Virucidal activity of WHO-recommended formulations against enveloped viruses including Zika, Ebola and emerging Coronaviruses

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Running title: Inactivation of emerging viruses by WHO formulations

Keywords: Enveloped viruses, Zika virus, Ebola virus, WHO, SARS, MERS

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Financial support

E.S. was supported by the Helmholtz Centre for Infection Research. M.P.W. and J.Y. were supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (2014K1A4A7A01074644), Gyeonggi-do, KISTI and the Institute Pasteur Ebola Task Force.

Conflict of interest: The authors do not have a conflict of interest.

Number of figures and tables: 2

Word count Abstract: 93

Word count text: 1989

Abstract

The World Health Organization (WHO) published two alcohol-based formulations to be used in healthcare settings and outbreak-associated infections, but inactivation efficacies of these products have not been determined against (re-) emerging viruses. In this study, we evaluated the virucidal activity of these WHO products in a comparative analysis. Zika virus (ZIKV), Ebola virus (EBOV), Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV) as (re-) emerging viral pathogens and other enveloped viruses could be efficiently inactivated by both WHO formulations implicating their use in healthcare systems and viral outbreak situations.

Introduction

Hygienic hand antisepsis is one of the most important measures in preventing healthcare and outbreak-associated viral infections. To reduce the spread of infections, biocides should be readily accessible with a proven virucidal efficacy. The World Health Organization (WHO) proposed in 2009 *Guidelines on Hand Hygiene in Health Care*, a document to implement the use of two alcohol-based hand rubs (formulation I and formulation II) for surgical and hygiene hand disinfection in healthcare settings and to reduce the transmission of pathogens by hands [1]. However, due to the recent outbreaks of emerging viruses in different parts of the world, limited data on the efficacy of disinfectants, including the WHO formulations, against these novel viruses exist. Most recently Zika virus (ZIKV), a *flavivirus* that had been discovered originally in Africa has raised considerable international concern. In 2013, the largest and most complex outbreak of Ebola virus (EBOV) occurred in West Africa, a *filovirus* spreading mainly through contact with body fluids of symptomatic patients or contaminated surfaces [2]. One year previously in 2012, a novel *Coronavirus* (CoV) named Middle East Respiratory Syndrome (MERS) emerged, preceded by the Severe Acute Respiratory Syndrome (SARS) in 2002/2003, with both viruses causing acute respiratory diseases in humans and displaying a high case-fatality rate.

We have previously evaluated the WHO formulations in a quantitative suspension test for chemical disinfectants and antiseptics in human medicine using different non-enveloped model viruses and observed that formulation I demonstrated a better activity than formulation II against these non-enveloped viruses [3]. However, both formulations did not meet the requirements for virucidal activity against poliovirus according to the European Guideline (EN14476) [3] and for surgical hand treatment

according to the European Norm (EN12971) [4]. Meanwhile, both WHO formulations were modified with higher alcohol content and lower glycerol concentration and are fulfilling the guideline requirements [5, 6].

In this study, we evaluated for the first time the modified WHO-recommended alcohol-based formulations against different enveloped viruses including emerging ZIKV, EBOV, SARS-CoV and MERS-CoV and performed a comparative inactivation analysis of these emerging and other important reference viruses.

Material and Methods

Cell culture and viral strains

An overview of the viruses and cell culture systems used in this study is given in supplementary Table 1. Hepatitis C virus (HCV) chimeric Jc1 virus was generated in the human hepatoma cell line (Huh7.5) as described [7]. The African lineage ZIKV strain (MP1751), isolated 1962 in Uganda, was propagated by using Vero-B4 cells like MERS-CoV strain EMC and SARS-CoV strain Frankfurt 1. Bovine CoV (BcoV) was produced in the human glioblastoma astrocytoma cells U373, human influenza A virus (H1N1) in Madin-Darby canine kidney epithelial cells (MDCK) and modified vaccinia Ankara strain (MVA) in baby hamster kidney cells (BHK-21). EBOV was propagated in Vero E6 cells as described before [8]. EBOV-like particles encoding a luciferase were generated using 239T cells as reported previously [9]. In general, cell lines were cultured in Dulbecco's modified minimal essential medium (DMEM) or Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and other additions (Table 1).

Quantitative suspension test and virus titrations

One part by volume of test virus suspension and one part by volume of the organic load were mixed with eight parts by volume of one of the two WHO formulations at different concentrations. Additional information are posted as supplementary information.

Statistical Analyses

Concentrations at which the formulations reached the half maximal virus inactivation effective concentration 50 (EC_{50}) were determined using nonlinear regression employing the robust fitting method on the normalized $TCID_{50}$ data implemented in GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The mean $TCID_{50}$ of two individual experiments and standard deviation of means were also calculated using GraphPad Prism. Significance of differences in mean EC_{50} obtained for the viruses between WHO formulations I and II was tested using two-tailed Wilcoxon matched-pairs signed rank test (**$p < 0.01$).

Results

Virucidal activity of WHO formulations against HCV and ZIKV

HCV and ZIKV are both belonging to the family of *Flaviviridae* (suppl. Table 1), but are transmitted in the environment by different routes. While HCV is a blood-borne virus [10], transmission of ZIKV occurs mainly through mosquitos, with the most important and common vectors of the *Aedes* genus. However, other modes of transmission, including sexual transmission have been reported. To determine the efficacy of WHO formulations I and II against HCV and ZIKV, we incubated the two viruses for 30 seconds with the formulations at final concentrations ranging from 10% to 80% (Fig. 1). In case of HCV, viral titers started to decline at 30% of WHO formulation II and 40% of WHO formulation I and were reduced to background levels with 60% of WHO formulation I and 40% of WHO formulation II, respectively (Fig. 1A). As depicted in Fig. 1B, a dose-dependent reduction of viral titers was also observed for ZIKV (Fig. 1B). Importantly, viral titers of 10^6 $TCID_{50}$/ml in the control decreased to undetectable levels with WHO formulation I at a concentration of 40%, whereas only 30% of the WHO formulation II were required for complete inactivation.

Susceptibility of BCoV, MERS-CoV and SARS-CoV to WHO formulations

Next, we investigated the susceptibility of emerging respiratory CoVs against the WHO formulations in the same experimental suspension assay setup. As reference for CoVs, which can be cultivated under lower biosafety levels, we included BCoV naturally infecting cattle. As depicted in suppl. Figure 1A, WHO formulation II at a 30% concentration was sufficient to completely inactivate BCoV, while for WHO formulation I higher concentrations of at least 40% were required (suppl. Fig. 1A). Similar inactivation profiles could be observed for MERS-CoV (Fig. 1C) and SARS-

CoV (Fig. 1D) demonstrating a high susceptibility of these emerging CoVs to WHO formulations. Furthermore, these results implicate BCoV as valid surrogate virus for inactivation studies with MERS-CoV and SARS-CoV.

Virucidal activity of WHO formulations against EBOV, H1N1 and MVA

Work with infectious EBOV is restricted to biosafety level 4 laboratories, significantly limiting studies with these viruses. In 2014, Watt et al. reported a novel life cycle modelling approach for EBOV, which can be performed at biosafety level 2 laboratories [9]. Inactivation of these transcription- and replication-competent virus-like particles (trVLPs) with WHO formulations showed a dose-dependent reduction of trVLP reporter activity with increasing WHO formulation I and II concentrations (Fig. 2A). Next, we tested full infectious EBOV cultured under biosafety level 4 for its susceptibility to WHO formulations for the potential usage in outbreak situations. Interestingly, viral titers of 10^7 $TCID_{50}$/ml in the control were reduced to background levels at 40% of WHO formulation II and 60% of WHO formulation I showing again a superior virucidal activity of WHO formulation II compared to WHO formulation I (Fig. 2B). Due to their importance in causing viral respiratory epidemics and pandemics, we also included the influenza A virus H1N1 in these inactivation experiments. H1N1 could be inactivated at concentrations of 60% WHO I and 40% WHO II, respectively (suppl. Fig. 1B). Furthermore, MVA was studied for its susceptibility to WHO formulations as it is the chosen test virus for all enveloped viruses in the European Guideline. In line with EBOV and H1N1, similar inactivation profiles could be observed with increasing WHO I and II concentrations (suppl. Fig. 1C).

Comparative inactivation profiles for WHO formulations against enveloped viruses

Based on the obtained virucidal activities of the WHO formulations against the different enveloped viruses, we next analysed the inactivation profiles in a comparative analysis (Fig. 2C, D). The most susceptible viruses to the WHO formulation I were the bovine and emerging CoVs (SARS-CoV, MERS-CoV and BCoV) and ZIKV (Fig. 2C). With a shift to increasing WHO I concentration, the more stable viruses included the full infectious EBOV (trVLPs excluded in this analysis) and HCV (Fig. 2C). The highest alcohol-based concentrations of WHO formulation I with more than 40% were required for H1N1 and MVA, which displayed nearly identical inactivation response curves (Fig. 2C). The results for the isopropanol-based WHO formulation II are depicted in Fig. 2D and demonstrated a similar pattern of susceptibility for the different enveloped viruses with an obvious shift towards lower concentrations (Fig. 2D). As for WHO formulation I, the CoVs and ZIKV showed the highest susceptibility to WHO formulation II, whereas HCV, EBOV, H1N1 and MVA demonstrated a more resistant inactivation profile (Fig. 2D). To also directly compare the performance of the two WHO formulations, we determined the concentrations at which the products reached the half maximal virus inactivation effective concentration 50 (EC_{50}) (suppl. Fig. 2). The WHO formulation II showed a significantly higher virucidal activity against the different viruses compared to WHO I ($p=0.0078$). In summary, CoVs and ZIKV showed the highest susceptibility to WHO formulations. EBOV and HCV were observed to be less susceptible than the CoV, whereas H1N1 and MVA were the most stable viruses. In addition, WHO formulation II demonstrated a higher virucidal effect compared to WHO formulation I.

Discussion

The WHO has recommended two formulations published in *Guidelines on Hand Hygiene in Health Care,* a document enforcing the use of cheap alcohol-based hand rubs to reduce the transmission of pathogens [1]. We aimed in this study to analyse the virucidal efficacies of these products, particularly against emerging or re-emerging viruses that caused severe epidemics in the recent past [2]. Importantly, both WHO formulations inactivated all tested viruses including ZIKV, EBOV and emerging CoVs in a suspension test with 30 second exposure time implicating their usability in viral outbreak situations. In case of ZIKV, specific viral inactivation data are lacking and consequently disinfection guidelines are based on data obtained from other members of the *flaviviruses*. So far, one recent study by Müller et al. reported that ZIKV was inactivated by classical inactivation methods including UV light [11]. ZIKV was readily reduced in viral titers by the WHO formulations, similar as the other member of the family of *Flaviviridae*, HCV. These findings are supported by earlier analyses of the environmental stability and inactivation profiles of HCV, which showed strong virucidal effects of the main WHO formulation ingredients ethanol and isopropanol [12]. For EBOV limited data on the efficacy of virucidal products are available as these viruses require high biosafety level laboratories. The Center for Disease Control (CDC) advises “suitable disinfectant solutions include 0.5% sodium hypochloride as well as 2% glutaraldehyde and phenolic disinfectants (0.5-3%) for EBOV inactivation [13]. The comparative inactivation analyses of all viruses tested revealed that the CoVs, in particular SARS-CoV, were the most susceptible viruses to WHO formulation treatment. The degree of susceptibility of the different viruses to the WHO formulation likely depends on the specific surface properties of the lipophilic envelope of the

respective virus. We could show by a comparative inactivation analysis that H1N1 and MVA showed the highest stability against alcohol-based inactivation, with higher concentrations of WHO formulation I and II being required compared to CoVs, ZIKV and EBOV. These results confirm MVA as the model surrogate viruses for all enveloped viruses for testing chemical disinfectants and antiseptics in human medicine [8]. When testing non-enveloped viruses like noro-, polio- or adenovirus a far higher level of resistance to both WHO formulations was observed, probably due to the more hydrophilic character of these viruses [3, 6]. Interestingly, WHO formulation I was superior compared WHO formulation II in inactivating these non-enveloped viruses, whereas in this study the opposite effect occurred with WHO formulation II exerting a higher virucidal activity against enveloped viruses. This discrepancy can be explained by the presence of the virus envelope, which likely renders enveloped viruses more susceptible to the isopropanol-based WHO II compared to the ethanol-based WHO I formulation [14]. Furthermore, isopropanol has one more carbon compared to ethanol giving it greater lipophilic properties and higher virucidal activities against lipophilic viruses [15].

In conclusion, WHO-recommended alcohol-based formulations were validated with different enveloped viruses. A strong virucidal effect against emerging pathogens including ZIKV, EBOV, SARS-CoV and MERS-CoV could be demonstrated implicating the usability of these WHO formulations in healthcare and outbreak-associated viral infections.

Acknowledgments.

We are grateful to Takaji Wakita and Jens Bukh for JFH1 and HCV isolates, to Charles Rice for Huh7.5 cells and the E9E10 monoclonal antibody, and to Thomas Hoenen for providing the EBOV trVLP system. We would like to thank Beatrice Zumkehr and Katharina Kowalski for technical assistance. Moreover, we thank all members of the Institute of Experimental Virology, Twincore, for helpful suggestions and discussions.

Figure legends

Figure 1

Virucidal activity of WHO formulations I and II against HCV, ZIKV, MERS-CoV and SARC-CoV. A) WHO formulations I and II were tested for their efficacy in inactivating HCV. The biocide concentrations ranged from 0% to 80% with an exposure time of 30 seconds. For this inactivation assay, one part virus and one part of organic load was mixed with eight parts of biocide. Residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infectious dose 50 ($TCID_{50}$) values (n.d.: not detected). The cytotoxicity was calculated in analogy to the determination of virus titer [$TCID_{50}$/ml] and is depicted as dashed line. The mean of two independent experiments with standard deviations are shown. Efficacy of WHO formulations I and II against ZIKV (B), MERS-CoV (C) and SARS-CoV (D) was addressed by a quantitative suspension assay as described for panel A.

Figure 2

Effect of WHO formulations I and II against EBOV and comparative viral susceptibility analysis

WHO formulations I and II were tested for their efficacy in inactivating EBOV trVLP (A) and EBOV (B). The biocide concentrations ranged from 0% to 80% with an exposure time of 30 seconds. For this inactivation assay, one part virus and one part of organic load was mixed with eight parts of biocide. For determination of the EBOV trVLP infectivity luciferase activity was measured 72 hours later. For EBOV, residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infectious dose 50 ($TCID_{50}$) values (n.d.: not detected). The

cytotoxicity was calculated in analogy to the determination of virus titer [$TCID_{50}$/ml] and is depicted as dashed line. The mean of two independent experiments with standard deviation are shown. Normalized values of percent inactivation of viral infectivity (y-axis) were plotted against WHO formulations I (C) or II (D) in dose-response curves (x-axis, log representation). Viruses are listed in each panel and are ranked from the most to the less stable. Normalization and non-linear regression calculation of all data were performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

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Figure 1

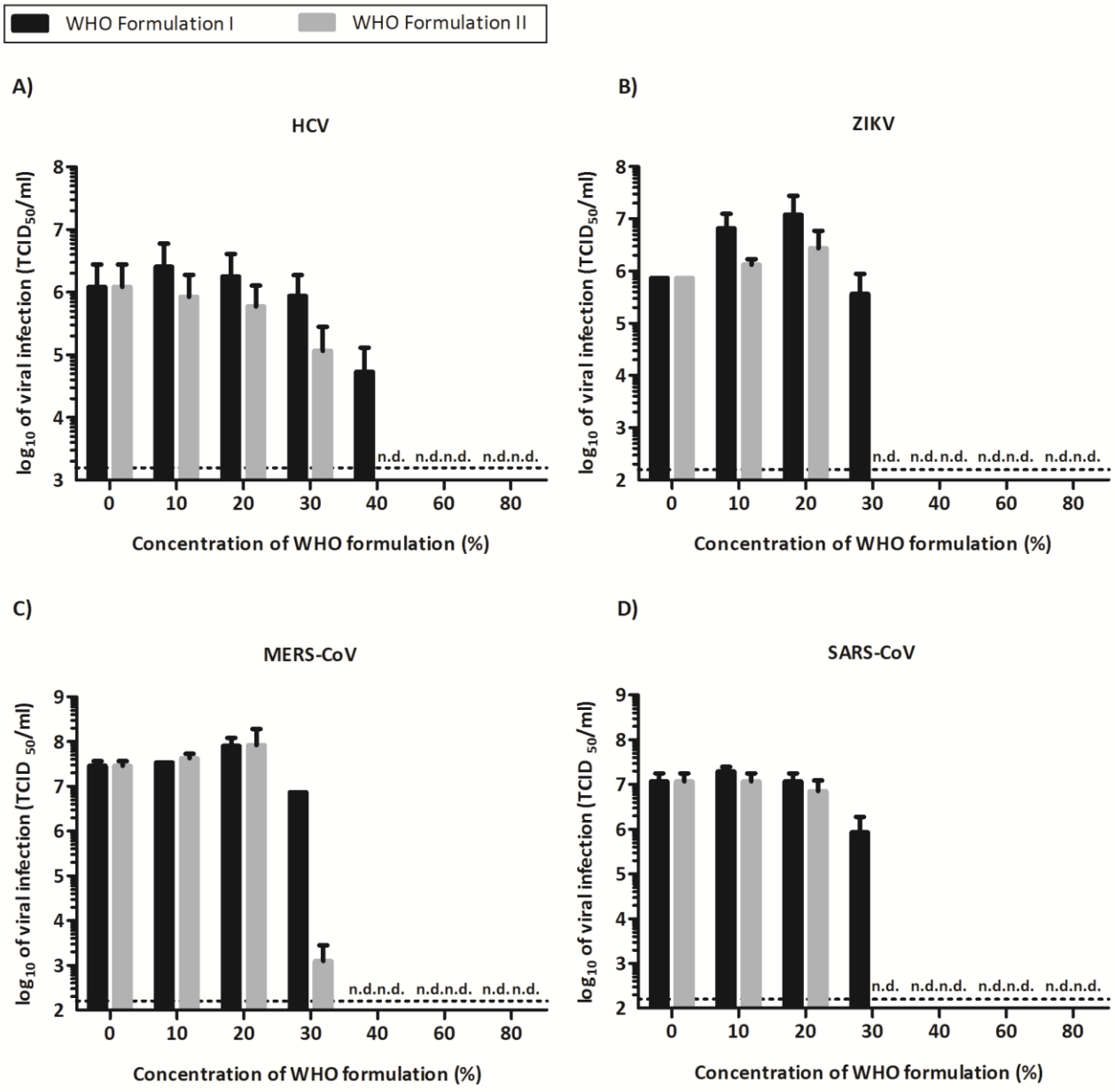
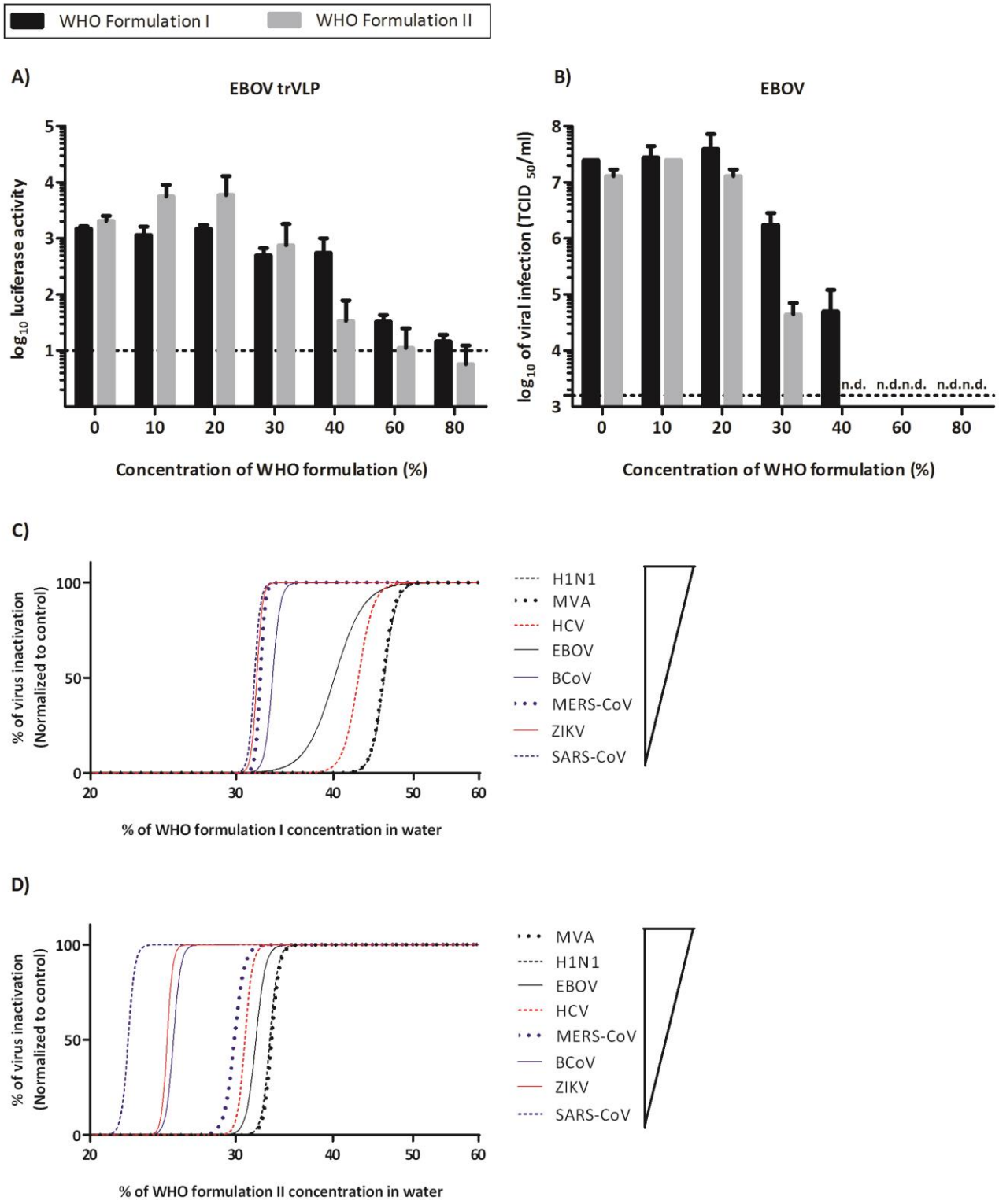

WHO Formulation I
WHO Formulation II
A)
HCV
log10 of viral infection (TCID50/ml)
n.d. n.d.n.d. n.d.n.d.
Concentration of WHO formulation (%)
B)
ZIKV
log10 of viral infection (TCID50/ml)
n.d. n.d.n.d. n.d.n.d. n.d.n.d.
Concentration of WHO formulation (%)
C)
MERS-CoV
log10 of viral infection (TCID 50/ml)
n.d.n.d. n.d.n.d. n.d.n.d.
Concentration of WHO formulation (%)
D)
SARS-CoV
log10 of viral infection (TCID 50/ml)
n.d. n.d.n.d. n.d.n.d. n.d.n.d.
Concentration of WHO formulation (%)

Figure 2


WHO Formulation I
WHO Formulation II
A)
EBOV trVLP
log10 luciferase activity
Concentration of WHO formulation (%)
B)
EBOV
log10 of viral infection (TCID 50/ml)
n.d. n.d.n.d. n.d.n.d.
Concentration of WHO formulation (%)
C)
% of virus inactivation (Normalized to control)
% of WHO formulation I concentration in water
H1N1
MVA
HCV
EBOV
BCoV
MERS-CoV
ZIKV
SARS-CoV
D)
% of virus inactivation (Normalized to control)
% of WHO formulation II concentration in water
MVA
H1N1
EBOV
HCV
MERS-CoV
BCoV
ZIKV
SARS-CoV