Dstl Porton Down
Salisbury
SP4 0JQ

Issued 30th July 2020

Experimental survival of SARS-CoV-2 on an insect-repellent-treated surface

Dstl was tasked by MoD Surgeon General to examine the anti-viral activity of Mosi-guard Natural® spray against SARS-CoV-2 virus. Two approaches were adopted: assessment of the anti-viral activity of the product (and selected individual components of the formulation) when applied directly to the virus as a liquid drop, and assessment of the product following its application to latex 'synthetic skin'.

This experimental work has not been externally peer-reviewed.

1.1 Summary

1.1 In a standard assay (liquid contact), mixing a virus suspension with Mosi-guard Natural® spray or selected constituent components resulted in a reduction in SARS-CoV-2 England-2 isolate titres after 1 minute.

- Mosi-guard Natural® (50% v/v) and Citriodiol® (50% v/v) decreased viral titre by approximately two to three $\log_{10}$ respectively, but not below the limit of quantification.
- At a higher concentration (90% v/v), Mosi-guard Natural® gave a significant decrease of over a four $\log_{10}$ drop in viral titre, resulting in no recoverable virus.

1.2 In a surface contact assay, some loss of recoverable virus was observed over 4 hours when SARS-CoV-2 England-2 isolate was placed on a latex 'synthetic skin' surface (sprayed ~1 hour beforehand with Mosi-guard Natural). SARS-CoV-2 England-2 isolate was recoverable from all Mosi-guard Natural®-treated surfaces tested.

- Pre-application of Mosi-guard Natural® to a latex synthetic skin resulted in a reduction of at least 1 $\log_{10}$ in viral titre of SARS-CoV-2 England-2 isolate ($p<0.001$) at all time-points and an approximate 2 $\log_{10}$ reduction after four hours on the surface.
- There was evidence that the Mosi-guard Natural® treatment on latex synthetic skin affected the survival of the virus over a four hour period ($p=0.015$).
- Virus was recoverable at all time points from Mosi-guard Natural®-treated latex synthetic skin at a level consistently above the limit of quantification.

2. Key insights

2.1 One minute liquid suspension tests indicated that Citriodiol®, ethanol, isopropanol and Mosi-guard Natural® have anti-viral activity against SARS-CoV-2 England-2 isolate if mixed with the virus in the liquid phase.

2.2 SARS-CoV-2 England 2 isolate survival studies on latex as a 'synthetic skin' (which was pre-treated with Mosi-guard Natural® approximately 1 hour before testing) provided evidence ($p=0.015$) that Mosi-guard Natural® has anti-viral properties against SARS-CoV-2 England-2 isolate.

2.3 SARS-CoV-2 England-2 isolate was recoverable from all Mosi-guard Natural®-treated latex synthetic skin' and did not result in complete virus loss over the four hour test period.

2.4 SARS-CoV-2 England-2 isolate was recoverable from one of three Mosi-guard Natural®-treated plastic surfaces at four hours.

3. Key caveats

3.1 The first test described in this report was a one minute contact time test to ascertain whether Mosi-guard Natural® or components (Citriodiol®, isopropanol, ethanol) had any antiviral properties. Fixed concentrations were chosen to reflect the reported formulation of Mosi-guard Natural®[1].

3.2 The second test presented here related to the survival of SARS-CoV-2 England-2 isolate (suspended in tissue culture medium) on two artificial surfaces, plastic and latex synthetic 'skin': both surfaces were sprayed with Mosi-guard Natural® (referred to herein as Mosi-guard) approximately 1 hour before being exposed to the virus.

3.3 There was a 52 and 57 minute delay between application of the test compound, to the synthetic skin and plastic respectively, and the application of the virus onto the treated surface ('time 0' in the figures). Thus, any antiviral-activity in the first hour of application was not accounted for. Due to this delay, it was assumed that most of the alcohol components of Mosi-guard evaporated prior to application of virus.

3.4 Only one application on the latex was made: the effect of varying the concentration of Mosi-guard on the latex, on virus survival was not investigated.

3.5 Tissue culture medium (TCM) is a matrix likely to be optimal in terms of viral survival.

3.6 Latex synthetic 'skin' was impregnated with Mosi-guard; latex is used as a representative surface but is unlikely to behave exactly as treated human skin We have no data relating the concentration applied experimentally to the latex to that resulting from a spray and rub application of Mosi-guard on human skin.

3.7 These tests do not test the effect of aging (i.e. change in performance) of the Mosi-guard on the latex synthetic skin over time on virus survival (other than the inherent aging effect in the test described above).

3.8 The wetting behaviour of the virus droplet on the Mosi-guard covered plastic and latex synthetic skin was observed to be different. On the Mosi-guard treated 24 well plate only, the virus droplet did not remain as a sessile droplet but completely wetted the surface of the well to the edges. Therefore under these conditions the

surface area of virus and Mosi-guard interaction dynamics were different to the untreated controls and treated latex skin

3.9 Testing was conducted at room temperature only (19°C-22°C), in class III microbiological safety cabinets with 1000 air changes per hour and ambient relative humidity (approximately 35%RH) and indoor light experimental conditions.

3.10 The mixed liquid product/virus droplet test followed a standard laboratory test which has been conducted on range of viruses at Dstl, and allows direct comparison of data. Dstl has previously published similar work on Ebola virus[2].

3.11 Dstl did not test p-menthane-3,8-diol (PMD) or any other individual components of Mosi-guard or Citriodiol®, other than the ethanol or isopropanol in the liquid product/virus droplet test.

3.12 Ethanol, rather than denatured ethanol, was used in these tests.

3.13 No British Standard disinfectant tests were carried out.

3.14 No material compatibility tests were carried out with items of personal protective equipment (PPE).

4. Key assumptions

4.1 This study was conducted using the SARS-CoV-2 England-2 isolate. This isolate was isolated from a patient in the UK and obtained from Public Health England (PHE) Colindale. Although other isolates may behave differently within the experimental design described; it is assumed that all isolates and subsequent passages will behave in a similar manner.

5. Results

Liquid contact disinfection test

5.1 Viral recovery was classified into three groups; i) SARS-CoV-2 recovered and quantifiable in the $TCID_{50}$ assay, ii) SARS-CoV-2 recovered but below the limit of quantification (LoQ) by flask passage and iii) no evidence of cytopathic effect (CPE) (Figures 1 & 2).

5.2 In this test, all treatments tested gave a significant decrease in viral titre compared to a SARS-CoV-2 England-2 isolate control ($p < 0.001$) in the liquid-surface mix test (Figure 1A).

5.3 Mosi-guard (final concentration 50% v/v) and Citriodiol® (50% v/v) decreased viral titre by approximately two to three log_{10} respectively, but not below the LOQ.

5.4 A three log_{10} reduction was observed after 40% ethanol treatment (growth of virus was only seen in the second passage in flask: Figure 2)

5.5 20% isopropanol only reduced the viral titre by approximately one log_{10}. 20% ethanol and 10% isopropanol combined in the same test reduced viral titre below the limit of quantification, a three log_{10} reduction. Viable virus was recovered from both test conditions (Figure 1A, 2).

5.6 A concentrated viral titre test, with a final concentration of 90% Mosi-guard (v/v), showed a significant decrease of over a four log_{10} drop in titre compared to a SARS-CoV-2 England-2 isolate control ($p < 0.001$), returning a result below the LoQ with no virus recovery after two rounds of flask passage (Figure 1B, 2).

Survival of SARS-CoV-2 on Mosi-guard-treated latex synthetic skin

5.7 There was no measureable reduction in viral titres on untreated samples (latex synthetic skin p=0.547 and plastic 24 well plates p=0.113: Figure 3).

5.8 There was a reduction in viral titre on Mosi-guard treated plastic and latex synthetic skin ($p<0.001$), compared to the untreated controls at all time-points (Figure 3).

5.9 Treated plastic 24 well plates had a larger reduction in titre but the spread dynamics of the experiment may have played a role. Treated plastic well viral titres fell to the LoQ at 3 and 4 hours, but live virus remained (demonstrated by subsequent positive flask recovery, data not shown).

5.10 Viral reduction due to Mosi-guard was of at least 1 $\log_{10}$ at all time-points with an approximate 2 $\log_{10}$ reduction by the end of the time-course for latex synthetic skin.

5.11 ANCOVA was used to compare decay curves. There was moderate statistical evidence that the Mosi-guard treatment affected the decay rate on the latex synthetic skin ($p=0.015$) and strong statistical evidence on the tissue culture 24 well plate ($p<0.001$). Little evidence was found for differences between surface conditions for untreated samples ($p=0.6302$). For Mosi-guard treated samples only, there was moderate statistical evidence of the surface type altering the decay of the samples ($p=0.006$).

5.12 Under the conditions tested, pre-application of Mosi-guard to a latex synthetic skin resulted in a reduction in viral tire of SARS-CoV-2 England-2 isolate ($p<0.001$). However, SARS-CoV-2 England-2 isolate was recoverable from all surfaces tested and did not result in complete viral inactivation on the latex synthetic skin.

5.13 In summary, SARS-CoV-2 England-2 was recovered from all treated samples.

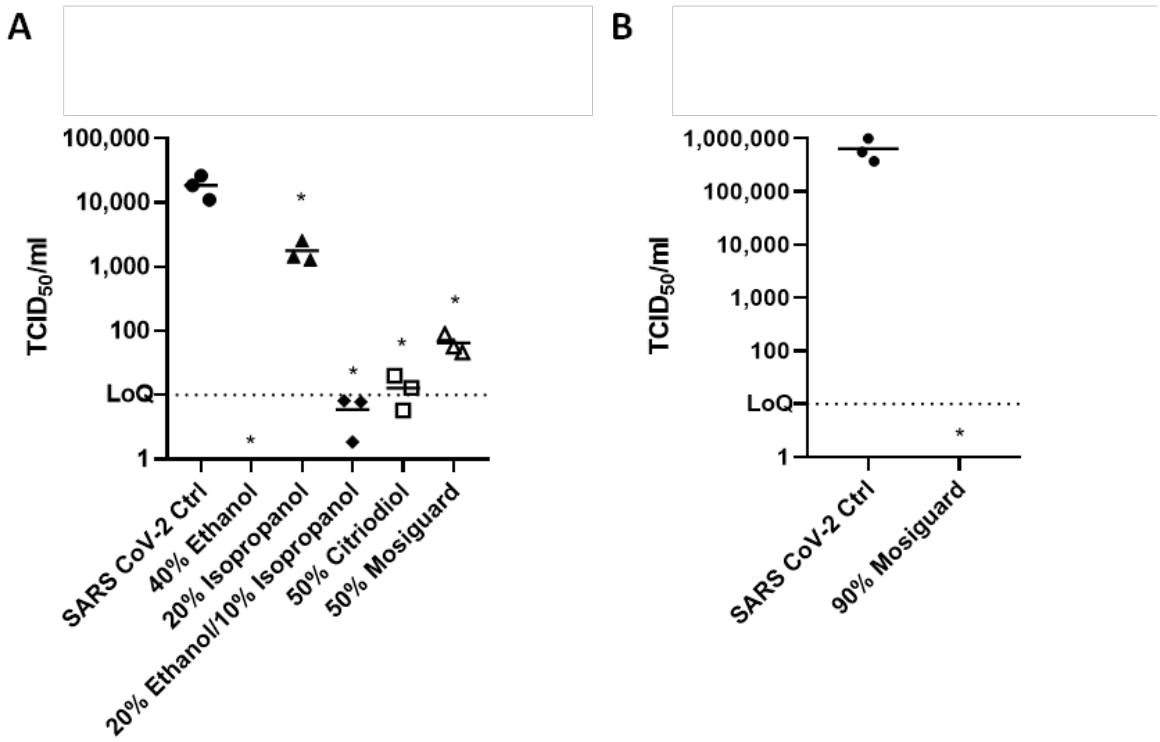

Figure 1: The effect of Mosi-guard Natural® and its constituent components on the survival of SARS-CoV-2 England-2 isolate. A) Working concentrations of test products have been calculated on a 1:1 ratio with virus. Statistical significance was determined by 1-way ANOVA ($p < 0.001$) and Dunnett's tests informed that each component was different from the virus only control (*$p < 0.001$) B) Working concentrations of test products have been calculated on a 1:9 ratio with virus. Statistical significance was determined by T test with a Welch's correction ($p < 0.001$). The dashed line indicates the limit of quantification (LoQ) of the $TCID_{50}$ assay.

	$TCID_{50}$ Assay	Flask Passage 1	Flask Passage 2
40% Ethanol	–	–	+
20% Isopropanol	+	+	ND
20% Ethanol / 10% Isopropanol	+	+	ND
50% Citriodiol	+	+	ND
50% Mosi-guard	+	+	ND
90% Mosi-guard	–	–	–

+ SARS-CoV-2 recovered
- SARS-CoV-2 not recovered
ND Not determined

Figure 2**:** Viral recovery of SARS-CoV-2 England-2 isolate from test samples. Where there is recorded evidence of the presence of virus it is denoted by "+". Where there is no evidence of virus recovery it is denoted by "-". Where positive recovery was achieved from the first passage in flasks further secondary passage in flasks was not performed, as indicated by "ND". The only test sample to show no evidence of the presence of virus was 90% Mosi-guard.

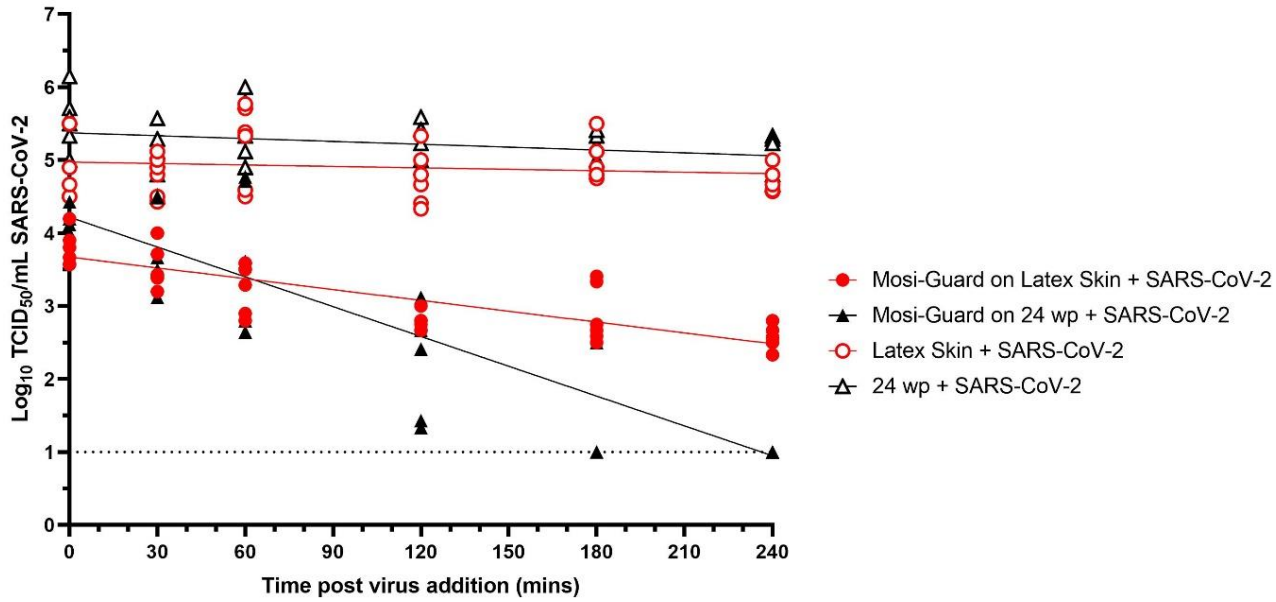

Figure 3. The effect of Mosi-guard Natural® against SARS-CoV-2 England-2 isolate on different surfaces over time. Virus was deposited on to Mosi-guard treated (full shapes) or un-treated (open shapes) surfaces of synthetic latex skin (red, circles) or tissue culture 24 well plates (wp: black, triangles). On the Mosi-guard treated well plate only, virus suspension dispersed over the surface of the well to the edges (i.e. did not remain as a droplet); thus the surface area of virus and Mosi-guard interaction dynamics were likely different to the untreated controls and treated latex skin.

Linear regression analysis is indicated by the red and black lines respectively. The dotted line at Log_{10} $TCID_{50}$=1 is the limit of detection of the $TCID_{50}$ assay: viable virus was still recoverable from Mosi-guard-treated plastic at 180 and 240 minutes. At four hours on plastic, only 1 of 3 had viable virus recovery, at a level below the LoQ.

6. Methods

6.1 Citriodiol® is a trade name for a commercial preparation of Eucalyptus citriodora oil. Formulations containing Citriodiol® are widely used as mosquito repellents. A major component of Citriodiol® is p-menthane-3,8-diol (PMD).

6.2 Mosi-guard Natural® is a commercial insect repellent spray that features Citriodiol® (30-50%) as the active ingredient (as well as ethanol 20-40%; water 10-30% and isopropanol: 5-20%[1]). Provision of Mosi-guard and Citriodiol® formulations to Dstl was facilitated by Army Health and Performance Research (AHPR)

6.3 SARS-CoV-2 England-2 isolate was provided by PHE. Passage 2 stocks were grown in Vero C1008 cells, therefore Passage 3 stocks of virus were used this these experiments. Enumeration was via 50% tissue culture infectious dose ($TCID_{50}$) assay[4] read 3 days post-infection.

6.4 Where data were returned below the limit of quantification of the $TCID_{50}$ assay, it was important to understand if complete viral clearance had occurred. As such, samples were also passaged through 2 rounds of cell culture flasks in order to see if any virus was recovered (as evidence by cytopathic effect following viral replication in cell culture).

6.5 A two phase experimental approach was taken to testing the antiviral activity of Mosi-guard: liquid/liquid disinfectant testing and viral survival on Mosi-guard-treated latex synthetic skin (liquid/surface test).

Disinfection testing

6.6 Mosi-guard Natural® and selected constituent components were tested in a liquid disinfection test. 25µl each of virus and the test preparation were mixed and placed on a surface for a single contact time (1 minute) on a single surface (flat bottom, sterile, polystyrene cell culture 24 well plate). Mean initial viral titres for these tests were approximately $1.88x10^4$ $TCID_{50}$ ml^{-1}.

6.7 Samples were stored in lidded 24 well plates within a class III microbiological safety cabinet environment.

6.8 A fixed concentration of test compound was compared individually: Mosi-guard (50% v/v), Citriodiol® (50% v/v), ethanol (40% final concentration), isopropanol (20% final concentration) and 20% ethanol and 10% isopropanol combined.

6.9 An additional experiment was performed using a concentrated virus at a higher viral titre, with a final concentration of 90% Mosi-guard (v/v). Mean initial viral titres for these tests were approximately $3.45x10^5$ $TCID_{50}$ ml^{-1}.

6.10 After 1 minute, the test sample was recovered in TCM, and centrifuged and washed in TCM three times. Triplicate samples of each test condition were tested for viable virus by $TCID_{50}$ assay and the presence of low levels of virus tested by subsequent passage in flasks.

6.11 Tissue culture media (TCM) was Leibovitz L-15 media supplemented with 2% (v/v) foetal calf serum, 2 mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin.

6.12 For initial tests statistical significance was determined by 1-way ANOVA ($p < 0.001$) and Dunnett's tests informed that each component was different from the virus only control ($p < 0.001$). Validity of these tests was assessed by Brown-Forsythe test (P=0.752), QQ plot and residual plot. For studies with concentrated virus a T test with a Welch's correction was used ($p < 0.001$).

Survival of SARS-CoV-2 on Mosi-guard-impregnated synthetic skin

6.13 0.05 mm thick latex (Hygenic Corporation medical grade latex, Hytone HPN-09814 variant, 42" wide, 0.020" thick) 'synthetic skin' was sprayed with Mosi-guard via a SprayCraft SP50K air brush (using a compressed air supply at 1 bar pressure) and cut into 10 mm diameter discs using a Samco cutting press.

6.14 Individual latex synthetic skin discs, were placed in each well of a 24 well tissue culture plate with the treated surface facing up. A second 24 well plate with no synthetic skin coupons added was also sprayed with Mosi-guard as a treated-surface control. Coating dynamics on each surface were different: on plastic, Mosi-guard spray lead to an even distribution of various size discrete droplets over the surface area of the plastic, whereas the application to latex skin appeared more evenly spread.

6.15 To assess the survival on the skin over time, SARS-CoV-2 England-2 isolate was added as a 10 µL droplet to each of 18 wells with Mosi-guard coated latex synthetic skin sections (52 minutes after initial coating), 18 wells of the Mosi-guard treated plastic plates (57 minutes after initial coating), and to the same number of untreated surface and latex synthetic skin controls. The droplet was not spread.

6.16 Mean initial viral titres for these tests were 7.03×10^3 $TCID_{50}$ ml^{-1} and 1.37×10^5 $TCID_{50}$ ml^{-1} for Mosi-guard and control latex synthetic skin respectively.

6.17 Behaviour of the virus droplet was different: on the Mosi-guard treated 24 well plate only, the virus droplet did not remain as a droplet but dispersed over the surface of the well to the edges. Therefore, under these conditions, the surface area of virus and Mosi-guard interaction dynamics will have been different to the untreated controls and treated latex skin.

6.18 10 µL of TCM was added to the remaining 6 wells/coupons on each place (without virus) as negative control for residual interference to the $TCID_{50}$ assay after the washing steps (rather than a direct toxicity test of the disinfectant on the cell line, which was carried out prior to testing, indicating the requirement for 3 washing steps).

6.19 At pre-determined time-points after the addition of virus (0, 30, 60, 120, 180 and 240 mins) triplicate samples from each condition plus a negative control from each condition were processed via recovery in TCM, 3 washes and viral quantification by $TCID_{50}$ assay.

6.20 The software Graphpad PRISM version 8 was used to graph, model and statistically analyse the data. The viral counts (estimated by $TCID_{50}$ assay) were transformed to the logarithm of 10 to better fit a Gaussian distribution. The mean for technical duplicates were used. Linear regression analysis on the logarithm-transformed data was used to model the data. Analysis of covariance (ANCOVA) was used to compare the data-sets from the four conditions tested. The data showed little evidence for non-Gaussian distribution or heteroscedasticity.

References

1. Citrefine Safety data sheet mosi-guard natural Spray http://ww.mosi-guard.com/Media/MSDS%20Spray.pdf.
2. Smither, S. *et al*. (2016). Effectiveness of four disinfectants against Ebola virus on different materials. *Viruses*, 8, 185.
3. Reed, L.J. & Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg*. 27, 493-497.