

1 **Rapid bedside inactivation of Ebola virus for safe nucleic acid tests**

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22 **ABSTRACT**

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

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44 INTRODUCTION

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

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

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

67 EVD diagnosis is primarily based on RT-PCR technology (3), and the current
68 methods for nucleic acid (NA) extraction include several handling steps with infectious material
69 before EBOV is inactivated. The steps in the QIAamp Viral RNA extraction method from QIAGEN
70 that was used during the recent outbreak (15) are: 1) sample collection; 2) triple packing systems (5)
71 for the shipment and transport of samples to high containment laboratories (16); 3) pipetting of
72 aliquots; 4) addition of AVL buffer; 5) incubation; 6) addition of ethanol; and 7) disinfection using
73 0.5% hypochlorite for 5 minutes before release from the glovebox (17). These handling steps can be
74 eliminated if efficient bedside inactivation of EBOV is obtained.

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

87

88 **METHODS**

89 **EBOV inactivation BSL-4 experiments**

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

94 Two million cultured infectious EBOV particles corresponding to a Cq-value of 15
95 (data not shown) (100 µl) were mixed 1:1 with MPLB buffer from the MagNA Pure LC DNA
96 isolation Kit I (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland) or mock-treated with
97 Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Inc. Waltham,
98 Massachusetts, United States). The mixtures were incubated for 20 minutes at room temperature
99 and then filtered through the Amicon 100K Ultra Centrifugal filters according to the manufacturer's
100 instructions, to wash out the toxic compounds in the MPLB buffer. The filters were resuspended
101 and diluted 1:500 in DMEM containing 2% fetal bovine serum (FBS) (Thermo Fisher Scientific,
102 Inc. Waltham, Massachusetts, United States) and 0.01% Penicillin-streptomycin (PEST)(Thermo
103 Fisher Scientific, Inc. Waltham, Massachusetts, United States) and added to (3×10^6 cells) VeroE6
104 cells. After 2 hours of infection at 37°C, the cells were washed twice and incubated with DMEM
105 containing 2% FBS and 0.01% PEST. At 24, 48, 144 and 336 hours post-infection the cells were
106 harvested by addition of TRIzol (Thermo Fisher Scientific, Inc. Waltham, Massachusetts, United
107 States) according to the manufacturer's instructions. At 24 and 48 hours post-infection cells were
108 also fixed with ice-cold methanol/acetone at -20°C for 30 minutes and processed for
109 immunofluorescence using in-house antibodies targeting the EBOV VP40 and GP. Three
110 independent experiments were performed. In addition, five cultured infectious EBOV particles were
111 mixed 1:1 with MPLB buffer or mock-treated with DMEM, filtrated and used to infect VeroE6 cells
112 as described above. Three independent experiments were performed.

113 The supernatant from day 6 (144 hours) post-infection was transferred to a new
114 monolayer of (3×10^6 cells) VeroE6 cells and fresh DMEM containing 2% FBS and 0.01% PEST
115 was added 1:1. The cells were kept for additional 7 days before the supernatant was transferred a
116 new monolayer of fresh cells. This procedure was repeated for three passages. After the last
117 passage, the cells were harvested by the addition of TRIzol according to the manufacturer's
118 instructions. Three independent experiments were performed.

119 Inactivation of EBOV in whole blood was performed by spiking healthy donor blood
120 (whole blood) (100 μ l) with 2×10^6 infectious EBOV particles (100 μ l). Spiked blood was mixed 1:1
121 with MPLB buffer or DMEM. The mixtures were incubated for 20 minutes at room temperature and
122 20 μ l was diluted 1:1000 in DMEM containing 2% FBS and 0.01% PEST, in order to dilute the
123 toxic compounds in the MPLB buffer. The solution was added to confluent VeroE6 cells. After 2
124 hours of infection at 37°C, the cells were washed twice and incubated with DMEM containing 2%
125 FBS and 0.01% PEST. At day 7 post-infection the cells were harvested by the addition of TRIzol.
126 Three independent experiments were performed.

127 For analysis of EBOV inactivation, the EBOV RNA expression level was evaluated.
128 NA-extraction was performed by adding chloroform to the TRIzol lysed cells (ratio 1:5), followed
129 by incubation for 3 minutes at room temperature and centrifugation at 12,000 x g for 15 minutes at
130 4°C. RNA was extracted from the aqueous phase using the QIAamp Viral RNA Mini Kit
131 (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The expression of
132 EBOV RNA was evaluated in duplicates with an in-house modified version of an assay published
133 by *Gibb et al.* (20). The TaqMan Fast Virus 1-Step MasterMix and the StepOnePlus Real-Time
134 PCR System (Thermo Fisher Scientific, Inc. Waltham, Massachusetts, United States) were used.
135 Human beta-actin mRNA was evaluated using a commercial endogenous control gene expression
136 assay (Thermo Fisher Scientific, Inc. Waltham, Massachusetts, United States). The EBOV RNA

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

139

140 **Preparation of blood collection vacuum tubes for rapid bedside inactivation**

141 MPLB buffer was added to ordinary blood collection Vacutainer K2 EDTA tubes (4 ml)(Becton
142 Dickinson, Franklin Lakes, New Jersey, United States) using a 25Gx1 needle (Becton Dickinson,
143 Franklin Lakes, New Jersey, United States) and a 3 ml LUER LOK syringe (Becton Dickinson,
144 Franklin Lakes, New Jersey, United States). The ratio of MPLB buffer to EDTA-blood was 1:1.
145 Repeated experiments showed that the addition of 1.6 ml of MPLB buffer to a 4 ml Vacutainer
146 EDTA-tube resulted in an automatic collection of 1.6 ml of blood due to the remaining vacuum in
147 these tubes. After intravenous blood collection using a butterfly needle with small-bore extension
148 tubing, the tube contents were mixed by flipping the tube 5-10 times by hand. The tubes were
149 disinfected on the outside using 1% Virkon (Wilmington, Delaware, United States) or 70% ethanol
150 and were ready to be spiked with the EBOV or used directly for NA extraction.

151

152 **EBOV RNA purification and stability experiments**

153 Healthy donor blood was collected at Statens Serum Institut (SSI) (Copenhagen, Denmark) using
154 the blood collection vacuum tubes containing the MPLB buffer, and spiked with a solution of the
155 gamma-irradiated and freeze-dried EBOV standard preparation for diagnostic purposes (ENIVD)
156 prepared from the recent outbreak in Guéckédou/Guinea. The freeze-dried EBOV standard
157 preparation was resolved in 100 µl of nuclease-free water and diluted 1:10, resulting in a stock
158 solution containing 2×10^6 copies/ml (genome copies). Aliquots of 50 µl were prepared in tubes with
159 gaskets and stored at -80°C. Ten-fold dilutions were prepared from the EBOV standard stock

160 solution in nuclease-free water. Fifty μ l of each dilution was spiked into 2 ml of MPLB buffer-
161 inactivated blood. Total NA was extracted from 1 ml of MPLB buffer-inactivated blood using the
162 MagNA Pure 96 DNA & Viral NA Large Volume kit and a MagNA Pure 96 Robot (Roche
163 Diagnostics A/S, Risch-Rotkreuz, Switzerland). The manufacturer's instructions were followed
164 except that the first step in the protocol (addition of MPLB buffer) was omitted. The samples were
165 eluted in 50 μ l of elution buffer. A slightly modified version of the QIAamp Viral RNA mini kit
166 protocol was also used. Briefly, the first four steps in the QIAamp Viral RNA mini kit spin protocol
167 were omitted and the RNA extraction protocol was followed from step 5 by adding 560 μ l of
168 ethanol (96-99%) to 700 μ l of the MPLB buffer-inactivated spiked sample. Hereafter, the spin
169 protocol was followed according to the manufacturer's instructions. The samples were eluted in 50
170 μ l of AVE elution buffer.

171 The expression of EBOV RNA was evaluated in duplicates with an in-house modified
172 version of an assay published by *Weidmann et al.* (21). The SensiFast probe NO-ROX One-Step kit
173 (Meridian Bioscience Inc., Cincinnati, Ohio, United States) and the Mx3005P thermal cycler
174 (Stratagene, California, United States) were used according to the manufacturer's instructions. The
175 commercial RealStar® Filovirus Screen RT-PCR MasterMix (Altona diagnostics, Hamburg,
176 Germany) (22) was also used according to the manufacturer's instructions. Purified EBOV RNA
177 was included in every RT-PCR reaction as a positive control to evaluate the integrity of the assay.
178 Nuclease-free water was used in all experiments as a negative control. In the RealStar® Filovirus
179 Screen RT-PCR assay the internal control was added to the reaction mixture to assess RT-PCR
180 inhibition.

181 EBOV positive clinical blood samples were obtained from a Norwegian patient
182 diagnosed with EVD in Sierra Leone and repatriated to Norway for treatment at the clinical BSL-4
183 isolation unit at Oslo University Hospital, Ullevål, Norway. Blood was collected using either the

184 MPLB buffer containing blood collection vacuum EDTA-tubes or normal EDTA-tubes without
185 MPLB-buffer. Total NA was isolated using MagNA Pure LC (Roche Diagnostics A/S, Risch-
186 Rotkreuz, Switzerland) with an elution volume of 100 μ l. For comparison, EBOV RNA was also
187 extracted with the QIAamp Viral RNA Mini kit using the automated QIAcube (QIAGEN, Hilden,
188 Germany). The expression of EBOV RNA was evaluated with an in-house modified version of an
189 assay published by Huang *et al.* (23). In this assay, the sequences for the forward primer and probe
190 were slightly modified to account for mismatches in the recent EBOV outbreak strain (Enp-F 5'-
191 GCAGAGCAAGGACTGATACA-3' and Enp-P FAM-5'-
192 CAACAGCTTGGAATCAGTAGGACA-3'-BHQ1). Two μ l of RNA was analysed in a 25 μ l
193 reaction using 500 nM primers and 200 nM probe. The RT-PCR was performed with the One-Step
194 RT-PCR Kit (QIAGEN, Hilden, Germany) using the RotorGene cyclor system (QIAGEN, Hilden,
195 Germany). The viral load in the clinical samples was estimated from a standard curve of the
196 inactivated EBOV standard preparation for diagnostic purposes (ENIVD).

197

198 RESULTS

199 Inactivation of EBOV using MPLB buffer

200 We evaluated whether MPLB buffer inactivated EBOV. Under BSL-4 conditions, 2×10^6 infectious
201 EBOV particles were either treated with MPLB buffer or mock-treated with DMEM, filtered and
202 used to infect VeroE6 cells. At 24, 48, 144 and 336 hours post-infection, EBOV and beta-actin
203 RNA expression was analysed by RT-PCR. Viral EBOV RNA levels were normalized to beta-actin
204 RNA levels and presented as relative expression compared to levels obtained with mock-treated
205 virus 24 and 48 hours post-infection (Figure 1A). The presence of EBOV particles were also

206 analysed by staining the cells 24 and 48 hours post-infection with specific antibodies targeting the
207 EBOV VP40 and GP (Figure 1B).

208 At 24 and 48 hours post-infection, the viral EBOV RNA level in the cells infected
209 with MPLB buffer-treated EBOV virus was 4-log units lower than in cells infected with mock-
210 treated EBOV (Figure 1A). The relative expression of EBOV RNA in the MPLB buffer-treated
211 EBOV-infected cells decreased to undetectable level (Cq over 40) at later post-infection time
212 points. In contrast, the relative EBOV RNA expression in the mock-treated EBOV-infected cells
213 increased by more than 5-log units post-infection (Figure 1A). EBOV specific staining of the cells
214 at 24 hours (data not shown) and 48 hours post-infection (Figure 1B) did not show any presence of
215 EBOV in the MPLB buffer-treated EBOV-infected cells in contrast to the mock-treated EBOV-
216 infected cells where EBOV could easily be detected (Figure 1B).

217 To investigate if the infectivity changed over a longer period of time the supernatant
218 from day 6 (144 hours) post-infection was passaged onto fresh VeroE6 cells three consecutive times
219 with 7 days intervals. To demonstrate the sensitivity of the assay an experiment was included in
220 which only five infectious EBOV particles were mock-treated with DMEM, filtered, used to infect
221 VeroE6 cells and passaged 3 times as described above. The cell monolayers from all the passages
222 were harvested and analysed for EBOV and beta-actin RNA expression by RT-PCR. The viral
223 EBOV RNA levels were normalized to beta-actin RNA levels and presented as relative expression
224 compared to levels obtained with mock-treated virus 24 hours post-infection (Figure 1C). The
225 relative expression of the EBOV RNA in the MPLB buffer-treated EBOV-infected cells did not
226 increase over time (three passages) (Figure 1C). We detected as low as five virus particles using
227 filtration and passaging, which demonstrates the sensitivity of this assay to detect live virus. These
228 results clearly demonstrate that the MPLB buffer inactivates EBOV and that MPLB buffer-treated
229 EBOV does not replicate.

230 Furthermore, the inactivation efficiency in blood was evaluated by spiking whole
231 blood with 2×10^6 infectious EBOV particles. The spiked blood were either mock-treated or treated
232 with MPLB buffer and used to infect VeroE6 cells. The MPLB buffer is very toxic and in the
233 previous experiments, Ambicon X100 filters were used to wash away, the toxic compounds.
234 However, Ambicon X100 filters could not be used in this experiment due to clogging of the filter by
235 cell debris from the blood sample. Instead, the MPLB inactivated blood sample was diluted 1:1000
236 in order to reduce the toxic concentration. At day 7 post-infection, EBOV and beta-actin RNA
237 expression was analysed by RT-PCR. The viral EBOV RNA levels were normalized to beta-actin
238 RNA levels and compared to levels obtained with mock-treated virus (Figure 1D).

239 At 7 days post-infection the viral EBOV RNA level in the cells infected with MPLB
240 buffer-treated EBOV spiked blood was 7-log units lower as compared with cells infected with
241 mock-treated EBOV spiked blood (Figure 1D). These results demonstrate that MPLB buffer
242 inactivates EBOV spiked whole blood.

243

244 **Preparation and test of stability of blood collection vacuum tubes for rapid bedside** 245 **inactivation**

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

253 To evaluate the stability of the blood collection vacuum tubes containing the MPLB
254 buffer, tubes were prepared and stored for 1-16 weeks under different temperatures (5°C, 25°C and
255 37°C). After storage intravenous blood was collected as described above and spiked with an
256 inactivated EBOV standard preparation, resulting in a final concentration of 5×10^3 copies/ml. RNA
257 was extracted using the MagNa Pure 96 Robot and EBOV RNA was analysed using the in-house
258 modified EBOV RT-PCR assay (21) and the RealStar® Filovirus Screen RT-PCR assay. The blood
259 collection vacuum tubes containing the MPLB buffer maintained the residual vacuum for at least 16
260 weeks and no adverse effect on EBOV RT-PCR detection was observed after sample collection
261 (data not shown).

262

263 **Analysis of RNA extraction methods**

264 MPLB buffer is produced for automated MagNA Pure nucleic acid extraction using the MagNA
265 Pure robot. Therefore, we investigated whether samples treated with the MPLB buffer could be
266 extracted manually with the QIAamp Viral RNA mini kit. Intravenous blood was collected using
267 the MPLB buffer-containing blood collection tubes. The MPLB buffer-treated samples were spiked
268 with a 10-fold dilution series of the inactivated EBOV standard, resulting in a final EBOV sample
269 concentration ranging between 50,000–50 copies/ml. RNA was extracted in a parallel workflow
270 using the MagNA Pure 96 robot and a modified version of the QIAamp Viral RNA mini kit. EBOV
271 RNA was analysed using the modified in-house EBOV RT-PCR assay (21) and the RealStar®
272 Filovirus Screen RT-PCR assay (Figure 3).

273 Decreased RNA levels was observed for blood samples purified with the QIAamp
274 Viral RNA mini kit compared to MagNA Pure purified samples (Figure 3). If RNA extraction was
275 not performed immediately or within 2 hours after blood collection, then extraction using the

276 QIAamp Viral RNA kit was very difficult due to clogging of the purification columns, resulting in a
277 reduced RNA yield. These results show that MPLB buffer-treated samples can be purified manually
278 using the QIAamp Viral RNA mini kit.

279 Differences in sensitivity were observed between the in-house EBOV RT-PCR assay
280 and the RealStar® Filovirus Screen RT-PCR assay (Figure 3). The sensitivity of the RealStar®
281 Filovirus Screen RT-PCR assay (Figure 3) was lower than the in-house EBOV RT-PCR assay
282 (Figure 3A). The in-house EBOV RT-PCR assay detected EBOV RNA in blood containing 50
283 copies/ml of EBOV (Figure 3), whereas the RealStar® Filovirus Screen RT-PCR assay had a cut-
284 off at 5000 copies/ml of EBOV (Figure 3). These results show that the commercial RealStar®
285 Filovirus Screen RT-PCR assay was less sensitive for blood samples than the in-house modified
286 EBOV RT-PCR assay in our setting.

287

288 **Stability of EBOV RNA after inactivation with MPLB buffer**

289 To analyse the stability of the EBOV RNA in the MPLB buffer, intravenous blood was collected
290 using the MPLB buffer-containing blood collection tubes. The MPLB buffer-treated samples were
291 spiked with the EBOV standard preparation, resulting in a final EBOV sample concentration of
292 5×10^3 copies/ml. The spiked samples were stored for 1-28 days at 5°C, 25°C and 37°C. After
293 storage, RNA was extracted using the MagNA Pure 96 robot and the QIAamp Viral RNA mini kit.
294 EBOV RNA was analysed using the in-house EBOV RT-PCR assay (21) (Figure 4).

295 EBOV RNA was stable in the MPLB buffer-treated blood for the entire 28-day test
296 period independent of the storage temperature (Figure 4). Small variations in EBOV RNA levels
297 was observed for samples stored at 25°C and 37°C when the RNA was extracted with the MagNA
298 Pure extraction method (Figure 4). In contrast, EBOV RNA extraction from these samples using the

299 QIAamp RNA method was very difficult due to clogging of the purification columns, and hence no
300 EBOV RNA could be detected after 24-48 hours. Clogging of the purification columns was not
301 observed when the blood samples were stored at 5°C (Figure 4). These results show that RNA is
302 preserved in MPLB buffer for at least 5 weeks independent of the storage temperature. However,
303 the QIAamp RNA extraction method is dependent on the storage temperature of the MPLB buffer-
304 treated blood samples.

305

306 **Stability of EBOV RNA using bedside inactivation of EBOV-positive patient samples**

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

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

321

322 DISCUSSION

323 Rapid bedside inactivation of EBOV is crucial for the safety of medical and technical
324 staff, risk containment and sample transport. Additionally, bedside inactivation removes the need
325 for sample handling under high containment conditions, which facilitates high-throughput testing
326 and rapid diagnosis of the disease. In this study, we demonstrated inactivation of EBOV by the
327 commercially available MPLB buffer used for NA extraction. EBOV inactivation was analysed by
328 examining viral replication in MPLB buffer-treated or mock-treated EBOV infected cells at
329 different post-infection time points (up to three passages). Using this assay, we could detect as low
330 as five infectious EBOV particles. We detected EBOV replication in cells infected with mock-
331 treated EBOV virus but not in cells infected with MPLB buffer-treated EBOV virus, which clearly
332 demonstrated that the MPLB buffer efficiently inactivated EBOV. We used two million infectious
333 virus particles of cultured virus, which corresponds to a Cq value of about 15 in our RT-PCR
334 settings. This corresponds to a higher concentration level of the EBOV compared to the most
335 reported cases from the 2014 outbreak (24, 25).

336 The use of commercially available NA extraction reagents for EBOV inactivation is
337 well known (13), and these reagents are currently used (15). A recent report by Smithers et al.,
338 2015 showed that the frequently used AVL buffer alone did not inactivate EBOV. EBOV spiked
339 marmoset blood treated with AVL buffer needed to be combined with either heating or ethanol to
340 ensure complete EBOV inactivation over the time of three passages (26). We showed that the
341 MPLB buffer inactivated EBOV over the time of three passages without the need for additional
342 treatment. However, these experiments were performed on EBOV cell cultures and not spiked
343 blood samples. We showed a 7-log reduction in EBOV RNA levels for MPLB buffer-treated spiked
344 blood samples compared to mock-treated blood samples at 7 days post infection; however, the

345 viability of EBOV in spiked blood samples was not tested over time. Nevertheless, the results after
346 the 7 days post-infection compared to the cultured virus are comparable.

347 There is a potential that the MPLB buffer does not have the same effectiveness on
348 inactivation of EBOV in blood as it has on EBOV in cell culture. MPLB buffer is very toxic to cells
349 and therefore a filtration step was used prior to addition to Vero6 cells. However, filtration could
350 not be used for the EBOV spiked blood samples due to clogging of the filters and a 1:1000 dilution
351 of the MPLB buffer treated or mock-treated blood samples were used instead. Using this high
352 dilution of the spiked blood samples, we might have lost the sensitivity of the assay and the lack of
353 passage over time could have the potential that small residues of infectious EBOV could remain.
354 However, since the results from 7 days post infection is comparable between the spiked blood and
355 the cultured virus, it may indicate that the MPLB treatment will inactivate Ebola virus even in the
356 spiked blood samples. Nevertheless, one should have it in mind that the inactivation is not only
357 dependent on the matrix of samples but also even on the concentration of the virus and this may
358 differ between individuals. However, this data demonstrate that using vacuum MPLB tubes increase
359 the biosafety aspects of the handling of samples significantly.

360 The MPLB buffer contains 20-25% Triton X-100 and 30-50% guanidinium
361 thiocyanate (GITC) (27), whereas the AVL buffer contains 50-70% GITC (28). Triton X-100 has
362 been shown to inactivate a wide range of enveloped viruses (29), and the combination of Triton X-
363 100 and GITC in the MPLB buffer might indicate that MPLB buffer is more efficient than the AVL
364 buffer. The MPLB buffer has been shown to inactivate Vaccinia virus and Cowpox virus (18). This,
365 combined with the results from the present study indicates that other viruses will be inactivated by
366 MPLB buffer as well, including risk group 4 pathogens such as Lassa virus, Marburg virus and
367 Crimean Congo haemorrhagic fever virus. However, this speculation is not within the scope of this
368 study and requires further investigation.

369 MPLB buffer is produced for automated MagNA Pure NA extraction using a MagNA
370 Pure robot. This NA extraction system is a high-throughput machine that can perform 96 NA
371 extractions in less than one hour. However, this machine is not always available in resource poor
372 settings, field laboratories or high containment facilities. We showed that EBOV RNA could be
373 extracted from MPLB buffer-inactivated blood samples using the manual QIAamp Viral RNA mini
374 kit. However, RNA extraction using the QIAamp Viral RNA mini kit is dependent on the time after
375 blood collection and the storage temperature. Often, there is a time span between sample collection
376 and laboratory analysis. Blood samples stored at 25°C or 37°C could easily be extracted using the
377 MagNA Pure RNA extraction system; however, RNA extraction using the QIAamp Viral RNA
378 extraction kit was very difficult due to clogging of the purification columns. This phenomenon was
379 not observed for blood samples stored at 5°C where EBOV RNA could be detected in the samples
380 28 days after blood collection using either extraction method without difficulty or loss of material.
381 The current WHO guidelines recommend a storage temperature between 0-5°C to preserve EBOV
382 RNA in EDTA blood samples (3), and rapid degradation of EBOV RNA has been observed when
383 samples are stored at room temperature compared to 4°C (17). However, our results demonstrate
384 that MPLB buffer preserves the EBOV RNA even when the samples are stored at 25-37°C. This
385 observation simplifies sample collection because it eliminates the need for cooling (*e.g.*, during
386 transport and shipment). However, the consequence of this stability is that MPLB buffer-treated
387 blood samples need to be processed by the MagNA Pure RNA extraction system.

388 We also show that the commercial WHO-approved RealStar® Filovirus Screen RT-
389 PCR assay (22, 30) was less sensitive than a modified in-house EBOV RT-PCR assay (21). This
390 difference might reflect incompatibility between the RealStar® Filovirus Screen RT-PCR assay and
391 the RNA extraction methods used in our experiments. The RealStar® Filovirus Screen RT-PCR
392 assay was optimized for the QIAamp Viral RNA mini kit (22, 30), whereas our QIAamp Viral RNA

393 extraction method is a modified version of the original protocol due to the use of MPLB buffer.
394 This discrepancy could explain the lower sensitivity of the RealStar® Filovirus Screen RT-PCR
395 assay in our experiments. Very few reports have been published regarding the performance of
396 commercial EBOV RT-PCR assays, but in a recent communication a similar low sensitivity of the
397 RealStar® Filovirus Screen RT-PCR assay (31) was reported. These results indicate the necessity
398 for confirmatory EBOV RT-PCR analysis when analysing clinical samples suspected to be positive
399 for EBOV (3).

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

411 This bedside inactivation method was applied in Norway on a case patient who had
412 been diagnosed with EVD in Sierra Leone and subsequently been repatriated for treatment. Oslo
413 University hospital have a small BSL-4 laboratory in connection to the clinical BSL-4 isolation
414 unit, but this laboratory lacks the facilities for NA extraction and molecular diagnostics. The
415 inactivation of EBOV using MPLB buffer containing blood collection tubes eliminated the need for
416 the shipment of samples to BSL-4 laboratories in other countries for analysis, thereby facilitating

417 rapid and daily monitoring of the patient's EBOV viral load in the primary laboratory, NA based
418 differential diagnostics of other pathogens and rapid sharing of inactivated material between
419 laboratories. However, MPLB buffer will have a negative impact on biochemical and serology tests
420 and therefore these tests will still require BSL-4 handling.

421 In summary, we present an easy, efficient and robust bedside inactivation method for
422 NA tests by adding MPLB buffer directly into ordinary vacuum blood collection tubes. These
423 inactivation tubes can be prepared and stored for at least 5 months independent of the storage
424 temperature without losing the vacuum and function. We suggest using this bedside inactivation
425 method for the collection of blood from patients suspected of EVD or other BSL-4 viruses, for the
426 safe transport of samples and safe routine NA testing without the need for BSL-4 facilities. In the
427 case of an outbreak situation these tubes can easily be prepared and transported to different
428 locations; however this has not been tested. It would be desirable to have the addition of MPLB
429 buffer to vacuum EDTA-blood collection tubes commercialized and manual preparation would
430 therefore only be a solution in case of a sudden emergency. Commercialization will also remove the
431 risk of sudden changes in buffer content or concentrations that might influence the inactivation
432 efficiency of the MPLB buffer.

433

434

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446

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549 **FIGURE LEGENDS**550 **Figure 1: Effect of MPLB buffer on EBOV infectivity.**

551 Two million cultured infectious EBOV particles were treated with MPLB buffer or DMEM (mock-
552 treated) (1:1) and used to infect VeroE6 cells. **A)** The cells were harvested at 24, 48, 144 and 336
553 hours post-infection and analysed for EBOV viral RNA. The EBOV viral RNA levels were
554 normalized to the beta-actin RNA levels (internal control) and presented as relative expression to
555 levels obtained with mock-treated virus at 24 hours post-infection. Black solid lines represents
556 relative EBOV RNA levels from mock-treated EBOV infected cells and dotted lines represents
557 relative EBOV RNA levels from MPLB buffer-treated EBOV infected cells. The mean \pm the
558 standard error of the mean (SEM) from the three independent experiments is shown. **B)** The cells
559 were fixed at 48 hours post-infection and stained for EBOV using specific in-house antibodies
560 targeting the EBOV VP40 and GP. **C)** Supernatant from mock-treated EBOV and MPLB buffer-
561 treated EBOV infected cells were passed every 7 days in VeroE6 cells for three passages. The
562 EBOV viral RNA levels were normalized to the beta-actin RNA levels (internal control) and
563 presented as relative expression to levels obtained with mock-treated virus at 24 hours post-
564 infection. Black solid lines represent relative EBOV RNA levels from Mock-treated EBOV infected
565 cells (2×10^6 infectious EBOV particles) and solid grey lines represent relative EBOV RNA levels
566 from mock-treated EBOV infected cells (5 infectious EBOV particles). The dotted lines represent
567 relative EBOV RNA levels from MPLB buffer-treated EBOV infected cells (2×10^6 EBOV
568 particles). The mean \pm SEM from the three independent experiments is shown. **D)** Whole blood was
569 spiked with 2×10^6 infectious EBOV particles, treated with MPLB buffer or DMEM (mock-
570 treated)(1:1), diluted 1:1000 and used to infect VeroE6 cells. The cells were harvested at day 7 post-
571 infection and analysed for EBOV viral RNA. The EBOV viral RNA levels were normalized to the

572 beta-actin RNA levels (internal control) and presented as relative expression to levels obtained with
573 mock-treated virus. The mean \pm SEM from the three independent experiments is shown.

574

575 **Figure 2: Preparation of blood collection vacuum tubes containing MPLB buffer**

576 **A)** Materials required for the preparation of blood collection tubes, including the BD Vacutainer K2
577 EDTA tubes (4 ml), the MagNA Pure LC DNA isolation Kit I lysis/binding buffer refill, the 3 ml
578 BD LUER LOK syringe and the BD 25Gx1 needle. **B-C)** A total of 1.6 ml of MPLB buffer is added
579 to the Vacutainer EDTA tube by puncturing the cap of the tube. The cap of the tube should not be
580 removed because the vacuum in the tube has to be preserved for subsequent blood collection. **D)**
581 Using the remaining vacuum in the Vacutainer tube, 1.6 ml of intravenous blood will be
582 automatically collected using a butterfly needle and directly inactivated by the presence of the
583 MPLB buffer in the tube.

584

585 **Figure 3: Analysis of RNA extraction methods.**

586 Intravenous blood was collected using MPLB buffer blood collection tubes and spiked with a 10-
587 fold dilution series of the inactivated EBOV standard preparation. RNA was extracted in a parallel
588 workflow using the MagNA Pure 96 robot and the QIAamp Viral RNA mini kit. EBOV RNA was
589 analysed using the in-house EBOV RT-PCR assay and the RealStar® Filovirus Screen RT-PCR
590 assay. The mean Cq \pm SD of replicates is shown (n=8).

591

592 **Figure 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 of 5×10^3 copies/ml) and stored at 5°C, 25°C and 37°C for 0-28 days. RNA was extracted in parallel using MagNA Pure RNA extraction and QIAamp Viral RNA extraction. EBOV RNA was analysed using the in-house EBOV RT-PCR assay. Purified EBOV RNA was included in every RT-PCR reaction as a positive control (Pos ctrl) and nuclease-free water was used in all experiments as a negative control (Neg ctrl). The mean Cq \pm SD of duplicates is shown. The mean Cq \pm SD of duplicates is shown.

Table 1. EBOV viral load from an EBOV-positive patient

Sample name	Collection date	Inactivation method	Extraction method	Extraction volume (μ l)	Cq values	Viral load (copies/ml)
FH10001	08-10-2014	Bedside inactivation	MagNA Pure	100	27.5	847000
			QiaCube	100	26.9	768000
FH10005	10-10-2014	Bench inactivation	MagNA Pure	100	27.7	734500

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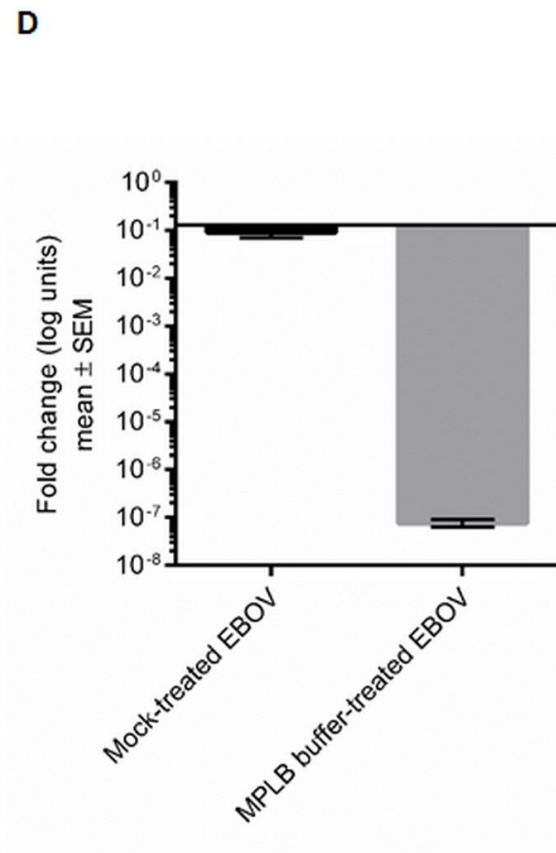
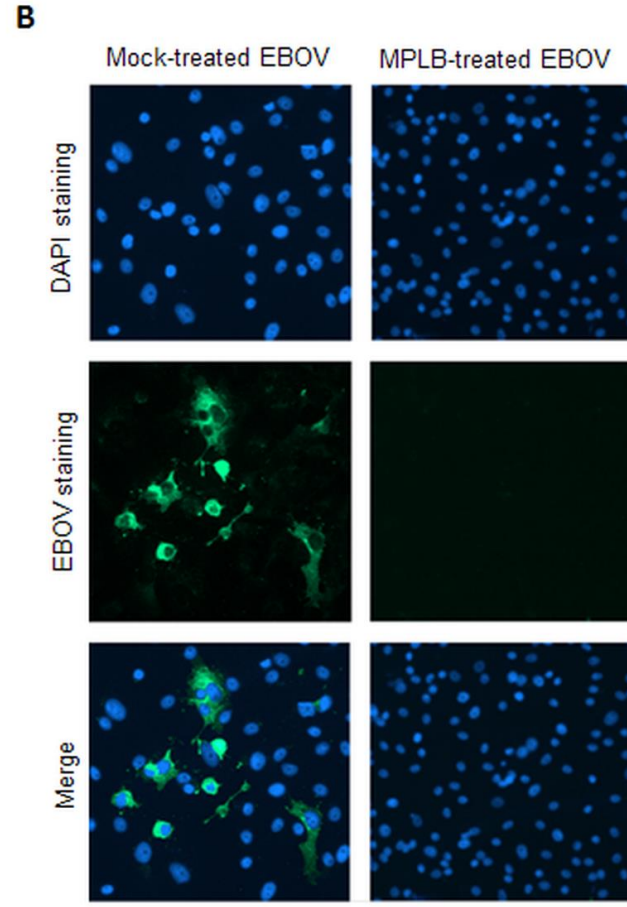
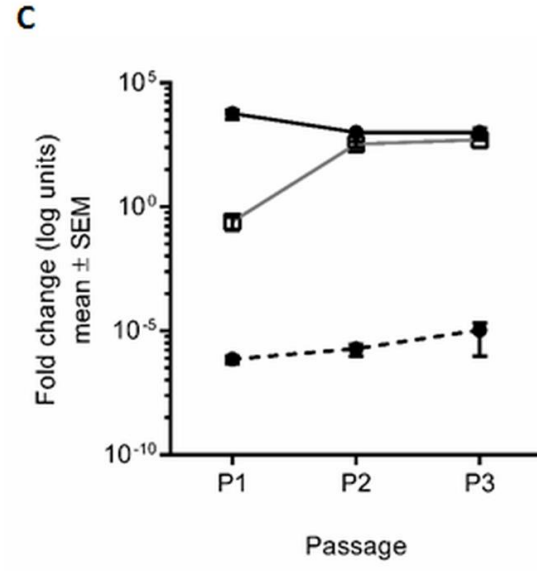
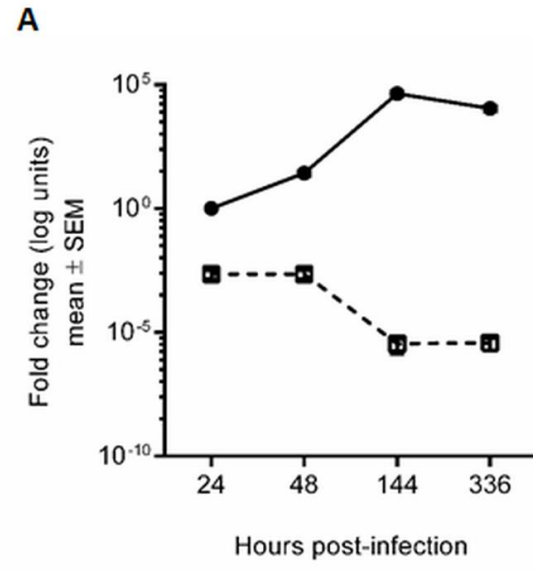


Figure 1: Effect of MPLB buffer on EBOV infectivity.

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Figure 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 I lysis/binding buffer refill, the 3 ml BD LUER LOK syringe and the BD 25Gx1 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 will be automatically collected using a butterfly needle and directly inactivated by the presence of the MPLB buffer in the tube.

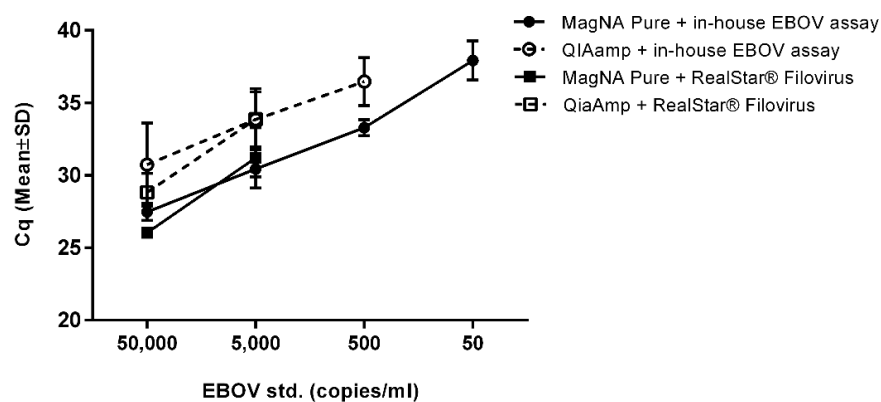


Figure 3: Analysis of RNA extraction methods.

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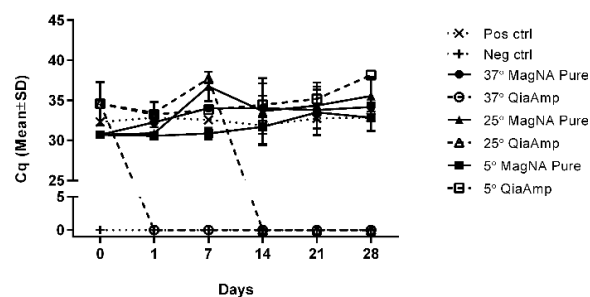


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