# Medical countermeasures during the 2018 Ebola virus disease outbreak in the North Kivu and Ituri Provinces of the Democratic Republic of the Congo: a rapid genomic assessment

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## Summary

**Background** The real-time generation of information about pathogen genomes has become a vital goal for transmission analysis and characterisation in rapid outbreak responses. In response to the recently established genomic capacity in the Democratic Republic of the Congo, we explored the real-time generation of genomic information at the start of the 2018 Ebola virus disease (EVD) outbreak in North Kivu Province.

**Methods** We used targeted-enrichment sequencing to produce two coding-complete Ebola virus genomes 5 days after declaration of the EVD outbreak in North Kivu. Subsequent sequencing efforts yielded an additional 46 genomes. Genomic information was used to assess early transmission, medical countermeasures, and evolution of Ebola virus.

**Findings** The genomic information demonstrated that the EVD outbreak in the North Kivu and Ituri Provinces was distinct from the 2018 EVD outbreak in Équateur Province of the Democratic Republic of the Congo. Primer and probe mismatches to Ebola virus were identified in silico for all deployed diagnostic PCR assays, with the exception of the Cepheid GeneXpert *GP* assay.

**Interpretation** The first two coding-complete genomes provided actionable information in real-time for the deployment of the rVSVΔG-ZEBOV-GP Ebola virus envelope glycoprotein vaccine, available therapeutics, and sequence-based diagnostic assays. Based on the mutations identified in the Ebola virus surface glycoprotein ($GP_{1,2}$) observed in all 48 genomes, deployed monoclonal antibody therapeutics (mAb114 and ZMapp) should be efficacious against the circulating Ebola virus variant. Rapid Ebola virus genomic characterisation should be included in routine EVD outbreak response procedures to ascertain efficacy of medical countermeasures.

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## Introduction

On July 28, 2018, the Provincial Health Division of North Kivu, in the northeast region of the Democratic Republic of the Congo, notified national authorities of a cluster of 26 suspected cases of acute viral haemorrhagic fever associated with 20 deaths. All 26 cases were reported from the Mabalako health zone in North Kivu Province,[1] which borders Uganda and Rwanda. Blood, plasma, saliva, and semen samples were diagnostically tested at the Institut National de Recherche Biomédicale (INRB) in Kinshasa (Democratic Republic of the Congo), which identified the aetiological agent as Ebola virus (EBOV; order Mononegavirales, family Filoviridae, genus Ebolavirus, species Zaire ebolavirus). On Aug 1, 2018, the Ministry of Health announced the country's tenth Ebola virus disease (EVD) outbreak (including Bundibugyo virus), 8 days after another EVD outbreak was declared over in Équateur Province (appendix).[2]

The benefits of whole-genome sequencing when applied to outbreak responses have been demonstrated frequently,[3–5] but the ability to apply these sequencing techniques to field settings is relatively new. Historically, diagnostic samples from EVD outbreaks were sent to established genomic facilities, typically overseas, for sequencing. The 2013–16 EVD outbreak in Western Africa—defined according to UN Statistics Division geographical locations—was the first EVD outbreak in which sequencing was a major component of the outbreak response. The first diagnostic samples were sent to Germany for confirmation and sequencing in

## Research in context

### Evidence before this study

The capacity for in-country whole-genome sequencing and the ability to obtain actionable information to strategically select diagnostic assays and immunotherapy have been important goals of rapid response teams in an Ebola virus disease (EVD) outbreak. Thus, we were interested in previous studies done in field settings that assessed medical countermeasures and target erosion in diagnostic assays in real-time. We searched PubMed from database inception to Aug 15, 2018, without language restrictions for articles using the terms "EVD", "Ebola", or "Zaire ebola", "treatment", "diagnostic", "genomic epidemiology", "Beni" or "Nord-Kivu." Our search yielded more than ten articles discussing the implications of an outbreak of EVD during military conflict and documentation of public misconceptions about infectious disease that can hinder disease prevention and contact tracing.

### Added value of this study

In this study, early and continuous evaluation of diagnostic assays and therapeutics was done using in-country genomic sequencing capacities. Two Ebola virus (EBOV)-positive samples provided evidence for substantial target erosion effects in some deployed diagnostic assays used to generate actionable information. The information also provided guidance for vaccine deployment. We also introduced a novel method for reducing the time from sample acquisition to sequence assembly that resulted in the generation of 48 EBOV genomes and provided proof-of-concept for the in-vivo evaluation of deployed immunotherapeutic treatments.

### Implications of all the available evidence

The establishment of in-country genomic capacities for real-time sequencing reduces the time from patient sample acquisition to assembly of EBOV viral genomes. Greater investment in in-country genomic and outbreak response teams can positively influence response times by generating actionable information for diagnostics and evaluating the efficacy of medical countermeasures.

March 2014,[3] with genomes shared on GenBank within 10 days of the start of the outbreak. The establishment of genomic capabilities in-country began with the use of desktop sequencers in Liberia and Sierra Leone,[4,5] followed by the deployment of portable hand-held devices.[6] By the end of the outbreak, several groups had deployed genomic capabilities to Western Africa, sequencing approximately 1500 samples, which represented 5% of individuals known to be infected by EBOV from the outbreak.[7] Once generated, data were used immediately to assess diagnostic assays and medical countermeasures, and inform the public health response.

The pre-emptive establishment of in-country genomic capacities, led by local scientists, could reduce the time from sample acquisition to sequence assembly and, therefore, accelerate the generation of actionable data. This capability could also be applied to more complex sequencing activities, such as resistance monitoring of prelicensed therapeutics and vaccines. In this analysis, we used a recently established sequencing capability at INRB[2] to provide genomic information in real-time for the 2018 EVD outbreak in North Kivu Province of the Democratic Republic of the Congo to assess early transmission, medical countermeasures, and EBOV evolution.

## Methods

### Sample collection and sequencing

Blood, plasma, saliva, and semen samples from patients with suspected EVD from the July 2018 outbreak in the North Kivu and Ituri Provinces were processed at mobile laboratories and INRB (appendix). The study was approved by the Ministry of Health of the Democratic Republic of the Congo, and oral consent was obtained from all patients before sample collection. Amplicon sequencing was done for virus identification using the MinION platform (Oxford Nanopore Technologies, Oxford, UK) as previously described (appendix).[8] Complete viral genome sequencing was done using the iSeq100 or MiSeq Desktop sequencer (Illumina Technologies, San Diego, CA, USA) using the KAPA RNA HyperPrep library preparation kit (KAPA Biosystems, Wilmington, MA, USA) followed by TruSeq Exome Enrichment, as previously described.[8] A subset of samples was prepared using the Nextera Flex for Enrichment method, which is under development by Illumina Technologies and shared as a research product in accordance with early access agreements (appendix). The method is based on library preparation by a bead-linked transposome, which produces libraries compatible with an additional single enrichment step. This protocol decreased the time from sample acquisition to assembled EBOV genome from 44 continuous hours (5·5 days with 8 working hours per day) to 28 continuous hours (3·5 days; appendix). For EBOV population reconstruction, we used unique molecular identifiers to calculate the true depth of coverage and MAGERI software to identify ultra-rare variants in sequencing data (appendix).[9] All other library preparation steps, raw read processing, and assembly are described in the appendix. Consensus EBOV genome sequences were deposited in GenBank (accession numbers MK007329-MK007344, MK088510, MK088515, and MK163644-MK163675; appendix).

### Genomic and diagnostic analysis

At the start of the July 2018 outbreak, INRB had several diagnostic assays readily available for deployment that had been used during the May 2018 EVD outbreak in Équateur Province: the GeneXpert Ebola Assay (nucleoprotein [*NP*] and glycoprotein gene [*GP*] targets; Cepheid, Sunnyvale, CA, USA),[10] a modified version of an EBOV *NP* assay,[11] and an assay that targets the EBOV

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For more on **UN standard country and area codes** see https://unstats.un.org/

See **Online** for appendix

For more on **Geneious 11.1.4** see www.geneious.com

RNA-dependent RNA polymerase (*L*) gene.[12] Primer3 (available in Geneious 11.1.4) was used for in silico analyses of 25 readily available and additional published diagnostic assays.[13] Primer and probe sequences for each molecular assay were mapped to the available EBOV genomes from the EVD outbreak in July 2018. Nucleotide mismatches were manually confirmed by a pairwise alignment. The number and position of mismatches defined for each primer and probe set are shown in figure 1 and the appendix. Since the GeneXpert Ebola Assay is multiplexed, we were able to compare the performance of the assay during the EVD outbreak in the North Kivu and Ituri Provinces with a previous field evaluation study done during the 2013–16 EVD outbreak in Western Africa.[14] We calculated and compared the mean difference in Ct values for the *NP* and *GP* targets from each outbreak. A median-joining haplotype network was estimated (appendix). All data were immediately disseminated to partners involved in the outbreak response and used to test deployed diagnostics and medical countermeasures in silico.

## Antibody binding confirmation

The EBOV *GP* gene from the EVD outbreak in North Kivu Province was cloned by introducing glycoprotein ($GP_{1,2}$) differences identified during the outbreak compared with the reference EBOV Kikwit (EBOV/Kik) isolate (Val003Ala, Glu047Asp, Arg314Gly, Leu368Pro, Pro378Leu, Pro429Thr, Ala432Thr, Thr435Ala, Gly440Ser, Glu457Lys, Lys478Arg, Ala499Thr, and Gly557Arg) into an expression plasmid encoding EBOV/Kik $GP_{1,2}$ (GenBank accession number AAQ55048.1) using site directed mutagenesis with Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA). The resulting *GP* gene fragments were assembled using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs), and sequences were confirmed using Sanger sequencing. The primers used, and transfection and analysis of data are described in the appendix.

## Molecular evolution and rate analysis

Additional EBOV sequences were downloaded from GenBank; long stretches of ambiguous bases or sequences derived from experimental infections or constructs were removed. Our initial species-rank dataset included 59 sequences (appendix). We constructed a representative dataset (n=15) by selecting a single sequence (the earliest or most complete) from each EVD outbreak. All alignments, maximum-likelihood trees, root-to-tip regression, and single nucleotide polymorphism analyses were generated as previously described.[2] Clade-level analyses using a fixed local clock were estimated (appendix).

For species-rank alignments, Bayesian analysis using BEAST (versions 1.8.4 and 1.10.0; appendix)[15] was used to generate maximum-clade credibility trees. Substitution models, partition schemes, clock, tree priors, and model testing were done as previously described.[2] Monophyly was enforced for all sequences sampled after 1977 to constrain tree topology consistent with previous EBOV studies. We expanded EBOV rooting by repeating the Bayesian procedure done previously, including all available EBOV *GP* sequences without topology constraints (appendix).[16] Each analysis consisted of up to $1{\cdot}0\times10^9$ Markov chain Monte Carlo steps ($2{\cdot}5\times10^8$ were discarded as burn-in). Parameters and trees were sampled every 100 000 generations.

## Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of

| | EBOV gene target | Primer or probe type | Primer or probe sequence | Primer or probe mismatch | Study |
|---|---|---|---|---|---|
| GeneXpert_NP | *NP* | Reverse | CTGTGGCGACTCCGAGTGCAA | | Pinsky et al (2015)[10] |
| | | Probe* | TGAGCATGGTCTTTTCCCTCAAC | | |
| | | Forward* | GCTCCTTTCGCCCGACTTTTGAA | ========T========== | |
| GeneXpert_GP | *GP* | Reverse | GGAAGCCCCGAATCCCGT | | Pinsky et al (2015)[10] |
| | | Probe | CCTGACGGGAGTGAGTGTCTACC | | |
| | | Forward | GGGCTGAAAACTGCTACAATCTTGAAATC | | |
| NP_ENZ_mod | *NP* | Reverse | AGGACCAAGTCATCTGGTGC | ========A========== | Weidmann et al (2004)[11]† |
| | | Probe | CARAGTTACTCGGAAAACGGCAT | ========T========== | |
| | | Forward | ATGATGGARGCTACGGCG | ==========T======= | |
| EBOV_L | *L* | Forward | CAGCCAGCAATTTCTTCCAT | | de Wit et al (2016)[12] |
| | | Probe 1 | ATCATTGGCRTACTGGAGGAGCAG | | |
| | | Probe 2 | TCATTGGCGTACTGGAGGAGCAGG | | |
| | | Reverse‡ | TTTCGGTTGCTGTTTCTGTG | =C================= | |

***Figure 1:*** **Evaluation of deployed diagnostic EBOV assays**

EBOV=Ebola virus. *Mismatches occurred in all 48 sequences tested, except where marked, whereby only 42 sequences were tested because the near-complete MAN035 sequence had missing data in the binding region. †An ambiguous base (R) was used to modify the original probe sequences identified in this study. ‡Mismatches occurred in all 48 sequences tested, except where marked, whereby only MAN028 exhibited the mismatch.

the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

## Results

On July 31, 2018, six patient samples from the Mabalako health zone in North Kivu Province were shipped to INRB for testing, of which four samples were positive for EBOV.[10] Partial EBOV polymerase cofactor (*VP35*) gene amplicons were generated for two samples (18FHV090 and 18FHV092) and sequenced using the MinION (Oxford Nanopore Technologies).[17] A phylogenetic tree including other EBOV sequences indicated that EBOV was the causative disease agent in the six patients (figure 2A).

Samples 18FHV089 and 18FHV090 (no additional sample remained for 18FHV092) were prepared for sequencing using the Illumina iSeq100 (appendix). The first two coding-complete genomes were obtained on Aug 6, 2018.[18] No differences in *VP35* consensus sequences were found using the MinION or Illumina portable sequencing platforms for sample 18FHV090. A phylogenetic tree using coding-complete EBOV sequences indicated that the July 2018 outbreak in North Kivu Province was distinct from the May 2018 outbreak in Équateur Province (caused by EBOV/"Tum") and all previously described EBOV variants, justifying the establishment of a new EBOV variant (figure 2B). Considering current conventions[19] and proximity of the EVD outbreak in July 2018 to the Ituri River, we propose to name the variant EBOV/"Ituri" (EBOV/"Itu").

Samples were processed using the TruSeq RNA Exome and the new Nextera Flex for Enrichment targeted sequencing strategies with custom filovirus probes. The Nextera Flex for Enrichment method shortened the time from sample acquisition to generation of consensus sequences by 16 continuous hours (44 to 28 h, 5·5 to 3·5 days with 8 working hours per day; appendix). On Aug 24, 2018, INRB received 38 samples obtained from 26 patients in the Mandima (Ituri Province) and Mabalako (North Kivu Province) health zones (figure 2C). By Oct 12, 2018, 44 coding-complete and four partial viral genomes from 48 patient samples were sequenced with a mean genome coverage of 97·93% (18 572 of 18 965 nucleotides), ranging from 74·22% (14 075 of 18 965 nucleotides) to 99·96% (18 957 of 18 965 nucleotides; appendix). Initial characterisation of the intra-outbreak data identified 45 single nucleotide polymorphisms, including 20 non-synonymous changes (appendix).

The reported index case for the EVD outbreak in North Kivu Province was an individual who resided in Masimbembe (Mangina health zone).[20] No samples were collected, and the case is only listed as probable. However, the first two coding-complete genomes (18FHV089 and 18FHV090) originated from a group of individuals who had been admitted to hospital in Mangina. Reports indicate one of the individuals was discharged from hospital and died a few days later at home. A number of family members who cared for the individual developed clinical signs, seven of whom died.[1,21,22] On Aug 13, 2018, an individual from Mandima in the Mandima health zone of Ituri Province, who was previously admitted to

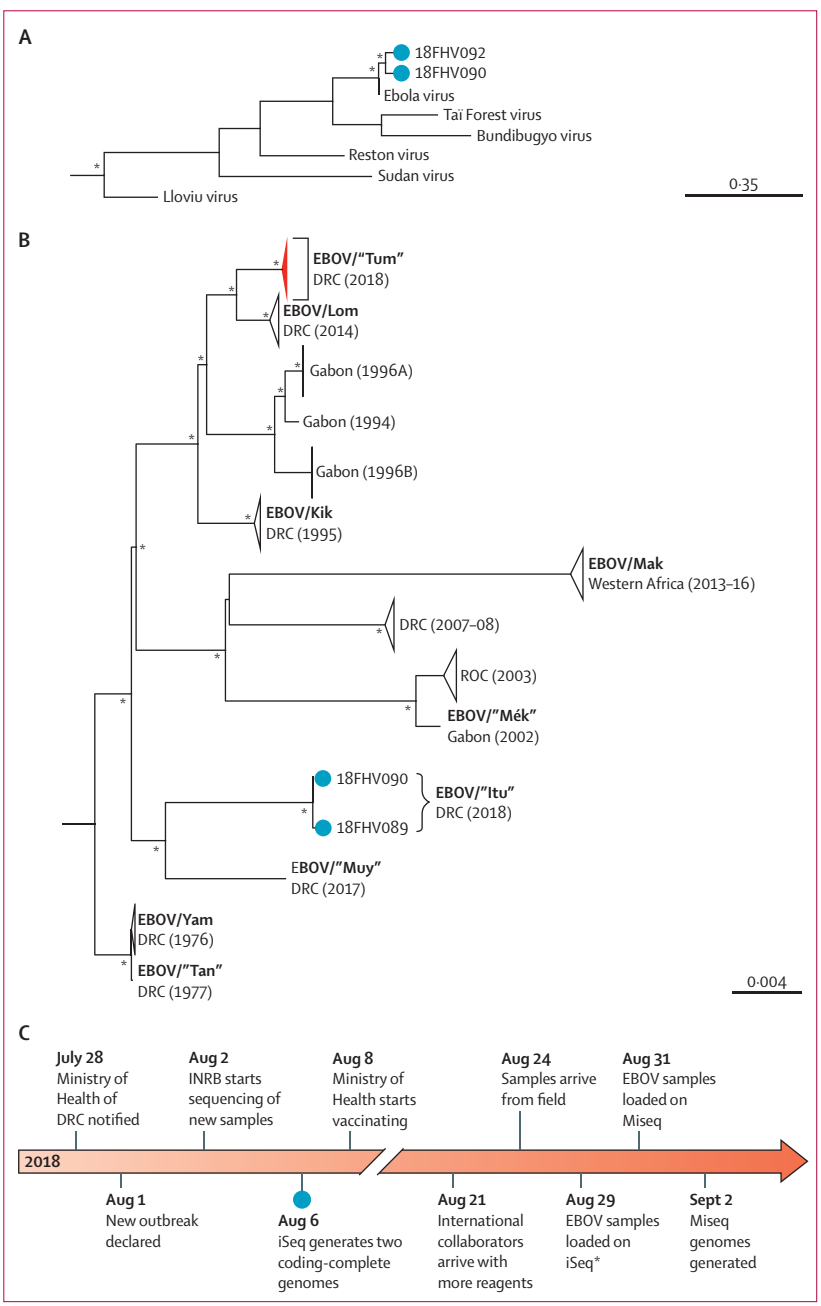


***Figure 2:*** **A summary of sequencing and notable events of the 2018 EVD outbreak in the North Kivu and Ituri Provinces**

(A) Maximum-likelihood tree using a 212-nucleotide sequence of the EBOV *VP35* gene showing the most likely Ebolavirus species in two early outbreak samples (blue circles). Asterisks indicate bootstrap support values greater than 70%. Scale is shown as substitutions per site. (B) Maximum-likelihood tree using coding-complete EBOV. Asterisks indicate bootstrap support values greater than 70%. Scale is shown as substitutions per site. (C) Timeline of the real-time response of in-country and international collaborators. The blue circle indicates the timepoint at which the two early outbreak samples were sequenced. EVD=Ebola virus disease. EBOV=Ebola virus. DRC=Democratic Republic of the Congo. ROC=Republic of the Congo. INRB=Institut National de Recherche Biomédicale. *Consensus sequences were assembled the following day.

hospital with heart problems, died and was later determined to be EVD positive. By Aug 15, 2018, six additional individuals were confirmed to have EVD in Mandima, demonstrating the spread of EBOV/"Itu" from North Kivu to Ituri Province.

Using the 48 genomes, a haplotype network was generated to provide information about the transmission chains (figure 3). Our analysis indicated that samples from Masimbembe (Mandima health zone), and one from the Beni health zone formed the core of a 13-case

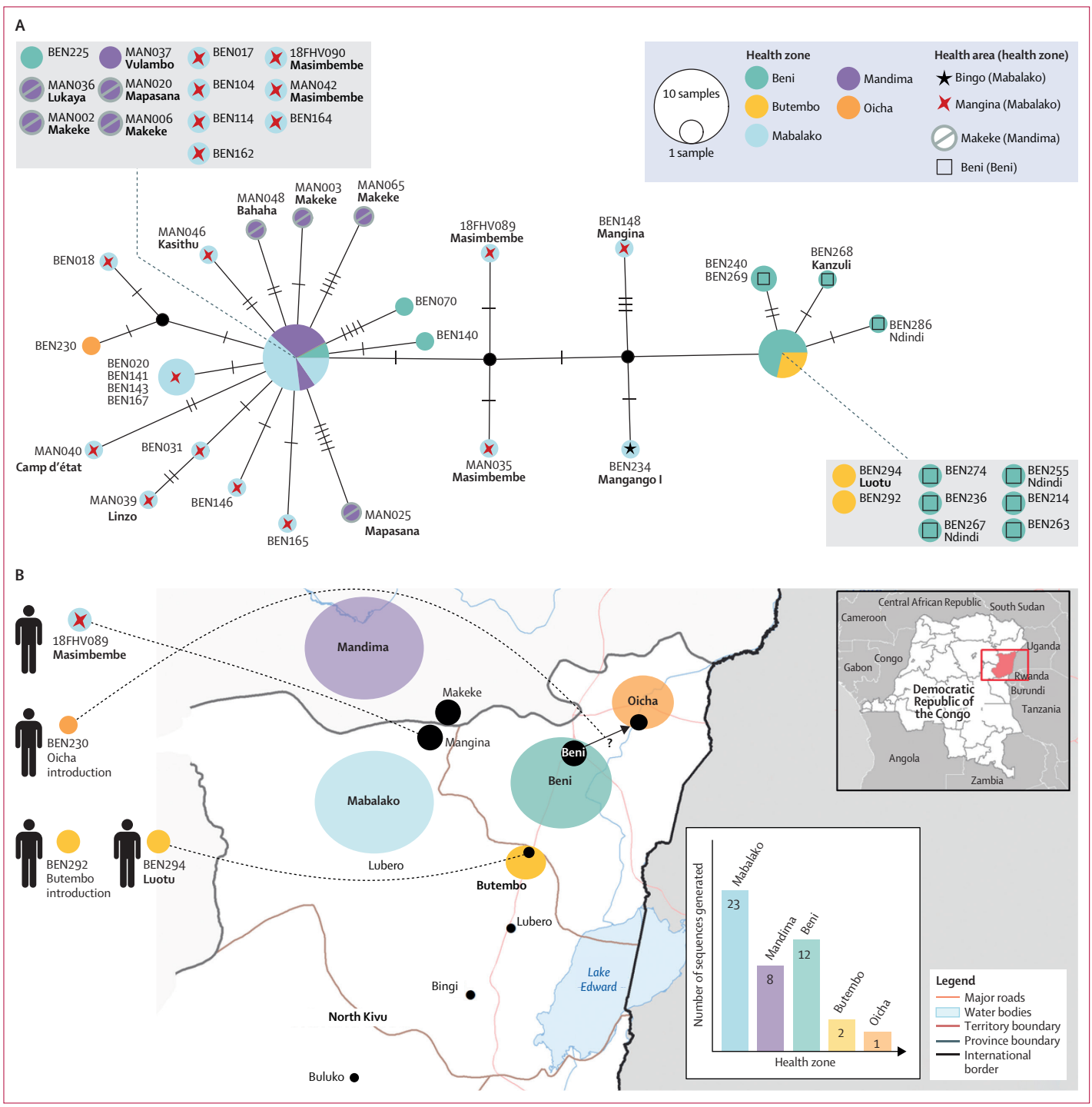


*Figure 3:* **Early transmission analysis of the 2018 EVD outbreak in the North Kivu and Ituri Provinces**
(A) Median-joining haplotype network using 46 EBOV/"Itu" sequences. Genomes are identified by health zone and health areas within health zones. Available patient villages are shown in bold. (B) Regional map of the Democratic Republic of the Congo provinces showing EVD cases of interest n health zones. EVD=Ebola virus disease.

cluster that circulated in the area from July 27, 2018, until approximately Aug 15, 2018 (figure 3; appendix), which was consistent with previously published reports.[1,20] The main cluster in the haplotype included one of the earliest samples analysed at INRB (18FHV090), supporting the epidemiological data. However, several probable EVD cases are considered likely to precede the earliest confirmed case, suggesting the outbreak might have started as early as May.[1] The genomic data further supports these hypotheses. In August 2018, an individual residing in Oicha (North Kivu Province) was readmitted to hospital after their partner was confirmed EVD positive after visiting Beni (North Kivu Province).[23] Retrospective analysis of the individual's medical history revealed clinical signs compatible with EVD that began on July 17, 2018, but the individual had tested negative for EBOV. However, during their readmittance to hospital in mid-August, the individual had an EBOV-positive semen sample (BEN230; figure 3), which provided evidence that EBOV persistence in survivors of EVD is not exclusive to a single variant (ie, EBOV/Mak).[24]

Our analysis inferred an EBOV transmission chain from Beni to a cluster of early cases in Butembo (North Kivu Province). These genomes, in addition to several others from Beni, established a second transmission cluster in the haplotype network. The respective EVD cases were detected after Aug 20, 2018. Several of the cases in Beni were detected in the Ndindi health area, which was a focal point of outbreak response teams during late August. On Sept 4, 2018, an EBOV-positive sample was obtained from an individual who had died in Butembo. The individual was known to have been in close proximity with an EBOV-positive individual from the Ndindi health area and fled to Butembo after becoming symptomatic. This was the first EVD case confirmed in Butembo. The attending nurse became the first local case and third fatality on Sept 5, 2018, in Luoto (Butembo).[25]

The generation of the first two coding-complete sequences enabled real-time in silico evaluation of available medical countermeasures at the beginning of the outbreak response. The three PCR-based detection assays deployed during the 2018 EVD outbreak in Équateur Province were evaluated for mismatches.[2] These included assays targeting the EBOV *L* gene,[12] the *NP* gene,[11] and the multiplex GeneXpert Ebola assay,[10] which amplifies both *NP* and *GP* genes (figure 1). No mismatches were found in the *L* gene assay, three mismatches (one in the forward primer, one in the probe, and one in the reverse primer) were found in the nucleoprotein assay, and a single mismatch was found in the forward primer of the GeneXpert Ebola assay at position 16 from the 3′ nucleoprotein gene end.

We compared the mean difference in Ct values between the *NP* and *GP* assay for positive EBOV/"Itu" and EBOV/Mak samples. The mean difference in Ct values between EBOV/"Itu" positive samples was –3·9 cycles (range –7·0 to 4·7), which is comparable to results from a field evaluation of positive EBOV/Mak samples (mean difference –4·1 cycles).[14] These results indicated that the single mismatch in the forward nucleoprotein primer of the GeneXpert assay did not affect the detection of EBOV/"Itu". We also evaluated an additional 21 published EBOV detection assays in silico (appendix).

The ZMapp monoclonal antibody cocktail is an experimental therapeutic that was first used to treat patients during the 2013–16 EVD outbreak in Western Africa.[26] We compared binding of the individual components of ZMapp to the EBOV/"Itu" $GP_{1,2}$ with that of the 1995 EBOV/Kik $GP_{1,2}$ using flow cytometry. mAb6D8 was used as a positive control since its well defined epitope was not altered in the EBOV/"Itu" $GP_{1,2}$ sequence (appendix). The individual ZMapp monoclonal antibodies (13C6, 2G4, and 4G7) bound to the EBOV/"Itu" $GP_{1,2}$ consensus sequence with similar efficiency to that of the EBOV/Kik $GP_{1,2}$, indicating that the monoclonal antibodies recognised the EBOV/"Itu" variant. Between Aug 28–31, 2018, downstream sequencing efforts revealed several changes in individual consensus sequences (appendix); none were located in amino acid residues previously found to disrupt the action of ZMapp monoclonal antibodies.[26,27] However, the Gly557Arg mutation partly affected binding of mAb4G7 to EBOV/"Itu" $GP_{1,2}$(appendix).

mAb114 is a monoclonal antibody therapeutic that was approved for compassionate use in the North Kivu and Ituri Province EVD outbreak. mAb114 protects rhesus monkeys (*Macaca mulatta*) from disease and death after an otherwise lethal dose of EBOV.[28,29] EBOV/"Itu" sequences were evaluated in silico for mutations that could alter mAb114 binding, focusing on residues located in the crystal structure interface between EBOV $GP_{1,2}$ and mAb114 (residues 113–121): no non-synonymous changes were found.[29]

We sequenced serum samples from five patients before and after mAb114 treatment to evaluate selection pressure on the EBOV population and changes in intra-host EBOV sequence diversity. Two of the patient samples had few EBOV-specific reads, which indicates a low viral load resulting in limited sequence coverage, and thus these two samples were excluded from further analysis. No amino acid changes were detected in the genomes of the three remaining samples. Furthermore, no single nucleotide polymorphisms or minority mutants were identified at residues 113–121 after treatment with mAb114. We evaluated intra-host variation during treatment to identify any treatment-induced changes in the viral population. No changes in diversity were identified after mAb114 treatment, but considering the small sample size, these results should be interpreted with caution (appendix). All five patients made a full recovery.

As the number of EBOV variants identified and characterised increases, broader patterns of EBOV evolution and spatiotemporal relationships can be

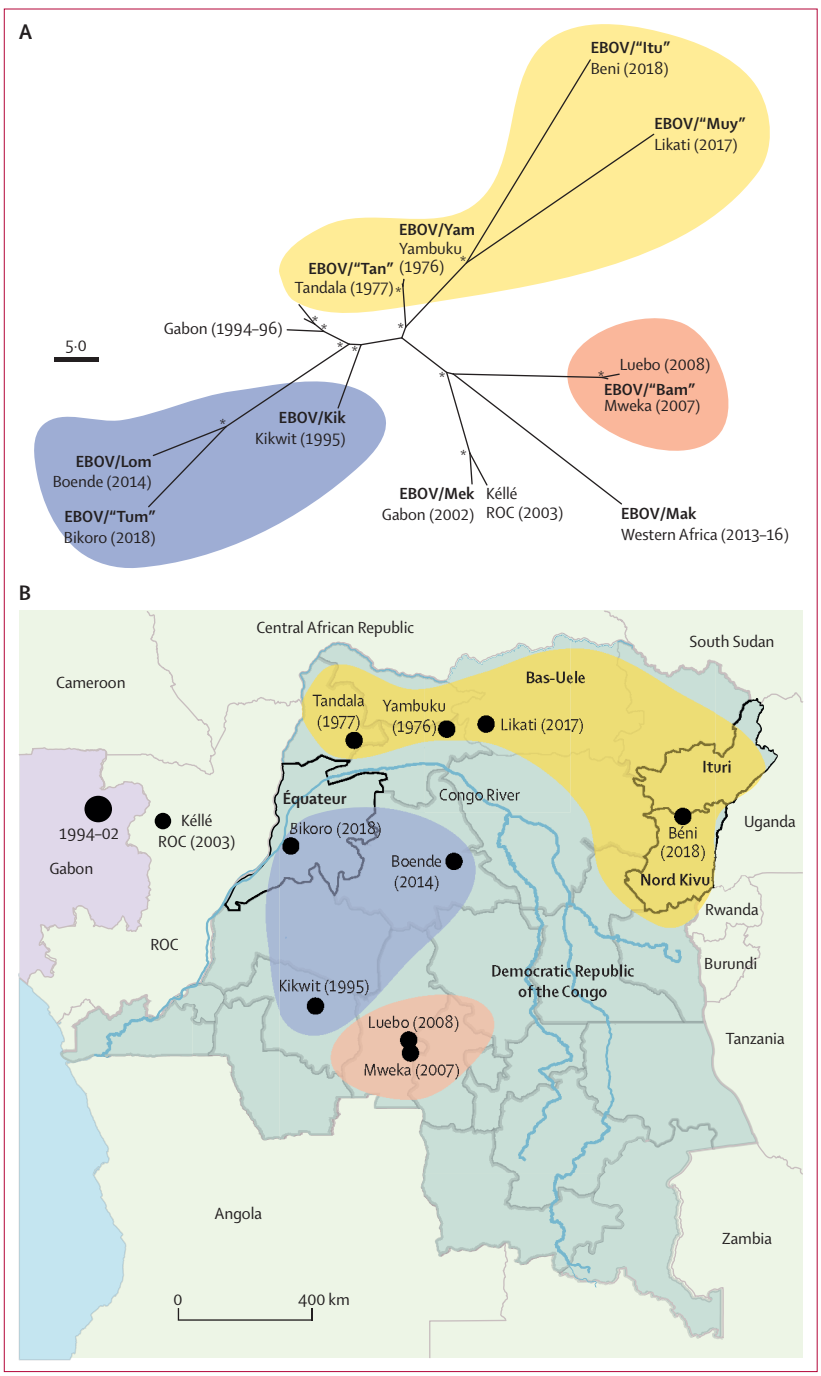


***Figure 4:*** **Unrooted maximum-clade credibility tree (A) and scaled map (B) labelled with the cities of EVD outbreaks**

Yellow, orange, and blue shading shows Ebola virus variants with geographical proximity. Scale is shown as substitutions per site per year. Asterisks at tree nodes indicate a posterior probability greater than 0·9. ROC=Republic of the Congo.

extrapolated from the available genomic data. Using a single, representative, coding-complete sequence from each EVD outbreak, we observed geographical clustering by phylogenetic lineage (figure 4). The four EVD outbreaks that occurred north of the Congo River in Yambuku, Tandala, Likati, and Beni (2018, EBOV/"Itu") cluster together. Similarly, to the south of the Congo River, the three northwest outbreaks cluster together (Kikwit, Boende, and Bikoro), as did the two unnamed variants identified further to the southeast. Moreover, these two clades had evolved independently during at least the past 30–40 years (figure 4; appendix).

EBOV/"Itu" sequences provided further evidence for lineage-specific rate variability. We did a root-to-tip analysis as an initial assessment of the inter-outbreak substitution rate (figure 5A). Single representative sequences from each outbreak were used to avoid sampling bias, giving each variant equal weight within the analysis. We estimated the substitution rate using data from all EVD outbreaks from Aug 22, 1976, to April 1, 2014, which included EBOV/Mak (slope $0{\cdot}76\times10^{-3}$, correlation coefficient 0·96; figure 5A). Since April 2014 , four additional EVD outbreaks have been described, which were caused by EBOV/Lom (August 2014), EBOV/"Muy" (May 2017), EBOV/"Tum" (May 2018), and EBOV/"Itu" (ongoing as of February 2019). EBOV/Lom and EBOV/"Tum" were previously found to evolve at a slower rate than the other EBOV variants.[2,30] The inclusion of EBOV/"Muy" and EBOV/"Itu" variants further reduced the estimated rate and linearity of the slope (slope $0{\cdot}37\times10^{-3}$, correlation coefficient 0·63; figure 5A).

We used a Bayesian approach to assess EBOV inter-outbreak evolutionary rate estimates for previous outbreaks (until April 2014) and current outbreaks (until August 2018) using both strict (figure 5B) and relaxed clocks (figure 5C; appendix). Consistent with our root-to-tip analysis, the addition of EBOV/"Itu" and three other variants significantly decreased the overall inter-outbreak evolutionary rate (August 1976–April 2014, $0{\cdot}75\times10^{-3}$ substitutions per site per year [95% highest posterior density $0{\cdot}69–0{\cdot}80\times10^{-3}$]; August 1976–August 2018, $0{\cdot}47\times10^{-3}$ substitutions per site per year [$0{\cdot}44–0{\cdot}49\times10^{-3}$; figure 5B]). No significant differences were identified between strict and relaxed clock mean estimates.

The relaxed clock allowed each branch to have a different evolutionary rate, enabling the detection of significant rate variations between EBOV lineages (figure 5C). Notably, the branch rate leading to the EBOV/Mak variant ($0{\cdot}86\times10^{-3}$ substitutions per site per year [95% highest posterior density $0{\cdot}66–1{\cdot}1\times10^{-3}$]) was higher than the tree's mean evolutionary rate. Faster rates were also observed in more basal branches leading to the Republic of the Congo and Gabon 2002–03 EVD outbreaks and the earlier Gabon 1994–96 EVD outbreaks, although slower rates were observed along more terminal branches. In contrast, slower-than-average evolutionary rates were observed for branches leading to EBOV/"Itu" ($0{\cdot}39\times10^{-3}$ substitutions per site per year [95% highest posterior density $0{\cdot}23–0{\cdot}73\times10^{-3}$]), EBOV/"Muy" ($0{\cdot}33\times10^{-3}$ substitutions per site per year [$0{\cdot}18–0{\cdot}62\times10^{-3}$]), EBOV/"Tum" ($0{\cdot}24\times10^{-3}$ substitutions per site per year [$0{\cdot}09–0{\cdot}41\times10^{-3}$]), and EBOV/Lom ($0{\cdot}27\times10^{-3}$ substitutions per site per year [$0{\cdot}07–0{\cdot}55\times10^{-3}$]; figure 5C). EBOV evolutionary rate

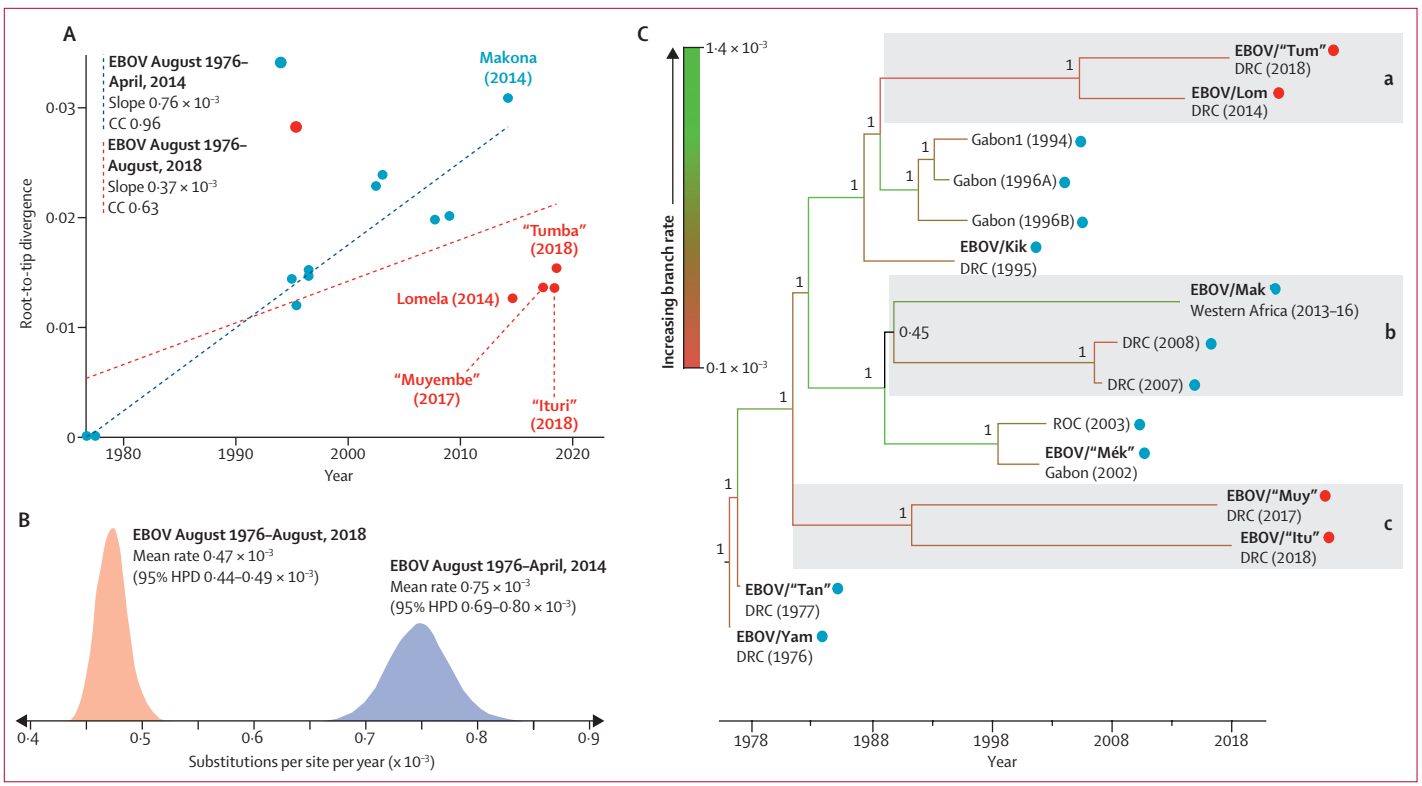


***Figure 5*: Root-to-tip and Bayesian analysis of inter-outbreak evolutionary rates with the addition of EBOV/"Itu" and EBOV/"Muy" variants**

Stacked root-to-tip analysis (A) and Bayesian strict clock rate estimate analysis (B) starting with EBOV sequences from August 1976, to April 2018, including EBOV/Mak (n=11; blue) and all four additional representatives as of August 2018, including EBOV/"Itu" (n=15; light red). (C) A Bayesian maximum-clade credibility tree of representative EBOV sequences (n=15) using an uncorrelated lognormal relaxed clock. Branches are coloured according to relative branch rate. Red circles at node tips correspond to points in panel A. Posterior probabilities are shown at tree nodes. The tree is scaled by substitutions per site per year. EBOV=Ebola virus. CC=correlation coefficient. HPD=highest posterior density. DRC=Democratic Republic of the Congo. ROC=Republic of the Congo.

trends were also confirmed using so-called clade-level fixed local clocks (appendix).

## Discussion

Rapid characterisation of coding-complete EBOV genomes was used for the first time during the 2013–16 EVD outbreak in Western Africa to assess medical countermeasures against EBOV and assist epidemiological efforts in real-time.[4–6,31] This global effort set a standard for the generation of pathogen genomic data to improve public health responses. We report the findings of an in-country effort that resulted in the real-time sequencing of the first two coding-complete EBOV genomes and the subsequent rapid generation of multiple coding-complete EBOV genomes from patients of the 2018 EVD outbreak in the North Kivu and Ituri Provinces of the Democratic Republic of the Congo. Together, the data identified the aetiological agent and confirmed the outbreak was epidemiologically distinct from the 2018 EVD outbreak in Équateur Province. The information was used to evaluate target erosion effects on available and deployed molecular detection assays and immuno-therapeutic antibodies. Early and continuous genomic sequencing helped direct public health responses, deployment of medical countermeasures, and retro-spectively provided novel insights into EBOV trans-mission chains and evolution.

Within 1 week of the EVD outbreak declaration, we assessed the available detection assays. Based on the results, the INRB selected the dual-target GeneXpert Ebola for deployment. The cartridge-based platform provides improved safety compared with other quantitative real-time-PCR assays when working in the mobile laboratory setting, simplicity, and speed of diagnosis. Although a single mismatch was identified in the forward primer in the *NP* assay, comparisons with previous field assessments of EBOV/Mak were similar. Additionally, the redundancy of the diagnostic assay was a consideration with the deployment of the vesicular stomatitis virus-based EBOV vaccine, which expresses the $GP_{1,2}$ protein and could lead to false positives.

We investigated whether available monoclonal antibody immunotherapies (mAb114 and ZMapp) could be used effectively against EBOV/"Itu". Early in silico analysis of the 12 synapomorphic amino acid changes in the EBOV/"Itu" $GP_{1,2}$ (compared with EBOV/Kik $GP_{1,2}$) and analysis of ZMapp using flow cytometry did not indicate changes in monoclonal antibody binding to $GP_{1,2}$. The $GP_{1,2}$ mutation (Gly557Arg), detected in a single patient, resulted in partly reduced binding

of mAb4G7 (ZMapp component). However, further evaluation is needed to determine if this reduced binding would affect ZMapp efficacy. Our analyses are ongoing as novel EBOV/"Itu" sequences are generated during the outbreak. In that context, generation of highly accurate EBOV genome drafts is desirable (facilitated by earlier establishment and training of local scientists to use the Illumina iSeq and MiSeq) to ensure accuracy of detected single nucleotide polymorphisms. The expedited protocols presented in this study could be used to indicate whether a treated patient with EVD is infected with an EBOV harbouring an escape mutation that disrupts key binding sites in deployed therapeutic monoclonal antibodies—a concept of precision medicine that might now become a reality for outbreak response.

Although we did not have access to mAb114 for in-vitro studies, several patients with EVD were treated and sampled before and after treatment. No single nucleotide polymorphisms (ie, changes to the consensus sequence) or increases in EBOV diversity (minority variant populations >2%) were identified within EBOV $GP_{1,2}$ residues (113–121) at the structural interface with mAb114. The small sample size and low frequency of patient sampling limit the value of these results. Additional sampling from more treated patients during this or future EVD outbreaks will allow evaluation of $GP_{1,2}$ minority variant populations at the intra-host level and provide information about the efficacy of treatment options in the early stages of an outbreak.

With continuous generation of coding complete EBOV/"Itu" sequences, we can further explore EBOV evolution in a broader, evolutionary context. EBOV/"Itu" belongs to a separate phylogenetic clade in addition to the 2017 EBOV/"Muy" sequence from Bas-Uele Province. This clade diverged from the other extant EBOV lineages approximately 30–40 years ago and seems to be endemic to northeastern areas of the Democratic Republic of the Congo. Geographical clustering of closely related EBOV variants is apparent and might help predict which variants are likely to cause outbreaks in specific areas. In this study, elucidating EBOV spread within the Democratic Republic of the Congo was not possible based on current sample diversity.

The apparent differences between EBOV inter-outbreak and intra-outbreak rates have been described.[2,7,31,32] The inclusion of EBOV/"Itu" and EBOV/"Muy" resulted in a slower mean inter-outbreak substitution rate than previously calculated, which could be explained by branch-level variability in evolutionary rate. Our findings indicate that evolutionary rates can vary along lineage branches leading to groups of individual variants regardless of their independent genetic diversity. Analysis of EBOV/"Itu" and other variants provides further support that multiple lineages have reduced substitution rates, and greater rate variability exists among EBOV variants than previously hypothesised.[2,30]

A large part of the rapid response was credited to an in-country capacity.[2] Our ongoing international partnership trained local personnel and invested in materials, reagents, and infrastructure to prepare INRB to independently respond, identify, and characterise viral outbreaks. During the ongoing EVD outbreak in North Kivu Province, the concept of initiating real-time genomics at the start of an outbreak was tested in full and led to the generation of actionable information about suitable diagnostics and medical countermeasures.

#### Contributors

PM-K, AAz, MRW, CBP, KP, OF, ED, MS-L, AAS, J-JM-T, MP, SAM, and GP conceived and designed the study. PM-K, AAz, MRW, SMM, KC, CP, KP, PAL, BB, AAr, NV, SK, MMD, MF, AAg, JN, FM, EP, JP, SS, SG, GPS, RT, AL, JJZ, ISF, BN, MM, and GP collected data. PM-K, ND, MRW, KC, CP, JTL, JRK, JC, CJ-VA, MD, MLB, MS-L, and GP did data analysis. PM-K, ND, MRW, KC, CP, JTL, JHK, MS-L, GP, and SAM wrote the manuscript.

#### Declaration of interests

Illumina paid the costs for attendance of PM-K at the American Society of Tropical Medicine & Hygiene 2018 conference. MRW reports non-financial support from Illumina during the conduct of the study. All other authors declare no competing interests.

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