.

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3 patients who died and in 3 of the 9 who recovered (the outcomes in 2 patients were not reported), which suggested a range of disease severity.[34] This finding is broadly consistent with clinical descriptions of Lassa fever: a sometimes fatal febrile illness with nonspecific symptoms that include fever, headache, malaise, and general weakness, often indistinguishable from malaria or common viral infections but with the potential for the development of hemorrhagic features.[34-36]

The analysis of this data set did not show evidence that the increase in cases in 2018 was driven by a particular viral strain or by human-to-human transmission. Instead, the viral genomes were distributed throughout lineage II, the predominant lineage in southwestern Nigeria, and they appear to be broadly representative of the genetic diversity that was seen in that region in previous years (Fig. S3 in the Supplementary Appendix). The genomes did not show substantial clustering according to the date of sampling or a "ladder-like" structure typical of human-to-human transmission; instead, they were consistent with multiple zoonotic transmissions from a genetically diverse reservoir. The only pair of genomes that clustered together on the phylogenetic tree and were also from the same geographic region had an estimated time to their most recent common ancestor of 12 years (95% credible interval, 9 to 14) (Fig. S3 in the Supplementary Appendix), which is not consistent with human-to-human transmission. We reported these findings to the NCDC and other local health authorities in real time to aid in their understanding of the factors accounting for the increase in Lassa fever cases[37] and shared the findings with the scientific community on virological.org (http://virological.org/t/2018-lassa-virus-sequencing-in-nigeria/201).

BROADER GENOMIC CONTEXT

After the analysis of the initial data set, we generated an additional 115 complete or partial Lassa virus genomes from the 2017–2018 transmission season to extend these findings, as well as 91 genomes from the 2015–2016 and 2016–2017 seasons to provide additional context for assessing the genomes from 2018 (BioProject accession number, PRJNA436552) (Table 1, and Table S2 in the Supplementary Appendix). The mean unambiguous assembly length of these genomes was 10,150 bases (range, 3878 to 10,649), and the mean coverage depth was 846× (range, 2 to 12,822) (Table 1, and Table S2 in the Supplementary Appendix).

Table 1. Sequencing Metrics in Each Lassa Virus Season from 2015 through 2018.*

Season and Laboratory	No. of Genomes	Assembly Length	Mean Coverage (Range)
		bp	
2017–2018			
ACEGID	14	10,258	207× (1–1,834)
ACEGID or Broad Institute	115	9,959	586× (4–12,822)
2016–2017			
Broad Institute	39	10,495	604× (12–4,801)
2015–2016			
ACEGID or Broad Institute	52	10,374	1,531× (12–10,195)
Total	220		

* Samples were sequenced at the African Center of Excellence for Genomics of Infectious Diseases (ACEGID), Redeemer's University, on an Illumina MiSeq or at the Broad Institute on an Illumina HiSeq2500 or NovaSeq. To confirm the reproducibility of the assemblies, 14 samples were sequenced at both sites, resulting in 220 unique genomes.

This extended 2017–2018 data set captures 29% of the RT-qPCR–confirmed Lassa fever cases over the period of our sample collection and spans 13 of the 21 states with reported cases, with 79% coming from southwestern Nigeria. Analysis of the 2017–2018 data set revealed an overall pattern similar to that of the initial 14 samples sequenced: maximum-likelihood phylogenies show that the genomes span previously known Lassa virus diversity in Nigeria (Fig. 2A, and Fig. S4 in the Supplementary Appendix), which suggests that the increase was not driven by a particular viral strain. The genomes do not display substantial clustering according to the date of sampling, a finding consistent with predominantly zoonotic rather than human-to-human transmission.

Although human-to-human transmission appeared to be uncommon, we were able to detect possible cases in this larger data set. We identified eight groups of two to three patients with the following properties: they were infected with identical or nearly identical viruses (i.e., that differed from one another by ≤7 nucleotides and had estimated times to the most recent common

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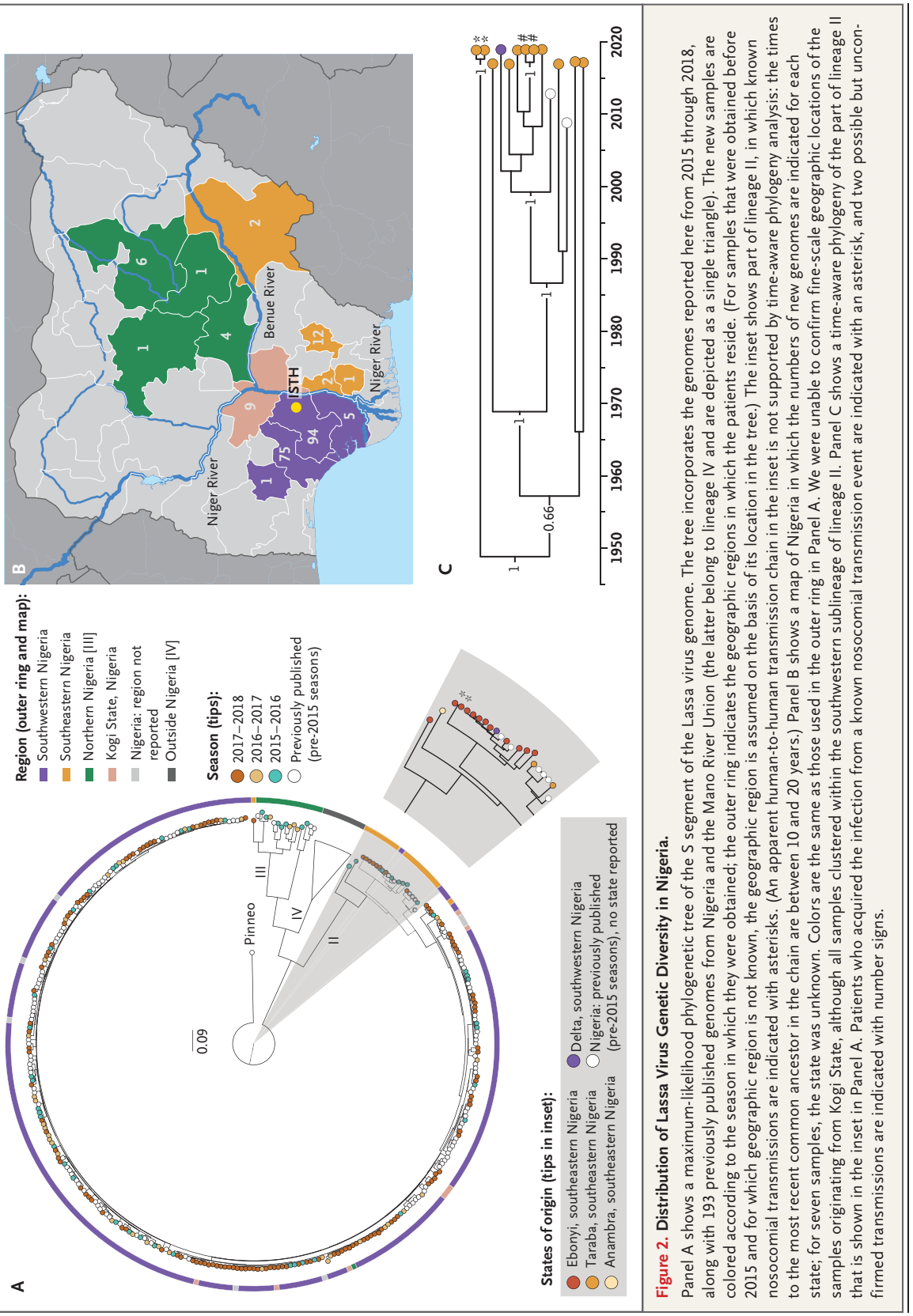

Figure 2. Distribution of Lassa Virus Genetic Diversity in Nigeria.

Panel A shows a maximum-likelihood phylogenetic tree of the S segment of the Lassa virus genome. The tree incorporates the genomes reported here from 2015 through 2018, along with 193 previously published genomes from Nigeria and the Mano River Union (the latter belong to lineage IV and are depicted as a single triangle). The new samples are colored according to the season in which they were obtained; the outer ring indicates the geographic regions in which the patients reside. (For samples that were obtained before 2015 and for which geographic region is not known, the geographic region is assumed on the basis of its location in the tree.) The inset shows part of lineage II, in which known nosocomial transmissions are indicated with asterisks. (An apparent human-to-human transmission chain in the inset is not supported by time-aware phylogeny analysis: the times to the most recent common ancestor in the chain are between 10 and 20 years.) Panel B shows a map of Nigeria in which the numbers of new genomes are indicated for each state; for seven samples, the state was unknown. Colors are the same as those used in the outer ring in Panel A. We were unable to confirm fine-scale geographic locations of the samples originating from Kogi State, although all samples clustered within the southwestern sublineage of lineage II. Panel C shows a time-aware phylogeny of the part of lineage II that is shown in the inset in Panel A. Patients who acquired the infection from a known nosocomial transmission event are indicated with an asterisk, and two possible but unconfirmed transmissions are indicated with number signs.

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ancestor of ≤1 year [Fig. S5 in the Supplementary Appendix]), were tested within 19 days of each other, and were from the same state (Table S2 in the Supplementary Appendix). These features suggest infection from a recent common source and might represent human-to-human transmission; for example, one of these pairs corresponds to a reported nosocomial human-to-human transmission event (Fig. 2A and 2C). Overall, only a limited number of cases consistent with common-source infections and no examples of sustained human-to-human transmission were found.

GEOGRAPHIC STRUCTURE OF DIVERSITY

Phylogenetic analysis of the full data set shows that Lassa virus genomes cluster geographically with the persistence of two main genetically distinct lineages. Lineage II, which includes 93% of the genomes in this data set, is found predominantly in southern Nigeria, and lineage III is found predominantly in northern Nigeria (Fig. 2A and 2B). Both lineage II and lineage III display relatively high genetic diversity, which is visible in their long internal branch lengths (Fig. S5 in the Supplementary Appendix); the estimated times to the most recent common ancestor for both lineages are more than 200 years. A third Nigerian lineage, lineage I, has rarely been observed and did not appear in our sampling.

This pattern of distinct geographic lineages, each internally diverse, suggests that Lassa virus has remained stably separated in these regions, possibly in the rodent populations. The observed Lassa virus populations largely segregate along the courses of the Niger and Benue Rivers (Fig. 2B). Lineage II genomes come predominantly from south of the Benue River, whereas lineage III genomes come from north of the Benue River and east of the Niger River. Within lineage II, our data set suggests internal geographic substructure separated by the Niger River. Genomes from the southwest (west of the Niger River) consist almost entirely of a distinct sublineage of lineage II (Fig. 2A and 2B), which is largely confined to that region. We hypothesize that the Niger and Benue Rivers have presented a natural barrier to the movement and mixing of mastomys rodents, leading to sequestering of their associated Lassa virus populations within these regions.

DISCUSSION

We investigated the genomic diversity of Lassa virus in humans to better understand the increase in Lassa fever cases that occurred in 2018 in Nigeria. In a data set of 220 genomes, including 129 from 2018, we found no evidence that a particular viral strain or extensive human-to-human transmission drove the increase. In particular, Lassa virus in 2018 was drawn from a wide range of previously observed viral diversity rather than from a single dominant strain, and we did not find extensive phylogenetic clustering of Lassa virus from samples that had been collected close together in time, as would be expected if this increase were driven by human-to-human transmission. The absence of these patterns suggests that Lassa virus transmission in 2018 continues to be sustained largely by numerous distinct cross-species transmission events from a genetically diverse reservoir.[16,17] These findings helped to guide the public health response by alleviating concerns about a new or more virulent strain of Lassa virus, perhaps with a higher potential for human-to-human transmission, as a potential explanation for the increase in cases. The reason for the unusual increase in Lassa fever cases remains unknown, but it may involve changes in the rodent reservoir population or improved surveillance and heightened public awareness.[38]

These data help resolve the geographic structure and recent population history of Lassa virus in Nigeria. For example, the most recent common ancestor in the viral population west of the Niger River is substantially younger than that east of the river (with estimated times to the most recent common ancestor of 74 and 219 years, respectively), which suggests that either the introduction of the virus or a population bottleneck occurred in the western areas within the past century. The persistent segregation of these two populations since that time suggests the importance of established local rodent populations in sustaining distinct viral populations.[16] Broader geographic sampling of Lassa virus, including from the poorly characterized rodent reservoir, is needed to fully understand viral diversity and may be useful for the development of diagnostics, therapeutics, and vaccines.[5,6]

The initial data set of Lassa fever cases from 2018 in this study was generated in Nigeria,

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which was made possible through longer-term investment in the capacity of local, responsive genomics laboratories.[39] Continued development of partnerships between local genomics laboratories and public health agencies will facilitate a more agile approach to the rapid integration of genomic and epidemiologic data, which will contribute to a better understanding of disease transmission and more informed response strategies.

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APPENDIX

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