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Ebola virus persistence in breast milk after no reported illness: a likely source of virus transmission from mother to child

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Running title: Ebola virus persistence in breast milk

Abstract

A nine-month-old infant died from Ebola virus (EBOV) disease with unknown epidemiological link. While her parents did not report previous illness, laboratory investigations revealed persisting EBOV RNA in the mother's breast milk and the father's seminal fluid. Genomic analysis strongly suggests EBOV transmission to the child through breastfeeding.

Keywords: Ebola virus; mother-to-child transmission; real-time sequencing; breast milk; asymptomatic carriage

On 18th August 2015, a 9-month-old breastfed female infant from Dubréka, Guinea, developed fever (38°C), diarrhoea, vomiting, and cough. The father, a trained nurse, administered erythromycin, paracetamol, amodiaquine, albendazole, and metopimazine. During the following five days, the clinical status of the child remained relatively stable. On 24th August, her condition rapidly deteriorated, with severe vomiting and diarrhoea. The family attended a primary health care center in Dubréka, which referred them to the University Hospital in Conakry. On the way to the hospital, the infant developed respiratory distress and passed away. A buccal swab was tested positive for Ebola virus (EBOV) by reverse transcription PCR (RT-PCR) on 25th August (Fig. 1A). The National Committee of Ethics in Medical Research of Guinea approved the use of diagnostic leftover samples and corresponding patient data for this study (permits Nº11/CNERS/14). All necessary consents required by applicable law from the patients whose information is included in the article have been obtained in writing.

An epidemiological investigation did not yield a known source of infection. Contact with known Ebola virus disease (EVD) patients or survivors could not be identified. The child was the first EVD case in over 42 days in the area where the family was living (Supplementary Fig. S1). She had never presented at a health care facility and did not receive routine vaccinations, as the mother feared contact with EVD patients in health centers. In addition, the family members reported only few social contacts with the exception of contacts to the 4-year-old stepbrother and the grandmother.

In an attempt to trace the source of infection by molecular epidemiology, the EBOV-positive RNA of the child was transferred to our sequencing facility at the Ebola Treatment Center in Coyah on 30th August and the viral genome was sequenced using MinION technology in Guinea (Oxford Nanopore, UK) [1]. Phylogenetic analysis revealed that the virus (European Nucleotide Archive [ENA] accession number LT630606) belonged to the Sierra-Leone 3

(SL3) lineage and clustered with strains of the large Conakry–Dubréka sublineage that were circulating between May and July 2015 in these prefectures (Fig. 1B) [1]. We identified a unique genomic signature consisting of three nucleotide polymorphisms at positions 6027, 12290, and 15660, which were not found elsewhere in the EBOV database.

Due to EVD confirmation in the child, the parents received the rVSV-ZEBOV vaccine on 27th August (Fig. 1A) [2]. On 30th August, both tested positive for EBOV-specific IgG but not IgM in ELISA, which was interpreted as a sign of past EBOV infection and raised the suspicion of asymptomatic virus carriage. Therefore, body fluids of the parents were tested for EBOV RNA using the RealStar Zaire Ebolavirus RT-PCR kit (Altona, Germany) on a Rotor-Gene instrument (Qiagen) [3]. On 9th September, the mother's breast milk was tested EBOV RNA-positive with a cycle threshold (Ct) value of 23·3, while urine and whole blood were negative (Fig. 1A). Sequencing and phylogenetic analysis demonstrated that the viruses from child and breast milk (ENA accession number LT630561) are closely related and share two of the unique single nucleotide polymorphisms (Fig. 1B). In the phylogenetic tree, the virus from the breast milk appears ancestral to that of the infant. On 15th September, a semen sample from the father tested EBOV RNA-positive with a Ct value of 35·6, whereas urine and whole blood were negative (Fig. 1A). The presence of EBOV RNA in the semen sample was confirmed by repeat RNA extraction and RT-PCR testing as well as genomic sequencing. The virus sequenced from the semen (ENA accession number LT630562) was also part of the SL3 lineage, but ancestral to the Conakry–Dubréka cluster and without close link to the viruses of mother and child (Fig. 1B). On 7th October, the father's semen tested negative for EBOV.

Epidemiological investigation could not reveal source, locality, or time period of EBOV infection in the parents. They did not report a severe febrile illness and denied contacts with known EVD cases or family deaths, or attending funerals since the beginning of the Ebola outbreak. The father, a 28-year-old nurse, had worked as community health care worker in the

district of Mali in the Northern part of Guinea from February 2014 to March 2015. Only five EVD cases have been reported in this district throughout the outbreak. From March to August 2015, he was unemployed and lived in Dubréka in a family with 4 members. With the exception of a motorcycle accident that required dental reconstructive surgery in a private clinic in Conakry, he denied any noticeable health episode in the past. The mother, a 23-year-old college student, did not report any medical episode except chronic headaches. She travelled daily between Dubréka and her school in Conakry by collective taxi.

The described family cluster highlights peculiarities of EVD, which are poorly understood and difficult to study, including EBOV infection in the absence of classical risk factors and virus persistence in patients with a mild or asymptomatic course of EVD associated with risk of virus transmission.

The molecular epidemiological data indicate that mother and father became infected in Conakry or Dubréka and that both infections are not directly linked. The lack of known risk factors suggests that sources and routes of transmission exist, which are not yet understood. The signs and symptoms of EVD in the parents are also difficult to assess, as intermittent febrile illness may be considered the norm in rural populations in Africa. In the absence of objective clinical investigations, we describe the disease here as “mild or asymptomatic”. Whether there is an association between this specific clinical manifestation and the (unknown) source and route of infection is a matter of speculation.

Mild or asymptomatic EBOV infections have been described [4]. However, it is not known whether this clinical manifestation, like severe non-fatal EVD, is associated with virus persistence [5, 6]. The cases of mother and father show that EBOV may persist in breast milk and seminal fluid in survivors with mild disease or asymptomatic infection. This raises the possibility of virus transmission by individuals who were not diagnosed with the disease, and

thus do not know they had EVD and are not listed in the national survivors databases, like the two parents.

The closely related EBOV sequences in mother and child, the ancestral position of the mother's virus relative to the child's virus in the phylogram, the epidemiological link between mother and child, and the absence of contact of the child to other EVD cases in the community or the hospital suggest that the mother transmitted the virus to the child via breastfeeding. Our findings are in line with the case of an asymptomatic mother, whose breast milk tested positive and who may have transmitted EBOV to her 13-month-old child [7]. In addition, there are anecdotal descriptions on presence of EBOV or Sudan virus RNA in breast milk of mothers with symptomatic EVD [8, 9]. The timing of events in our case remains speculative. Given that the child was breast-fed for 9 months before she became infected, it is plausible to assume that the infection in the mother occurred rather recently before the child's infection. However, due to the mild or asymptomatic course, the accumulation of EBOV in the mammary gland may have been delayed relative to blood.

The detection of EBOV RNA in seminal fluid of the father suggests that the number of survivors with persisting EBOV in seminal fluid, as predicted from the incidence of hospitalised cases only [10], may be an underestimate. The mechanisms of EBOV persistence in immunologically privileged or glandular tissue, in particular in the presence of circulating antibodies against EBOV, are hardly understood [5, 11]. The cases described here at least suggest a lack of correlation between severity of disease and virus persistence. Whether persistence is associated with reduced levels of neutralising antibodies or poor T cell memory response remains to be studied.

In conclusion, the described findings call for efforts to estimate the prevalence of EVD survivors who had not been diagnosed during the acute phase and improve counselling for this group of survivors. Fortunately, transmission of virus via breastfeeding many months

after recovery is likely to be an extremely rare phenomenon. Among the high number of female survivors, there are presumably hundreds of mothers who have given birth and nursed healthy babies in the post-outbreak period.

Notes

Author contributions. D.S., M.K., B.D., N.A., D.L.F., and B.A.D. performed the epidemiological investigations. J.A.B., F.R.K., K.S., S.M., T.E., A.M., V.A., O.F., N.M., and S.D. performed the laboratory investigations. N.J.L. performed the sequence analysis. A.A.S., M.W.C., X.A., D.M., P.F., R.B.A., S.K., M.H.D., S.G., and S.D. coordinated case management, laboratory investigations, and fieldwork. D.S., S.D., and S.G. wrote the manuscript. All authors reviewed the final draft. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Potential conflicts of interests. N.J.L. is on the speakers' bureau of and has received travel

fees from Oxford Nanopore Technologies. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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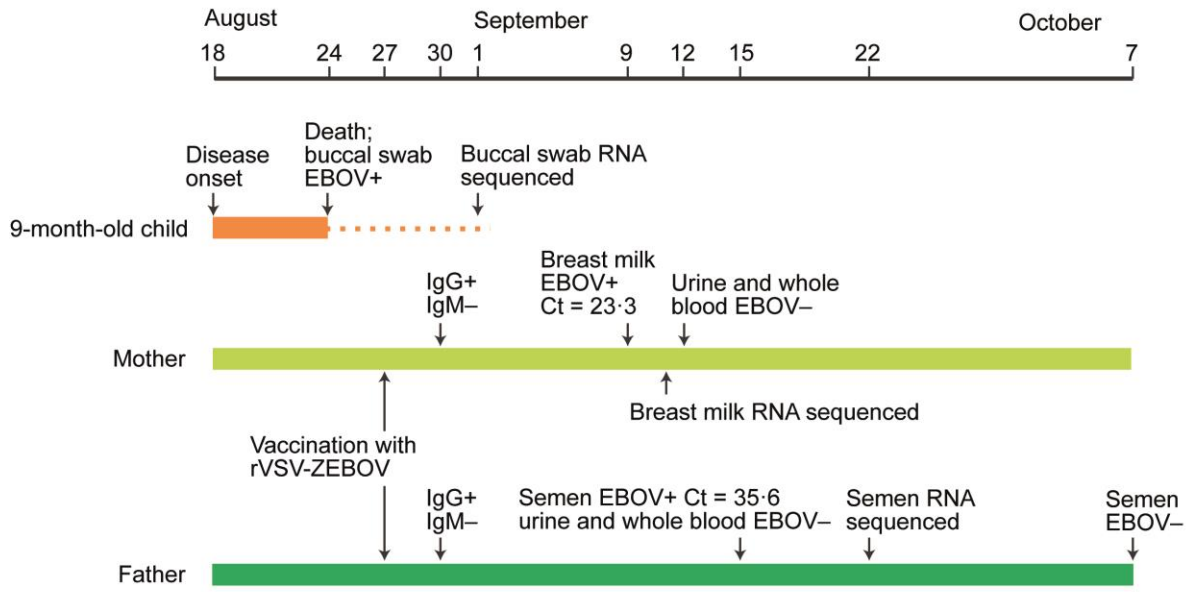
Figure 1. Timeline of clinical events, interventions, and laboratory investigations and phylogenetic tree of the EBOV sub-lineage containing the sequences from the family. (A) The three cases are depicted by horizontal bars with events indicated by arrows. Results of laboratory investigations are indicated as positive (+) and negative (–). For positive real-time PCR results, the cycle threshold (Ct) is given, which is an indirect measure of viral RNA concentration. (B) Phylogenetic reconstruction was performed by maximum likelihood under the GTR+Gamma model using RAxML as described [1, 12]. The sub-tree shown in the figure depicts the sequences that share a common ancestor with the sequences from the mother and father; the complete tree is shown in [1]. The geographic origin of the cases is indicated by colour. ENA accession numbers are LT630562 for the father's semen, LT630561 for the mother's breast milk, and LT630605 for the child's swab. The length of the branches roughly corresponds to the number of single nucleotide polymorphisms (SNP) in the respective strain(s) that distinguish them from the ancestral virus.

A



B