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The effect of a non-denaturing detergent and a guanidinium-based inactivation agent on the viability of Ebola virus in mock clinical serum samples

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ABSTRACT

The 2014 Ebola outbreak in West Africa required the rapid testing of clinical material for the presence of potentially high titre Ebola virus (EBOV). Safe, fast and effective methods for the inactivation of such clinical samples are required so that rapid diagnostic tests including downstream analysis by RT-qPCR or nucleotide sequencing can be carried out. One of the most commonly used guanidinium − based denaturing agents, AVL (Qiagen) has been shown to fully inactivate EBOV once ethanol is added, however this is not compatible with the use of automated nucleic acid extraction systems. Additional inactivation agents need to be identified that can be used in automated systems. A candidate inactivation agent is Triton X-100, a non-denaturing detergent that is frequently used in clinical nucleic acid extraction procedures and has previously been used for inactivation of EBOV. In this study the effect of 0.1% and 1.0% Triton X-100 (final concentration 0.08% and 0.8% respectively) alone and in combination with AVL on the viability of EBOV (10^6 $TCID_{50}$/ml) spiked into commercially available pooled negative human serum was tested. The presence of viable EBOV in the treated samples was assessed by carrying out three serial passages of the samples in Vero E6 cells (37 °C, 5% CO_2, 1 week for each passage). At the end of each passage the cells were observed for evidence of cytopathic effect and samples were taken for rRT-PCR analysis for the presence of EBOV RNA. Before cell culture cytotoxic components of AVL and Triton X-100 were removed from the samples using size exclusion spin column technology or a hydrophobic adsorbent resin.

The results of this study showed that EBOV spiked into human serum was not fully inactivated when treated with either 0.1% (v/v) Triton X-100 for 10 mins or 1.0% (v/v) Triton X-100 for 20 mins (final concentrations 0.08% and 0.8% Triton X-100 respectively). AVL alone also did not consistently provide complete inactivation. Samples treated with both AVL and 0.1% Triton X-100 for 10 or 20 mins were shown to be completely inactivated. This treatment is compatible with downstream analysis by RT-qPCR and next generation sequencing.

1. Introduction

The Ebola outbreak in West Africa required the testing of unprecedentedly high numbers of high titre clinical samples for the presence of Zaire ebolavirus (EBOV, family Filoviridae) (Carroll et al., 2015; Shiwani et al., 2017). After inactivation of the clinical samples within high containment facilities (Containment Level4/CL3 or a flexible film isolator in field laboratories) downstream sample processing was performed at a lower level of containment (CL2) (Logue et al., 2017; Bailey et al., 2016). For processing the samples at CL2 there is a requirement to demonstrate that procedures used to inactivate the virus potentially present within the clinical sample are effective. In addition, inactivated material from high consequence pathogens such as EBOV is a valuable resource for the development of new diagnostic assays or therapeutic reagents. Recent failures in inactivation have caused significant concern, particularly where ACDP 3 and 4 pathogens are involved ('CDC Report on the Potential Exposure to Anthrax' 2014; Weiss et al., 2015). For these reasons proof of effective inactivation of virus is an increasingly important component of study design.

The effectiveness of inactivation is dependent on factors including the nature and concentration of the pathogen, the sample matrix and the concentration and contact time of the inactivation agent with the sample. For PCR-based techniques, guanidine-based denaturing agents are commonly used, although each buffer uses different concentrations of active ingredients and ratios of inactivation agent to sample. The precise formulations of inactivation buffers are generally proprietary

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and the efficiency of a particular buffer against a specific pathogen cannot be predicted with complete confidence.

During the Ebola outbreak in West Africa many laboratories employed manual nucleic acid extractions using the QIAamp viral nucleic acid extraction kit (Qiagen, 52904) which uses guanidinium-based AVL buffer as the inactivation agent (Reusken et al., 2015). Data have shown that buffer AVL will inactivate high titre EBOV in cell culture medium (Blow et al., 2004). When EBOV is present in clinical samples, however, complete inactivation is not guaranteed (Smither et al., 2015; Haddock et al., 2016) and ethanol also needs to be added before full inactivation is achieved. For manual extraction methods ethanol can be added to the AVL treated samples before removal from primary containment. For high sample throughput using the AVL compatible automated nucleic acid extraction system (Qiagen EZ1) ethanol cannot be added before the samples are removed from containment and loaded onto the machine. In PHE laboratories in Sierra Leone samples were removed from containment and inactivation was completed using heat (60 °C for 15 min) (Bhagat et al., 2000; Bailey et al., 2016). The potential problem with using heat inactivation in such a high throughput situation is that constant temperature monitoring of the samples is required to ensure that the temperature is held and full inactivation is achieved. The addition of a defined volume of a chemical inactivation agent such as Triton X-100 allows consistent treatment of the samples.

Triton X-100, a non-denaturing detergent that solubilises lipid membranes, is a commonly used inactivation agent that has been shown to reduce infectivity of samples without affecting blood chemistry results (Tempestilli et al., 2015; Lau et al., 2015). Triton X-100 was recommended by CDC as an inactivation agent which reduces the titre of haemorrhagic fever viruses. The Guidelines, however, state that 100% inactivation should not be assumed. https://www.cdc.gov/hai/pdfs/bbp/vhfinterimguidance05_19_05.pdf. Triton-X 100 is also included in many commercial nucleic acid extraction buffers at various concentrations such as the Roche Magnapure 96 external lysis buffer (Rosenstierne et al., 2016). The addition of 1.0% (v/v, final concentration) Triton X-100 to mock clinical serum samples prior to RNA extraction has been shown to have no effect on downstream processing for RT-qPCR and PCR amplification of longer DNA fragments (Lewandowski et al., 2016). The generation of larger amplicons (up to 2874 bp) shows that samples treated with 1.0% (v/v) Triton X-100 produces high quality nucleic acid material that is suitable for next generation whole genome sequencing applications, an approach that is increasingly used in viral diagnostics (Kwong et al., 2015). The addition of Triton X-100 to the inactivation buffer before downstream processing of a sample on an automated extraction platform could provide an additional inactivation stage. The use of two different inactivation agents is recommended by the World Health Organisation (WHO); Ideally two different methods of inactivation should be used (e.g. one physical method and one chemical method) although two chemical methods may be just as effective www.who.int/bloodproducts/publications/WHO_TRS_924_A4.pdf. It has recently been shown that 0.1% Triton X-100 effectively reduced EBOV infectivity, although complete inactivation was not achieved (Colavita et al., 2017).

The aim of this study was to determine the effectiveness of Triton X-100 for inactivation of EBOV in serum samples, alone or in combination with guanidinium thiocyanate containing AVL buffer. The addition of Triton X-100 could be used as a well defined additional inactivation treatment before nucleic acid extraction.

2. Materials and Methods

2.1. Virus

Work with live virus was carried out in the CL4 laboratories at PHE Porton Down. The virus stock used was Ebola virus/H.sapiens-tc/COD/1976/Yambuku-Ecran, obtained from the first Ebola haemorrhagic fever outbreak in Zaire in 1976, passaged through guinea pigs. To generate mock positive patient serum the EBOV stock virus was diluted (1: 10) in negative human serum (Sigma H4522) to a concentration of 10^6 $TCID_{50}$/ml.

2.2. Effect of Triton X-100 alone and in combination with AVL on the viability of EBOV in serum

EBOV in serum (140 µl) was treated with 560 µl volumes of the following inactivation agents 1) 0.1% Triton X-100; 2) 1.0% Triton X-100; 3) AVL; 4) 0.1% Triton X–100 + AVL, each with a 10 min contact time and 5) 1.0% Triton X-100 with a 20 min contact time. As a positive control, EBOV in serum was added to 560 µl of tissue culture medium and passed through size exclusion columns or treated with SM2 adsorbent resin in parallel with inactivated samples. For comparison the same volume of EBOV in serum was added directly to cells. The concentration of Triton X-100 alone was increased to 1.0% with a contact time of 20 min to determine whether it could be used as an inactivation agent in the absence of AVL. Each treatment was set up in duplicate or triplicate.

2.2.1. Removal of AVL or Triton X-100 before cell culture

AVL and Triton X-100 are toxic to cells (Colavita et al., 2017; Kumar et al., 2015) and the addition of 100–200 µl volumes to Vero E6 cells in 25 cm^2 flasks with 5 ml medium was sufficient to cause cell death. Work up experiments carried out at CL2 showed that AVL could be removed from samples using size exclusion spin columns. Initially Vivaspin 2.0 columns (100 000 MW CO PES VS0201) that were able to hold 2 ml of liquid were used, however the protocol that worked successfully at CL2 did not translate well to use within the CL4 cabinet line, so after the first experiment AVL was removed using Amicon 50 000 MWO, UFC50500 Ultra-0.5 centrifugal filter devices (Kumar et al., 2015) in a microcentrifuge. These held a smaller volume of liquid (0.5 ml) but were easier to handle within the cabinet line. Triton X-100 was most effectively removed from samples using SM2 resin. This was used alone and in combination with the size exclusion spin columns as required.

2.2.2. Removal of AVL and 0.1% Triton X-100 using Sartorius Vivaspin 2 columns (100 000 MW CO PES VS0201)

AVL and 0.1% Triton X-100 were removed from the samples using Sartorius Vivaspin columns. The entire sample (700 µl) was added to the spin columns and centrifuged at 2000 x g for 20 min. The eluate (approximately 500 µl) was removed and phosphate buffered saline (PBS, Gibco 10010-015) added to the column to a final volume of 2 ml. The samples were centrifuged a further three times at 2000 x g for 15 min and after each spin the eluate removed and PBS added to the column to a final volume of 2 ml. The liquid from the column reservoir after the final spin was approximately 50 µl. PBS (200 µl) was added to the concentrated virus to allow the liquid to be efficiently removed from the column.

2.2.3. Removal of 1.0% Triton X-100 using SM2 resin

To remove 1.0% Triton X-100 the samples were treated with a hydrophobic resin SM2 beads (Bio Rad, 152–8920) using a modification of a batch method (Holloway 1973). SM2 resin (0.15 g) was added to each sample containing 1.0% Triton X-100. The tubes were mixed by inversion for 1 min followed by 5 min static incubation at room temperature. This was repeated 10 times (total of 10 mins of mixing and 1 h of static incubation). The resin was allowed to settle to the bottom of the tube and the sample carefully removed using a pipette.

2.2.4. Removal of AVL buffer using Amicon ultra-0.5 centrifugal filter devices (50 000 MWO, UFC505008)

To prepare the column 500 µl 70% ethanol was added to the column, centrifuged and the eluate removed. This was repeated using 500 µl sterile water. All centrifugation steps were 10 mins at 11 000 x g