

BRIEF REPORT

Emergence of Zaire Ebola Virus Disease in Guinea

Sylvain Baize, Ph.D., Delphine Pannetier, Ph.D., Pharm.D., Lisa Oestereich, M.Sc., Toni Rieger, Ph.D., Lamine Koivogui, Ph.D., N'Faly Magassouba, Ph.D., Barrè Soropogui, M.Sc., Mamadou Saliou Sow, M.D., Sakoba Keita, M.D., Hilde De Clerck, M.D., Amanda Tiffany, M.P.H., Gemma Dominguez, B.Sc., Mathieu Loua, M.D., Alexis Traoré, M.D., Moussa Kolié, M.D., Emmanuel Roland Malano, M.D., Emmanuel Heleze, M.D., Anne Bocquin, M.Sc., Stephane Mély, M.Sc., Hervé Raoul, Ph.D., Valérie Caro, Ph.D., Dániel Cadar, D.V.M., Ph.D., Martin Gabriel, M.D., Meike Pahlmann, Ph.D., Dennis Tappe, M.D., Jonas Schmidt-Chanasit, M.D., Benido Impouma, M.D., Abdoul Karim Diallo, M.D., Pierre Formenty, D.V.M., M.P.H., Michel Van Herp, M.D., M.P.H., and Stephan Günther, M.D.

SUMMARY

In March 2014, the World Health Organization was notified of an outbreak of a communicable disease characterized by fever, severe diarrhea, vomiting, and a high fatality rate in Guinea. Virologic investigation identified *Zaire ebolavirus* (EBOV) as the causative agent. Full-length genome sequencing and phylogenetic analysis showed that EBOV from Guinea forms a separate clade in relationship to the known EBOV strains from the Democratic Republic of Congo and Gabon. Epidemiologic investigation linked the laboratory-confirmed cases with the presumed first fatality of the outbreak in December 2013. This study demonstrates the emergence of a new EBOV strain in Guinea.

The authors' affiliations are listed in the Appendix. Address reprint requests to Dr. Günther at the Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Str. 74, 20359 Hamburg, Germany, or at guenther@bni.uni-hamburg.de.

Drs. Baize and Pannetier contributed equally to this article.

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OUTBREAKS CAUSED BY VIRUSES OF THE GENERA EBOLAVIRUS AND MARBURGVIRUS represent a major public health issue in sub-Saharan Africa. Ebola virus disease is associated with a case fatality rate of 30 to 90%, depending on the virus species. Specific conditions in hospitals and communities in Africa facilitate the spread of the disease from human to human. Three ebolavirus species have caused large outbreaks in sub-Saharan Africa: EBOV, *Sudan ebolavirus*, and the recently described *Bundibugyo ebolavirus*.^{1,2} Epidemics have occurred in the Democratic Republic of Congo, Sudan, Gabon, Republic of Congo, and Uganda. *Reston ebolavirus* circulates in the Philippines. It has caused disease in nonhuman primates but not in humans.³ The fifth species, *Tai Forest ebolavirus*, was documented in a single human infection caused by contact with an infected chimpanzee from the Tai Forest in Ivory Coast.⁴ Although this event indicated the presence of *Tai Forest ebolavirus* in West Africa, this subregion was not considered to be an area in which EBOV was endemic.

On March 10, 2014, hospitals and public health services in Guéckédou and Macenta alerted the Ministry of Health of Guinea and — 2 days later — Médecins sans Frontières in Guinea about clusters of a mysterious disease characterized by

fever, severe diarrhea, vomiting, and an apparent high fatality rate. (Médecins sans Frontières had been working on a malaria project in Guéckédou since 2010.) In Guéckédou, eight patients were hospitalized; three of them died, and additional deaths were reported among the families of the patients. Several deaths were reported in Macenta, including deaths among hospital staff members. A team sent by the health ministry reached the outbreak region on March 14 (Fig. 1). Médecins sans Frontières in Europe was notified and sent a team, which arrived in Guéckédou on March 18. Epidemiologic investigation was initiated, and blood samples were collected and sent to the biosafety level 4 laboratories in Lyon, France, and Hamburg, Germany, for virologic analysis.

METHODS

PATIENTS

Blood samples were obtained from 20 patients who were hospitalized in Guéckédou, Macenta, and Kissidougou because of fever, diarrhea, vomiting, or hemorrhage. Demographic and clinical data for the patients were provided on the laboratory request forms. Clinical data were not collected in a systematic fashion. This work was performed as part of the public health response to contain the outbreak in Guinea; informed consent was not obtained.

DIAGNOSTIC ASSAYS

Viral RNA was extracted from 50 to 100 μ l of undiluted plasma and 1:10 diluted plasma with the use of the QIAmp viral RNA kit (Qiagen). Nucleic acid amplification tests for detection of filoviruses and arenaviruses were performed with the use of commercially available kits and published primers and probes⁵⁻¹¹ (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

VIRAL SEQUENCING

Fragments amplified by filovirus L gene-specific primers were sequenced with the use of polymerase-chain-reaction (PCR) primers. Complete EBOV genomes were sequenced directly with the use of RNA extracted from serum obtained from three patients with high levels of viral RNA, as measured on real-time reverse-transcriptase-PCR (RT-PCR) analysis. The genome was ampli-



Figure 1. Map of Guinea Showing Initial Locations of the Outbreak of Ebola Virus Disease.

The area of the outbreak is highlighted in red. The main road between the outbreak area and Conakry, the capital of Guinea, is also shown. The map was modified from a United Nations map.

fied in overlapping fragments with the use of EBOV-specific primers. The fragments were sequenced from both ends with the use of conventional Sanger techniques. The sequence of the contigs was verified by visual inspection of the electropherograms.

VIRAL ISOLATION

About 100 μ l of all serum samples was used to inoculate Vero E6 cells maintained in 25-cm² flasks in Dulbecco's modified Eagle's medium containing 2 to 5% fetal-calf serum and penicillin-streptomycin. Cells and supernatant were passaged several times. Virus growth in the cells was verified on immunofluorescence with the use of polyclonal mouse anti-EBOV-specific antibodies in the serum of mice challenged with EBOV or on the basis of an increase in viral levels in the cell-culture supernatant over several orders of magnitude, as measured on real-time RT-PCR.

ELECTRON MICROSCOPY

Specimens from two patients were prepared for electron microscopy with the use of a conventional negative-staining procedure. In brief, a drop of 1:10 diluted serum was adsorbed to a glow-discharged carbon-coated copper grid and stained

Table 1. Demographic, Clinical, and Virologic Characteristics of 15 Patients with Confirmed Ebola Virus Disease during the 2014 Outbreak in Guinea.*

Patient No.	Age (yr)	Sex	Hospital	Date of Sampling	Symptoms	Outcome	Date of Death	Virus Isolation	GenBank Accession No.
C1	20	F	Guéckédou	March 12	Fever, diarrhea, vomiting	Died	March 18	No	ND
C2	25	F	Guéckédou	March 13	Fever, diarrhea, vomiting	Died	March 25	No	ND
C3	35	M	Guéckédou	March 13	Fever, vomiting	Died	March 17	No	ND
C4	25	M	Guéckédou	March 18	Fever, diarrhea, vomiting, hemorrhage	Died	March 18	No	ND
C5	16	F	Guéckédou	March 19	Spontaneous abortion	Survived	—	Yes	KJ660348
C6	27	F	Guéckédou	March 20	Fever, diarrhea, vomiting	Died	ND	No	ND
C7	47	F	Guéckédou	March 20	Fever, diarrhea, vomiting	Died	March 22	Yes	KJ660347
C8	29	M	Macenta	March 16	Fever, hemorrhage	Died	March 16	No	ND
C9	55	F	Macenta	March 16	Fever, diarrhea, vomiting	Died	March 19	No	ND
C10	17	M	Macenta	March 16	Fever, diarrhea, vomiting	ND	ND	No	ND
C11	7	M	Macenta	ND	Fever, diarrhea, vomiting	Died	March 26	No	ND
C12	30	M	Macenta, Nzérékoré	February 28	Fever, vomiting	Died	February 28	Yes	ND
C13	50	M	Macenta	March 12	Fever, diarrhea, vomiting	Died	March 12	Yes	ND
C14	41	M	Macenta, Nzérékoré	March 13	Fever, diarrhea, vomiting, hemorrhage	Died	March 16	No	ND
C15	28	F	Kissidougou	March 17	Fever, diarrhea, vomiting, hemorrhage	Survived	—	Yes	KJ660346

* All sampling and recording of patients' status were performed in 2014. ND denotes not determined.

with freshly prepared 1% phosphotungstic acid (Agar Scientific). Images were taken at room temperature with the use of a Tecnai Spirit electron microscope (FEI) equipped with a LaB6 filament and operated at an acceleration voltage of 80 kV.

PHYLOGENETIC ANALYSIS

We obtained all 48 complete genome sequences of filoviruses that are currently available from GenBank and aligned them with the new EBOV Guinea sequences (18,959 nucleotides). We used software designed to perform statistical selection of best-fit models of nucleotide substitution (jModelTest¹²) to identify the general time-reversible model of se-

quence evolution with gamma-distributed rate variation among sites (GTR+gamma) as the model that best describes the phylogenetic data. We used the Bayesian Markov Chain Monte Carlo method, as implemented in MrBayes 3.1.2 software,¹³ to infer the composition of one phylogenetic tree, using two runs of four chains with 1 million steps with a burn-in rate of 25% and the GTR+gamma model. A second tree was inferred for the same alignment with a maximum-likelihood method implemented in PhyML software¹⁴ under the GTR+gamma model with 1000 bootstrap replications. A reconstruction of the EBOV phylogenetic tree with the use of molecular clock

models is provided in Figure S1 in the Supplementary Appendix.

EPIDEMIOLOGIC INVESTIGATIONS

We gathered data on possible transmission chains from hospital records and through interviews with patients in whom EBOV infection was suspected and their contacts, affected families, inhabitants of villages in which deaths occurred, attendants of funerals, public health authorities, and hospital staff members.

RESULTS

IDENTIFICATION OF THE EBOV STRAIN

To detect the causative agent, we used conventional Filoviridae-specific RT-PCR assays targeting a conserved region in the L gene to test samples obtained from 20 hospitalized patients who were suspected of being infected with a hemorrhagic fever virus.^{5,6,9} In addition, we performed EBOV-specific real-time RT-PCR assays targeting the glycoprotein (GP) or nucleoprotein (NP) gene.^{7,10} Samples from 15 of 20 patients tested positive on the conventional L gene PCR assay and the real-time assays (Table 1). EBOV was identified in the serum of one patient on electron microscopy (Fig. 2, inset) and was isolated in cell culture from 5 patients. None of the samples were positive for Lassa virus on Lassa virus-specific RT-PCR assays.^{8,11} Sequencing of the fragments amplified by the L gene RT-PCR assays revealed EBOV sequences. The partial L gene sequences were identical for all confirmed cases, except for a synonymous T-to-C polymorphism at position 13560, which was found in Patients C12 and C14.

SEQUENCING OF SAMPLES FROM PATIENTS

The EBOV in samples obtained from three patients was completely sequenced with the use of conventional Sanger techniques (GenBank accession numbers, KJ660346, KJ660347, and KJ660348; the sequences in the preliminary report have been updated). The three sequences, each 18,959 nucleotides in length, were identical, with the exception of a few polymorphisms at positions 2124 (G→A, NP552 glycine→glutamic acid), 2185 (A→G, synonymous), 6909 (A→T, sGP291 arginine→tryptophan), 9923 (T→C, synonymous), 13856 (A→G, L759 aspartic acid→glycine), and 15660 (T→C, synonymous). The Guinean EBOV

strain showed 97% identity to EBOV strains from the Democratic Republic of Congo and Gabon. Phylogenetic analysis of the full-length sequences by means of Bayesian and maximum-likelihood methods revealed a separate, basal position of the Guinean EBOV within the EBOV clade (Fig. 3).

CLINICAL AND EPIDEMIOLOGIC ANALYSIS

The prominent clinical features of the EBOV infection in the confirmed cases were fever, severe diarrhea, and vomiting; hemorrhage was less frequent. The case fatality rate in the initial cases was 86% (12 of 14 patients with a known outcome died). Confirmed cases originated from hospitals in Guéckédou, Macenta, Nzérékoré, and Kissidougou prefectures (Fig. 1). We performed an epidemiologic look-back investigation of the transmission chains by reviewing hospital documentations and interviews with affected families, patients with suspected disease, and inhabitants of villages in which cases occurred.

According to the initial epidemiologic investigation, the suspected first case of the outbreak was a 2-year-old child who died in Meliandou in Guéckédou prefecture on December 6, 2013 (Fig. 2). A second investigation confirmed the origin of the outbreak in Meliandou but revealed a somewhat different timing of the early events (including the death of Patient S1 at the end of December and the deaths of Patients S2, S3, and S4 in January). Patient S14, a health care worker from Guéckédou with suspected disease, seems to have triggered the spread of the virus to Macenta, Nzérékoré, and Kissidougou in February 2014. As the virus spread, 13 of the confirmed cases could be linked to four clusters: the Baladou district of Guéckédou, the Farako district of Guéckédou, Macenta, and Kissidougou. Eventually, all clusters were linked with several deaths in the villages of Meliandou and Dawa between December 2013 and March 2014.

INITIAL PHASE OF THE OUTBREAK

This report is focused on the initial phase and geographic origin of the EBOV outbreak. Before the end of March 2014 (week 13), a total of 111 clinically suspected cases with 79 deaths (71% case fatality rate on the basis of clinical suspicion) had been recorded in the prefectures of Guéckédou, Macenta, and Kissidougou. According to the timeline of the transmission chains (Fig. 2), the outbreak of confirmed disease start-

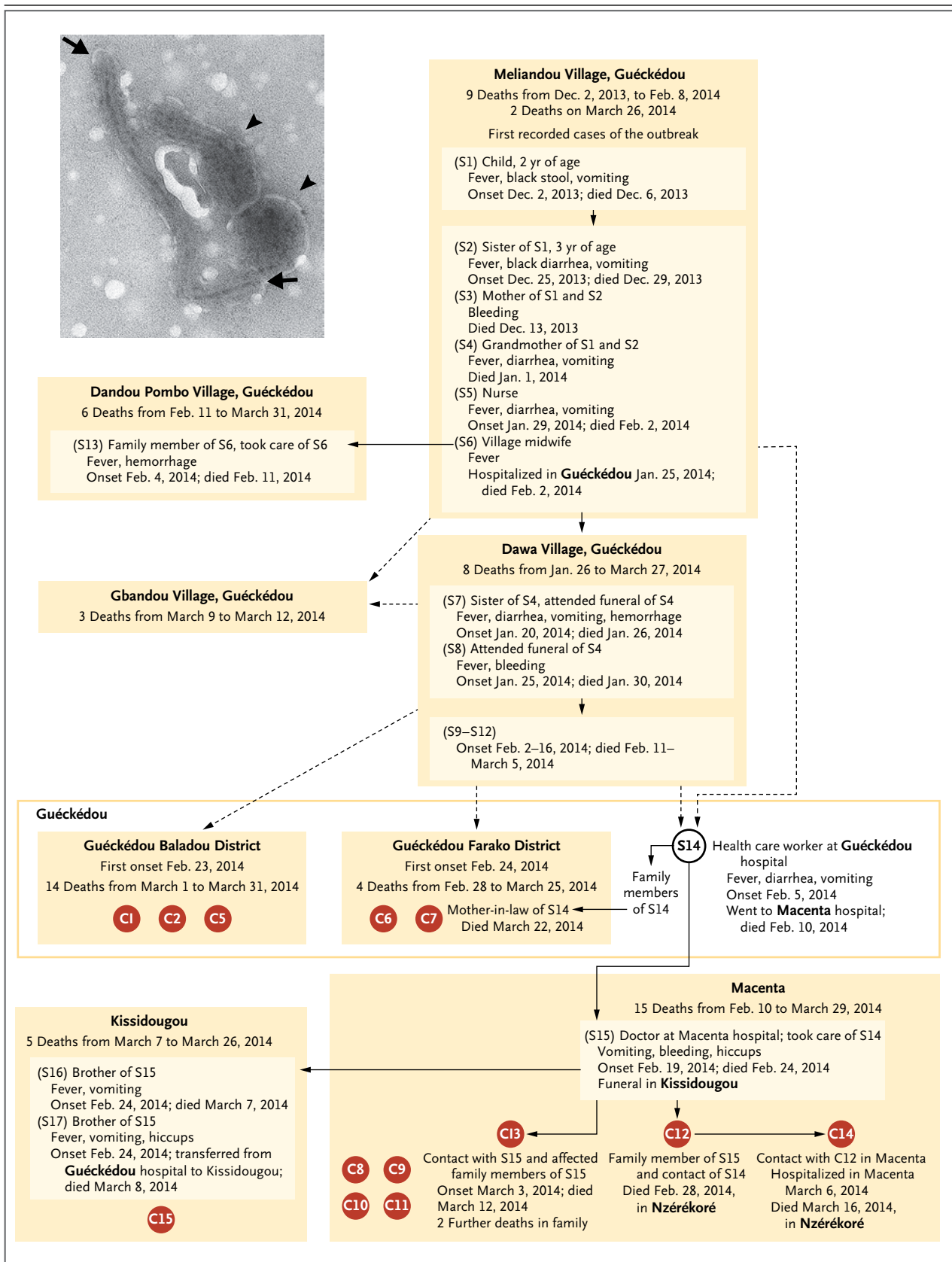


Figure 2 (facing page). Transmission Chains in the Outbreak of Ebola Virus Disease in Guinea.

Shown are transmission chains in the Ebola virus disease outbreak involving laboratory-confirmed cases. The presumed means of transmission of *Zaire ebolavirus* (EBOV), as revealed by epidemiologic investigation, are indicated by solid arrows. Dashed arrows indicate that the epidemiologic links are not well established. Laboratory-confirmed cases (C) are indicated with red circles, and suspected cases (S) are indicated with the case number. The inset image is an electron microscopic scan of the Guinean strain of EBOV in blood obtained from a patient. A typical complete virus particle, with the ends marked by arrows, and two degraded particles (arrowheads) are shown.

ed in the prefecture Guéckédou and then spread to Macenta and Kissidougou (Fig. 4). The male-to-female ratio among patients who died was 41:59; the median age was 35 years (interquartile range, 25 to 51).

DISCUSSION

This study demonstrates the emergence of EBOV in Guinea. The high degree of similarity among the 15 partial L gene sequences, along with the three full-length sequences and the epidemiologic links between the cases, suggest a single introduction of the virus into the human population.

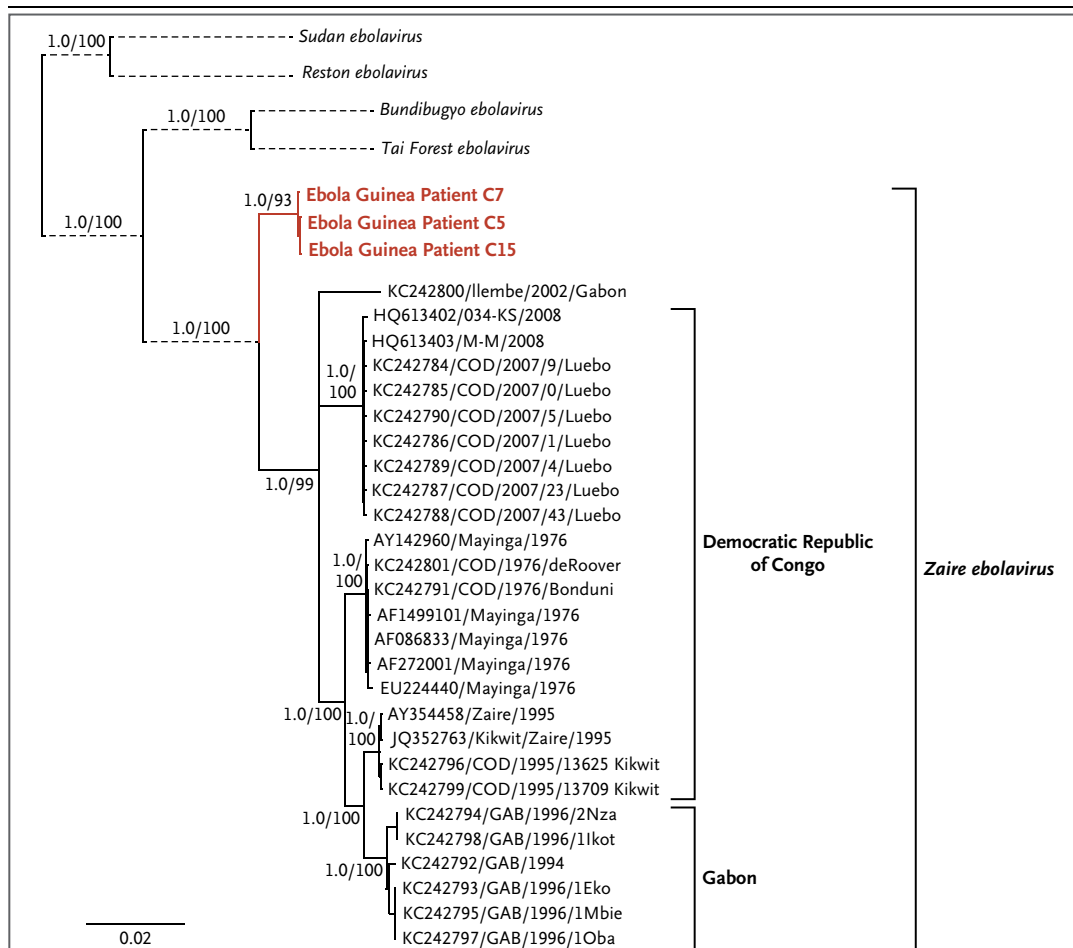
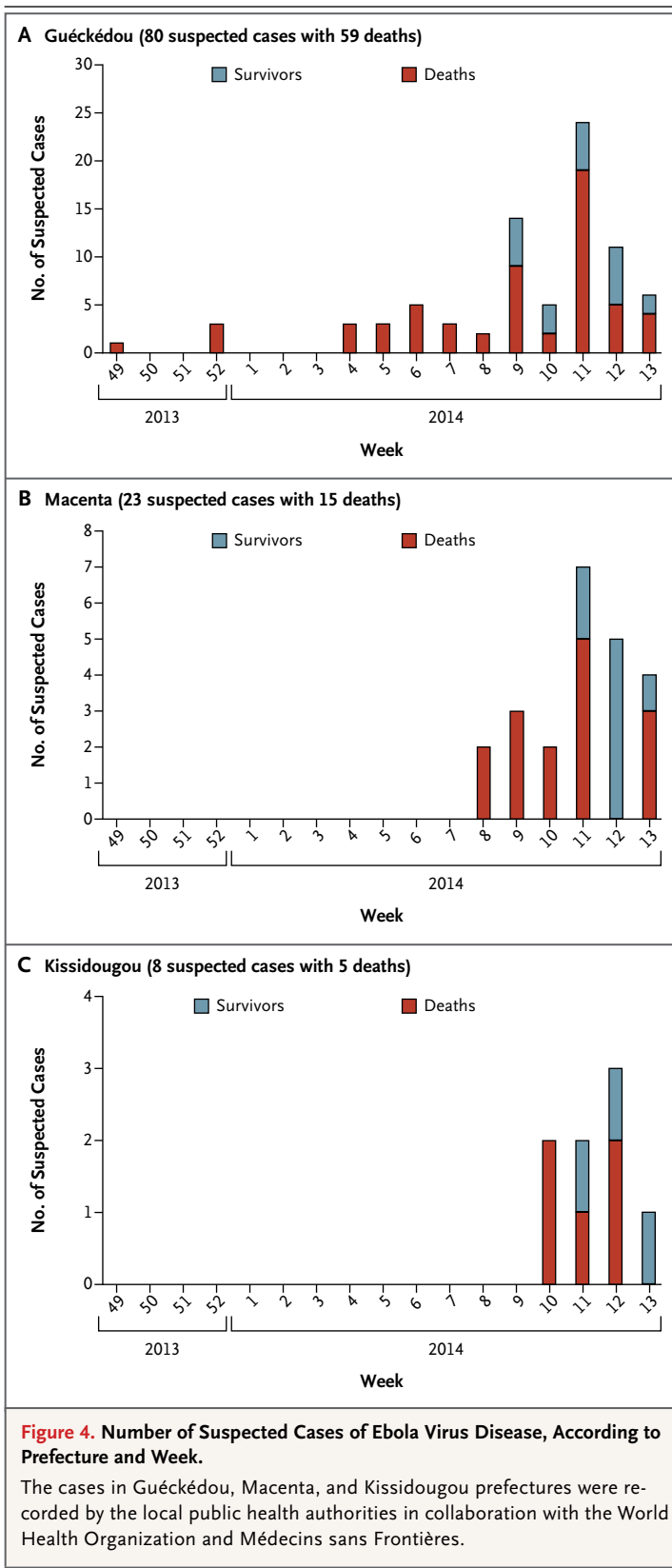


Figure 3. Phylogenetic Analysis of the Ebolavirus Genus, Including the EBOV Strains from Guinea.

The phylogenetic tree was inferred with the use of the Bayesian Markov Chain Monte Carlo method. A second tree that was inferred for the same set of sequences with a maximum-likelihood method confirmed the Bayesian tree (data not shown). Bayesian posterior probabilities and bootstrap percentages (1000 replicates of the maximum-likelihood tree) are shown on the branches. For clarity of presentation, the branches for the non-EBOV species were shortened and condensed (dashed branches). The GenBank accession number, strain designation, country of origin, and year of isolation are indicated on the EBOV branches. The EBOV Guinea strain is available from the European Virus Archive (www.european-virus-archive.com).



This introduction seems to have happened in December 2013. Further epidemiologic investigation is ongoing to identify the presumed animal source of the outbreak. It is suspected that the virus was transmitted for months before the outbreak became apparent because of clusters of cases in the hospitals of Guéckédou and Macenta. This length of exposure appears to have allowed many transmission chains and thus increased the number of cases of Ebola virus disease.

The clinical picture of the initial cases was predominantly fever, vomiting, and severe diarrhea. Hemorrhage was not documented for most of the patients with confirmed disease at the time of sampling but may have developed later in the course of the disease. The term Ebola virus disease (rather than the earlier term Ebola hemorrhagic fever) takes into account that hemorrhage is not seen in all patients¹⁵ and may help clinicians and public health officials in the early recognition of the disease. The case fatality rate was 86% among the early confirmed cases and 71% among clinically suspected cases, which is consistent with the case fatality rates observed in previous EBOV outbreaks.¹⁵⁻¹⁷

Phylogenetic analysis of the full-length sequences established a separate clade for the Guinean EBOV strain in a sister relationship with other known EBOV strains. This suggests that the EBOV strain from Guinea has evolved in parallel with the strains from the Democratic Republic of Congo and Gabon from a recent ancestor and has not been introduced from the latter countries into Guinea. However, the determination of both the timing of the introduction of the virus into Guinea and its phylogenetic origin also depend on our understanding of the evolutionary rate of EBOV in nature (Fig. S1 in the Supplementary Appendix). A reliable estimate for this rate, such as one derived from archaeological calibration,¹⁸ may be required to answer these questions.

Potential reservoirs of EBOV, fruit bats of the species *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*, are present in large parts of West Africa.¹⁹ Therefore, it is possible that EBOV has circulated undetected in this region for some time. The emergence of the virus in Guinea highlights the risk of EBOV outbreaks in the whole West African subregion.

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APPENDIX

The authors' affiliations are as follows: the National Reference Center for Viral Hemorrhagic Fevers (S.B., D.P., A.B., S.M.), Unité de Biologie des Infections Virales Emergentes, Institut Pasteur (S.B.), Centre International de Recherche en Infectiologie (CIRI), Université de Lyon, INSERM Unité 1111, Ecole Normale Supérieure de Lyon, Université Lyon 1 (S.B.), and Laboratoire P4 INSERM-Jean Mérieux (D.P., A.B., S.M., H.R.), Lyon, and Epicentre (A. Tiffany) and Pole de Génomique des Pathogènes, Unité de Recherche et d'Expertise Environnement et Risques Infectieux, Institut Pasteur (V.C.), Paris — all in France; Bernhard Nocht Institute for Tropical Medicine, World Health Organization (WHO) Collaborating Center for Arbovirus and Hemorrhagic Fever Reference and Research, and the German Center for Infection Research (DZIF), Partner Site Hamburg — both in Hamburg, Germany (L.O., T.R., D.C., M.G., M.P., D.T., J.S.-C., S.G.); Institut National de Santé Publique (L.K.), Université Gamal Abdel Nasser de Conakry, Laboratoire des Fièvres Hémorragiques en Guinée (N.M., B.S.), Hôpital National Donka, Service des Maladies Infectieuses et Tropicales (M.S.S.), Ministry of Health Guinea, Prevention and Disease Control (S.K.), and WHO (E.R.M., E.H., A.K.D.), Conakry, Section Prévention et Lutte contre la Maladie à la Direction Régionale de la Santé de Nzérékoré, Nzérékoré (M.L.), and Section Prévention et Lutte contre la Maladie à la Direction Préfectorale de la Santé de Guéckédou (A. Traoré) and Hôpital Préfectoral de Guéckédou (M.K.), Guéckédou — all in Guinea; Médecins sans Frontières (H.D.C., M.V.H.); Médecins sans Frontières (A.T., G.D.) and WHO (P.F.) — both in Geneva; and WHO, African Regional Office, Brazzaville, Republic of Congo (B.I.).

REFERENCES

1. Feldmann H, Geisbert TW. Ebola haemorrhagic fever. *Lancet* 2011;377:849-62.
2. Hartman AL, Townner JS, Nichol ST. Ebola and Marburg hemorrhagic fever. *Clin Lab Med* 2010;30:161-77.
3. Miranda ME, Ksiazek TG, Retuya TJ, et al. Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. *J Infect Dis* 1999;179:Suppl 1:S115-S119.
4. Formenty P, Hatz C, Le Guenno B, Stoll A, Rogenmoser P, Widmer A. Human infection due to Ebola virus, subtype Côte d'Ivoire: clinical and biologic presentation. *J Infect Dis* 1999;179:Suppl 1:S48-S53.
5. Sanchez A, Ksiazek TG, Rollin PE, et al. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. *J Infect Dis* 1999;179:Suppl 1:S164-S169.
6. Leroy EM, Baize S, Volchkov VE, et al. Human asymptomatic Ebola infection and strong inflammatory response. *Lancet* 2000;355:2210-5.
7. Huang Y, Wei H, Wang Y, Shi Z, Raoul H, Yuan Z. Rapid detection of filoviruses by real-time TaqMan polymerase chain reaction assays. *Virol Sin* 2012;27:273-7.
8. Vieth S, Drosten C, Lenz O, et al. RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. *Trans R Soc Trop Med Hyg* 2007;101:1253-64.
9. Panning M, Laue T, Olschlager S, et al. Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. *J Infect Dis* 2007;196:Suppl 2:S199-S204.
10. Gibb TR, Norwood DA Jr, Woollen N, Henchal EA. Development and evaluation of a fluorogenic 5' nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J Clin Microbiol* 2001;39:4125-30.
11. Olschlager S, Lelke M, Emmerich P, et al. Improved detection of Lassa virus by reverse transcription-PCR targeting the 5' region of S RNA. *J Clin Microbiol* 2010;48:2009-13.
12. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 2012;9:772.
13. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003;19:1572-4.
14. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 2010;59:307-21.
15. Kortepeter MG, Bausch DG, Bray M. Basic clinical and laboratory features of filoviral hemorrhagic fever. *J Infect Dis* 2011;204:Suppl 3:S810-S816.
16. Ebola haemorrhagic fever in Zaire, 1976. *Bull World Health Organ* 1978;56:271-93.
17. Khan AS, Tshioko FK, Heymann DL, et al. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. *J Infect Dis* 1999;179:Suppl 1:S76-S86.
18. Smith O, Clapham A, Rose P, Liu Y, Wang J, Allaby RG. A complete ancient RNA genome: identification, reconstruction and evolutionary history of archaeological Barley Stripe Mosaic Virus. *Sci Rep* 2014;4:4003.
19. Leroy EM, Kumulungui B, Pourrut X, et al. Fruit bats as reservoirs of Ebola virus. *Nature* 2005;438:575-6.

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