Persistence of Ebola virus after the end of widespread transmission in Liberia: an outbreak report

Emily Kainne Dokubo, Annika Wendland*, Suzanne E Mate*, Jason T Ladner*, Esther L Hamblion, Philomena Raftery, David J Blackley, A Scott Laney, Nuha Mahmoud, Gloria Wayne-Davies, Lisa Hensley, Eric Stavale, Lawrence Fakoli, Christopher Gregory, Tai-Ho Chen, Augustine Koryon, Denise Roth Allen, Jennifer Mann, Andrew Hickey, John Saindon, Mehboob Badini, April Baller, Peter Clement, Fatorma Bolay, Yatta Wapoe, Michael R Wiley, James Logue, Bonnie Dighero-Kemp, Elizabeth Higgs, Alex Gasasira, Desmond E Williams, Bernice Dahn, Francis Kateh, Tolbert Nyenswah, Gustavo Palacios†, Mosoka P Fallah†*

Summary

Background **Outbreak response efforts for the 2014–15 Ebola virus disease epidemic in west Africa brought widespread transmission to an end. However, subsequent clusters of infection have occurred in the region. An Ebola virus disease cluster in Liberia in November, 2015, that was identified after a 15-year-old boy tested positive for Ebola virus infection in Monrovia, raised the possibility of transmission from a persistently infected individual.**

Methods **Case investigations were done to ascertain previous contact with cases of Ebola virus disease or infection with Ebola virus. Molecular investigations on blood samples explored a potential linkage between Ebola virus isolated from cases in this November, 2015, cluster and epidemiologically linked cases from the 2014–15 west African outbreak, according to the national case database.**

Findings **The cluster investigated was the family of the index case (mother, father, three siblings). Ebola virus genomes assembled from two cases in the November, 2015, cluster, and an epidemiologically linked Ebola virus disease case in July, 2014, were phylogenetically related within the LB5 sublineage that circulated in Liberia starting around August, 2014. Partial genomes from two additional individuals, one from each cluster, were also consistent with placement in the LB5 sublineage. Sequencing data indicate infection with a lineage of the virus from a former transmission chain in the country. Based on serology and epidemiological and genomic data, the most plausible scenario is that a female case in the November, 2015, cluster survived Ebola virus disease in 2014, had viral persistence or recurrent disease, and transmitted the virus to three family members a year later.**

Interpretation **Investigation of the source of infection for the November, 2015, cluster provides evidence of Ebola virus persistence and highlights the risk for outbreaks after interruption of active transmission. These findings underscore the need for focused prevention efforts among survivors and sustained capacity to rapidly detect and respond to new Ebola virus disease cases to prevent recurrence of a widespread outbreak.**

Funding **US Centers for Disease Control and Prevention, Defense Threat Reduction Agency, and WHO.**

Lancet Infect Dis 2018

Published Online
July 23, 2018
http://dx.doi.org/10.1016/S1473-3099(18)30417-1

See Online/Comment
http://dx.doi.org/10.1016/S1473-3099(18)30435-3

*Joint first authors

†Contributed equally

US Centers for Disease Control and Prevention, Atlanta, GA, USA (E K Dokubo MD, D J Blackley DrPH, A S Laney PhD, C Gregory MD, T-H Chen MD, D Roth Allen PhD, J Mann MPH, A Hickey PhD, J Saindon DrHSc, D E Williams MD); **World Health Organization, Monrovia, Liberia** (A Wendland MPH, E L Hamblion PhD, P Raftery MSc, N Mahmoud MD, G Wayne-Davies MPH, M Badini MBBS, A Baller MD, P Clement MD, A Gasasira MBChB); **US Army Medical Research Institute of Infectious Diseases. Frederick, MD, USA** (S E Mate PhD, J T Ladner PhD, M R Wiley PhD, G Palacios PhD); **US National Institutes of Health, Bethesda, MD, USA** (L Hensley PhD, E Stavale BSc, J Logue BSc, B Dighero-Kemp BSc, E Higgs MD); **National Public Health Institute of Liberia, Monrovia, Liberia** (L Fakoli BSc, F Bolay PhD, T Nyenswah MPH, M P Fallah PhD); **International Rescue Committee, Monrovia, Liberia** (A Koryon BSc); **Ministry of Health, Monrovia, Liberia** (Y Wapoe MD, B Dahn MD, F Kateh MD); **and Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, USA** (J T Ladner)

Correspondence to:
Dr Emily Kainne Dokubo, US Centers for Disease Control and Prevention, Atlanta, GA 30333, USA
vic8@cdc.gov

Introduction

The 2014–15 Ebola virus disease outbreak in west Africa was unprecedented in magnitude, resulting in more than 28 000 cases and 11 000 deaths[1] and devastating the health systems in Liberia, Guinea, and Sierra Leone. After the first reported Ebola virus disease case in Liberia on March 30, 2014,[2] infection spread rapidly across the country and led to the death of more than 4800 people.[1] With support from international organisations and multilateral partners, the Government of Liberia developed outbreak response capacity and implemented measures that were effective in limiting continued spread of the virus and bringing widespread transmission to an end.[3,4]

In March, 2015, Ebola virus disease was confirmed in a woman after unprotected sexual intercourse with a male survivor, providing evidence of viral persistence after convalescence and the first documentation of sexual transmission of Ebola virus.[5,6] Response efforts prevented the further spread of infection. On May 9, 2015, Liberia was the first of the three most affected countries to be declared free of human-to-human Ebola virus disease transmission.[7] This was followed by a 90-day period of enhanced surveillance to rapidly identify any new cases or missed transmission chains in the country. A cluster of cases detected in June, 2015, also showed Ebola virus transmission from a persistently infected source within Liberia, with a reduced rate of viral evolution.[8] Implementation of a rapid and robust response was effective in limiting additional infections and Liberia was again declared free of Ebola virus transmission on Sept 3, 2015—42 days after the last confirmed case had two consecutive negative blood samples for Ebola virus.[9] Both flare-ups showed the potential for Ebola virus disease resurgence in the absence of active transmission chains.

Research in context

Evidence before this study

We searched PubMed without language restrictions using the terms "ebola" or "filovirus" combined with both "persistence" and "transmission" for articles published up to Jan 1, 2018. Previous studies used a combination of epidemiological investigation, laboratory, and next-generation sequencing (NGS) data to study routes of transmission of Ebola virus from persistently infected individuals. In those studies, when identified, the route of transmission was attributed to sexual transmission from a persistently infected male survivor. These studies also showed the important role that persistently infected so-called human reservoirs had in prolonging the Ebola virus disease outbreak in west Africa.

Added value of this study

An Ebola virus disease cluster in Liberia in November, 2015, after the end of widespread transmission, raised the possibility of transmission from a persistently infected individual. Investigations showed that a female patient survived Ebola virus disease in 2014, had viral persistence or recurrent disease, and transmitted the virus to other family members a year later. Based on serology and epidemiological and genomic data, our study is to our knowledge the first to provide evidence suggesting Ebola virus transmission from a persistently infected female survivor of Ebola virus disease.

Implications of all the available evidence

The investigation of the source of infection for this cluster provides additional evidence of Ebola virus persistence after recovery from acute infection and highlights the risk of Ebola virus disease outbreaks even after interruption of active transmission. Risk assessment and focused prevention efforts are needed for survivors and their close contacts. These findings also highlight the need for sustained capacity to rapidly detect and respond to new Ebola virus disease cases to prevent recurrence of a widespread outbreak. Ebola virus disease transmission from persistently infected male survivors is well documented. However, data are scant for Ebola virus disease transmission from persistently infected female survivors. A better understanding of the dynamics of viral clearance and sequelae among this population is needed.

On Nov 17, 2015, a 15-year-old boy presented to a hospital in Monrovia, Liberia, with symptoms consistent with Ebola virus infection. A blood specimen obtained from the patient tested positive on Nov 19, 2015, for the presence of Ebola virus RNA and health authorities were notified immediately of the confirmed diagnosis of Ebola virus disease. The Liberia Incident Management System was activated and response efforts were focused on identifying the source of infection, detecting additional cases and contacts, and preventing further spread of the virus.

Methods

Study design

We did epidemiological and laboratory investigations to ascertain the source of infection for the cluster of Ebola virus disease cases identified in November, 2015, and assess possible transmission scenarios. Investigation of the source of infection was led by a team of epidemiologists from the Liberia Ministry of Health, US Centers for Disease Control and Prevention (CDC), WHO, and other partner organisations. A non-research determination was issued by the CDC and the US Army Medical Research Institute of Infectious Diseases Office of Human Use and Ethics for this public health outbreak investigation.

Procedures

We defined cases based on WHO case-definition recommendations during an Ebola outbreak.[10] We defined a suspected case as either: an individual with sudden onset of high fever and reported contact with a suspected, probable, or confirmed Ebola case or a sick or dead animal; an individual with sudden onset of high fever and at least three Ebola-associated symptoms; an individual with unexplained bleeding; or sudden and unexplained death. We defined a probable case as either: a suspected case assessed by a clinician; or a deceased suspected case linked epidemiologically to a confirmed case. We defined a confirmed case as a suspected or probable case with a positive laboratory result for Ebola virus antigen by reverse transcriptase qualitative PCR (RT-qPCR) detection of virus RNA or by detection of anti-Ebola IgM antibodies. We defined a non-case as a suspected or probable case with no specific antibodies, RNA, or detectable antigens on laboratory testing. We defined contacts based on interaction with an Ebola case, and we classified them by risk status.[11]

Using standardised case investigation forms, we did detailed investigations of confirmed cases to identify potential sources of infection, including exposure to a previously unrecognised case or Ebola virus disease survivor. We gathered information by interviewing Ebola virus disease cases, family members, community members, and other key informants identified during the investigations. Interviewers adapted questions according to information provided by key informants. We used open-ended questions to seek information on previous exposure to or infection with Ebola virus, including contact with known Ebola virus disease cases, survivors, or people with Ebola virus disease symptoms, and we ascertained travel histories and attendance at funerals. To facilitate active case-finding, we listed people who had been in contact with confirmed cases and monitored these people closely for development of Ebola virus disease symptoms. We searched the Liberian

Ministry of Health's national Ebola virus disease case database and laboratory database to establish if cases in this cluster or people identified during interviews were previously recorded as confirmed or suspected Ebola virus disease cases. Data obtained from field investigations were discussed during daily meetings of the epidemiology team. Any new leads were examined and investigated further to obtain as complete a picture as possible based on the information provided by key informants. We used these data to create transmission chain diagrams for every potential scenario, which were classified further according to likelihood.

The laboratory team coordinated specimen collection and transportation to a central laboratory (Liberian Institute for Biomedical Research, Charlesville, Margibi County, Liberia), diagnostic testing, data management, and results reporting. We did diagnostic assessments to facilitate targeted and effective response measures. We obtained specimens from all suspected cases in accordance with the WHO phase III surveillance strategy.[12] We obtained blood specimens from suspected Ebola virus disease cases admitted to the suspect ward of the Ebola treatment unit (ETU) and we transferred these samples directly to the on-site mobile Ebola virus disease testing laboratory for diagnosis. In line with WHO guidelines,[13] we took a repeat specimen more than 48 h later if the initial test was negative. We transported specimens from suspected cases under investigation using an established specimen transport system (Riders for Health, Liberia) and following procedures for priority specimens. We analysed all specimens for Ebola virus RNA using either RT-qPCR[14] or the GeneXpert Ebola assay,[15] allowing for rapid diagnosis with results within 90 min. We reported results of priority specimens to relevant stakeholders by telephone as soon as they were available, followed by an official daily report as per Liberia Ministry of Health protocol. This procedure allowed for real-time evidence-based case investigation and response. We maintained line-lists of suspected and confirmed cases and these were updated daily with laboratory results, including RT-qPCR cycle threshold (Ct) values of specimens from patients admitted to the ETU.

We did serological testing using ELISA for the detection and semi-quantification of Ebola virus-specific IgG and IgM antibody levels. We used two commercially available assays to ascertain the antibody response against Ebola virus glycoproteins from individuals exposed to Ebola virus and an assay designed for the detection of antibody responses in vaccinated individuals. We quantified IgG antibody against Ebola virus glycoprotein using the Filovirus Animal Nonclinical Group (FANG) assay, which has been described previously.[16] We measured IgM antibody levels against Ebola virus glycoproteins using ADI ELISA (Alpha Diagnostics International, San Antonio, TX, USA). We processed ADI ELISA plates as per manufacturer's instructions and we calculated the relative abundance (rel[Ab]) of immunoglobulin in patient samples reflecting level of immune response for every sample dilution based on the line of best fit through the optical densities recorded for four premade assay calibrators. We assigned a rel[Ab] of 0 EU/mL if all dilution points had an optical density below the optical density of the 1 EU/mL calibrator.

We extracted Ebola virus genomic RNA from blood samples of laboratory-confirmed Ebola virus disease cases and enriched these samples using the TruSeq RNA Access kit (Illumina, San Diego, CA, USA) with custom capture probes designed against Ebola virus.[17] This library preparation method included 17 cycles of PCR amplification after ligation of sequencing adapters and ten cycles of PCR amplification after enrichment. We prepared two libraries for every sample, using a 1-min and 2-min fragmentation time to generate RNA fragments of optimum length; we processed every library separately and did not pool according to the enrichment protocol as previously described.[8] We assessed the quality of sequencing libraries using an Agilent 2100 Bioanalyzer and a DNA1000 chip (Agilent, Santa Clara, CA, USA) and quantified libraries using KAPA Library Quantification Kit (KapaBiosystems, Wilmington, MA, USA) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Every library was normalised to 2 nmol before pooling. A 10 pmol/L pool with 10% PhiX diversity control was sequenced on MiSeq (Illumina) at the LIBR Genomics Laboratory (Charlesville, Margibi County, Liberia) using V2 reagent kits (Illumina) with a minimum of 2×101 cycles per run. We assembled Ebola virus genomes by aligning them to a reference, and we analysed them as described previously.[8,18] Additional details on genetic sequencing and analyses are provided in the appendix.

See Online for appendix

Role of the funding source

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

The index case was a 15-year-old boy (individual A) who reported 4 days of fatigue, generalised myalgia, arthralgia, fever, diarrhoea, and haematemesis, with onset of symptoms on Nov 13, 2015. He had been treated at home with medications provided by a local drugstore owner, without improvement of his symptoms, and continued to attend the local school in his community. The patient was admitted to the paediatric ward of a hospital on Nov 17, 2015, and subsequently put into isolation based on clinical suspicion of Ebola virus disease. The presence of Ebola virus RNA was confirmed by RT-qPCR in a blood sample from the patient on Nov 19, 2015.

After confirmation of Ebola virus disease in individual A, the patient was transferred to the ETU on

	RT-qPCR		Ebola virus antibodies		Genome coverage (%)*	Ebola virus lineage
	Initial test (glycoprotein Ct, nucleoprotein Ct); date	Second test (glycoprotein Ct, nucleoprotein Ct); date	IgM (EU/mL)	IgG (EU/mL)		
Individual A, index case (male, aged 15 years)	Positive (23·6, 19·2); Nov 19, 2015	Positive (24·0, 20·3); Nov 23, 2015	ND	ND	99·83%	LB5
Individual B (father of individual A)	Positive (39·5, 35·6); Nov 20, 2015	Negative† (ND, ND); Nov 25, 2015 Indeterminate (ND, 40·8); Nov 27, 2015	590	2313	19·5%	Undetermined; one read supports LB5 SNP at 6056
Individual C (male, aged 8 years, brother of individual A)	Positive (34·4, 30·5); Nov 20, 2015	Positive (40·3, 37·1); Nov 23, 2015	ND	ND	99·79%	LB5
Individual D (mother of individual A)						
Blood sample	Negative	Negative	1436	851 334	NA	NA
Breastmilk sample	Negative	Negative	ND	827	NA	NA
Individual E (male, aged 2 months, brother of individual A)	Negative	Negative	ND	4524	NA	NA
Individual F (male, aged 5 years, brother of individual A)	Negative	Negative	ND	ND	NA	NA

Ct=cycle threshold. NA=not available. ND=not detected. RT-qPCR=reverse transcriptase qualitative PCR. *GenBank accessions: individual A=KY744596; individual B=SAMN05916118; individual C=KY744597. †Specimens were tested at the National Reference Laboratory using RT-PCR on ABI7500, a different methodology and technology from the GeneXpert used at ELWA III laboratory, where all other specimens were tested. This difference might reflect a lower sensitivity than GeneXpert technology.

Table 1: **Laboratory results for Ebola virus disease cases and family members (November, 2015)**

Nov 19, 2015, for further management. His father, mother, and three younger brothers (aged 8 years, 5 years, and 2 months) who resided in the same household were judged high-risk contacts and were transferred on the same day to the suspect ward of the ETU for close monitoring and diagnostic assessment. On Nov 20, 2015, the presence of Ebola virus RNA was confirmed in blood samples from the father (individual B) and the 8-year-old brother (individual C; table 1). Individual B did not acknowledge any Ebola virus disease symptoms, but members of his extended family reported that he might have had a mild illness in the weeks before admission to the ETU. Individual C had reported headache, lethargy, myalgia, dyspnoea, and fever 2 days after the onset of symptoms in individual A and was treated with local herbs. Ebola virus RNA was not detected in samples from the mother (individual D), the 2-month-old brother (individual E), or the 5-year old brother (individual F). Both the 15-year-old boy (individual A) and his 8-year-old brother (individual C) received ZMapp (Mapp Biopharmaceutical, San Diego, CA, USA) as part of the Partnership for Research on Ebola Virus in Liberia (PREVAIL) medical countermeasures study by the Liberia Ministry of Health and US National Institutes of Health. Individual A died on Nov 23, 2015.

Serological testing of blood samples was negative in the index case (individual A) and his 8-year-old brother (individual C), whereas the father (individual B) was positive for Ebola virus-specific IgM and IgG antibodies, suggesting that he was infected before his sons (table 1). Serology also revealed a high titre of Ebola virus IgG and a low level of IgM in a blood sample from the mother (individual D) of the index case (individual A) and presence of IgG in her breast milk, and a low level of IgG in the 2-month old brother (individual E), which was probably attributable to transfer of maternal antibodies. The 5-year-old brother (individual F) did not show any serological evidence of infection. Ebola virus antibodies were not detected in any other blood specimens obtained. All family members of the deceased index case (individual A) were discharged after two consecutive RT-qPCR negative tests from specimens obtained more than 48 h apart. Additional serological testing on a blood sample obtained 6 months after the initial tests still showed the presence of low levels of IgM and remarkably high levels of IgG in the mother (individual D). Efforts to further characterise the IgM response were unsuccessful. A semen specimen from the father (individual B) and vaginal fluid from the mother (individual D) also obtained 6 months after detection of the cluster were negative for Ebola virus on RT-qPCR.

Different sources of infection and many possible transmission scenarios were assessed (table 2). Case investigations did not reveal travel to other Ebola-affected countries, association with cases from other areas with recent Ebola virus transmission, or contact with potential non-human reservoirs of Ebola virus, raising the possibility that the source of infection for this cluster was an unrecognised transmission chain or a persistent viral source within Liberia. In subsequent interviews, no reports were made of contact within the 21-day Ebola virus disease incubation period between the confirmed cases and people with Ebola virus disease symptoms, funeral attendance, or sexual contact with known survivors of Ebola virus disease. Possible transmission

through blood transfusion or during a medical procedure were investigated but deemed unlikely. Based on the scant supporting evidence for other sources of infection, the investigation then focused on identification of a potential source of transmission from a persistently infected individual.

In view of the serological results (positive IgG and IgM) for the mother (individual D), indicating previous Ebola virus infection, an in-depth investigation was done to establish a history of illness due to Ebola virus disease or past viral exposure (table 2), which is visualised in figure 1. Interviews from several sources showed that in July, 2014, the mother (individual D) had cared for her sick brother (individual G), who worked as a nurse's aide at a local clinic. Individual G and his supervising officer at the clinic (individual H) reportedly cared for a patient (individual X) in July, 2014, who had symptoms consistent with Ebola virus disease and subsequently died at the health facility. Individuals G and H both became ill with Ebola virus disease symptoms; individual G was cared for by his sister (individual D) and eventually succumbed to his illness without being laboratory-tested for Ebola virus disease. Based on strong suspicion for Ebola virus disease, individual H was tested and confirmed as positive for Ebola virus RNA in August, 2014. Individual G's wife (individual I) and child (individual J) also became symptomatic during the same period and were confirmed to have Ebola virus disease. The three confirmed cases were admitted to an ETU and recovered. After caring for her brother in July, 2014, individual D reportedly became very ill with symptoms consistent with Ebola virus disease. She did not seek care at an ETU and no admission or laboratory records for her were identified in the national databases for that period. She suffered a miscarriage in August, 2014, and eventually recovered from her illness. No contact was reported between individuals A, B, and C with individual G during his period of illness and death.

Individual D gave birth to a baby boy (individual E) in September, 2015, via home delivery by a trained traditional midwife. Individual D presented to a local hospital on Oct 24, 2015, with complaints of a 2-week history

	Potential source of infection	Investigation findings
Individual D (mother of index case)		
Viral persistence or recrudescence	Individual G (brother of individual D)	Individual D became very ill in July, 2014, with symptoms consistent with Ebola virus disease after caring for her brother (individual G), a probable Ebola virus disease case who died on July 26, 2014; individual D had a miscarriage in August, 2014, and eventually recovered from her illness without admission to an ETU; no admission records were found for individual D at any of the operational ETUs at the time and no laboratory results were found in the national laboratory database for that period; individual G worked as a nurse's aide at a clinic under the supervision of individual H (the officer in charge of that clinic), and both cared for a patient meeting the probable case definition for Ebola virus disease, but who was never tested (individual X); individual H was later admitted to an ETU and confirmed positive by PCR for Ebola virus disease; the spouse of individual G (individual I) and his child (individual J) also developed symptoms of Ebola virus disease, were admitted to an ETU, and tested positive by PCR for Ebola virus disease
New infection	Trained traditional midwife	The trained traditional midwife who attended individual D during delivery was tested by serology and no Ebola virus antibodies were detected
New infection	Contaminated medical instrument	Transmission scenario judged unlikely because no further cases of Ebola virus disease were identified outside the family cluster
New infection	Blood transfusion	The hospital did not have access to a blood bank at the time of the Ebola virus disease outbreak but kept a list of recurring donors; seven potential donors with a blood type matching that of individual D were identified; two of these donors were listed in the blood bank log during the period when individual D received a transfusion; both donors were tested by PCR and serology and no Ebola virus RNA or antibodies were detected
Individual A (index case)		
New infection	Sexual contact with a survivor	Investigations did not identify any sexual partners for individual A
Individual B (father of index case)		
New infection	Sexual contact with a survivor	A sexual partner was mentioned during investigations but intense follow-up did not identify anyone with a previous history of Ebola virus disease; because of the sensitive nature of this transmission scenario, gaps existed in the information provided
New infection	Contact with an ill individual	A colleague of individual B was reported as ill on Oct 5, 2015; the colleague was tested by PCR and serology and no Ebola virus RNA or antibodies were detected
Individuals A–D		
New infection	Contact with a recent case	No recent case meeting the clinical case definition of Ebola virus disease outside this family cluster could be identified with whom any individual in the family cluster had been in contact
New infection	Attendance at funeral	No one reported recent attendance at a funeral
New infection	Contact with an animal source	No contact with non-human primate or consumption of bushmeat was reported
New infection	Travel to an area with known active Ebola virus disease transmission	No one reported recent travel to areas with known active transmission of Ebola virus disease
New infection	Contact with Ebola virus disease survivors (individuals I and J)	Individuals I and J visited the family in early-to-mid September, 2015 (duration of visit was unclear); neither of the visiting survivors reported an illness during the visit, and no contact with body fluids of the survivors was reported during the visit

ETU=Ebola treatment unit.

Table 2: **Epidemiological investigation of postulated source case scenarios for the Ebola cluster in Liberia (November, 2015)**

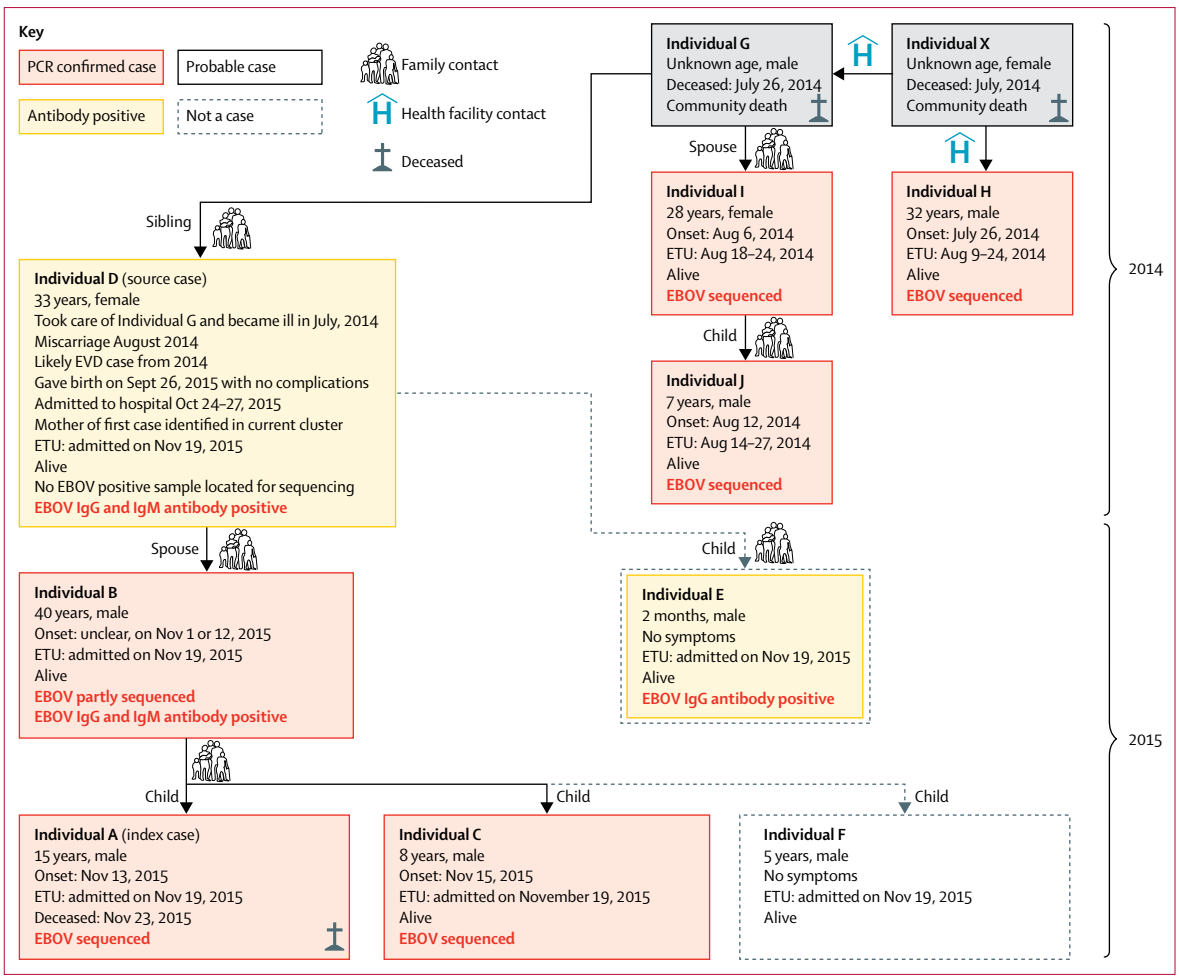

Figure 1: **Transmission chain for Ebola cluster (November, 2015)**
EBOV=Ebola virus. ETU=Ebola treatment unit. EVD=Ebola virus disease.

of fatigue, dyspnoea, and increasing lower extremity oedema. She was admitted and treated for anaemia and malaria and received a blood transfusion using banked blood provided by the hospital. Her symptoms improved slightly and she was discharged home a few days later but continued to feel unwell. The traditional midwife who delivered her baby did not report any symptoms consistent with Ebola virus disease and did not have detectable antibodies to Ebola virus on serological testing. Moreover, no reports were made of Ebola virus disease symptoms among hospital staff or patients during individual D's admission. Two blood donors—who donated blood to the hospital during the period of individual D's transfusion—tested negative for Ebola virus by RT-qPCR and antibodies were not detected on serology. The possibility that individual D's illness might have been due to Ebola virus disease recrudescence was investigated. Without a blood sample from individual D during the time of her suspected acute infection in August, 2014, the samples taken from confirmed cases from the July, 2014, cluster (individuals H, I, and J)—with whom individual D was linked—were used as a proxy to investigate a potential link between the virus that might have infected individual D with the viruses isolated from individuals A, B, and C (table 3).

The near-complete Ebola virus genomes assembled from individuals A, C, and H grouped phylogenetically within the LB5 sublineage that circulated in Liberia for many months, starting around August, 2014 (figure 2A).[18] Viruses from sublineage LB5 are known to have circulated in Montserrado, Nimba, Grand Kru, Bomi, Sinoe, Lofa, Grand Bassa, and Grand Cape Mount counties in Liberia during August, 2014, to December, 2014, and these viruses represent about 20% of all Ebola virus genomes sequenced from Liberia.[18] However, before this investigation, the last time an LB5 sublineage virus was detected was during the March, 2015, flare-up in Montserrado county, which occurred roughly 8 months before the flare-up investigated here. Because of low viral titres, only partial genomes were obtained from individual B,

	Sample type	Initial RT-qPCR test	Second RT-qPCR test	Ebola IgM (EU/mL)	Ebola IgG (EU/mL)	Genome coverage (%)	Ebola virus lineage
Individual H, supervising officer of individual G*	Blood	Positive	Positive	Not tested	Not tested	99·88%	LB5
Individual I, wife of individual G	Blood	Positive	Positive	Not tested	Not tested	1·87%	Undetermined; one read supported LB5 SNP at position 6056
Individual J, child of individual G	Blood	Positive	Positive	Not tested	Not tested	16·8%	Undetermined

RT-qPCR=reverse transcriptase qualitative PCR. SNP=single nucleotide polymorphism. *Individual G is a probable Ebola virus disease case who was cared for by his sister (individual D) during his illness.

Table 3: **Laboratory results for Ebola virus disease cases linked to individual D (July, 2014)**

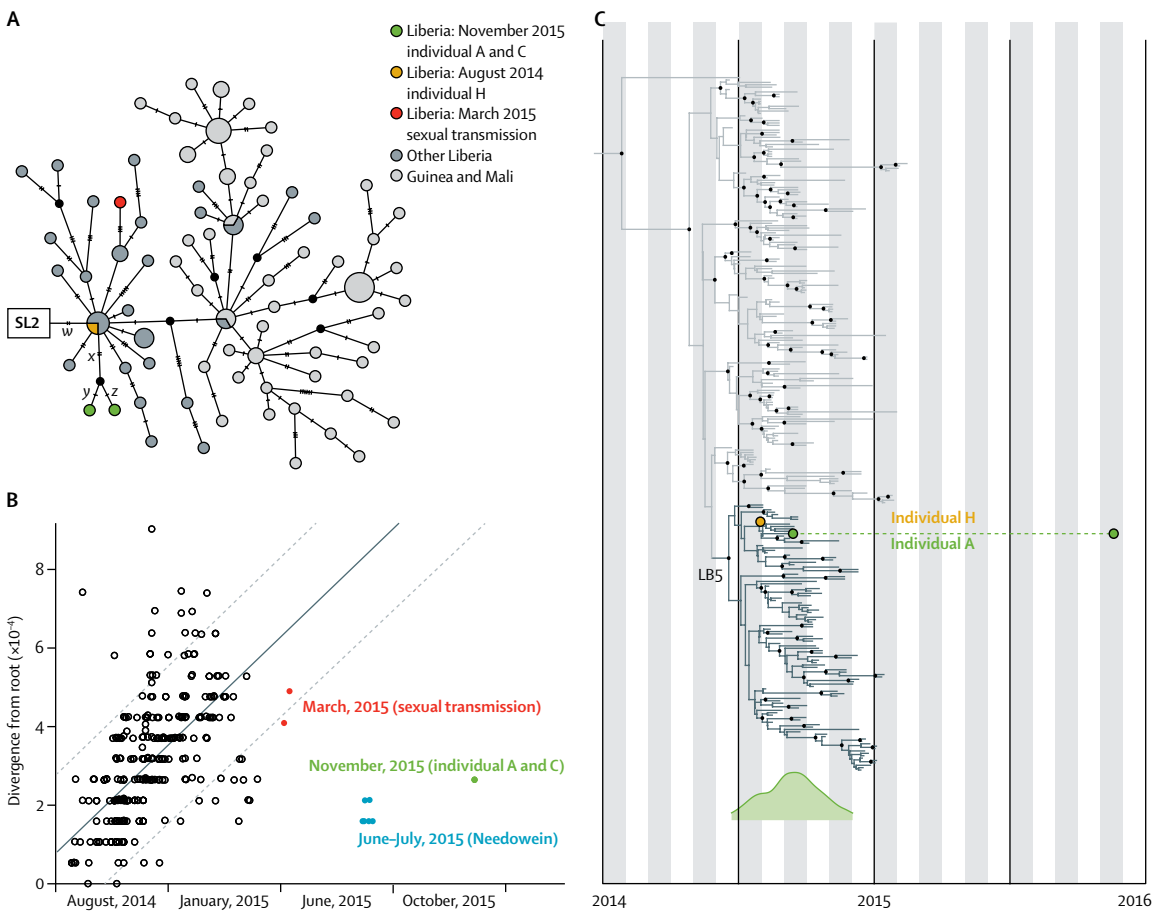

Figure 2: **Comparison of Ebola virus genomes from individuals A, C, and H with those from earlier cases in Liberia, and genetic divergence analysis**
(A) Median-joining haplotype network depicting sequence similarity of the Ebola virus genomes from the March, 2015, and November, 2015, flare-ups in Liberia, and those from 96 additional cases sampled during August, 2014, to December, 2014, all of which belong to the LB5 sublineage (GenBank accessions are in the appendix). Every coloured circle represents a sampled Ebola virus haplotype, the size of every circle is proportional to the number of samples, and the hatch marks indicate the number of substitutions along each edge. The white box represents the basal SL2 haplotype, which was introduced to Liberia early in the summer of 2014.[18] Information about the substitutions on the labelled branches (w, x, y, and z) is in table 4. (B) Root-to-tip distance versus sampling date for genomes from the first three Ebola virus disease flare-ups in Liberia (coloured red, blue, and green)[5,6,8] and 289 additional genomes from the Liberian portion of the SL2 lineage, including sequences from Guinea and Mali linked to reintroductions from Liberia (GenBank accessions are in the appendix). The solid line represents the overall trend and dashed lines the 95% prediction interval for the linear regression, which was calculated excluding genomes from the three Ebola virus disease flare-ups. Viral genomes from individual A and individual C from the November, 2015, cluster are included (coloured green). (C) Time-structured maximum clade credibility phylogeny including Ebola virus genomes from individual A (green circles) and individual H (yellow circle) and 289 additional genomes from the Liberian portion of the SL2 lineage, including sequences from Guinea and Mali linked to reintroductions from Liberia (GenBank accessions are in the appendix). The right green circle indicates the true sampling date and the left green circle represents the median value for the estimated sampling date. The 95% highest posterior distribution for the estimated sampling date is shown below the tree (green line and shading). Black circles indicate well-supported nodes with posterior probability of 0·9 or higher. The dark grey branches indicate the LB5 sublineage.

	Reference nucleotide	Alternative nucleotide	Individuals with alternative nucleotide	Individual B depth of coverage†	Branch of median-joining haplotype network‡
Position* 6056	A	C	A, B, C	1	w
Position* 16 514	G	A	A, C	0	w
Position* 11 125	G	A	A, B, C	2	x
Position* 13 185	G	A	A, C	0	x
Position* 11 048	C	T	C	0	y
Position* 12 267	T	C	A	3	z

The basal SL2 haplotype was introduced to Liberia early in the summer of 2014.[18] *Positions are relative to reference genome Ebola virus/*Homo sapiens*-wt/GIN/2014/Makona-C15 (KJ660346.2). †Depth of coverage at each position in individual B after correcting for PCR duplicates. ‡Branches depicted in figure 2A.

Table 4: **Substitutions recorded in individuals A, B, and C relative to the basal SL2 haplotype**

individual I, and individual J. The partial genomes from individual B and individual I were also consistent with placement in the LB5 sublineage, whereas the partial assembly of individual J's genome provided no information about sublineage placement. The genome from individual H was equivalent to the basal LB5 haplotype—ie, it contained only the two substitutions that define this sublineage. In addition to the two substitutions shared by all LB5 sublineage viruses, the genomes from individual A and individual C shared two additional substitutions and each contained a third substitution unique to each case (table 4). Using our standard coverage threshold (threefold or more depth of coverage after duplicate removal), the Ebola virus assembly for individual B covered about 20% of the genome, including the nucleotide position at which individual A contained a unique substitution (position 12 267T→C); individual B showed the reference genotype at this position. We obtained between onefold and twofold the depth of coverage from individual B at two other positions showing substitutions in individual A and individual C—one unique to these two cases (11 125G→A) and one shared by all LB5 sublineage viruses (6056A→C). Genomic data from individual B matched those from individual A and individual C at both positions. Despite low levels of sequencing depth, the detection of multiple variants placing the genomes from individual A (the index case) and individual C (his 8-year-old brother) phylogenetically close to the genome from individual B (their father) provides strong support for an epidemiological link between these cases. However, the exact placement of the genome from individual B in the haplotype network (figure 2A) is uncertain.

The Ebola virus genomes from individual A and individual C showed significantly lower amounts of nucleotide-level sequence divergence than expected, based on their sampling date (figure 2B). A similar reduction in divergence was noted in a previous cluster associated with a persistently infected source.[8] Based on the overall rate of sequence divergence noted during the west African outbreak, we estimated a median expected sampling date for the Ebola virus genome from individual A of about 14 months before the actual sampling date (figure 2C), which is consistent with a mean decrease in the rate of evolution along this branch of 6·4-fold (95% highest posterior density, 1–16·4 fold).

Discussion

The persistence of Ebola virus has been shown in immunologically protected sites of the body among individuals who have recovered from acute Ebola virus disease, and in body fluids of people who are convalescing.[19,20] Ebola virus RNA has been detected in semen,[21,22] breastmilk,[23] aqueous humor,[24] and cerebrospinal fluid[25] of survivors after resolution of the initial infection. The presence of viral particles in body fluids of survivors has resulted in Ebola virus infection among previously uninfected individuals and accounted for the sexual transmission of Ebola virus from a male survivor to his female partner in Liberia after unprotected sexual intercourse.[5,6] Despite no active Ebola virus transmission chains in west Africa, Ebola virus persistence poses a continued risk for resurgence of cases[26,27] and has potential for a large-scale outbreak if not detected rapidly and controlled.

The investigation into the source of infection for the cluster of Ebola virus disease cases reported in Liberia in November, 2015, identified a link to a lineage of Ebola virus that began circulating in Liberia in 2014, and is currently the last known cluster of infections from an endogenous source in that country. Sequencing of Ebola virus RNA extracted from serum samples of the confirmed cases in this cluster showed that the flare-up was not caused by independent introduction from an unknown non-human reservoir but was a continuation of the west African Ebola virus disease outbreak that began in 2013. More specifically, this flare-up represents the re-emergence of a Liberian Ebola virus transmission chain, not re-introduction of Ebola virus into Liberia from a neighbouring country. Furthermore, the level of sequence divergence noted for the Ebola virus genomes in this cluster was substantially lower than expected based on sampling date and other Ebola virus genomes from the Liberian portion of the outbreak.[6,8,17] The evidence supports viral persistence and transmission from a previously infected individual.

Based on the serological profile of individual D (the mother of the index case), evidence suggests that she might have been the first member of the family to be infected with Ebola virus. Ebola virus-specific IgG antibodies indicate previous exposure to the virus,[28] and with no history of having received an Ebola virus-preventive vaccine, this presence was most probably attributable to an immune response to Ebola virus infection. Additional detection of Ebola virus IgM antibodies with the available serological assays could indicate recent infection or disease recrudescence, but might also be a laboratory artifact and reflect the

unusually high IgG titres seen in this individual. Cross-reactivity between IgM antibodies to Ebola virus and anti-IgG detection antibodies has been described previously.[29] Despite stringent lot-release criteria, cross-over can still occur with detection antibodies. Serological testing done 6 months after the initial testing of the family members still detected IgM and high levels of IgG. In view of the very high levels of IgG noted in individual D, additional testing would be needed to confirm if the relatively low levels of IgM detected in the same sample were truly IgM or spillover in the assays from the overwhelming titres of IgG recorded. Based on epidemiological and genomic data, individual D—the mother of the 15-year-old boy (index case)—was the likely source of infection for this case cluster. She seemingly survived an acute infection in 2014 based on presence of Ebola virus disease symptoms, her positive serology, and epidemiological linkage to a confirmed Ebola virus disease patient.

Few occurrences of Ebola virus disease recrudescence have been reported, manifesting as uveitis from Ebola virus in the aqueous humor of a survivor and meningitis caused by virus detected in a survivor's cerebrospinal fluid months after recovering from the initial infection.[24,25] Disease recrudescence can be associated with changes in the immune status of a patient. Pregnancy results in an immunosuppressive state, with a rebound of inflammatory responses during the postpartum period, which can result in latent infections manifesting as symptomatic disease or worsening during that period.[30] It is plausible that the mother (individual D) had viral persistence or recrudescent disease after her pregnancy and childbirth in September, 2015, became ill in October, 2015, transferred protective antibodies to her newborn baby (individual E) and transmitted the virus to her husband (individual B)—who in turn transmitted the virus to two of their three sons (individuals A and C). No reports have been published of Ebola virus transmission due to disease recrudescence. Furthermore, we have no clear indication of the mode of transmission from the mother to her husband. Although persistence of Ebola virus in vaginal fluid has been described,[31] no recorded evidence exists of sexual transmission from female survivors. Most likely, transmission between family members occurred during close physical interaction or contact with other body fluids.

Several factors limit empirical inference of transmission directionality for this cluster based on molecular evidence. These include an incomplete genome assembly for the virus infecting the father (individual B) and no available samples from 2014 for the mother (individual D), her brother (individual G), or the source patient (individual X). In August, 2014, ETUs had limited capacity for epidemiological investigations, diagnostics, and treatment, so many patients died in the community without full investigations of transmission chains and laboratory confirmation of Ebola virus disease to aid subsequent investigations. Further characterisation of the serological response in the mother (individual D) to confirm a relapse of Ebola virus disease was unsuccessful. However, similar to the preceding two Ebola virus disease clusters in Liberia in March, 2015, and June, 2015, epidemiological and molecular evidence exists that the November, 2015, cluster resulted from a persistent viral source within the country.

The rapid containment of flare-up-associated Ebola virus disease cases indicates the increased public health and outbreak response capacity in west Africa. After the end of widespread Ebola virus transmission, the Ebola virus disease outbreak was declared to no longer be a Public Health Emergency of International Concern on March 29, 2016. However, the findings from this and recent Ebola virus disease clusters highlight the risk of Ebola virus disease flare-ups even after an outbreak is declared over. Risk assessment and focused prevention efforts are needed for Ebola survivors and their close contacts. Further studies are also needed to better understand viral persistence and transmission dynamics among survivors. Efforts should continue to focus on strengthening health systems to prevent, rapidly detect, and respond to Ebola virus infections in the region.

Contributors

EKD, AW, ELH, DJB, ASL, GW-D, NM, MB, AK, CG, T-HC, DRA, JM, DEW, and MPF did epidemiological investigations. PR, JS, and AH did laboratory investigations. MRW, JTL, SEM, LF, GP, and FB did sequencing and genome analysis. LH, ES, JL, and BD-K did serology. YW, PC, EKD, TN, AG, DEW, MPF, BD, and FK contributed to response coordination. EKD, AW, SEM, JTL, ELH, PR, GP, and MF wrote the initial draft of the report. AB and EH were involved in case management. All authors reviewed and edited the final report.

Declaration of interests

We declare no competing interests.

Acknowledgments

Funding for this work was provided by the US Centers for Disease Control and Prevention (CDC), the Defense Threat Reduction Agency, and WHO. We acknowledge the patients and key informants in this investigation; Montserrado county health team; Liberia Ministry of Health incident management system; CDC-Liberia and WHO-Liberia Ebola response teams; other technical partners; and staff from Illumina for providing filovirus hybrid capture probes. The content of this publication reflects the views of the authors and does not necessarily reflect the views or policies of the CDC, the US Department of Defense, the US Department of the Army, or the institutions and companies affiliated with the authors.

References

1 Centers for Disease Control and Prevention. Ebola (Ebola virus disease): case counts. Dec 27, 2017. https://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/case-counts.html (accessed June 27, 2018).

2 Centers for Disease Control and Prevention. Ebola (Ebola virus disease): previous updates—2014 west Africa outbreak. March 24, 2016. https://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/previous-updates.html (accessed June 27, 2018).

3 Pillai SK, Nyenswah T, Rouse E, et al. Developing an incident management system to support Ebola response: Liberia, July–August 2014. *MMWR Morb Mortal Wkly Rep* 2014; **63:** 930–33.

4 Bell BP, Damon IK, Jernigan DB, et al. Overview, control strategies, and lessons learned in the CDC response to the 2014–2016 Ebola epidemic. *MMWR Suppl* 2016; **65** (suppl 3)**:** 4–11.

5 Christie A, Davies-Wayne GJ, Cordier-Lassalle T, et al. Possible sexual transmission of Ebola virus: Liberia, 2015. *MMWR Morb Mortal Wkly Rep* 2015; **64:** 479–81.

6 Mate SE, Kugelman JR, Nyenswah TG, et al. Molecular evidence of sexual transmission of Ebola virus. *N Engl J Med* 2015; **373:** 2448–54.

7 WHO. Ebola situation report: 13 May 2015. http://apps.who.int/ebola/current-situation/ebola-situation-report-13-may-2015 (accessed June 27, 2018).

8 Blackley DJ, Wiley MR, Ladner JT, et al. Reduced evolutionary rate in reemerged Ebola virus transmission chains. *Sci Adv* 2016; **2:** e1600378.

9 WHO. Ebola situation report: 9 September 2015. http://apps.who.int/ebola/current-situation/ebola-situation-report-9-september-2015 (accessed June 27, 2018).

10 WHO. Case definition recommendations for Ebola or Marburg virus diseases. Aug 9, 2014. http://apps.who.int/iris/bitstream/10665/146397/1/WHO_EVD_CaseDef_14.1_eng.pdf?ua=1&ua=1 (accessed June 27, 2018).

11 Wolfe CM, Hamblion EL, Schulte J, et al. Ebola virus disease contact tracing activities, lessons learned and best practices during the Duport Road outbreak in Monrovia, Liberia, November 2015. *PLoS Negl Trop Dis* 2017; **11:** e0005597.

12 WHO. Surveillance strategy during phase 3 of the Ebola response. Nov 5, 2015. http://www.who.int/csr/resources/publications/ebola/surveillance-strategy-phase3/en/ (accessed June 27, 2018).

13 WHO. Laboratory diagnosis of Ebola virus disease. Sept 19, 2014. http://apps.who.int/iris/bitstream/handle/10665/134009/WHO_EVD_GUIDANCE_LAB_14.1_eng.pdf?sequence=1 (accessed June 27, 2018).

14 Joint Project Manager Medical Countermeasure Systems. Ebola Zaire (EZ1) rRT-PCR (TaqMan) Assay on ABI 7500 Fast Dx, LightCycler, and JBAIDS instruction booklet, version 3.0. Oct 10, 2014. https://www.fda.gov/downloads/medicaldevices/safety/emergencysituations/ucm418802.pdf (accessed July 5, 2018).

15 Cepheid Innovation. Xpert Ebola assay: instructions for use—for use under an Emergency Use Authorization (EUA) only. March, 2015. http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM439578.pdf (accessed June 27, 2018).

16 Kennedy SB, Bolay F, Kieh M, et al. Phase 2 placebo-controlled trial of two vaccines to prevent Ebola in Liberia. *N Engl J Med* 2017; **377:** 1438–47.

17 Kugelman JR, Wiley MR, Mate S, et al. Monitoring of Ebola virus Makona evolution through establishment of advanced genomic capability in Liberia. *Emerg Infect Dis* 2015; **21:** 1135–43.

18 Ladner JT, Wiley MR, Mate S, et al. Evolution and spread of Ebola virus in Liberia, 2014–2015. *Cell Host Microbe* 2015; **18:** 659–69.

19 Clark DV, Kibuuka H, Millard M, et al. Long-term sequelae after Ebola virus disease in Bundibugyo, Uganda: a retrospective cohort study. *Lancet Infect Dis* 2015; **15:** 905–12.

20 Rowe AK, Bertolli J, Khan AS, et al. Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. *J Infect Dis* 1999; **179** (suppl 1)**:** S28–35.

21 Deen GF, Broutet N, Xu W, et al. Ebola RNA persistence in semen of Ebola virus disease survivors: final report. *N Engl J Med* 2017; **377:** 1428–37.

22 Soka MJ, Choi MJ, Baller A, et al. Prevention of sexual transmission of Ebola in Liberia through a national semen testing and counselling programme for survivors: an analysis of Ebola virus RNA results and behavioural data. *Lancet Glob Health* 2016; **4:** e736–43.

23 Sissoko D, Keïta M, Diallo B, et al. Ebola virus persistence in breast milk after no reported illness: a likely source of virus transmission from mother to child. *Clin Infect Dis* 2017; **64:** 513–16.

24 Varkey JB, Shantha JG, Crozier I, et al. Persistence of Ebola virus in ocular fluid during convalescence. *N Engl J Med* 2015; **372:** 2423–27.

25 Jacobs M, Rodger A, Bell DJ, et al. Late Ebola virus relapse causing meningoencephalitis: a case report. *Lancet* 2016; **388:** 498–503.

26 Diallo B, Sissoko D, Loman NJ et al. Resurgence of Ebola virus disease in Guinea linked to a survivor with virus persistence in seminal fluid for more than 500 days. *Clin Infect Dis* 2016; **63:** 1353–56.

27 MacIntyre CR, Chughtai AA. Recurrence and reinfection: a new paradigm for the management of Ebola virus disease. *Int J Infect Dis* 2016; **43:** 58–61.

28 Ksiazek TG, Rollin PE, Williams AJ, et al. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. *J Infect Dis* 1999; **179:** S177–87.

29 MacNeil A, Reed Z, Rollin PE. Serologic cross-reactivity of human IgM and IgG antibodies to five species of Ebola virus. *PLoS Negl Trop Dis* 2011; **5:** e1175.

30 Singh N, Perfect JR. Immune reconstitution syndrome and exacerbation of infections after pregnancy. *Clin Infect Dis* 2007; **45:** 1192–99.

31 Rodriguez LL, De Roo A, Guimard Y, et al. Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. *J Infect Dis* 1999; **179:** S170–76.