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Rapid Bedside Inactivation of Ebola Virus for Safe Nucleic Acid Tests

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Rapid bedside inactivation of Ebola virus would be a solution for the safety of medical and technical staff, risk containment, sample transport, and high-throughput or rapid diagnostic testing during an outbreak. We show that the commercially available Magna Pure lysis/binding buffer used for nucleic acid extraction inactivates Ebola virus. A rapid bedside inactivation method for nucleic acid tests is obtained by simply adding Magna Pure lysis/binding buffer directly into vacuum blood collection EDTA tubes using a thin needle and syringe prior to sampling. The ready-to-use inactivation vacuum tubes are stable for more than 4 months, and Ebola virus RNA is preserved in the Magna Pure lysis/binding buffer for at least 5 weeks independent of the storage temperature. We also show that Ebola virus RNA can be manually extracted from Magna Pure lysis/binding buffer-inactivated samples using the QIAamp viral RNA minikit. We present an easy and convenient method for bedside inactivation using available blood collection vacuum tubes and reagents. We propose to use this simple method for fast, safe, and easy bedside inactivation of Ebola virus for safe transport and routine nucleic acid detection.

The most recent Ebola virus disease (EVD) outbreak began in West Africa in December 2013. As of March 2016, the number of confirmed, probable, and suspected EVD cases reported worldwide was 28,646. Guinea, Liberia, and Sierra Leone were the most affected countries with 3,804, 10,666 and 14,122 cases, respectively (1).

Ebola virus (EBOV) is classified as a risk group 4 pathogen that requires handling under biosafety level 4 (BSL-4) conditions. To meet this requirement, several mobile BSL-4 facilities were used during the recent West Africa outbreak (1, 2). However, extensive safety precautions and training of medical and technical staff are needed to ensure personal safety (2–6). As of August 2015, 880 health care workers had been diagnosed with EVD, and 512 had died from the disease (7). Rapid bedside inactivation of EBOV would be a solution for the safety of medical and technical staff, risk containment, and easier transport of samples without requiring expensive category A shipping. Additionally, this process removes the need for sample handling under high-containment environments and facilitates high-throughput and rapid testing under nonbiosafety laboratory conditions and, thus, a rapid diagnosis of the disease.

There is a need for a simple, efficient, and safe bedside inactivation method for EBOV. Presently, laboratory EBOV inactivation is accomplished by gamma irradiation (8), UV radiation (9), nanoemulsion (10), and photoinducible alkylating agents (11), but these methods are not applicable in outbreak situations or as bedside inactivation methods. Other EBOV inactivation methods, such as acetic acid (12), heat (12), AVL buffer (13), TRIzol (13) or the combination of heat and Triton X-100 (14), are more applicable in outbreak situations and are currently used in field laboratories. Unfortunately, all of these methods require hands-on handling and manipulation of the sample before EBOV is inactivated.

EVD diagnosis is primarily based on RT-PCR technology (3), and the current methods for nucleic acid (NA) extraction include several handling steps with infectious material before EBOV is inactivated. The steps in the QIAamp viral RNA extraction

method from Qiagen that was used during the recent outbreak (15) are (i) sample collection; (ii) triple packing systems (5) for the shipment and transport of samples to high-containment laboratories (16); (iii) pipetting of aliquots; (iv) addition of AVL buffer; (v) incubation; (vi) addition of ethanol; and (vii) disinfection using 0.5% hypochlorite for 5 min before release from the glove box (17). These handling steps can be eliminated if efficient bedside inactivation of EBOV is obtained.

The commercially available Magna Pure lysis/binding (MPLB) buffer from Roche was shown to inactivate two species of Orthopox virus (Vaccinia virus and Cowpox virus) (18). In this report, we show that MPLB buffer also inactivates EBOV. When MPLB buffer is directly injected into ordinary vacuum blood collection EDTA tubes using a needle and syringe, a residual vacuum is maintained, thereby allowing the direct drain of blood from the patient into the inactivation tube. Thus, a rapid bedside inactivation method is obtained, and handling of the sample under high-containment conditions is eliminated. MPLB buffer is produced for automated Magna Pure NA extraction using a Magna Pure robot, but we show that the EBOV RNA can also be extracted from MPLB buffer-inactivated blood samples using a slightly modified version of the manual QIAamp viral RNA minikit. Furthermore, the EBOV RNA is stable in the MPLB buffer blood collection tubes for more than 5 weeks independent of the temperature.

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MATERIALS AND METHODS

EBOV inactivation BSL-4 experiments. All of the EBOV inactivation experiments were conducted at the BSL-4 laboratory in Stockholm, Sweden. EBOV from the recent outbreak (Ebola virus/H.sapiens-tc/SLE/2014/Makona) was isolated and cultured, and infectivity was quantified by fluorescence forming units, as previously described (19) (unpublished data).

Two million cultured infectious EBOV particles corresponding to a quantification cycle (C_q) value of 15 (data not shown) (100 μ l) were mixed 1:1 with MPLB buffer from the Magna Pure LC DNA isolation kit I (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland) or mock-treated with Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Inc., Waltham, MA, United States). The mixtures were incubated for 20 min at room temperature and then filtered through the Amicon 100K Ultra centrifugal filters according to the manufacturer's instructions to wash out the toxic compounds in the MPLB buffer. The filters were resuspended and diluted 1:500 in DMEM containing 2% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, United States) and 0.01% penicillin-streptomycin (PEST) (Thermo Fisher Scientific, Inc., Waltham, MA, United States) and added to (3×10^6 cells) Vero E6 cells. After 2 h of infection at 37°C, the cells were washed twice and incubated with DMEM containing 2% FBS and 0.01% PEST. At 24, 48, 144, and 336 h postinfection, the cells were harvested by addition of TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, United States) according to the manufacturer's instructions. At 24 and 48 h postinfection, cells were also fixed with ice-cold methanol-acetone at -20°C for 30 min and processed for immunofluorescence using in-house antibodies targeting the EBOV viral matrix protein (VP40) and glycoprotein (GP). Three independent experiments were performed. In addition, five cultured infectious EBOV particles were mixed 1:1 with MPLB buffer or mock treated with DMEM, filtered, and used to infect Vero E6 cells, as described above. Three independent experiments were performed.

The supernatant from day 6 (144 h) postinfection was transferred to a new monolayer of (3×10^6 cells) Vero E6 cells, and fresh DMEM containing 2% FBS and 0.01% PEST was added 1:1. The cells were kept for additional 7 days before the supernatant was transferred to a new monolayer of fresh cells. This procedure was repeated for three passages. After the last passage, the cells were harvested by the addition of TRIzol according to the manufacturer's instructions. Three independent experiments were performed.

Inactivation of EBOV in whole blood was performed by spiking healthy donor blood (whole blood) (100 μ l) with 2×10^6 infectious EBOV particles (100 μ l). Spiked blood was mixed 1:1 with MPLB buffer or DMEM. The mixtures were incubated for 20 min at room temperature, and 20 μ l was diluted 1:1,000 in DMEM containing 2% FBS and 0.01% PEST in order to dilute the toxic compounds in the MPLB buffer. The solution was added to confluent Vero E6 cells. After 2 h of infection at 37°C, the cells were washed twice and incubated with DMEM containing 2% FBS and 0.01% PEST. At day 7 postinfection, the cells were harvested by the addition of TRIzol. Three independent experiments were performed.

For analysis of EBOV inactivation, the EBOV RNA expression level was evaluated. NA extraction was performed by adding chloroform to the TRIzol-lysed cells (ratio, 1:5) followed by incubation for 3 min at room temperature and centrifugation at $12,000 \times g$ for 15 min at 4°C. RNA was extracted from the aqueous phase using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The expression of EBOV RNA was evaluated in duplicates with an in-house modified version of an assay published by Gibb et al. (20). The TaqMan fast virus 1-step master mix and the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, United States) were used. Human beta-actin mRNA was evaluated using a commercial endogenous control gene expression assay (Thermo Fisher Scientific, Inc., Waltham, MA, United States). The EBOV RNA levels were normalized to

beta-actin levels and presented as the relative expression compared to normalized data obtained from mock-treated cells.

Preparation of blood collection vacuum tubes for rapid bedside inactivation. MPLB buffer was added to ordinary blood collection vacutainer K2 EDTA tubes (4 ml) (Becton Dickinson, Franklin Lakes, NJ, United States) using a 25G \times 1 needle (Becton Dickinson, Franklin Lakes, NJ, United States) and a 3-ml Luer Lok syringe (Becton Dickinson, Franklin Lakes, NJ, United States). The ratio of MPLB buffer to EDTA-blood was 1:1. Repeated experiments showed that the addition of 1.6 ml of MPLB buffer to a 4-ml vacutainer EDTA tube resulted in an automatic collection of 1.6 ml of blood due to the remaining vacuum in these tubes. After intravenous blood collection using a butterfly needle with small-bore extension tubing, the tube contents were mixed by flipping the tube 5 to 10 times by hand. The tubes were disinfected on the outside using 1% Virkon (Wilmington, DE, United States) or 70% ethanol and were ready to be spiked with the EBOV or used directly for NA extraction.

EBOV RNA purification and stability experiments. Healthy donor blood was collected at the Statens Serum Institut (SSI) (Copenhagen, Denmark) using the blood collection vacuum tubes containing the MPLB buffer and spiked with a solution of the gamma-irradiated and freeze-dried EBOV standard preparation for diagnostic purposes (ENIVD) prepared from the recent outbreak in Guéckédou/Guinea. The freeze-dried EBOV standard preparation was resolved in 100 μ l of nuclease-free water and diluted 1:10, resulting in a stock solution containing 2×10^6 copies/ml (genome copies). Aliquots of 50 μ l were prepared in tubes with gaskets and stored at -80°C . Tenfold dilutions were prepared from the EBOV standard stock solution in nuclease-free water. Then, 50 μ l of each dilution was spiked into 2 ml of MPLB buffer-inactivated blood. Total NA was extracted from 1 ml of MPLB buffer-inactivated blood using the Magna Pure 96 DNA and viral NA large-volume kit and a Magna Pure 96 robot (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland). The manufacturer's instructions were followed except that the first step in the protocol (addition of MPLB buffer) was omitted. The samples were eluted in 50 μ l of elution buffer. A slightly modified version of the QIAamp viral RNA minikit protocol was also used. Briefly, the first four steps in the QIAamp viral RNA minikit spin protocol were omitted, and the RNA extraction protocol was followed from step 5 by adding 560 μ l of ethanol (96 to 99%) to 700 μ l of the MPLB buffer-inactivated spiked sample. Here, the spin protocol was followed according to the manufacturer's instructions. The samples were eluted in 50 μ l of AVE elution buffer.

The expression of EBOV RNA was evaluated in duplicates with an in-house modified version of an assay published by Weidmann et al. (21). The SensiFast probe No-Rox one-step kit (Meridian Bioscience, Inc., Cincinnati, OH, United States) and the Mx3005P thermal cycler (Stratagene, CA, United States) were used according to the manufacturers' instructions. The commercial RealStar filovirus screen RT-PCR master mix (Altona Diagnostics, Hamburg, Germany) (22) was also used according to the manufacturer's instructions. Purified EBOV RNA was included in every RT-PCR as a positive control to evaluate the integrity of the assay. Nuclease-free water was used in all experiments as a negative control. In the RealStar filovirus screen RT-PCR assay, the internal control was added to the reaction mixture to assess RT-PCR inhibition.

EBOV-positive clinical blood samples were obtained from a Norwegian patient diagnosed with EVD in Sierra Leone and repatriated to Norway for treatment at the clinical BSL-4 isolation unit at Oslo University Hospital, Ullevål, Norway. Blood was collected using either the MPLB buffer-containing blood collection vacuum EDTA tubes or normal EDTA tubes without MPLB buffer. Total NA was isolated using Magna Pure LC (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland) with an elution volume of 100 μ l. For comparison, EBOV RNA was also extracted with the QIAamp viral RNA minikit using the automated QIAcube (Qiagen, Hilden, Germany). The expression of EBOV RNA was evaluated with an in-house modified version of an assay published by Huang et al. (23). In this assay, the sequences for the forward primer and probe were slightly modified to account for mismatches in the recent EBOV outbreak strain

(Enp-F: 5'-GCAGAGCAAGGACTGATACA-3' and Enp-P: FAM-5'-CAA CAGCTTGGCAATCAGTAGGACA-3'-BHQ1). Then, 2 μ l of RNA was analyzed in a 25- μ l reaction using 500-nM primers and a 200-nM probe. The RT-PCR was performed with the one-step RT-PCR kit (Qiagen, Hilden, Germany) using the RotorGene cycler system (Qiagen, Hilden, Germany). The viral load in the clinical samples was estimated from a standard curve of the inactivated EBOV standard preparation for diagnostic purposes (i.e., ENIVD).

RESULTS

Inactivation of EBOV using MPLB buffer. We evaluated whether MPLB buffer inactivated EBOV. Under BSL-4 conditions, 2×10^6 infectious EBOV particles were either treated with MPLB buffer or mock treated with DMEM, filtered, and used to infect Vero E6 cells. At 24, 48, 144, and 336 h postinfection, EBOV and beta-actin RNA expressions were analyzed by RT-PCR. Viral EBOV RNA levels were normalized to beta-actin RNA levels and presented as the relative expression compared to levels obtained with mock-treated virus at 24 and 48 h postinfection (Fig. 1A). The presence of EBOV particles was also analyzed by staining the cells 24 and 48 h postinfection with specific antibodies targeting the EBOV VP40 and GP (Fig. 1B).

At 24 and 48 h postinfection, the viral EBOV RNA level in the cells infected with MPLB buffer-treated EBOV was 4-log units lower than in the cells infected with mock-treated EBOV (Fig. 1A). The relative expression of EBOV RNA in the MPLB buffer-treated EBOV-infected cells decreased to undetectable level ($C_q > 40$) at later postinfection time points. In contrast, the relative EBOV RNA expression in the mock-treated EBOV-infected cells increased by more than 5-log units postinfection (Fig. 1A). EBOV-specific staining of the cells at 24 h (data not shown) and 48 h (Fig. 1B) postinfection did not show any presence of EBOV in the MPLB buffer-treated EBOV-infected cells, in contrast to the mock-treated EBOV-infected cells, where EBOV could easily be detected (Fig. 1B).

To investigate if the infectivity changed over a longer period of time, the supernatant from day 6 (144 h) postinfection was passaged onto fresh Vero E6 cells 3 consecutive times with 7-day intervals. To demonstrate the sensitivity of the assay, an experiment was included in which only 5 infectious EBOV particles were mock treated with DMEM, filtered, used to infect Vero E6 cells, and passaged 3 times as described above. The cell monolayers from all of the passages were harvested and analyzed for EBOV and beta-actin RNA expression by RT-PCR. The viral EBOV RNA levels were normalized to beta-actin RNA levels and presented as the relative expression compared to levels obtained with mock-treated virus 24 h postinfection (Fig. 1C). The relative expression of the EBOV RNA in the MPLB buffer-treated EBOV-infected cells did not increase over time (3 passages) (Fig. 1C). We detected as low as 5 virus particles using filtration and passaging, which demonstrates the sensitivity of this assay to detect live virus. These results clearly demonstrate that the MPLB buffer inactivates EBOV and that MPLB buffer-treated EBOV does not replicate.

Furthermore, the inactivation efficiency in blood was evaluated by spiking whole blood with 2×10^6 infectious EBOV particles. The spiked blood samples were either mock treated or treated with MPLB buffer and used to infect Vero E6 cells. The MPLB buffer is very toxic and, in the previous experiments, Ambicon X100 filters were used to wash away the toxic compounds. However, Ambicon X100 filters could not be used in this experiment due to clogging of the filter by cell debris from the blood sample.

Instead, the MPLB-inactivated blood sample was diluted 1:1,000 in order to reduce the toxic concentration. At day 7 postinfection, EBOV and beta-actin RNA expressions were analyzed by RT-PCR. The viral EBOV RNA levels were normalized to beta-actin RNA levels and compared to levels obtained with mock-treated virus (Fig. 1D).

At 7 days postinfection, the viral EBOV RNA level in the cells infected with MPLB buffer-treated EBOV-spiked blood was 7-log units lower than cells infected with mock-treated EBOV-spiked blood (Fig. 1D). These results demonstrate that MPLB buffer inactivates EBOV-spiked whole blood.

Preparation and test of stability of blood collection vacuum tubes for rapid bedside inactivation. Bedside inactivation tubes were prepared by injecting MPLB buffer directly into vacutainer EDTA tubes using a thin needle and syringe (Fig. 2A to C). The vacuum in the tube has to be preserved to maintain the ability for a direct drain of blood from the patient into the tube containing the MPLB buffer (Fig. 2D). After blood collection using a butterfly needle with small-bore extension tubing, the tube contents were mixed by flipping the tube 5 to 10 times by hand. The outside surface of the tubes was disinfected using 1% Virkon or 70% ethanol, and the samples were ready for NA extraction.

To evaluate the stability of the blood collection vacuum tubes containing the MPLB buffer, tubes were prepared and stored for 1 to 16 weeks under different temperatures (5°C, 25°C, and 37°C). After storage, intravenous blood was collected as described above and spiked with an inactivated EBOV standard preparation, resulting in a final concentration of 5×10^3 copies/ml. RNA was extracted using the Magna Pure 96 robot, and EBOV RNA was analyzed using the in-house modified EBOV RT-PCR assay (21) and the RealStar filovirus screen RT-PCR assay. The blood collection vacuum tubes containing the MPLB buffer maintained the residual vacuum for at least 16 weeks, and no adverse effect on EBOV RT-PCR detection was observed after sample collection (data not shown).

Analysis of RNA extraction methods. MPLB buffer is produced for automated Magna Pure nucleic acid extraction using the Magna Pure robot. Therefore, we investigated whether samples treated with the MPLB buffer could be extracted manually with the QIAamp viral RNA minikit. Intravenous blood was collected using the MPLB buffer-containing blood collection tubes. The MPLB buffer-treated samples were spiked with a 10-fold dilution series of the inactivated EBOV standard, resulting in a final EBOV sample concentration ranging between 50 and 50,000 copies/ml. RNA was extracted in a parallel workflow using the Magna Pure 96 robot, and a modified version of the QIAamp viral RNA minikit. EBOV RNA was analyzed using the modified in-house EBOV RT-PCR assay (21) and the RealStar filovirus screen RT-PCR assay (Fig. 3).

Decreased RNA levels was observed for blood samples purified with the QIAamp viral RNA minikit compared to Magna Pure purified samples (Fig. 3). If RNA extraction was not performed immediately or within 2 h after blood collection, then extraction using the QIAamp viral RNA minikit was very difficult due to clogging of the purification columns, resulting in a reduced RNA yield. These results show that MPLB buffer-treated samples can be purified manually using the QIAamp viral RNA minikit.

Differences in sensitivity were observed between the in-house EBOV RT-PCR assay and the RealStar filovirus screen RT-PCR assay (Fig. 3). The sensitivity of the RealStar filovirus screen RT-

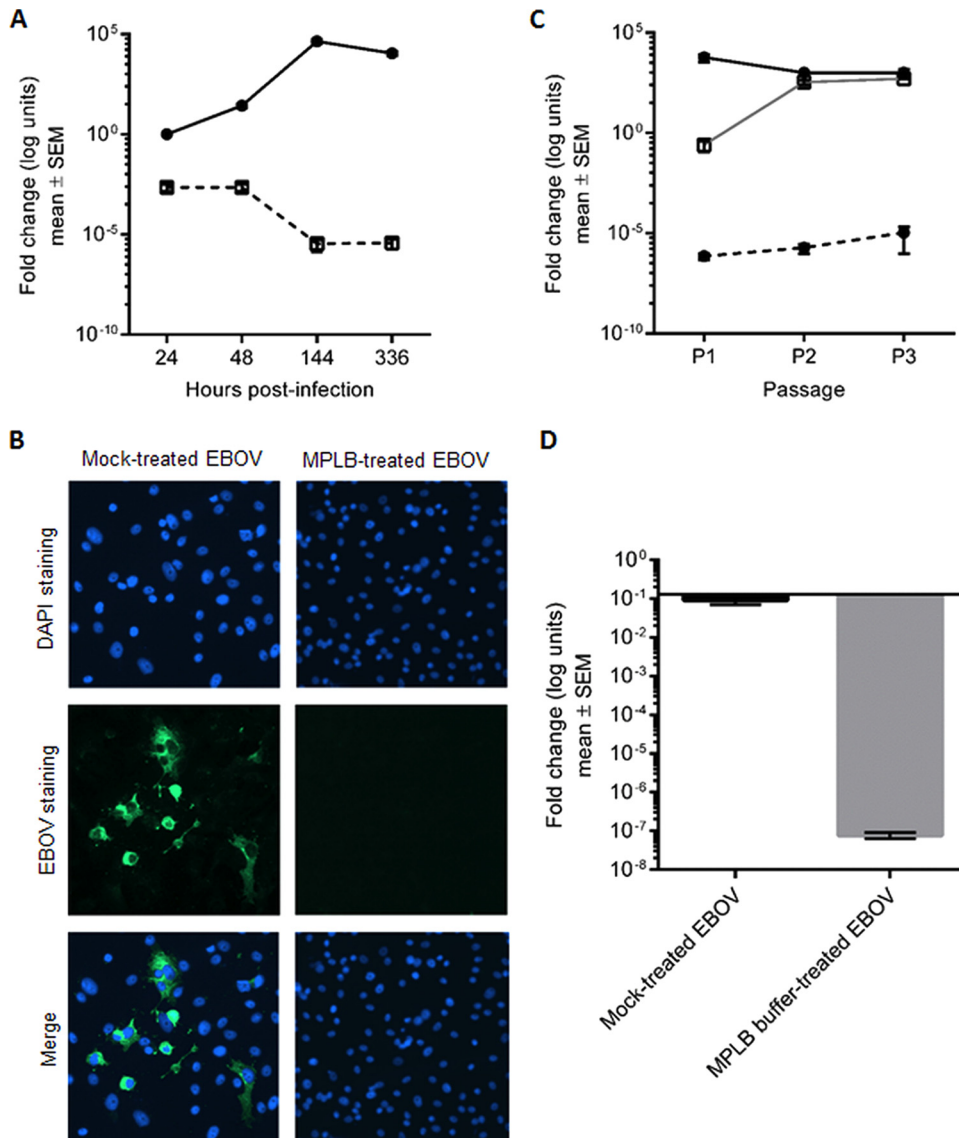


FIG 1 Effect of MPLB buffer on EBOV infectivity. Two million cultured infectious EBOV particles were treated with MPLB buffer or DMEM (mock-treated) (1:1) and used to infect Vero E6 cells. (A) The cells were harvested at 24, 48, 144, and 336 h postinfection and analyzed for EBOV viral RNA. The EBOV viral RNA levels were normalized to the beta-actin RNA levels (internal control) and presented as the relative expression to levels obtained with mock-treated virus at 24 h postinfection. Black solid lines represents relative EBOV RNA levels from mock-treated EBOV-infected cells; dotted lines represents relative EBOV RNA levels from MPLB buffer-treated EBOV-infected cells. The mean \pm the standard error of the mean (SEM) from the 3 independent experiments is shown. (B) The cells were fixed at 48 h postinfection and stained for EBOV using specific in-house antibodies targeting the EBOV VP40 and GP. (C) Supernatant from mock-treated EBOV and MPLB buffer-treated EBOV-infected cells were passed every 7 days in Vero E6 cells for 3 passages. The EBOV viral RNA levels were normalized to the beta-actin RNA levels (internal control) and presented as the relative expression to levels obtained with mock-treated virus at 24 h postinfection. Black solid lines represent relative EBOV RNA levels from mock-treated EBOV-infected cells (2×10^6 infectious EBOV particles); solid gray lines represent relative EBOV RNA levels from mock-treated EBOV-infected cells (5 infectious EBOV particles). The dotted lines represent relative EBOV RNA levels from MPLB buffer-treated EBOV-infected cells (2×10^6 EBOV particles). The mean \pm SEM from the 3 independent experiments is shown. (D) Whole blood was spiked with 2×10^6 infectious EBOV particles, treated with MPLB buffer or DMEM (mock-treated) (1:1), diluted 1:1,000, and used to infect Vero E6 cells. The cells were harvested at day 7 postinfection and analyzed for EBOV viral RNA. The EBOV viral RNA levels were normalized to the beta-actin RNA levels (internal control) and presented as the relative expression to levels obtained with mock-treated virus. The mean \pm SEM from the 3 independent experiments is shown.

PCR assay (Fig. 3) was lower than that of the in-house EBOV RT-PCR assay (Fig. 3A). The in-house EBOV RT-PCR assay detected EBOV RNA in blood containing 50 copies/ml of EBOV (Fig. 3), whereas the RealStar filovirus screen RT-PCR assay had a cutoff of 5,000 copies/ml of EBOV (Fig. 3). These results show that the commercial RealStar filovirus screen RT-PCR assay was less sensitive for blood samples than the in-house modified EBOV RT-PCR assay in our setting.

Stability of EBOV RNA after inactivation with MPLB buffer.

To analyze the stability of the EBOV RNA in the MPLB buffer, intravenous blood was collected using the MPLB buffer-containing blood collection tubes. The MPLB buffer-treated samples were spiked with the EBOV standard preparation, resulting in a final EBOV sample concentration of 5×10^3 copies/ml. The spiked samples were stored for 1 to 28 days at 5°C, 25°C, and 37°C. After storage, RNA was extracted using the Magna Pure 96 robot and

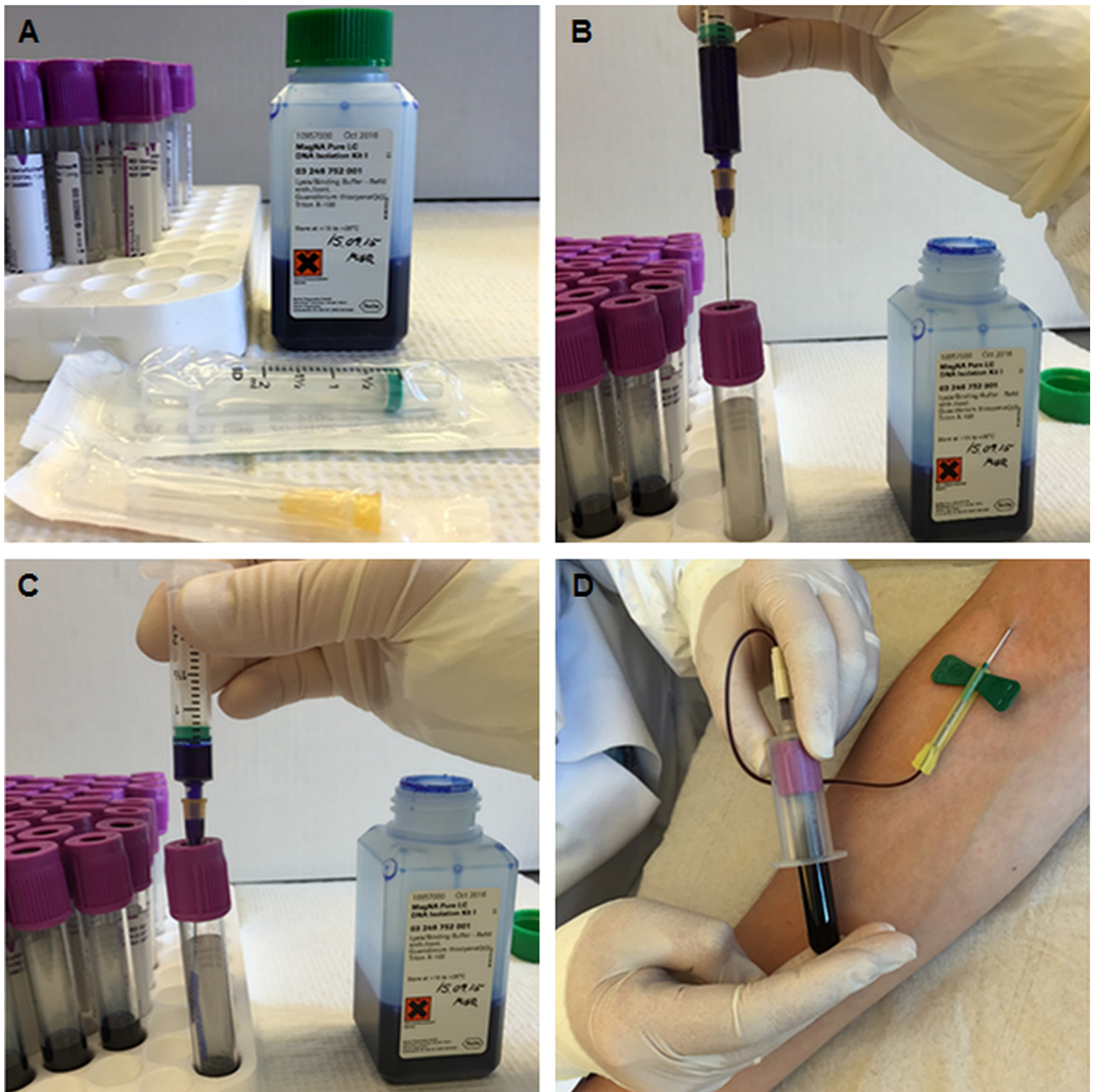


FIG 2 Preparation of blood collection vacuum tubes containing MPLB buffer. (A) Materials required for the preparation of blood collection tubes, including the BD vacutainer K2 EDTA tubes (4 ml), the Magna Pure LC DNA isolation kit lysis/binding buffer refill, the 3-ml BD Luer Lok syringe, and the BD 25G × 1 needle. (B, C) A total of 1.6 ml of MPLB buffer is added to the vacutainer EDTA tube by puncturing the cap of the tube. The cap of the tube should not be removed, because the vacuum in the tube has to be preserved for subsequent blood collection. (D) Using the remaining vacuum in the vacutainer tube, 1.6 ml of intravenous blood is automatically collected using a butterfly needle and directly inactivated by the presence of the MPLB buffer in the tube.

the QIAamp viral RNA minikit. EBOV RNA was analyzed using the in-house EBOV RT-PCR assay (21) (Fig. 4).

EBOV RNA was stable in the MPLB buffer-treated blood for the entire 28-day test period independent of the storage temperature (Fig. 4). Small variations in EBOV RNA levels were observed for samples stored at 25°C and 37°C when the RNA was extracted with the Magna Pure extraction method (Fig. 4). In contrast,

EBOV RNA extraction from these samples using the QIAamp RNA method was very difficult due to clogging of the purification columns; hence, no EBOV RNA could be detected after 24 to 48 h. Clogging of the purification columns was not observed when the blood samples were stored at 5°C (Fig. 4). These results show that RNA is preserved in MPLB buffer for at least 5 weeks independent of the storage temperature. However, the QIAamp RNA extrac-

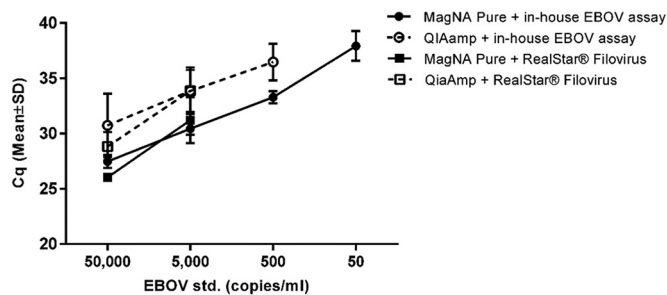


FIG 3 Analysis of RNA extraction methods. Intravenous blood was collected using MPLB buffer blood collection tubes and spiked with a 10-fold dilution series of the inactivated EBOV standard (std) preparation. RNA was extracted in a parallel workflow using the Magna Pure 96 robot and the QIAamp viral RNA minikit. EBOV RNA was analyzed using the in-house EBOV RT-PCR assay and the RealStar filovirus screen RT-PCR assay. The mean $C_q \pm$ standard deviation (SD) of replicates is shown ($n = 8$).

tion method is dependent on the storage temperature of the MPLB buffer-treated blood samples.

Stability of EBOV RNA using bedside inactivation of EBOV-positive patient samples. In October 2014, a Norwegian physician working in Sierra Leone who tested positive for EBOV was transported to Oslo University Hospital, Ullevål, Norway, for treatment. For daily monitoring of the EBOV viral load, the patient's blood was inactivated within the BSL-4 isolation unit using MPLB buffer prior to shipping to the Department of Microbiology at Oslo University Hospital and the Norwegian Institute of Public Health for analysis. To evaluate the effectiveness of bedside inactivation, blood inactivated in MPLB buffer-containing vacuum tubes (8 ml) was compared to MPLB buffer bench-treated blood. For this analysis, 4 ml of blood was mixed with 3 ml of MPLB buffer. Total RNA was extracted using the Magna Pure system and an automated QIAamp viral RNA extraction system using the QIAcube. EBOV was quantified using a standard curve of the EBOV standard preparation and the EBOV RT-PCR assay (23) (Table 1).

Similar EBOV RNA levels were observed between the bedside-inactivated and the bench-inactivated samples and between the two RNA extraction methods (Table 1). These results demonstrate the real-life use of MPLB buffer-containing blood collection tubes as an easy bedside inactivation procedure for EBOV-positive clinical samples.

DISCUSSION

Rapid bedside inactivation of EBOV is crucial for the safety of medical and technical staff, risk containment, and sample transport. Additionally, bedside inactivation removes the need for sample handling under high-containment conditions, which facilitates high-throughput testing and rapid diagnosis of the disease. In this study, we demonstrated inactivation of EBOV by the commercially available MPLB buffer used for NA extraction. EBOV inactivation was analyzed by examining viral replication in MPLB buffer-treated or mock-treated EBOV-infected cells at different postinfection time points (up to three passages). Using this assay, we could detect as low as five infectious EBOV particles. We detected EBOV replication in cells infected with mock-treated EBOV but not in cells infected with MPLB buffer-treated EBOV, which clearly demonstrated that the MPLB buffer efficiently inactivated EBOV. We used two million infectious virus particles of

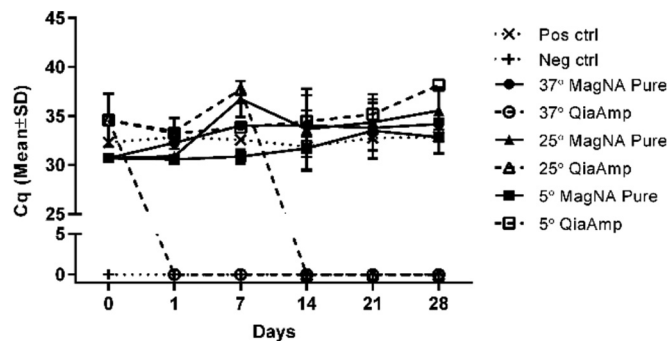


FIG 4 Stability of EBOV RNA after inactivation with MPLB buffer. Intravenous blood was collected using MPLB buffer blood collection tubes. The collected samples were spiked with the inactivated EBOV standard (final concentration, 5×10^3 copies/ml) and stored at 5°C, 25°C, and 37°C for 0 to 28 days. RNA was extracted in parallel using Magna Pure RNA extraction and QIAamp viral RNA extraction. EBOV RNA was analyzed using the in-house EBOV RT-PCR assay. Purified EBOV RNA was included in every RT-PCR as a positive control (Pos ctrl), and nuclease-free water was used in all experiments as a negative control (Neg ctrl). The mean $C_q \pm$ SD of duplicates is shown.

cultured virus, which corresponded to a C_q value of about 15 in our RT-PCR settings. This corresponded to a higher concentration level of the EBOV compared to the most reported cases from the 2014 outbreak (24, 25).

The use of commercially available NA extraction reagents for EBOV inactivation is well known (13), and these reagents are currently used (15). A recent report by Smither et al., in 2015, showed that the frequently used AVL buffer alone did not inactivate EBOV. EBOV-spiked mouse blood treated with AVL buffer needed to be combined with either heat or ethanol to ensure complete EBOV inactivation over the time of three passages (26). We showed that the MPLB buffer inactivated EBOV over the time of three passages without the need for additional treatment. However, these experiments were performed on EBOV cell cultures and not spiked blood samples. We showed a 7-log reduction in EBOV RNA levels for MPLB buffer-treated spiked blood samples compared to mock-treated blood samples at 7 days postinfection; however, the viability of EBOV in spiked blood samples was not tested over time. Nevertheless, the results after the 7 days postinfection compared to the cultured virus are comparable.

There is a potential that the MPLB buffer does not have the same effectiveness on inactivation of EBOV in blood as it has on EBOV in cell culture. MPLB buffer is very toxic to cells; therefore, a filtration step was used prior to the addition to Vero E6 cells. However, filtration could not be used for the EBOV-spiked blood samples due to clogging of the filters, so a 1:1,000 dilution of the MPLB buffer-treated or mock-treated blood samples was used instead. Using this high dilution of the spiked blood samples, we might have lost the sensitivity of the assay, and the lack of passage over time could introduce the potential for small residues of infectious EBOV to remain. However, since the results from 7 days postinfection are comparable between the spiked blood and the cultured virus, it may indicate that the MPLB treatment will inactivate Ebola virus even in the spiked blood samples. Nevertheless, one should have it in mind that the inactivation is dependent not only on the matrix of samples but also on the concentration of the virus, and this concentration may differ between individuals. However, these data demonstrate that using vacuum MPLB tubes

TABLE 1 EBOV viral load from an EBOV-positive patient

Sample name	Collection date	Inactivation method	Extraction method	Extraction volume (μ l)	C_q value	Viral load (copies/ml)
FHI0001	8 October 2014	Bedside inactivation	Magna Pure Qiacube	100 100	27.5 26.9	847,000 768,000
FHI0005	10 October 2014	Bench inactivation	Magna Pure	100	27.7	734,500

increases the biosafety aspects of the handling of samples significantly.

The MPLB buffer contains 20 to 25% Triton X-100 and 30 to 50% guanidinium thiocyanate (GITC) (27), whereas the AVL buffer contains 50 to 70% GITC (28). Triton X-100 has been shown to inactivate a wide range of enveloped viruses (29), and the combination of Triton X-100 and GITC in the MPLB buffer might indicate that the MPLB buffer is more efficient than the AVL buffer. The MPLB buffer has been shown to inactivate Vaccinia virus and Cowpox virus (18). This, combined with the results from the present study, indicates that other viruses will be inactivated by MPLB buffer as well, including risk group 4 pathogens such as Lassa virus, Marburg virus, and Crimean Congo hemorrhagic fever virus. However, this speculation is not within the scope of this study and requires further investigation.

MPLB buffer is produced for automated Magna Pure NA extraction using a Magna Pure robot. This NA extraction system is a high-throughput machine that can perform 96 NA extractions in less than 1 h. However, this machine is not always available in resource-poor settings, field laboratories or high-containment facilities. We showed that EBOV RNA could be extracted from MPLB buffer-inactivated blood samples using the manual QIAamp viral RNA minikit. However, RNA extraction using the QIAamp viral RNA minikit is dependent on the time after blood collection and the storage temperature. Often, there is a time span between sample collection and laboratory analysis. Blood samples stored at 25°C or 37°C could easily be extracted using the Magna Pure RNA extraction system; however, RNA extraction using the QIAamp viral RNA extraction kit was very difficult due to clogging of the purification columns. This phenomenon was not observed for blood samples stored at 5°C, where EBOV RNA could be detected in the samples 28 days after blood collection using either extraction method without difficulty or loss of material. The current WHO guidelines recommend a storage temperature between 0 and 5°C to preserve EBOV RNA in EDTA-blood samples (3), and rapid degradation of EBOV RNA has been observed when samples are stored at room temperature compared to 4°C (17). However, our results demonstrate that MPLB buffer preserves the EBOV RNA even when the samples are stored at 25 to 37°C. This observation simplifies sample collection, because it eliminates the need for cooling (e.g., during transport and shipment). However, the consequence of this stability is that MPLB buffer-treated blood samples need to be processed by the Magna Pure RNA extraction system.

We also show that the commercial WHO-approved RealStar filovirus screen RT-PCR assay (22, 30) was less sensitive than a modified in-house EBOV RT-PCR assay (21). This difference might reflect incompatibility between the RealStar filovirus screen RT-PCR assay and the RNA extraction methods used in our experiments. The RealStar filovirus screen RT-PCR assay was optimized for the QIAamp viral RNA minikit (22, 30), whereas our QIAamp viral RNA extraction method is a modified version of the

original protocol due to the use of MPLB buffer. This discrepancy could explain the lower sensitivity of the RealStar filovirus screen RT-PCR assay in our experiments. Very few reports have been published regarding the performance of commercial EBOV RT-PCR assays, but, in a recent communication, a similar low sensitivity of the RealStar filovirus screen RT-PCR assay (31) was reported. These results indicate the necessity for confirmatory EBOV RT-PCR analysis when analyzing clinical samples suspected to be positive for EBOV (3).

A bedside inactivation method is easily obtained by adding MPLB buffer directly into vacutainer EDTA-blood collection tubes. However, a few safety precautions are important when establishing procedures for the use of these MPLB buffer-containing tubes in a clinical setting. First, it is essential to prevent backflow of the MPLB buffer from the collection tube into the vein. Therefore, we recommend that sample collection always be performed with a butterfly needle with small-bore extension tubing, with the patient's arm in a downward position and the collection tube positioned lower than the butterfly needle. Second, if MPLB buffer is spilled, the contaminated surface must never be disinfected with chloramine or sodium hypochlorite (the active ingredients in bleach) as the first cleaning step, because this action may lead to the formation of toxic cyanide. Instead, first wipe up the spilled MPLB buffer, clean the surface with 70% ethanol, then clean with water, and then use chloramine or sodium hypochlorite according to the manufacturers' recommendations.

This bedside inactivation method was applied in Norway on a case patient who had been diagnosed with EVD in Sierra Leone and subsequently been repatriated for treatment. The Oslo University hospital has a small BSL-4 laboratory in connection with the clinical BSL-4 isolation unit, but this laboratory lacks the facilities for NA extraction and molecular diagnostics. The inactivation of EBOV using MPLB buffer-containing blood collection tubes eliminated the need for the shipment of samples to BSL-4 laboratories in other countries for analysis, thereby facilitating rapid and daily monitoring of the patient's EBOV viral load in the primary laboratory, NA-based differential diagnostics of other pathogens, and rapid sharing of inactivated material between laboratories. However, MPLB buffer will have a negative impact on biochemical and serology tests; therefore, these tests will still require BSL-4 handling.

In summary, we present an easy, efficient, and robust bedside inactivation method for NA tests by adding MPLB buffer directly into ordinary vacuum blood collection tubes. These inactivation tubes can be prepared and stored for at least 5 months independent of the storage temperature without losing the vacuum and function. We suggest using this bedside inactivation method for the collection of blood from patients suspected of EVD or other BSL-4 viruses, for the safe transport of samples, and for safe routine NA testing without the need for BSL-4 facilities. In the case of an outbreak situation, these tubes can easily be prepared and transported to different locations;

however, this has not been tested. It would be desirable to have the addition of MPLB buffer to vacuum EDTA-blood collection tubes commercialized, and manual preparation would therefore only be a solution in case of a sudden emergency. Commercialization would also remove the risk of sudden changes in buffer content or concentrations that might influence the inactivation efficiency of the MPLB buffer.

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