

Added Value of an Anti-Ebola Serology for the Management of Clinically Suspected Ebola Virus Disease Patients Discharged as Negative in an Epidemic Context

Antoine Nkuba-Ndaye,^{1,2,3} Daniel Mukadi-Bamuleka,^{2,3} Junior Bulubula-Penge,^{2,3} Guillaume Thaurignac,¹ François Edidi-Atani,^{2,3} Fabrice Mambu-Mbika,^{2,3} Bernice Danga-Yema,³ Meris Matondo-Kuamfumu,^{2,3} Eddy Kinganda-Lusamaki,^{1,2,3} Nella Bisento,² Raphaël Lumembe-Numbi,^{2,3} Gabriel Kabamba-Lungenyi,^{2,3} Divine Kitsa-Mutsumbirwa,⁴ Nelson Kambale-Sivihwa,⁵ François Boillot,⁶ Eric Delaporte,¹ Placide Mbala-Kingebeni,^{2,3} Ahidjo Ayouba,^{1,6} Martine Peeters,^{1,6} and Steve Ahuka-Mundeke^{2,3}

¹Recherches Translationnelles sur le Virus de l'Immunodéficience Humaine et Maladies Infectieuses, Université de Montpellier/Institut National de la Santé et de la Recherche Médicale U1175, Institut de Recherche pour le Développement, Montpellier, France, ²Département de Virologie, Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of the Congo, ³Département de Biologie Médicale, Université de Kinshasa, Kinshasa, Democratic Republic of the Congo, ⁴Laboratoire Mobile pour Maladie à Virus Ebola, Institut National de Recherche Biomédicale, Butembo, Nord-Kivu, Democratic Republic of the Congo, ⁵Laboratoire Mobile pour Maladie à Virus Ebola, Institut National de Recherche Biomédicale, Beni, Nord-Kivu, Democratic Republic of the Congo, ⁶Alter-Santé Internationale & Développement, Montpellier, France

Background. Survivors from Ebola virus disease (EVD) may be at the origin of EVD resurgence.

Methods. Simultaneous reactivity to at least 2 *Ebola virus* or *Zaire ebolavirus* (EBOV) antigens was detected in 11 of 488 (2.3%; 95% confidence interval [CI], 1.1–4.0) suspected EVD patients who were discharged as negative after 2 consecutive negative tests during the 10th *Ebola* outbreak in the Democratic Republic of the Congo.

Results. After extrapolating the total number of individuals discharged as negative during the entire outbreak, we estimated a total of 1314 additional missed *Ebola* cases.

Conclusions. These findings emphasize the usefulness of an EBOV serology analysis and the importance of extending epidemic surveillance to clinically suspected cases who were discharged as negative.

Keywords. DRC; *Ebola*; Ituri; North-Kivu; serology.

Ebolaviruses are among the most virulent viruses and has a high mortality rate. Since the first recognition of *Zaire ebolavirus* (EBOV) in 1976 in the Democratic Republic of the Congo (DRC), the country has registered 13 outbreaks [1]. The 10th Ebola virus disease (EVD) outbreak in Eastern DRC, declared on first August 2018, was the longest (2 years) and the most devastating ever recorded in the country with a total of 3470 cases with 2287 deaths [2]. Although it is generally assumed that each EVD outbreak results from an independent zoonotic transmission of the virus from wildlife to humans [3], recently 2 outbreaks in DRC have resurged from individuals who recovered from EVD [4]. Moreover, genomic analysis of the 2021 EVD outbreak in Guinea showed that resurgence from humans can even occur more than 5 years after the end of the epidemic [5].

During the large EVD outbreak in West Africa (2013–2016), several independent studies have shown that some EBOV infections remain asymptomatic or paucisymptomatic and therefore go undiagnosed [6, 7]. As part of the outbreak response in Eastern DRC, a recombinant vesicular stomatitis virus-*Zaire ebolavirus* vaccine (rVSV-ZEBOV-GP, *Mayinga* strain 1976) has been widely used in a ring vaccination strategy to prevent transmission [2] that may increase the possibility of developing mild *Ebolavirus* infection. In addition, insecurity in this affected area may have delayed access to reverse-transcription polymerase chain reaction (RT-PCR) testing, and some patients could have been referred to the Ebola treatment center after the viremic period. More importantly, viral relapse or resurgence due to the persistence of the virus in body fluids, such as semen and breast milk, or in immune-privileged sites can also occur in undiagnosed EVD patients [5]. To estimate the number of possible missed EVD patients during the 10th EBOV outbreak in Eastern DRC, we studied the presence of antibodies for EBOV in clinically suspected EVD cases with a negative RT-PCR test result.

PATIENTS AND METHODS

Study Design and Sample Collection

We performed a retrospective study on leftover blood samples of clinically suspected EVD patients who tested negative in 2 consecutive EBOV RT-PCR tests. Samples were collected in the 3 provinces affected by the 10th Ebola outbreak in the DRC, that is, North-Kivu, South-Kivu, and Ituri. Samples were centralized and stored frozen in the Biobank of the Institut National de Recherche Biomédicale. We used the national EBOV database containing clinical, demographic, and laboratory results of available blood samples (132 051) of 4 health zones where almost all confirmed cases >90% (3315 of 3461) occurred (Figure 1): Beni, Butembo, Katwa, and Mabalako. With the *Random* function of Microsoft Office Excel 2016, we selected 600 samples

Received 15 December 2021; editorial decision 10 February 2022; accepted 16 February 2022; published online 18 February 2022.

Correspondence: Antoine Nkuba-Ndaye, MD, MPH, Institut de Recherche pour le Développement, Université de Montpellier, 911 Avenue Agropolis, 34394 Montpellier Cedex 5, France (antoinkuba@gmail.com).

The Journal of Infectious Diseases® 2022;XX:1–5

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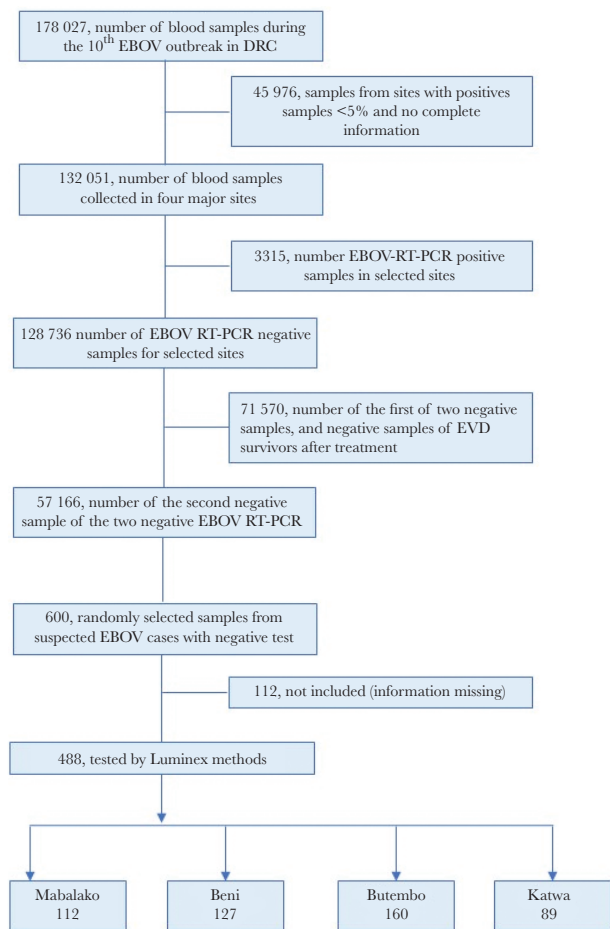


Figure 1. Flowchart of clinical Ebola virus disease (EVD) suspected patients and their stored samples selection for 4 health zones: Beni, Mabalako, Butembo, and Katwa. DRC, Democratic Republic of the Congo; EBOV, *Zaire ebolavirus*; RT-PCR, reverse-transcription polymerase chain reaction.

corresponding to the second negative EBOV RT-PCR samples. For a control group, we used archived samples collected in 2011 as part of human immunodeficiency virus monitoring in the same geographic area before Ebola vaccination was used or an EVD outbreak was reported [8]. We considered clinical variables related to acute phase symptoms, epidemiological variables (contact to index case), and sociodemographic variables (age, sex, and diagnostic site).

Laboratory Analysis

Serological analysis was performed using the Luminex technology (Luminex Corp., Austin, TX) and following the protocol previously published [7, 9]. In brief, 4 commercially available recombinant EBOV proteins were coupled to magnetic beads; 2 glycoproteins, GP-EBOV-k (Kissidougou strain 2014) and GP-EBOV-m (*Mayinga* strain 1976); 1 nucleoprotein, NP-EBOV (Kissidougou strain 2014); and 1 40-kDa viral protein (VP40-EBOV, Kissidougou strain 2014). Antigens coupled to beads were mixed with the patient sample (1/1000 sample

to dilution buffer), and the reaction signal for anti-EBOV immunoglobulin G (anti-IgG) was read and recorded on Bio-Plex 200 equipment (Bio-Rad, Marnes-la-Coquette, France). Results were expressed as median fluorescence intensity. Samples with positive signal to at least 2 antigens simultaneously were considered as positive EVD cases [9].

Statistical Analysis

We expressed categorical data in frequencies, whereas continuous data were expressed in median and quantiles. Chi-square Fisher's exact tests were used. We considered $P < .05$ as the significant threshold.

Ethical Considerations

The sample collection process in the field was exempted by the review from the ethical committee, because it was part of the EBOV outbreak response. Permission to analyze and use data was obtained by the Ethical Committee of the Ecole de Santé Publique de l'Université de Kinshasa (ESP-UNIKIN, Number ESP/CE/172/2021).

RESULTS

Sociodemographic Characteristics of Clinically Suspected Ebola Virus Disease Patients

Among the 600 randomly selected samples from negative EBOV RT-PCR samples, 488 with sufficient clinical information were tested for EBOV antibodies. Among these, 127 samples were from Beni, 160 from Butembo, 112 from Mabalako, and 89 from Katwa (Figure 1); 244 (50.3%) and 241 (49.7%) were from male and female patients, respectively. There was no age and sex information for 3 individuals. The median age was 22 years (interquartile range, 12–32), and 86.6% (420 of 485) were aged below 40 years (Supplementary Table S1).

Serological Characteristics of Clinically Suspected Ebola Virus Disease Patients

Of 488 samples analyzed, 7 (1.4%) were reactive with NP antigen, 54 (11.1%) with GP, and 39 (8%) with VP40 versus 2 (0.7%), 15 (5.4%), and 5 (1.8%) to NP, GP, and VP40 EBOV antigens, respectively, in the control group. Differences were significant for GP and VP40 EBOV antigens ($P < .01$) (Table 1). By applying the previously defined positivity criteria for EBOV infection with this assay, that is, simultaneous reactivity to at least 2 antigens, 11 (2.3%; 95% CI, 1.1–4.0) (Table 1) clinically suspected EVD patients were positive versus 1 sample in the control group (0.4%) ($P = .064$). We observed 2 (0.44%) samples positive to NP and GP, 5 (1.0%) samples positive to GP and VP40, 1 (0.2%) sample positive to NP and VP40, and 3 (0.6%) samples that had IgG antibodies to NP, GP, and VP40 EBOV antigens simultaneously.

During the outbreak, mobile laboratories in Beni, Butembo, Mabalako, and Katwa received and analyzed 132 051 blood

Table 1. Proportion of Anti-IgG EBOV Antibodies for NP, GP, and Matrix Protein VP-40

Variables (EBOV Antigens)	Ebola Suspected Cases (n = 488)		Negative Control (n = 280)		P Value
	n Positive	% (95% CI)	n Positive	% (95% CI)	
NP	7	1.4 (0.6–2.9)	2	0.7 (0.0–2.6)	.498
GP	54	11.1 (8.4–14.2)	15	5.4 (3.0–8.7)	.007 ^a
VP-40	39	8.0 (5.7–10.8)	5	1.8 (0.6–4.1)	.001 ^a
At least 2 antigens	11	2.3 (1.1–4.0)	1	0.4 (0.0–2.0)	.064
NP + GP	2	0.4 (0.0–1.4)	0	0.0 (0.0–1.3)	.536
GP + VP-40	5	1.0 (0.3–2.4)	1	0.4 (0.0–2.0)	.424
NP + VP-40	1	0.2 (0.0–1.1)	0	0.0 (0.0–1.3)	.999
NP + GP + VP-40	3	0.6 (0.0–1.8)	0	0.0 (0.0–1.3)	.557

Abbreviations: CI, confidence interval; EBOV, *Zaire ebolavirus*; GP, glycoprotein; NP, nucleocapsid.

^aSignificant difference at 1%.

samples from patients with clinical suspicion of EVD, and only 3315 (2.5%) were confirmed positive by PCR (unpublished observations). Because the rate of patients positive for at least 2 antigens was 2.3% in our study, we extrapolated that 1314 additional patients could have EBOV antibodies among the 57 166 who were tested twice and discharged as negative and thus could be undiagnosed EVD patients (Supplementary Table S2).

Clinical Characteristics of the Clinically Suspected Ebola Virus Disease Patients

The case definition used for active surveillance was broad and unspecific, and symptoms reported by clinically suspected EVD patients were not specific to only EBOV infection. We analyzed all reported symptoms when admitted for EBOV RT-PCR test and assessed whether some were associated with antibody positivity, that is, presence of antibodies to at least 1 EBOV antigen. Symptoms such as asthenia 312 (63.9%), headache 299 (61.3%), anorexia 278 (57%), fever 262 (53.7%), abdominal pain 245 (50.2%), and arthralgia 166 (34%) were the most prevalent symptoms expressed. Antibody positivity was not associated with particular clinical symptoms ($P > .05$) (Supplementary Table S3).

Information on epidemiological link with confirmed EVD patients was available for 324 patients. Reactivity with 2 EBOV antigens was higher in patients reporting a link (2 of 46; 4.3%) versus those who did not (6 of 278; 2.2%), but the difference was not significant ($P > .05$) (Supplementary Table S3). Among patients positive for at least 2 EBOV antigens, 6 (54.5%) reported symptom onset of less than 6 days, and 5 (45.5%) reported symptom onset greater than 6 days (Supplementary Table S4).

DISCUSSION

There is increasing evidence that EVD can re-emerge in patients who recovered from the disease, as illustrated in 2021 by at least 2 relapses from the 10th EBOV outbreak that ended in August 2020 in the DRC, but also from reports during the 11th outbreak in the Equateur province in 2020 in the DRC, and

from the outbreak in 2021 in Guinea, West Africa [2, 4, 5]. With the magnitude of 2 recent outbreaks in Eastern DRC and West Africa, the number of patients who recovered from the disease increased and thus also the risk for re-emergence from humans. Therefore, it is important to estimate the proportion of possible missed EVD patients.

The 10th EBOV outbreak in the DRC occurred in a region experiencing civil war for more than a decade, which hindered the EVD response team to list and perform follow-up of all clinically suspected EVD patients during certain periods [10]. Therefore, we cannot exclude that national security challenges also delayed access to diagnostic and clinical care. In the present study, we determined the presence of anti-EBOV antibodies in patients who attended the clinics with clinical symptoms of suspected EVD but tested negative after 2 consecutive RT-PCR tests during this outbreak.

We found that 2.3% (11 of 488) of samples were positive for anti-IgG antibodies to at least 2 EBOV antigens, which suggests previous EBOV infection. After extrapolating data for 57 166 individuals who were discharged as negative during the outbreak, we estimate that 1314 additional patients may have recovered from EBOV infection. This number is likely underestimated because asymptomatic household contacts of EVD cases were not tested for EBOV antibodies, and this was also not done for the additional cases that we uncovered in this report. During the West African outbreak, viral infection was reported in 2.6% (10 of 388) of household contacts without clinical symptoms related to EVD in Sierra Leone [6, 11]. Among highly exposed contacts, even 7.5% (20 of 267) with positive serology for at least 2 antigens were reported in Sierra Leone [12]. Another study in Guinea reported that 4% (57 of 1390) of asymptomatic and paucisymptomatic contacts of EBOV patients had antibodies [7]. It is interesting to note that a study conducted on 24 close contacts of symptomatic patients in Gabon showed that 11 (45.8%) patients developed both IgM and IgG response to EBOV antigens, indicating viral infection confirmed by detection of genomic ribonucleic acid

in peripheral blood mononuclear cells [13], but there are no follow-up data for these patients.

An additional factor of underestimation is that we did not test for IgM antibodies, although IgG antibodies start to develop rapidly after symptom onset and are generally present after viral clearance from blood [14]. In analogous observations with the same antibody assay in EVD survivors in Guinea showing simultaneous presence of IgG antibodies to multiple antigens after EBOV infection in 99% of patients who recovered [15], we used the same criteria in our study. It is possible that this could be too strict shortly after symptom onset especially in paucisymptomatic patients for whom almost no data on antibody kinetics are available [14]. Finally, we cannot exclude the possibility that those IgG-positive patients are from an unnoticed outbreak in the past. Therefore, we compared our samples with samples from 2011 in the same area, and 1 (0.4%) sample had antibodies to GP and VP40 simultaneously. Moreover, the proportion of reactivity to a single antigen was also significantly lower. The higher proportion of individuals with antibodies to GP antigens could be partially due to the vaccination strategy with the rVSV-ZEBOV-GP during the outbreak, which induces antibodies to GP proteins only, but information on vaccination was not recorded for the samples analyzed in our study and thus could not be further analyzed.

CONCLUSIONS

Despite the broad and unspecific case definition used for active surveillance to not miss any EBOV infection during the outbreak, more than 1000 additional EBOV cases could have gone undetected, that is, approximately one third of the official number of RT-PCR-confirmed cases. More importantly, the index case in the EVD outbreak in February 2021 in Guinea was not registered as an EVD survivor, whereas the genomic analysis of the virus showed that the virus strain was related to the previous outbreak [5]. This is the first study on anti-EBOV antibodies among clinically suspected EVD patients who were discharged as negative after RT-PCR tests, and it suggests that the proportion of paucisymptomatic EBOV infection can be higher than suspected from previous outbreaks. These findings emphasize the importance of extending epidemic surveillance during and after outbreaks to related EVD household contacts and to suspected cases discharged as negative with an additional accurate and specific serological assay, which ideally can also discriminate vaccine-related antibodies given the large use of vaccines in EVD outbreak response.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of

all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the laboratory teams for Ebola virus disease response in North-Kivu and Ituri provinces: Fyfy Mbelu-Matulu, Noella Mulopo-Mukanya, Elie Kavoyo-Mbayahi, Elias Mumbere-Kalemekwa, Sheila Kavira-Muhesi, Marie-Anne Kavira-Muhindo, Joseph Nyombe, Reagen Kakule-Baswagha, Victoire Katembo-Tasi, Callixte Kakule-Sadiki, Adrienne Amuri-Aziza, Lebon Matendo-Kakina, Servet Kimbonza-Sumba, Jean-Bosco Ntalu-Mayanga, and Angele Mpalaba-Nyembue. We also thank the Institut National de Recherche Biomédicale Biobanking team: Emmanuel Lokilo-Lofiko, Bellicia Disashi-Kamwanya, and André Citenga-Kabanga.

Financial support. The study was funded by the European and Developing Countries Clinical Trials Partnership Grant RIA2018EF-2087 Prise en charge améliorée de maladie à virus Ebola en situation d'urgence en République Démocratique du Congo (PEAU-EBOV) INSERM/ANRS-MIE C19-59.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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