MAJOR ARTICLE

Detection of Ebola Virus in Oral Fluid Specimens during Outbreaks of Ebola Virus Hemorrhagic Fever in the Republic of Congo

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Background. Patients who have refused to provide blood samples has meant that there have been significant delays in confirming outbreaks of Ebola virus hemorrhagic fever (EVHF). During the 2 EVHF outbreaks in the Republic of Congo in 2003, we assessed the use of oral fluid specimens versus serum samples for laboratory confirmation of cases of EVHF.

Methods. Serum and oral fluid specimens were obtained from 24 patients with suspected Ebola and 10 healthy control subjects. Specimens were analyzed for immunoglobulin G antibodies by enzyme-linked immunosorbent assay (ELISA) and for Ebola virus by antigen detection ELISA and reverse-transcriptase polymerase chain reaction (RT-PCR). Oral fluid specimens were collected with a commercially available collection device.

Results. We failed to detect antibodies against Ebola in the oral fluid specimens obtained from patients whose serum samples were seropositive. All patients with positive serum RT-PCR results also had positive results for their oral fluid specimens.

Conclusions. This study demonstrates the usefulness of oral fluid samples for the investigation of Ebola outbreaks, but further development in antibodies and antigen detection in oral fluid specimens is needed before these samples are used for filovirus surveillance activities in Africa.

Early detection and confirmation of viral hemorrhagic fever outbreaks have frequently been hampered by the difficulties in obtaining the appropriate clinical samples from subjects with suspected Ebola and transporting the samples [1–3]. In particular, the requirement for blood or serum samples has often led to significant delays in the notification and diagnosis of filovirus hemorrhagic fever cases, because of the lack of available sampling equipment, the weaknesses in the infrastructure for communication and transportation, the absence of properly trained personnel, and the cultural objections to the taking of blood or to other pre- or postmortem invasive sampling. These conditions are prevalent in many areas in Africa where outbreaks of Ebola virus hemorrhagic fever (EVHF) and Marburg virus hemorrhagic fever have occurred in the past decade.

During an outbreak of EVHF in the Republic of Congo in 2003 [4], strong cultural objections to collection of blood or postmortem skin biopsy specimens delayed the definitive diagnosis of the outbreak. On 28 January 2003, the Ministry of Health of the Republic of Congo and the World Health Organization were alerted of suspected cases of EVHF in the towns of Mbomo and Kéllé, which are situated in the Cuvette Ouest region. They immediately sent a team to investigate the rumors and to collect initial clinical samples

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for diagnosis. For several days, the patients and their families did not want to provide any tissue specimens for analysis, and only 5 serum samples were obtained by 12 February. Ebola virus infection was subsequently confirmed by ELISA and RT-PCR testing at the Centre International de Recherche Médicale de Franceville (Franceville, Gabon).

The reluctance to allow invasive methods of obtaining clinical samples led to the query as to whether noninvasive obtainment of oral fluid samples would be acceptable to patients who were unwilling to permit obtainment of a blood sample or to families who refused to allow collection of postmortem skin biopsies. Oral fluid sampling offers many advantages over serum sampling: the procedure is noninvasive and safe for patients and collectors, and some cultural barriers to obtainment of other samples are absent [5]. However, to our knowledge, no study thus far has assessed the use of oral fluid samples to determine the antigen and antibody status in patients with Ebola.

During 2 EVHF outbreaks, we assessed the use of oral fluid specimens, compared with serum specimens, for the confirmation of EVHF. The main objective of the study was to determine the sensitivity and specificity of tests that used oral fluid samples, compared with use of serum samples. This article reports virus detection and antibody detection findings for oral fluid specimens collected from 24 patients with suspected Ebola and from 10 healthy control subjects during 2 outbreaks of Ebola.

MATERIALS AND METHODS

Patients. The study was conducted during 2 outbreaks of EVHF in the Republic of Congo. In early February 2003, the World Health Organization and other international partners dispatched an international team to aid the government of the Republic of Congo in containing the outbreak in the town of Kéllé, which is in the Cuvette Ouest region. The team included medical anthropologists with expertise in social mobilization who succeeded in restoring community compliance with outbreak-control measures. An isolation ward was set up in the Kéllé hospital and began receiving patients. From 18 February to 6 March 2003, 11 patients who fit the case definition for EVHF were admitted in the Kéllé hospital isolation ward. For diagnostic purposes, 9 of these patients (age, 28–75 years) provided verbal consent to provide both an oral fluid sample and a venous blood sample. Serum and oral fluid samples were collected from the 9 patients. Later, 10 healthy volunteer subjects (age, 30–45 years) provided control samples.

In late November 2003, a national and international medical team was deployed in Mbomo to help local authorities control an EVHF outbreak. From 24 November to 8 December, 15 patients who fit the case definition were investigated by the epidemiological team. All patients but 2 gave verbal consent to provide both an oral fluid sample and a venous blood sample. The 2 remaining patients provided an oral fluid specimen only.

Sample collection and processing. Oral fluid is a complex fluid consisting of several components, including saliva and crevicular fluid. Crevicular fluid is a component of oral fluid that contains plasma-derived IgG and IgM, which transude from the capillary beds in the gingival crevice between the teeth and gums [6, 7].

Oral fluid specimens were collected using a commercially available device (OraSure; Epitope). The collection fiber pad was placed between the lower cheek and gum and was gently rubbed back and forth, until it became moist (figure 1). The pad was kept in place for 2–5 min and was then placed in a transport vial with preservative. The same day, venous blood was also collected into Vacutainer tubes (Becton Dickinson). The blood and oral fluid specimens from the same patient were labelled with the same unique identification number.

The samples were stored in a cool-box, packaged with a triple packaging system, and transported to the Centre International de Recherche Médicale de Franceville, where they arrived in an average of 5 days after collection. All of the specimens were subjected to the same handling and storage conditions.

When the oral fluid samples arrived in the laboratory, the pads were dry, and there was no preservative left in the vial; consequently, it was not possible to obtain any fluid from the specimen after centrifugation. This was most probably because of evaporation of the stopper liquid. The pads were subsequently put in suspension with 400 μL of PBS for 1 h. Then, the supernatant was divided into 2 parts: one part was used for RNA extraction using the QIamp Viral RNA kit (Qiagen),

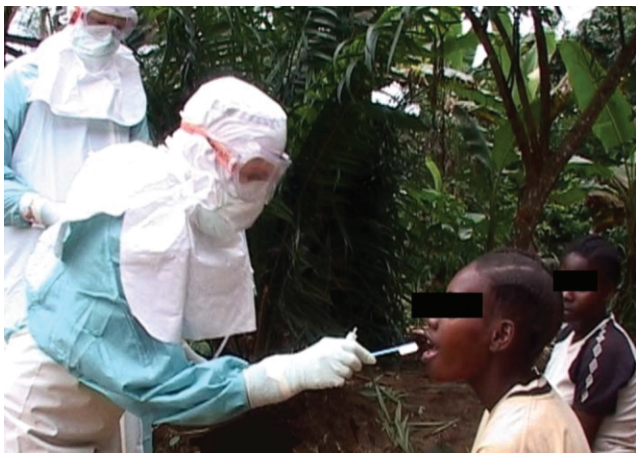

Figure 1. Collection of oral fluid specimens from subjects with suspected Ebola in Mbomo, the Republic of Congo, November 2003. (Photo was taken by Alain Epelboin of Centre National de la Recherche Scientifique/World Health Organization).

Table 1. Results of analysis of oral fluid and serum specimens collected from patients with laboratory-confirmed Ebola in Kéllé (February–March) and Mbomo (November–December), Republic of Congo, 2003.

Location, patient	Date of disease onset	Date that samples were obtained	Status (date of outcome)[a]	Gingival bleeding	Oral fluid specimen result			Serum sample result		
					IgG	Ag	RT-PCR	IgG	Ag	RT-PCR
Kéllé										
K1	6 Feb	28 Feb	Survivor	No	−	−	−	1:6400	−	−
K2	6 Feb	28 Feb	Survivor	No	−	−	−	1:1600	−	−
K3	20 Feb	28 Feb	Death (2 Mar)	Yes	−	−	+	−	>1:256	+
K4	23 Feb	28 Feb	Death (2 Mar)	Yes	−	>1:256	+	−	>1:256	+
K5	23 Feb	28 Feb	Death (3 Mar)	No	−	>1:64	+	−	>1:256	+
K6	21 Feb	28 Feb	Death (8 Mar)	Yes	−	−	+	−	>1:256	+
K7	24 Feb	1 Mar	Death (7 Mar)	No	−	>1:4	+	−	>1:256	+
K8	24 Feb	1 Mar	Death (5 Mar)	No	−	−	+	−	>1:256	+
K9	24 Feb	6 Mar	Death (8 Mar)	No	−	ND	+	−	ND	+
K10	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K11	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K12	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K13	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K14	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K15	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K16	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K17	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K18	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
K19	…	20 Mar	Healthy subject	No	−	−	−	−	−	−
Mbomo										
M1	15 Nov	24 Nov	Survivor	No	−	>1:64	+	−	>1:256	+
M2	20 Nov	24 Nov	Death (29 Nov)	No	−	>1:64	+	ND	ND	ND
M3	22 Nov	28 Nov	Survivor	No	−	>1:64	+	ND	ND	ND
M4	22 Nov	2 Dec	Survivor	No	−	−	−	1:1600	−	−
M5	16 Nov	2 Dec	Survivor	No	−	−	−	1:400	−	−
M6	15 Nov	3 Dec	Survivor	No	−	−	−	1:400	−	−
M7	15 Nov	5 Dec	Survivor	No	−	−	−	1:1600	−	−
M8	15 Nov	3 Dec	Noncase	…	−	−	−	−	−	−
M9	16 Nov	2 Dec	Noncase	…	−	−	−	−	−	−
M10	18 Nov	28 Nov	Noncase	…	−	−	−	−	−	−
M11	20 Nov	24 Nov	Noncase	…	−	−	−	−	−	−
M12	20 Nov	4 Dec	Noncase	…	−	−	−	−	−	−
M13	23 Nov	3 Dec	Noncase	…	−	−	−	−	−	−
M14	23 Nov	8 Dec	Noncase	…	−	−	−	−	−	−
M15	29 Nov	5 Dec	Noncase	…	−	−	−	−	−	−

NOTE. IgG antibodies against Ebola virus and Ebola virus antigens (Ag) were detected by ELISA. ND, not done; +, positive; −, negative.

[a] Noncases were initially classified as suspected cases.

and the other part was incubated in water at 60°C for 1 h to inactivate the virus and was then used for IgG detection and antigen detection. The tests were performed with this fluid, with a 1:10 dilution used for IgG detection and a 1:4 used dilution for antigen detection. From the serum samples, we used a dilution series of 1:100–1:6400 for the ELISA antibody test and a dilution series of 1:4–1:256 for the antigen detection test.

Laboratory tests. Both the oral fluid sample and the serum sample were analyzed for IgG antibodies using the ELISA assay and for Ebola virus using the antigen detection ELISA [8]. The specimens were examined for Ebola RNA by RT-PCR using primers derived from the L gene, as described elsewhere [9]. During the outbreak in Mbomo, we also detected Ebola RNA in the samples using the SmartCycler Technology (Cepheid) with L and NP gene-specific primers [10].

Table 2. Detection of Ebola IgG antibodies in oral fluid specimens versus serum samples during 2 outbreaks of Ebola virus hemorrhagic fever in the Republic of Congo, 2003.

Oral fluid sample results	Serum sample results		
	Positive	Negative	Total
Positive	0	0	0
Negative	6	26	32
Total	6	26	32

NOTE. Data are no. of patients. The sensitivity of the IgG antibody test for oral fluid samples was 0%, and the specificity was 100%; the positive predictive value could not be calculated, and the negative predictive value was 81%.

RESULTS

The results of the tests are presented in table 1. During the February outbreak, all 9 suspected cases were confirmed to be Ebola, whereas only 7 of the 15 suspected cases were confirmed to be Ebola during the November outbreak. The 8 remaining cases were classified as "noncases."

Serologic testing for IgG antibodies. The results of tests for the detection of IgG antibodies in oral fluid specimens versus serum specimens are presented in table 2. Six patients (K1, K2, M4, M5, M6, and M7), whose samples were obtained 10–22 days after the onset of symptoms, tested positive (at the 1:400–1:6400 dilution) for serum IgG antibodies against Ebola virus but negative for IgG antibodies in oral fluid specimens. We failed to detect IgG antibodies against Ebola in the oral fluid specimens collected from these patients. The sensitivity of the IgG antibody test for oral fluid samples was 0%, and the specificity was 100%; the positive predictive value could not be calculated, and the negative predictive value was 81%.

Antigen detection. Findings regarding antigen detection in oral fluid and serum samples are presented in table 3. Seven patients (K3, K4, K5, K6, K7, K8, and M1) had detectable antigen in their serum samples, but we were able to detect antigen in the oral fluid samples for only 4 of them. In addition, all 7 patients positive serum test results had high titers (superior to 1:256) of Ebola virus antigen, whereas the 4 oral fluid specimens had low titers. For oral fluid samples, the sensitivity of the antigen detection test was 57%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 89%.

RT-PCR results. RT-PCR results for oral fluid and serum samples are compared in table 4. For 8 seriously ill subjects (K3, K4, K5, K6, K7, K8, K9, and M1) whose samples were obtained 5–10 days after the onset of symptoms, the serum samples all tested positive for Ebola virus RNA. Their oral fluid samples were all positive for Ebola virus by RT-PCR. For oral fluid specimens, the sensitivity of RT-PCR was 100%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 100%.

Two patients who consented to provide an oral fluid sample refused to provide a serum sample. Both patients tested positive for Ebola virus by RT-PCR and antigen detection but negative for Ebola-specific oral fluid IgG antibodies.

The PCR products derived from oral fluid and serum specimens were identical. PCR products were analyzed to characterize the incriminated strain. Analysis of the sequenced PCR products (420 bp) showed only few synonymous substitutions, compared with Mayinga-76 (DR Congo, 1976), Kikwit-95 (DR Congo, 1995), and Gabon-94 sequences. This strain, therefore, belongs to the species *Zaire ebolavirus*.

DISCUSSION

Oral fluid specimens have been used for the surveillance of vaccine-preventable diseases, such as measles, mumps, and rubella [5, 11], and for individual diagnosis of HIV infection [12] by detecting antibodies against the target pathogens. During this study, we failed to detect Ebola IgG antibodies in 6 oral fluid samples, but numerous studies have testified to the suitability of oral fluid samples as a substitute for serum samples for the detection of specific antibodies to a variety of viral infections. The specimen pads arrived in poor condition (with small quantity of preservative fluid) at Centre International de Recherche Médicale de Franceville, thus not allowing an optimal extraction of the oral fluid. But, in itself, this cannot explain the total absence of antibody detected during this study. Very low levels of antibodies against Ebola in the crevicular fluid may be an explanation of the reported study. If future attempts/studies should try to extract the oral fluid in the field on the basis of the manufacturer's instructions, IgG antibody detection needs further development before being used for individual diagnoses or for seroepidemiological studies.

We were able to detect Ebola virus antigen only in 4 oral fluid specimens obtained from 7 patients whose serum samples were positive for Ebola virus antigen. The absence of antigen detection in the oral mucosal transudate may partly be explained by the bad storage and transportation conditions of the specimens. Studies of other RNA viruses have shown that the manipulation of and the storage conditions for oral fluid

Table 3. Detection of Ebola antigen in oral fluid specimens versus serum specimens during 2 outbreaks of Ebola virus hemorrhagic fever in the Republic of Congo, 2003.

Oral fluid sample results	Serum sample results		
	Positive	Negative	Total
Positive	4	0	4
Negative	3	24	27
Total	7	24	31

NOTE. Data are no. of patients. The sensitivity for detection of Ebola antigen for oral fluid samples was 57%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 89%.

Table 4. Detection of Ebola RNA by RT-PCR of oral fluid specimens versus serum specimens during 2 outbreaks of Ebola virus hemorrhagic fever in the Republic of Congo, 2003.

	Serum sample result		
Oral fluid result	Positive	Negative	Total
Positive	8	0	8
Negative	0	24	24
Total	8	24	32

NOTE. Data are no. of patients. The sensitivity for detection of Ebola antigen for oral fluid samples was 100%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 100%.

specimens can influence the stability of Ebola virus antigen [13]. It is also possible that the transudation of the antigen into the oral fluid is very low. Gingival bleeding was reported by 3 of the 7 antigen-positive patients; surprisingly, the results of ELISA antigen detection test of the oral fluid specimens were negative for 2 of them. Patients with blood in their oral fluid should have been the most likely to have positive antigen test results, which was not the case. Therefore, we cannot rule out a technical problem with the antigen detection ELISA of oral fluid samples. The assessment of the sensitivity (57%) and the specificity (100%) of standard antigen detection testing of oral fluid specimens for Ebola virus indicates that these samples cannot be used for confirmation until additional studies have been performed.

RT-PCR assays for the detection of Ebola virus yielded consistent results with both oral fluid specimens and serum specimens. RT-PCR of oral fluid samples confirmed all of the results found with blood specimens. The assessment of sensitivity (100%) and specificity (100%) of standard RT-PCR diagnostic tools that use oral fluid samples for the detection of Ebola virus indicates that these samples can be used for confirmation when blood/serum collection is not possible.

Given the current diagnostic algorithm for laboratory-confirmed cases of Ebola that involve confirmation by antigen detection, RT-PCR, or detection of IgM antibodies in the blood, detection of Ebola virus by RT-PCR in oral fluid specimens seems to be sufficiently reliable as a diagnostic tool in outbreak investigations. Individual confirmation of isolated cases of EVHF will be difficult to interpret with use of oral fluids, compared with serum samples, but it will not be as critical for confirmation of EVHF outbreaks in which several samples may be collected and tested. Oral fluid samples obtained from patients with isolated cases that are to be used for diagnosis should ideally be collected during the acute phase of illness, within the first 10 days of the disease (table 1).

In Africa, and especially in Central Africa, where the last epidemics of Ebola and Marburg infection occurred, blood is one of the essential constituents of the "vital force." According to a number of autochtonous representations, it is an object of greed, "devoured" by the man-eater sorcerers. Collection of blood specimens, regardless of the volume, is very poorly accepted and is considered to decrease a person's "vital force." Several times during the Ebola outbreaks in the Republic of Congo in 2002 and 2003, physicians, nurses, and Red Cross volunteers were verbally attacked and compared with *mondenge* (i.e., bloodsuckers). In such regions, replacement of blood samples with oral fluid specimens appears to be a very positive step. This sampling technique will cause less anxiety than would the sampling of blood and will help to convince the community of the exclusive viral etiology of the disease.

But in a system of "magic" thoughts that involve denials of the viral responsibility for illness, it is indispensable to analyze the oral fluid sampling from an anthropological point of view. For local perceptions, the saliva is a vector of the "good" word as well as the "bad" word. The partisan behavior of some social groups (e.g., religious, social, economic, and political groups) during previous Ebola epidemics [14] taught us that one may want to persuade the populations that oral fluid specimens are "a grip of the word" of the community. We should stay cautious with regard to any sampling methods, and we should further investigate the acceptability of oral fluid collection devices in rural African populations.

Oral fluid sampling is safer than blood sampling, eliminates the risk of disease transmission associated with needlestick injuries [15], and is easier and more economical. Oral sampling offers a considerable compliance advantage to the patient and seems to be a more culturally accepted procedure. This study demonstrated that oral fluid specimens can be used as an alternative sample for diagnosis of Ebola when patients refuse to provide blood samples. However, use of oral fluid samples does not permit for a wide range of biological investigations; thus, it is still necessary to obtain blood samples when physicians need to investigate the biological status of the patient (e.g., by biochemical and hematological tests) or the immune response of the patient, to optimize their treatment schemes.

Greater acceptability of diagnostic testing and the absence of the need for cold chain and rapid transfer of samples improve the prospects for the design of sensitive early-warning surveillance systems for viral hemorrhagic fevers in areas with less advanced technologically. Use of oral fluid samples has a wide application for case management and outbreak control, but laboratory techniques need further development.

Given that antibody- and antigen-detection techniques for oral fluid specimens will improve in the future—using, for example, an ELISA amplification system (e.g., Biotine-Streptavidine [Dako])—these findings may change the approach to surveillance and response to viral hemorrhagic fever, and they may improve our ability to detect suspected cases early and to design more-sensitive early-warning surveillance for these dis-

eases, to permit more-rapid responses to disease and implementation of disease-control measures.

The use of oral fluid samples could make the earlier detection of outbreaks much easier. We need to perform additional studies to clearly define the limitations and appropriate applications of this technology with regard to the diagnosis of EVHF and other viral hemorrhagic fevers.

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