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SUPPLEMENT ARTICLE

# Preliminary Evaluation of the Effect of Investigational Ebola Virus Disease Treatments on Viral Genome Sequences

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***Background.*** Several patients with Ebola virus disease (EVD) managed in the United States have received ZMapp monoclonal antibodies, TKM-Ebola small interfering RNA, brincidofovir, and/or convalescent plasma as investigational therapeutics.

***Methods.*** To investigate whether treatment selected for Ebola virus (EBOV) mutations conferring resistance, viral sequencing was performed on RNA extracted from clinical blood specimens from patients with EVD following treatment, and putative viral targets were analyzed.

***Results.*** We observed no major or minor EBOV mutations within regions targeted by therapeutics.

***Conclusions.*** This small subset of patients and clinical specimens suggests that evolution of resistance is not a direct consequence of antiviral treatment. As EVD antiviral treatments are introduced into wider use, it is essential that continuous viral full-genome surveillance is performed, to monitor for the emergence of escape mutations.

**Keywords.** molecular epidemiology; antiviral agents; Ebola virus; high-throughput nucleotide sequencing; directed molecular evolution.

From December 2013 to January 2016, Western Africa experienced an unprecedented outbreak of Ebola virus (EBOV) disease (EVD) caused by the novel Makona variant of EBOV [1, 2]. After its initial appearance, in the Guéckédou region of Guinea, cases of EVD quickly spread to other African countries, including Liberia, Sierra Leone, Mali, Senegal, and Nigeria [1, 3]. Patients with EVD (imported, medically evacuated, or locally acquired) were also cared for in the United States and Europe [4–7]. As of February 2016, 28 603 total confirmed, probable, and suspected EVD cases have been reported in Western Africa [3].

Previous outbreaks of EVD have not exhibited the scale and duration observed during the current epidemic, suggesting that ongoing human-to-human EBOV transmission could influence viral evolution. Several authors recently sequenced EBOV from patients in Sierra Leone, Mali, and Guinea and observed the appearance of distinct viral lineages evolving during the course of the outbreak [8–13]. These viral lineages suggest that extended human transmission of EBOV is influencing viral diversity. EBOV transmission appears to have stopped in Western Africa [3], but this outbreak demonstrated the need for changes in community behavior, effective therapeutics, and vaccines to quickly respond to future EVD cases.

Several cases of EVD were introduced into the United States following medical evacuations and personal travel from Western Africa. Nosocomial EBOV transmission from a single patient also resulted in secondary EVD cases in the United States [14, 15]. In an attempt to reduce disease severity, US patients with EVD were managed with supportive care, including critical care management and advanced organ support, and experimental EVD treatments, including convalescent plasma, ZMapp monoclonal antibodies (mAbs), TKM-Ebola small interfering RNA (siRNA), and/or brincidofovir for compassionate use.

ZMapp is an investigational EVD therapeutic composed of human-mouse chimeric mAbs c13C6, c2G4, c4G7, which recognize glycoprotein (GP) from EBOV variant Mayinga [16–19]. Individually, in vitro these mAbs can neutralize EBOV virions from both the 1995 Kikwit and 2013–present Guinean outbreaks [16] and together increase survival in nonhuman primates following experimental EBOV infection [16–19]. Antibody 2G4 binds to membrane-associated trimeric GP, and the epitope may be within GP2 amino acids 502–516 (REAIVNAQPKCNPNL) [18, 19]. Antibody 4G7 can also bind to GP2 amino acids 502–516; however, Qiu et al demonstrated that 4G7 can immunoprecipitate GP1 and predicted that it may bind near the GP1/GP2 cleavage site (amino acids 458–501) [16, 18]. Antibody

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13C6 immunoprecipitates both GP and secreted GP (sGP) from infected cells but cannot bind denatured and reduced GP, suggesting that 13C6 binds to a specific conformational epitope located within the 295 amino acids shared between sGP and GP1 [20].

Additional EVD therapeutics that were provided to US patients with EVD include TKM-Ebola and brincidofovir [21, 22]. TKM-Ebola is an experimental EBOV treatment composed of lipid-encapsulated 2′-*O*-methyl modified siRNA designed to target the polymerase (L) and viral protein 35 (VP35) RNA of the Mayinga variant [23]. Geisbert et al previously demonstrated that treatment with siRNA targeting L, VP35, and VP24 RNA provided postexposure protection following EBOV infection in a nonhuman primate model [23]. The TKM-Ebola product that has been used to treat patients only consists of siRNA targeting the L and VP35 genes. Brincidofovir (HDP-CDV; CMX001) is a lipidated analogue of cidofovir with antiviral activity against herpes simplex virus type 1, cytomegalovirus, poxviruses, and EBOV [24–27]. The metabolized active structures of cidofovir and brincidofovir act as nucleoside analogues, and long-term treatment can generate cidofovir-resistant poxviruses and cytomegalovirus with mutations mapping to the viral polymerases [28–30].

While viral sequence data from the West African outbreak are rapidly accumulating, few EBOV sequence data are available from patients with EVD who received investigational therapeutics. Additionally, the influence of experimental EVD treatments on EBOV evolution has not been assessed. To evaluate whether these therapeutics selected for viral genetic changes, we sequenced EBOV directly from clinical blood specimens from patients with EVD and investigated the putative viral targets of these experimental treatments.

## MATERIALS AND METHODS

### Overview of Patient Samples

EBOV was confirmed in plasma or blood samples submitted to the Centers for Disease Control and Prevention (CDC) through the detection of viral RNA by EBOV-specific quantitative reverse-transcription polymerase chain reaction (RT-PCR) and/or the detection of EBOV-specific immunoglobulin M and immunoglobulin G (IgG) antibodies by enzyme-linked immunosorbent assay (ELISA). US patients with EVD received single or multiple treatments with therapeutics targeting EBOV, and, when possible, we analyzed data collected at pretreatment and posttreatment time points from a single patient. All but one of the patients with available samples for this study recovered from EVD. An overview of patient information can be found in Supplementary Table 1. This project was determined by CDC institutional review to be a nonresearch public health response activity, and institutional review board review was not required.

### Whole-Genome Sequencing and Bioinformatics Analysis

RNA from patient 3 was isolated from a blood sample collected into an ethylenediaminetetraacetic acid–containing tube, using Tripure (Roche) and the RNeasy Mini kit (Qiagen), and treated with recombinant RNase-free DNase I (Roche). Complementary DNA (cDNA) was created with random primers [31] and 1-step SSIII RT-PCR (Invitrogen) with EBOV-specific primers [32]. DNA was fragmented to a mean target size of 300 base pairs by Covaris shearing, formed into libraries by using NEBNext Ultra kits (NEB), and deep sequenced by using Illumina MiSeq Reagents v2, generating 250–base pair paired-end reads. Consensus whole-genome sequences were initially assembled by mapping reads from patient 3 to the reference virus, Ebola virus/H.sapiens-wt/SLE/2014/Makona-G3724 (KM233053), with CLC Genomics Workbench. Regions with incomplete coverage were resequenced using Makona-specific primers (F, A, A2, A3, B, C, D, D1, E, E2, F, and G; Supplementary Table 2) and 1-step SSIII RT-PCR (Invitrogen). RNA was isolated from patients 1, 2, and 4, using the MagMAX Pathogen RNA/DNA isolation kit (Invitrogen) and BeadRetriever (Invitrogen), and was treated with recombinant DNase I RNase-free (Roche). To generate cDNA, a series of 7 overlapping Makona-specific PCR fragments were generated using 1-step SSIII RT-PCR (Invitrogen; F, A, B, C, D, E, and G; Supplementary Table 2). PCR amplicons were fragmented to a mean target size of 300 base pairs by Covaris shearing, formed into libraries by using NEBNext Ultra kits (NEB), and deep sequenced by using Illumina MiSeq Reagents v2 or MiSeq Reagents v2 nano, generating 250–base pair paired-end reads.

Whole-genome sequences for all patient samples were assembled by mapping reads to reference genomes Ebola virus/H.sapiens-wt/LBR/2014/Makona-201403007 (KP178538) and Ebola virus/H.sapiens-wt/SLE/2014/Makona-G3686.1 (KM034562), using BWA mem, SAMtools mpileup (-d 10000000), bcftools, and vcfutils.pl vcf2fq. Duplicates were removed with Picard. We did not observe consensus sequence differences when reads were mapped to different reference genomes. Any nucleotide variants in viral consensus sequences were resolved by hand with Integrated Genome Viewer (Broad Institute) to ensure that variants were randomly mapped in reads and not due to PCR amplification of a single copy. Only major variants are included in viral consensus sequences. Viral regions targeted by experimental treatments were aligned and compared using CLC Genomics Workbench. Variant calling was performed using the IRMA v0.5.9 pipeline (manuscript in preparation), which principally relied on BLAT, SAM, and SSW. Single-nucleotide variants were restricted to those with a heuristic frequency of ≥1.5%, while indels were those with a heuristic frequency of ≥0.45%. For a more detailed explanation of phylogenetic tree construction and data analysis, see the Supplementary Technical Appendix. Genomes acquired from clinical specimens were deposited into GenBank (accession numbers KP178538, KP240932-5, and KT589389-90).

## RESULTS

### ZMapp Treatment

To investigate the effect of ZMapp on viral epitopes, we generated sequence EBOV/Mak-201403007 (KP178538) from patient 3, who received 1 dose of ZMapp 2 days before specimen collection. All other viral sequences generated from clinical material were from ZMapp-untreated patients or from clinical material collected before ZMapp treatment. At the 2G4 epitope (amino acids 502–516), we observed nearly 100% consensus across patient isolates from the current outbreak, independent of whether the patients received ZMapp treatment (Figure 1*A*). These sequences only differed from those of the 1976 Yambuku and 1995 Kikwit variant isolates by 1 amino acid (A504→V), which was present in previously published Guinean and Sierra

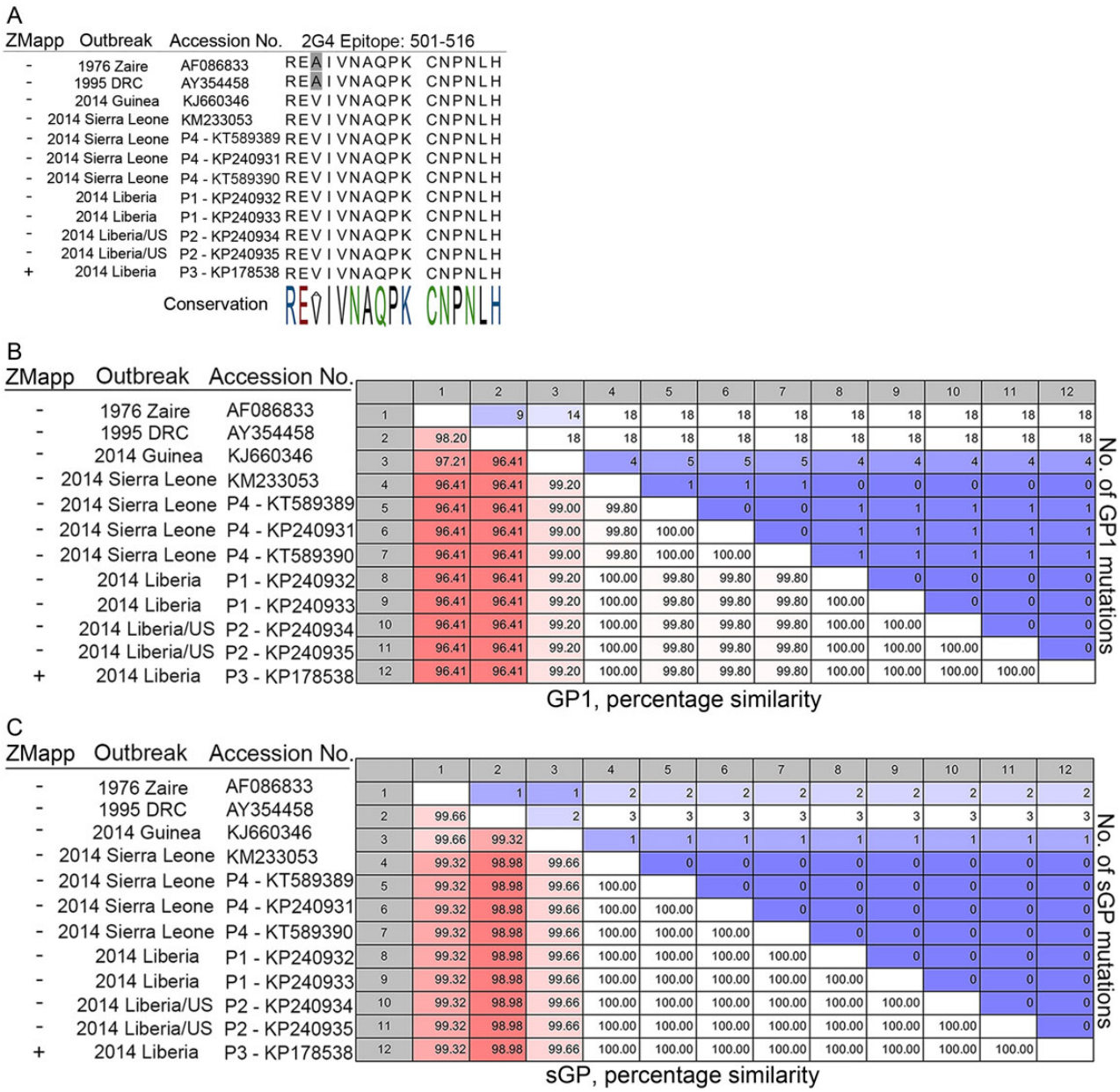


**Figure 1.** ZMapp monoclonal antibody effect on US Ebola virus (EBOV) sequences from US patients with EBOV disease. *A*, Alignment of patient EBOV sequences at the 2G4 epitope site in glycoprotein 2 (GP2). Patients who received ZMapp are designated with a plus sign, and those who were not treated with ZMapp are designed with a minus sign. Amino acids that differ from current 2014 outbreak sequences are highlighted in gray. *B*, Comparison of GP1 sequences from patients' clinical specimens at the putative 4G7 GP1 epitope site. Cells highlighted in blue indicate <5 amino acid changes, and cells highlighted in pink indicate changes in GP1 percentage similarity. *C*, Comparison of amino-terminal GP1 and secreted GP (sGP) sequences from patient clinical material at the putative 13C6 epitope. Only the amino-terminal 295 amino acids shared between GP1 and sGP sequences constituting the putative 13C6 epitope were compared. Cells highlighted in blue indicate <3 amino acid changes, and cells highlighted in pink indicate changes in sGP percentage similarity.

Leone sequences. When we compared the GP1 sequences between isolates (4G7 epitope), we observed only 1 amino acid change among the viruses identified from US patients with EVD (Figure 1*B* and Supplementary Figure 2). At the GP1/GP2 cleavage site (4G7 epitope; amino acids 453–501), there was nearly 100% sequence conservation between strains, independent of ZMapp treatment (Supplementary Figure 3). The closest mutation that we observed between isolates near this epitope was found at position 454, which was similar between the 1976 Yambuku and 1995 Kikwit variant isolates (454H) but differed in the 2014 Guinea, Sierra Leone, and patient sequences (454Y; Supplementary Figure 2). When we compared the amino acids shared between sGP and GP1 (13C6 epitope), we found 100% amino acid similarity between the US EBOV sequences (Figure 1*C* and Supplementary Figure 4).

#### TKM-Ebola Treatment

To investigate the effect of TKM-Ebola on viral nucleotide sequences, we sequenced EBOV from patient 4, who received 5 doses of TKM-Ebola. EBOV/Mak-201409581 (KT589389) was acquired before treatment, while EBOV/Mak-201403147 (KP240931) was acquired immediately after TKM-Ebola treatment and EBOV/Mak-201403164 (KT589390) was acquired 4 days after treatment. All other viral sequences generated from clinical material were from TKM-Ebola–untreated patients or from clinical material collected before TKM-Ebola treatment. We did not observe any nucleotide changes associated with TKM-Ebola treatment at either the EK-1 polymerase or VP35-855 binding sites (Figure 2*A* and 2*B*). However, we observed 2 nucleotide mutations associated with the current Western Africa outbreak that are within the 5′ 2–8 nucleotides located within the putative seed site of the siRNA guide, in agreement with Thi et al [33].

#### Brincidofovir Treatment

Since brincidofovir/cidofovir-resistance frequently maps to a viral DNA polymerase [28–30] we investigated whether EBOV from patients treated with brincidofovir also exhibit mutations

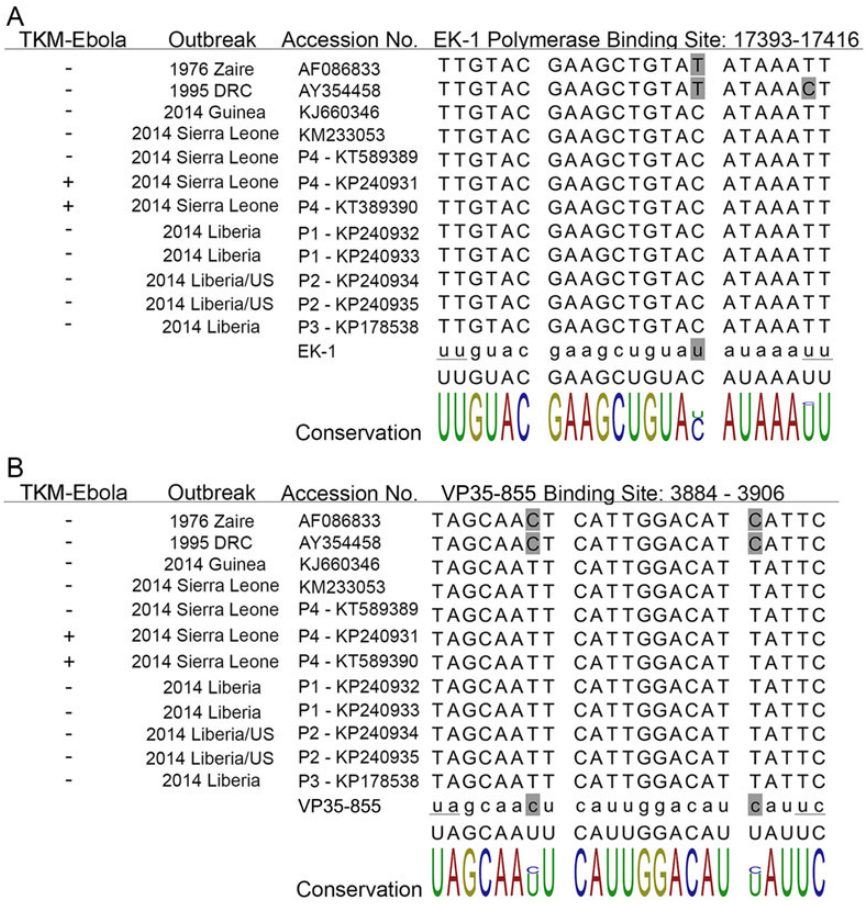


**Figure 2.** TKM-Ebola small interfering (siRNA) effect on imported US Ebola virus (EBOV) sequences. *A*, Alignment of patient EBOV sequences at the TKM-Ebola EK-1 polymerase-binding site. Patients who received TKM-Ebola are designated with a plus sign, and those who were not treated with ZMapp are designed with a minus sign. Nucleotides that differ from current 2014 outbreak sequences are highlighted in gray. siRNAs contain 3′ single-base-paired dinucleotide overhangs (underlined). *B*, Alignment of patient EBOV sequences at the TKM-Ebola VP35-855 binding site. Patients who received TKM-Ebola are designated with a plus sign. Nucleotides that differ from current 2014 outbreak sequences are highlighted in gray. siRNAs contain 3′ single-base-paired dinucleotide overhangs (underlined).

| Brincidofovir | Outbreak | Accession No. |
|---|---|---|
| - | 1976 Zaire | AF086833 |
| - | 1995 DRC | AY354458 |
| - | 2014 Guinea | KJ660346 |
| - | 2014 Sierra Leone | KM233053 |
| - | 2014 Sierra Leone | P4 - KT589389 |
| - | 2014 Sierra Leone | P4 - KP240931 |
| - | 2014 Sierra Leone | P4 - KT589390 |
| - | 2014 Liberia | P1 - KP240932 |
| + | 2014 Liberia | P1 - KP240933 |
| - | 2014 Liberia/US | P2 - KP240934 |
| + | 2014 Liberia/US | P2 - KP240935 |
| - | 2014 Liberia | P3 - KP178538 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 11 | 26 | 27 | 29 | 29 | 29 | 28 | 28 | 28 | 28 | 27 |
| 2 | 99.50 | | 25 | 26 | 28 | 28 | 28 | 27 | 27 | 27 | 27 | 26 |
| 3 | 98.83 | 98.87 | | 1 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 1 |
| 4 | 98.78 | 98.83 | 99.95 | | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 0 |
| 5 | 98.69 | 98.73 | 99.86 | 99.91 | | 0 | 0 | 3 | 3 | 3 | 3 | 2 |
| 6 | 98.69 | 98.73 | 99.86 | 99.91 | 100.00 | | 0 | 3 | 3 | 3 | 3 | 2 |
| 7 | 98.69 | 98.73 | 99.86 | 99.91 | 100.00 | 100.00 | | 3 | 3 | 3 | 3 | 2 |
| 8 | 98.73 | 98.78 | 99.91 | 99.95 | 99.86 | 99.86 | 99.86 | | 0 | 0 | 0 | 1 |
| 9 | 98.73 | 98.78 | 99.91 | 99.95 | 99.86 | 99.86 | 99.86 | 100.00 | | 0 | 0 | 1 |
| 10 | 98.73 | 98.78 | 99.91 | 99.95 | 99.86 | 99.86 | 99.86 | 100.00 | 100.00 | | 0 | 1 |
| 11 | 98.73 | 98.78 | 99.91 | 99.95 | 99.86 | 99.86 | 99.86 | 100.00 | 100.00 | 100.00 | | 1 |
| 12 | 98.78 | 98.83 | 99.95 | 100.00 | 99.91 | 99.91 | 99.91 | 99.95 | 99.95 | 99.95 | 99.95 | |

No. of polymerase mutations

Polymerase, percentage similarity

**Figure 3.** Brincidofovir effect on Ebola virus (EBOV) sequences. Comparison of EBOV polymerase amino acid sequences from multiple US patients with EBOV disease who were (+) or were not (–) treated with brincidofovir. Cells highlighted in blue indicate <11 amino acid changes, and cells highlighted in pink indicate changes in polymerase percentage similarity.

within the RNA polymerase. Patients 1 and 2 were both treated with a single 200-mg oral dose of brincidofovir. EBOV/Mak-201403261 (patient 1: KP240932) and EBOV/Mak-201403293 (patient 2: KP240934) were acquired before treatment and samples EBOV/Mak-201403275 (patient 1: KP240933) and EBOV/Mak-201403305 (patient 2: KP240935) were acquired 2 days after treatment [15]. EBOV/Mak-201403305 (KP240935) was also acquired after patient 2 received 2 units of intravenous convalescent plasma. Compared with EBOV sequences obtained from the same patients before treatment, we did not observe any differences in the polymerase sequences after brincidofovir treatment for either patient (Figure 3). Compared with the other EBOV sequences from the current Western Africa outbreak, we observed ≤3 amino acid changes between the viral polymerase sequences (Figure 3). To investigate whether brincidofovir-associated mutations may arise within regions outside of the viral polymerase, we compared whole-genome sequences between patient samples that were pre- and post-brincidofovir treatment. For patient 1 we observed only a single nucleotide change (3946), resulting in a silent mutation (EBOV/Mak-201403261 vs EBOV/Mak-201403275). For patient 2, we did not observe any nucleotide changes in viral sequences acquired before and after brincidofovir treatment (EBOV/Mak-201403293 vs EBOV/Mak-201403305).

#### Minor Viral Populations in Drug Target Sites

Most patient clinical samples were acquired within a short treatment period; therefore, resistant alleles may appear at low frequencies within the viral population. Thus, we investigated minor viral populations within clinical samples (Figure 4 and Supplementary Tables 4–6). The variant-calling algorithm identified the GP transcriptional editing site (indel 6917) [34] within the majority of clinical samples at or near the established heuristic values (Supplementary Tables 5 and 6). Minor alleles were observed at frequencies as high as 46%, but indels were observed at much lower frequencies. Most indels occurred near homopolymer tracts but at frequencies (0.475%–6.3%) greater than those commonly observed for MiSeq indel error rates (<0.001%) [35]. Genomes acquired from a single patient (patient 1: EBOV/Mak-201403261 vs EBOV/Mak-201403275; patient 2: EBOV/Mak-201403293 vs EBOV/Mak-201403305) exhibited dissimilar allele and indel frequencies (Figure 4 and Supplementary Tables 4–6) despite direct transmission of virus between patients and collection times spanning 11 and 2 days, respectively. Genomes acquired from patient 4 exhibited several conserved minor variants (G2814A-NP, G7364T-GP, C10410A-VP24, and C11543T-L), but none of these alleles generated nonsynonymous mutations. A C or T transition at site 3947 was observed at high frequency in the majority of

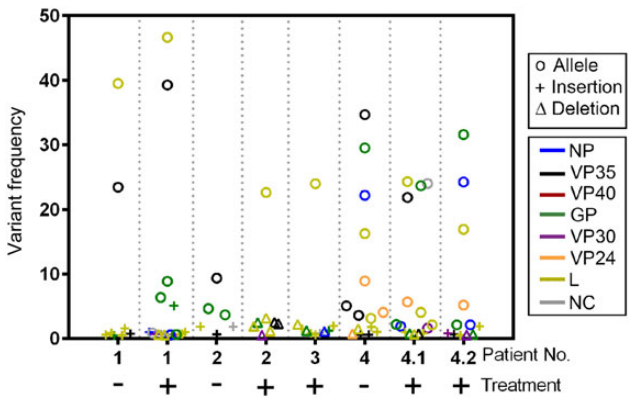


**Figure 4.** Minor viral populations from US patients with Ebola virus (EBOV) disease. Variant calling was performed using the IRMA v0.5.9 pipeline. Viral alleles were included if their frequency was >1.5%, while indels were included if their frequency was >0.45%. Data from patients 4.1 and 4.2 are from isolates EBOV/Mak-201409581(KT589389) and EBOV/Mak-201403164 (KT589390), respectively. For a more detailed listing of data analysis, see the Supplementary Technical Appendix. Abbreviations: GP, glycoprotein; L, polymerase; NC, non-coding region; NP, nucleoprotein; VP, viral protein; –, treated; +, untreated.

genomes, resulting in a silent mutation within VP35. Overall, we observed no minor alleles or indels in viral regions targeted by investigational therapeutics, and variants were also not enriched in polymerase sequences acquired from brincidofovir-treated patients.

#### EBOV Genome Comparison From West African and US Patients With EVD

Next, we investigated how EBOV sequences related phylogenetically with those from Western Africa and observed that they clustered with sequences from similar geographic and temporal lineages. EBOV/Mak-201403147 (patient 4) clustered with SL4 viral sequences acquired from Kenema Government Hospital (KGH) in August (EBOV/Mak-G4982, KR105288; EBOV/Mak-G4981, KR105287; and EBOV/Mak-G4698, KR105263; Figure 5). This patient developed symptoms in late August 2014 and was likely infected at KGH [21]. Next, viral sequences from US patients with EVD who were infected in Liberia appeared more similar to Liberian and SL2 sequences than other clades (Figure 5) but did not cluster together, supporting independent infections from distinct Liberian lineages. EBOV/Mak-201403007 from patient 3 (KP178538) (acquired on 3 August 2014) was most closely related to viral sequences acquired from Margibi and Montserrado on 26 August 2014. Sequences of EBOV from patients 1 and 2, who were epidemiologically related, clustered together on a separate lineage, consistent with known virus transmission from patient 1 to 2 (Figure 5). We observed that sequences from patients 1 and 2 contain an early stop codon within VP30, which eliminates the C-terminal 6 amino acids. This mutation was also observed in closely related viral sequences, EBOV/Mak-LIBR0090 (KR006945) and EBOV/Mak-LIBR10053 (KR006963), collected from Liberia in early October and November 2014. An identical mutation in VP30 was observed from a sequence acquired from Guinea as early as 30 September 2014 (EBOV/Mak-EM_000958, KR817084), and a similar VP30 C-terminal mutation was observed in a Guinean sequence from 31 January 2015 (EBOV/Mak-EM_004589, KR817106). Together, these data suggest that the VP30 truncation was newly acquired around September and continued to persist in the population until at least January 2015.

## DISCUSSION

We assessed whether experimental EVD treatments can influence early EBOV evolution after human infection, and overall, we did not observe any major or minor viral mutations in the specimens tested within regions targeted by these compounds. This data suggests that investigational therapeutics administered to US patients with EVD did not exert a strong selective force leading to the production of viral escape mutants. However, we have not directly tested whether viral isolates from these patients exhibit antiviral resistance in vitro and few samples were available for testing. In fact, these therapeutics may have reduced viral load, and, as a consequence, reduced the probability of generating resistant viral populations.

Development of viral resistance to these and similar antivirals is not without precedents. For example, EBOV GP escape mutations were generated in a ZMAb-treated nonsurviving nonhuman primate [17] and using a recombinant vesicular stomatitis virus ΔG-EBOVGP repeatedly passaged in cells in the presence of mAbs 2G4, 4G7, and 1H3 [36]. Additionally, cytomegalovirus DNA polymerase escape mutations generating brincidofovir resistance occurred when the virus was grown in tissue culture with increasing concentrations of brincidofovir over a 10-month period [29]. Finally, mutations within regions targeted by lipid encapsulated 2′-*O*-methyl modified synthetic short hairpin RNAs (sshRNAs) occurred in mice infected with hepatitis C virus (HCV) but did not appear in mice treated with scrambled sshRNA controls [37]. Furthermore, the escape mutations occurred in HCV within sshRNA seed regions (nucleotides 2–8 of the guide sequence) and reduced sshRNA efficacy [29]. Nucleotides 2–8 of the guide sequence are exposed to the solvent by the Argonaut proteins and mediate target recognition [38]. These mutations are similar to the sequence changes that we and others [33, 39] have observed in VP35 sequences from the current outbreak and suggest that TKM-Ebola VP35-855 siRNA may not effectively target current outbreak strains. Thi et al observed that TKM-Ebola can reduce Makona virus RNA levels but not as efficiently as siRNA specific to the Makona variant [33]. To address these concerns, Tekmira modified their TKM-Ebola product to target the Makona variant (TKM-Ebola-Guinea), but their clinical trial was recently terminated due to a lack of overall therapeutic benefit [40]. Chimerix also halted their clinical trials of brincidofovir in Western Africa [41].

Still unresolved is why we did not observe similar EBOV escape mutations within antiviral-treated patients. With the exception of patient 4 (who received 5 doses of TKM-Ebola), all patient samples were analyzed 2 days after they had received only a single dose of an investigational therapeutic (patients 1 and 2, brincidofovir; patient 3, ZMapp). Thus, the sampling period may have been too short to permit drug-resistant viruses to appear, or the virus was unable to produce escape mutants due to constraints or other epistatic interactions. However, additional sequences from patient 3 (13 days after onset) and patient 4 (2, 4, and 6 days after onset) were identical to sequences generated here (Whitmer et al, unpublished data). Prolonged exposure to suboptimal antiviral treatment may also lead to an increased potential to generate viral mutations. For example, previous in vitro ZMab, brincidofovir, and in vivo sshRNA escape mutants were generated over a longer period (28 days–10 months) [29, 36, 37], with the exception of the ZMab-treated nonsurviving nonhuman primate (8 days after infection, following 3 ZMab treatments) [17]. While brincidofovir/cidofovir-resistance maps to poxviral and cytomegalovirus DNA polymerases [28–30], the EBOV RNA polymerase only has

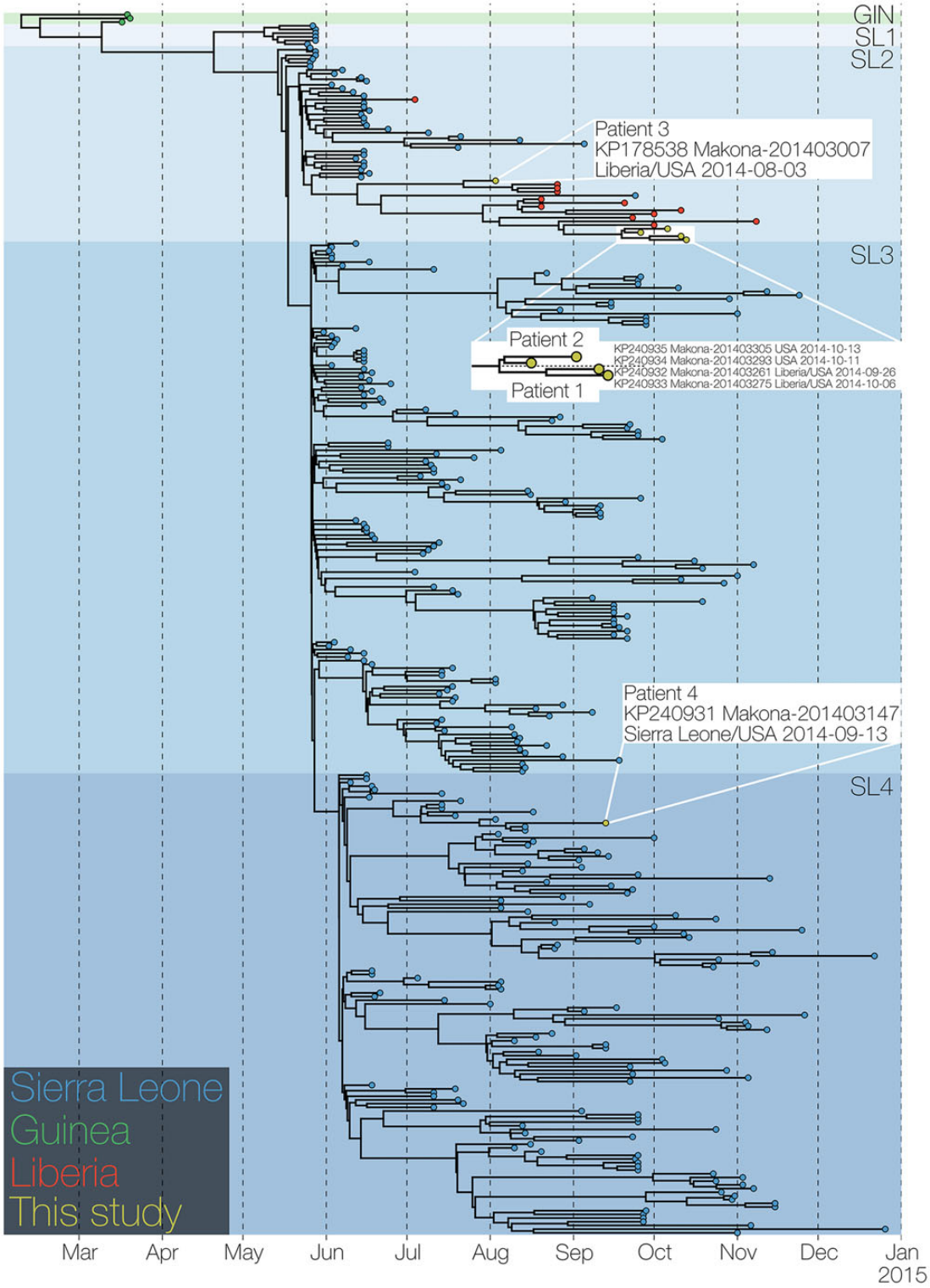


**Figure 5.** A phylogenetic tree was inferred with BEAST, using 331 Ebola virus genomes from the 2014 Western African Ebola virus disease outbreak. Sequences new to this project are labeled in yellow. Additional sequences from Sierra Leone are from Gire et al [8] and Park et al [13], Guinean sequences are from by Baize et al [1], and Liberian sequences are from Kugelman et al [12].

10%–11% homology with these polymerases and exhibits reduced similarity in regions conferring resistance. The therapeutic dose provided to patients likely remained high during the sampling period, reducing the possibility of generating viral mutations under suboptimal antiviral concentrations. Patient 3 likely still had therapeutic ZMapp levels, because IgG against

EBOV lysate (a mixture of ZMapp and endogenous antibodies) confirmed the presence of ZMapp IgG before nucleoprotein-specific IgG (endogenous antibodies) levels peaked [5]. These ELISAs were titered with the same serum sample that was used for viral sequencing. While we cannot assess brincidofovir levels or effectiveness within patients 1 and 2, we suspect that brincidofovir plasma levels were likely still high at the time point sampled (approximately 48 hours after treatment), since the plasma half-life at 2.0 mg/kg is 24 hours [42].

While treatments likely remained at a therapeutic level within patients, clinical samples were acquired following a short treatment period, which may not have allowed resistant viral populations enough time to develop as the major allele. We did not observe any minor mutations associated with viral resistance, but additional sequencing following longer treatment periods will elucidate whether extended in vivo treatment can mimic the purifying selection observed in vitro. Minor alleles are likely a reflection of variation due to EBOV replication but could also reflect additional variation due to sample preparation and sequencing errors. We observed that intrahost variant frequencies were unstable in 2 individual patients following direct viral transmission, but they were more stable in a third patient. Since minor variant populations can support epidemiological data [13], these observations highlight the need for additional emphasis on viral population dynamics. Ongoing surveillance by in-country sequencers could recognize minor viral allele populations in real time before their evolution into major populations.

Most of the patient samples we analyzed were collected from patients who ultimately recovered from EVD, excepting 1 fatal outcome. The 2 samples that were analyzed from the deceased patient were obtained before drug treatment (EBOV/Mak-201403261, KP240932) and after brincidofovir treatment and 2 days before the patient died (EBOV/Mak-201403275, KP240933). We only observed a single synonymous nucleotide change between the sequences acquired at these 2 separate time points, suggesting that the patient's death was likely due to advanced disease, infectious dose received, host genetics, or an inability of the immune response to contain viral infection and was not a consequence of viral mutation. Furthermore, patient 2 acquired EBOV infection from patient 1, and we did not observe any differences in the viral sequences isolated from patient 1 (EBOV/Mak-201403261, KP240932) and patient 2 pretreatment and posttreatment time points (EBOV/Mak-201403293, KP240934; EBOV/Mak-201403305, KP240935). We were intrigued by the observation that sequences from patients 1 and 2 contain an early stop codon within VP30, which eliminates the C-terminal 6 amino acids (282–288). A similar mutation was also observed within 2/25 Liberian sequences by Kugelman et al [12]. The C-terminal domain of VP30 (residues 142–272) has previously been shown to participate in nucleocapsid interaction and transcription activation [43], but the significance of the extreme C-terminal residues are currently unknown. Additional experiments will need to be conducted to investigate whether these residues have any impact on viral replication or pathogenesis.

Since we did not observe experimental drug-related mutations with viruses sequenced directly from a small number of patients, we hypothesize that, if effective at reducing viral load or improving clinical course and clinical outcomes in patients, investigational EVD therapeutics may be considered for broader use in EVD therapy. Most patients with EVD hospitalized in the United States and Europe received investigational therapies on an uncontrolled basis [15]. Some trials of investigational therapeutics for EVD were conducted in West Africa during 2014 and 2015, but only 1 randomized clinical trial was conducted (ZMapp). As EBOV-specific treatments are used more widely in the future for patients with EVD, it is essential that continuous EBOV full-genome surveillance is performed to monitor for the emergence of escape mutations.

## Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

## Notes

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