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Hand hygiene: virucidal efficacy of a liquid hand wash product against Ebola virus

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SUMMARY

Background: Hand washing is an important targeted hygiene intervention for limiting the spread of infectious agents, including the Ebola virus, which continues to re-emerge. We have assessed the virucidal efficacy of a commercially available liquid hand wash product (LHW) for inactivating Ebola virus.

Methods: The ASTM E1052-11 Standard was used to evaluate the efficacy of an LHW containing the microbicidal active salicylic acid for inactivating Ebola virus - Makona variant suspended in an organic load. Three concentrations (12.5%, 25%, 50%) of three lots of LHW prepared in 440 ppm hard water were evaluated at room temperature for 20, 30, and 60 s contact time.

Results: A 25% solution of the LHW caused 4.5 $\log_{10}$ and 4.8 $\log_{10}$ reduction in Ebola virus titer within 20 and 30 s, respectively. The efficacy of a 12.5% LHW solution was lower (1.9 and 2.0 $\log_{10}$ reduction in titer within 20 and 30 s, respectively). The efficacy of the 50% LHW solution could not be measured, due to inability to sufficiently neutralize the LHW at the end of exposure.

Conclusion: These results suggest the potential utility of an appropriately formulated liquid hand wash agent during Ebola virus disease outbreaks for use within healthcare, community, and home settings. Such an LHW should also be effective against other enveloped viruses, such as the pandemic coronavirus SARS-CoV-2.

Introduction

Ebola virus disease (EVD) outbreaks continue to occur periodically in the equatorial regions of Africa and have caused unprecedented morbidity and mortality around the world, with case fatality rates reaching 25%–90% [1]. Of these, 18 have involved the Ebola virus Zaire strain. The most recent outbreak

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associated with this strain occurred in the Democratic Republic of the Congo in 2018 [2]. Ebola virus transmission occurs through the direct contact with contaminated blood or body fluids of an infected person [1]. When outbreaks occur, there is a higher risk of transmission of the Ebola virus from infected patients to healthy persons and healthcare workers providing care and then from infected healthcare workers to the uninfected population [3,4]. This can contribute to virus dissemination.

The infection prevention and control (IPAC) of Ebola virus and other emerging-re-emerging viruses such as SARS-CoV-2, for which vaccines are not yet available, involves important interventions such as targeted hygiene products for decontaminating environmental surfaces and the use of appropriate hand hygiene practices [5–7].

Efficacy testing of microbicides such as hand hygiene agents through the study of inactivation of surrogate viruses theoretically should demonstrate their efficacy for inactivation of the Ebola virus. However, direct testing against high-risk pathogens is also needed to confirm these results to provide assurance to critical facilities and personnel, especially when dealing with a high consequence pathogen such as the Ebola virus. In the present investigation, Ebola virucidal assessments were performed at the Canadian Science Centre for Human and Animal Health, Public Health Agency of Canada BSL4 facility. Three concentrations of a commercially available liquid hand wash, LHW, were evaluated for inactivation of EBOV/Mak virus suspended in an organic soil load using methods specified in the ASTM International E1052-11 Standard [8]. This commercially available product is proposed for use as a prophylactic hand washing agent for community healthcare workers and the public-at-large interacting with EVD patients. Consistent with this application, we have evaluated the effectiveness of three different lots of an LHW for inactivating EBOV/Mak under ambient conditions for relatively short contact times (20, 30 and 60 s), such as might be applicable to the washing of hands [9].

Materials and methods

Cell line, virus, and medium

African green monkey Vero E6 cells (ATCC CRL-1586; American Type Culture Collection) were maintained at 37°C/5% CO_2 in Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal calf serum (FCS; Gibco) and 10 units/mL penicillin/streptomycin (Gibco). Ebola virus (Makona C07 variant; EBOV/Mak) (Ebola virus/H. sapiens-tc/GIN/2014/Makona-C05; GenBank accession no. KJ660348) was obtained from a clinical isolate. All manipulations involving EBOV/Mak were carried out at ambient temperature (21°C) in a BSL4 laboratory at the Canadian Science Centre for Human and Animal Health, Public Health Agency of Canada, Winnipeg, MB.

Stock virus preparation

A characterized stock of EBOV/Mak was prepared by infecting ten T-75 flasks of African green monkey Vero E6 cells at nearly full confluency at a multiplicity of infection of 0.01. At ~9 days post-infection, marked cytopathic effects (CPE) were evident in the Vero E6 cells, at which time the flasks were frozen at -70°C. The flasks were thawed the following day and the conditioned medium from the flasks was clarified by low-speed centrifugation (4500 × g) for 10 min. The supernatants were pooled and overlaid onto 20% weight/volume sucrose cushions prepared in Tris-NaCl-EDTA buffer. After centrifugation at 133000 × g for 2 h, the viral pellets were resuspended in virus culture medium (VCM; DMEM containing 2% FCS and 10 units/mL penicillin/streptomycin) overnight at 4°C. The resuspended virus was pooled and usable amounts aliquoted into containers and stored at -70°C until needed. Stock virus titers were determined to be >9 $\log_{10}$/mL by $TCID_{50}$ assay, with titer calculation based on the Reed-Muench method [10].

Liquid hand wash product test concentrations

Three concentrations (12.5%, 25%, 50%) of three lots of LHW (Dettol Liquid Hand Wash, Reckitt Benckiser, LLC, lot# P030216B16, P130116B16, and P280116B16) were prepared from the ready-to-use commercial product by dilution in 440 ppm hard water[11] (prepared as 1 L deionized water supplemented with 0.4 g calcium carbonate) on the day of assay performance. Use of hard water as diluent was included in the study design to simulate water hardness in the field. The resulting solutions were inverted to mix and were used within 4 h of preparation.

Evaluation of neutralizer effectiveness

A neutralization assay was performed to evaluate the ability of candidate neutralizing reagents to terminate the virucidal effects of LHW. The reagents evaluated included 100% FCS, VCM, and 1× Letheen broth in VCM (10× Letheen broth, BD Difco, diluted 1:10 in VCM). Ebola-Makona virus was diluted to approximately 10^4 to 10^5 $TCID_{50}$ per mL with only 10 μL virus evaluated per control in replicates of three. Candidate reagents were evaluated for neutralization efficacy and cytotoxicity to Vero E6 cells using the methods shown in Supplemental Materials.

LHW virucidal efficacy testing

Inactivation efficacy testing for LHW was performed in suspension studies (Figure 1) conducted at ambient temperature per ASTM E1052-11 [8], the version of the Standard which was effective at the time the testing was performed. A new version, ASTM-1052-20, has since been published [12]. On the day of the assay, concentrated stocks of EBOV/Mak (~$1\times10^{9.3}$ $TCID_{50}$/ml) were mixed with tripartite soil load [11,13] and used for efficacy assays. "Tripartite soil load" is a term used in the ASTM Standard [8] to signify an organic matrix used to challenge the virucidal efficacy of a microbicide. It is intended to mimic secretions or excretions in which the virus would be released from an infected person. Briefly, 140 μl of stock virus were added to 12.5 μL of 5% bovine serum albumin [Millipore-Sigma], 17.5 μL of 5% tryptone [BD Difco], 50 μL of 0.4% bovine mucin [Millipore-Sigma]) to compose a "Tripartite soil load" of ~10^9 $TCID_{50}$/mL. A volume of 10 μL of virus in tripartite soil load (~10^7 $TCID_{50}$ units) was added to 90 μL of test concentration of LHW or to 90 μL VCM (positive virus control). The resulting solutions were mixed and held at room temperature for 20, 30, and 60 s contact time after which they were

neutralized by adding 900 μL of 100% FCS to the test solutions, with pipetting to mix.

A 500-μL portion of each treatment neutralized with 100% FCS was 10-fold serially diluted in VCM, and 50 μL of the resulting dilutions were added to 96-well plates (n = 5 replicates per dilution) containing Vero E6 cells and allowed to adsorb to the cells over 45 min at 37°C. After adsorption, 150 μL of VCM were added to each well and the wells were incubated for 14 days. The wells were then scored for CPE and the virus titer was calculated according to the Reed-Muench procedure [10]. A separate 500-μL portion of each treatment neutralized with 100% FCS was loaded onto a pre-equilibrated GB S10 5000 detergent removal columns (Figure 1). These columns were allowed to stand for 2 min at ambient temperature and were then centrifuged according to manufacturer's recommendations (200 × g, 2 min). The eluates were collected, and 10-fold serially diluted in VCM. A 50 μL portion of the resulting dilutions (n = 5 replicates per dilution) was added to 96-well plates containing Vero E6 cells and allowed to adsorb to the cells for 45 min at 37°C. After adsorption, 150 μL of VCM were added to each well and the wells were incubated for 14 days. The wells were then scored for CPE and the virus titer calculated according to the Reed-Muench procedure (Figure 1) [10].

In addition to the efficacy testing described above, a single cytotoxicity control was run for the 25% LHW dilution in each experiment. This control was included to detect any cytotoxicity caused by the neutralizer used in the study (100% FCS) and LHW. This control consisted of 900 μl of neutralizer and 100 μl of 25% LHW solution. The resulting solution was 10-fold serially diluted in VCM, and 50 μL of the resulting solution was added to Vero E6 cells and incubated for 45 min. The wells were then topped up with 150 μL of VCM. Cells were scored for cytotoxicity 14 days post-inoculation.

Analysis of virucidal efficacy

The $\log_{10}$ reduction values achieved by the various LHW test lots and concentrations and exposure timepoints were calculated by subtracting the post-LHW exposure $\log_{10}$ $TCID_{50}$ values from the $\log_{10}$ titers obtained for the corresponding positive virus controls. Statistical comparison of the mean (n = 5 replicates) viral titers obtained in the neutralization effectiveness studies was performed using a non-parametric unpaired t-test, with statistical significance set at $P < 0.05$.

Results

Results of neutralizer effectiveness testing

During the evaluation of candidate neutralizing agents, it was determined that 100% VCM and 1x Letheen broth in VCM failed to adequately terminate the virucidal effects of LHW (data not shown). On the other hand, 100% FCS added to the 25% LHW concentration prior to introduction of EBOV/Mak in tripartite soil load prevented inactivation of the virus (See Supplemental Materials).

EBOV/Mak virucidal efficacy results obtained without use of detergent column

Three lots of LHW were evaluated per ASTM E1052-11 [8] at two concentrations (12.5%, 25%) each diluted in hard water, with contact times of 20, 30, and 60 s at ambient temperature (21°C) in a BSL4 facility. An initial EBOV/Mak challenge of ~7 $\log_{10}$ in 10 μl of tripartite soil load was exposed to the LHW concentrations in hard water for the three contact times. The post-exposure/neutralization titers for the positive virus

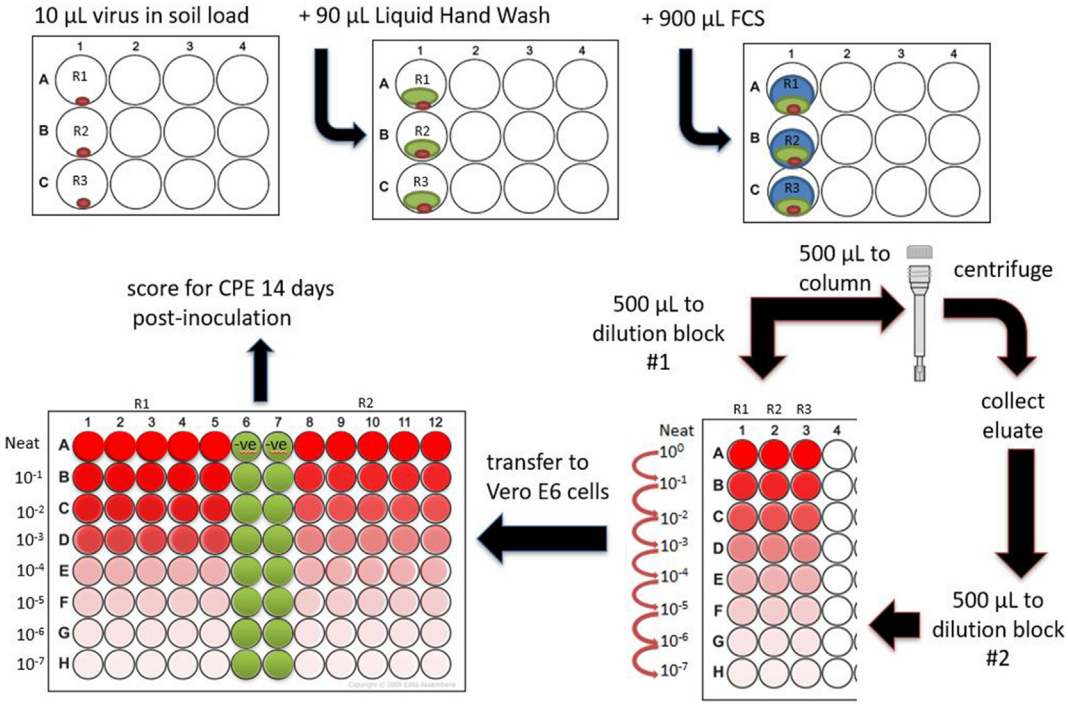

Figure 1. Schematic representation of the virucidal efficacy testing methodology employed. The entire procedure was performed once for each LHW lot. Abbreviations used: FCS, fetal calf serum; CPE, viral cytopathic effect.

controls (virus without LHW) and virucidal efficacy of the LHW test conditions were calculated. The $\log_{10}$ reduction values for each time point were calculated by subtracting the virus titers obtained for the LHW test conditions from the titers of the corresponding positive virus controls.

Incomplete inactivation of EBOV/Mak was observed with 12.5% LHW after 20, 30, and 60 s contact time (Figure 2). The $\log_{10}$ reductions in virus titer achieved were 1.9, 2.0, and 2.4, respectively, from an initial titer of 7 $\log_{10}$ $TCID_{50}$/mL in the neutralization solutions. A substantial (3.8 $\log_{10}$) reduction of EBOV/Mak titer was observed with 25% LHW after 20 s contact time (Figure 2). After 30 and 60 s contact time with 25% LHW, the limit of detection (2.8 $\log_{10}$ $TCID_{50}$/ml) was reached, indicating a virus titer reduction of $\geq$ 4.2 $\log_{10}$.

EBOV/Mak virucidal efficacy results obtained with use of detergent column

An alternate assessment of the efficacy of 25% LHW for inactivating EBOV/Mak was obtained through the use of a detergent-removal column (GBS-10 5000). This approach was intended to reduce to the remaining cytotoxicity to Vero E6 cells of LHW following neutralization with 100% FCS. The use of the column effectively lowered the limit of detection of the $TCID_{50}$ assay by 1 $\log_{10}$ (i.e., from 2.8 to 1.8 $\log_{10}$ $TCID_{50}$/mL). As shown in (Figure 3), passing the neutralized 25% LHW + EBOV/Mak virus through the detergent column allowed us to determine that viral inactivation at all time points evaluated was not complete after 20 s contact time for each LHW lot. In addition, for one of the LHW lots (P030216B16), inactivation was incomplete for the 30 and 60 s contact times as well. Having said this, as shown in (Figure 3), the $\log_{10}$ reduction values achieved with 25% LHW after the 20, 30, and 60 s contact times were substantial (4.5, 4.8, and 4.8 $\log_{10}$, respectively).

Discussion

The recent 2018 Ebola Zaire outbreak in the Democratic Republic of the Congo serves as a clear reminder that a prophylactic strategy, including immunizations, where available, and frequent hand washing utilizing highly efficacious hand hygiene agents, is required to limit and prevent the spread of Ebola virus and other infectious agents [1]. The criticality of hand hygiene in IPAC for reducing the dissemination of infectious viruses also has been emphasized during the recent SARS-CoV-2 pandemic, which has claimed more than 690,000 lives globally as of August 3, 2020 [14].

Since BSL4 facilities are required in order to handle these types of viruses, there is limited evidence in the scientific literature of the virucidal efficacy of microbicidal products able to inactivate Ebola virus and other hemorrhagic fever viruses. This is especially true in the case of antiseptics/skin cleansing agents.

Efficacy for viral inactivation is typically claimed in units of $\log_{10}$ reduction value. For instance, the US Environmental Protection Agency states in the disinfectant product guidance [15] that "The product should demonstrate complete inactivation of the virus at all dilutions. If cytotoxicity is present, the virus control titer should be increased to demonstrate a $\geq$3 $\log_{10}$ reduction in viral titer beyond the cytotoxic level. Similarly, 21 CFR part 310 [16] states that "To establish that a particular active ingredient is GRAE [generally recognized as effective] for use in health care antiseptics, clinical simulation studies using the parameters described in this section should be evaluated using log reduction criteria required for Health care personnel hand wash or health care personnel hand rub (reduction of 2.5 $\log_{10}$ on each hand within 5 minutes after a single wash or rub)".

Evaluation of the efficacy of hand wash agents described in the CDC guidance: Hand Hygiene in West African General (Non-Ebola Treatment Unit) Healthcare Settings [7], including alcohol-based hand sanitizers, soap and water, and mild (0.05%) chlorine solutions, has been undertaken by several groups using surrogate viruses *in vitro* instead of Ebola virus, as evaluated in the current study. For instance, Wolfe *et al.* [17] used the bacteriophage Φ6 and the bacterium *Escherichia coli* to challenge these hand wash agents in the presence or absence of an organic load. Their data suggested that efficacy

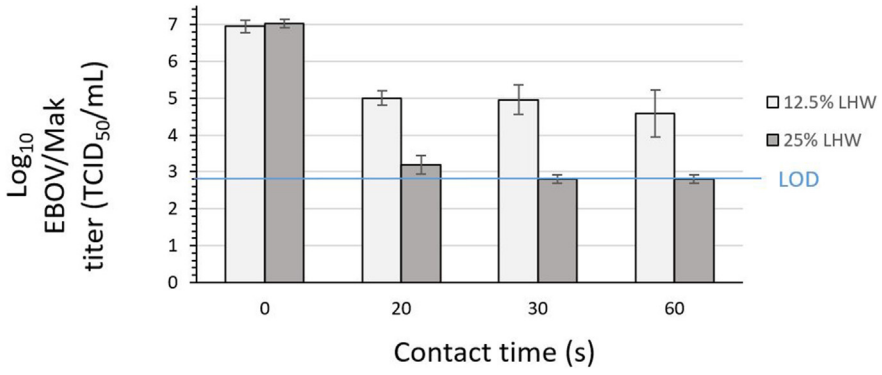

Figure 2. Inactivation of EBOV/Mak by 12.5% and 25% liquid hand wash (LHW). The values represent the mean ± standard deviation (n = three replicates, one for each LHW lot) of the $\log_{10}$ titer of the positive virus control (0 s contact time) and the post-neutralization samples (20, 30, and 60 s contact time). Individual viral titers were calculated based on three replicate wells per dilution. The limit of detection (LOD) of the assay was 2.8 $\log_{10}$ $TCID_{50}$/mL (shown in the plot as a blue line extending from y = 2.8 $\log_{10}$ $TCID_{50}$/mL). This was due to the cytotoxicity of the LHW dilution to the Vero E6 cells. Abbreviations used: EBOV/Mak, Ebola virus – Makona variant; FCS, fetal calf serum, LOD, limit of detection, s, seconds, $TCID_{50}$, tissue culture infectious dose$_{50}$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

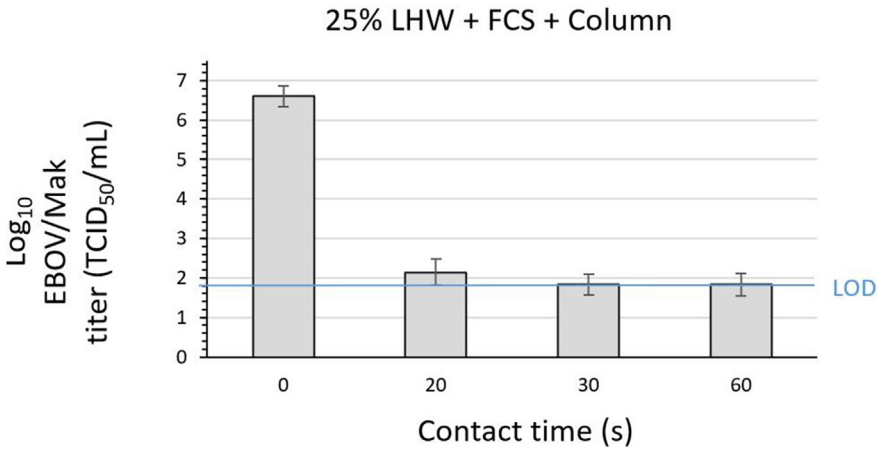

Figure 3. Inactivation of EBOV/Mak by 25% liquid hand wash (LHW) assessed following elution from a detergent column. The values represent the mean ± standard deviation (n = three replicates, one for each LHW lot) of the $\log_{10}$ titer of the positive virus control (0 s contact time) and the post-neutralization samples (20, 30, and 60 s contact time). Individual viral titers were calculated based on three replicate wells per dilution. The limit of detection (LOD) of the assay was 1.8 $\log_{10}$ $TCID_{50}$/mL (shown in the plot as a blue line extending from y = 1.8 $\log_{10}$ $TCID_{50}$/mL). This was due to the residual cytotoxicity of the LHW dilution to the Vero E6 cells. Abbreviations used: EBOV/Mak, Ebola virus — Makona variant; FCS, fetal calf serum, LOD, limit of detection, s, seconds, $TCID_{50}$, tissue culture infectious $dose_{50}$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for removal of bacteriophage Φ6 from a glove surface was similar for the various hand wash protocols, with 2.4—3.7 $\log_{10}$ removal occurring. Effectiveness for inactivation of bacteriophage Φ6 in rinse water was lower (1.3—2.2 $\log_{10}$). This was also suggested by the work of Cook *et al.* [18], which suggested that most of the virucidal efficacy of the hand wash protocols was due to physical removal, and not inactivation, of Ebola virus. Eggers *et al.* [19] evaluated the virucidal efficacy of iodine- and iodine/alcohol-containing hand wash agents for inactivating modified vaccinia virus Ankara (MVA) and Ebola virus strain Zaire (EBOV/Zaire) in suspension in the presence or absence of an organic load. After a 15-s contact time, MVA and EBOV/Zaire were inactivated by >4 to >5 $\log_{10}$ in the absence and presence of an organic load.

Physical removal is considered to be a major contributing factor for minimizing the risk of spreading infectious agents when washing hands. Removal is enhanced by hand wash products containing surfactants, when compared with water-based products such as those containing iodine or chlorine as active agents. The Ebola virus has been shown to be relatively stable in water, with the time required for 90% reduction in titer (D_{90}) being 2.1 days at 20°C in waste water [20] and 0.9 days (27°C) or 1.8 days (27°C) in protein-free water [21]. Considering the possible survival of EBOV for days in waste water, inactivation of the infectious agent being removed from (washed off of) contaminated hands and other surfaces becomes more critical during emerging pathogen outbreaks, such as those involving Ebola virus. This is especially important, considering the high lethality rate of the virus (the overall case fatality rate for outbreaks occurring between 1976 and 2017 has been calculated as 67%) [22], and the relatively low minimum infectious dose in humans (generally considered to be 1—10 $TCID_{50}$/mL) [23,24]. The target virucidal efficacy for a microbicidal product for use against Ebola virus should exceed the typical expectation of ≥3 $\log_{10}$ inactivation. A hand wash agent ideally will afford complete inactivation of the virus removed from the hands, so that introduction of infectious virus to the waste water generated through hand washing may be minimized. The utility of a hand wash agent as an intervention for preventing the spread of the Ebola virus is dependent both upon the ability of the agent to remove virus from the hands and environmental surfaces (not investigated in this paper), the $\log_{10}$ inactivation conferred, as well as the contact time that is required to achieve this level of virucidal activity. When washing hands with soap and water, the CDC recommends scrubbing hands for at least 20 s [9].

The virucidal efficacy of an LHW for inactivating EBOV/Mak in suspension was evaluated in this study per the ASTM International E1052-11 Standard [8]. Our data, generated with EBOV/Mak using 25% LHW and the use of the detergent removal column to minimize cytotoxicity (thereby decreasing the assay limit of detection), indicate that at least a 4.8 $\log_{10}$ reduction in titer was achieved within 30 s contact time at 21°C. While the 60 s contact time with 25% LHW also was found to completely inactivate EBOV/Mak, it is acknowledged that this duration of hand washing is not likely to be practiced by users.

The Ebola virucidal results for the higher LHW concentration (50%) could not be interpreted (data not shown), due to cytotoxicity to the Vero E6 cells when this concentration of the hand wash agent was tested, and the inability of the FCS and detergent removal column to mitigate this cytotoxicity. The 25% LHW concentration provided greater efficacy for inactivating EBOV/Mak than the 12.5% LHW concentration, so it is reasonable to expect that the 50% concentration or the full-strength (undiluted) LHW product might possess even greater efficacy than that empirically demonstrated for the 25% concentration. Unfortunately, this expectation could not be confirmed in the present study due to the limitations discussed above.

The microbicidal actives/ingredients in LHW are salicylic acid and citric acid, with ammonium lauryl sulfate, sodium lauryl sulfate, sodium lauryl ether sulfate, and cocamide monoethanolamine included as surfactants and foaming agents. Previous studies have shown LHW to be effective for

inactivating a variety of viruses and bacteria [25—27]. Salicylic acid, the major active ingredient in the LHW evaluated in this study, has been reviewed by the Cosmetic Ingredient Review Expert Panel [28], and found to be safe for use in cosmetic products. Salicylic acid is used in LHW at pH 4.1, which has not been found to negatively impact skin microbiota [29,30]. The virucidal efficacy of organic acids such as salicylic acid for non-enveloped viruses has been demonstrated previously [31]. Also, viruses are known to be sensitive to low pH, and the residual rhinovirucidal activity of salicylic acid has been shown *in vivo* for hours post-application on human hands [31].

The mechanisms of virucidal activity of microbicides are not completely understood [32]. It is likely that the susceptibility of enveloped viruses such as Ebola virus to microbicides such as salicylic acid is attributed to disruption/lysis of the lipid envelope, which is derived from the host cell during the viral maturation and release process (termed budding) [32,33]. This proposed mechanism is supported by the known efficacy of alcohol hand rubs against Ebola virus [34] and by the efficacy of a commercial antiseptic liquid for inactivating EBOV/Mak in suspensions containing a tripartite soil load [35,36]. Additional mechanisms of viral inactivation may play roles in the case of this particular LHW. For instance, low pH may lead to conformational changes in Ebola viral capsid proteins, altering capsid integrity. The surfactants in this LHW may also tend to disperse viral particles [37], making them vulnerable to the multiple synergistic virucidal mechanisms attributed to this LHW.

One limitation of this study is the fact that only the virucidal activity of the LHW was studied *in vitro*. We did not evaluate the removal of EBOV/Mak through the hand washing process, due to biosafety concerns. We focused on inactivation of the virus by LHW, since simple removal of virus from the hand may not be an effective means of limiting the spread of EVD if the virus is not subsequently inactivated prior to introduction to waste rinse water generated through hand washing. As mentioned above, infectious Ebola virus contained in waste water generated during the hand washing process may still remain infectious for days.

Taken together, the efficacy of hardwater solutions of the LHW for inactivation of EBOV/Mak in the presence of an organic load demonstrated above suggests that appropriately formulated LHW could play a role in limiting the spread of infectious Ebola virus during EVD outbreaks. The utility of LHW in limiting viral spread during outbreaks of EVD should be explored further in controlled field studies. Such a commercially available product may also be useful as an intervention for decontaminating spills on surfaces and excretions from infected patients and deceased Ebola patients.

Conclusion

A liquid hand wash applied in suspension as a 25% solution in hard water inactivated $\sim$5 $\log_{10}$ of EBOV/Mak within 30 s at room temperature in the presence of an organic load. These results suggest that the use of an appropriately formulated LHW should afford effective inactivation of EBOV from skin surfaces and in waste water resulting from hand hygiene practices during Ebola virus outbreaks. These data also suggest the potential benefits of the use of such a hand wash product in limiting spread of EVD in outbreaks.

Author statement

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Conflict of interest statement

R.W.N. received a fee for his role in authoring and editing the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.infpip.2021.100122.

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