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Evaluating the antimicrobial efficacy of long-lasting hand sanitisers on skin

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Summary

Background

The microbicidal efficacy of hand sanitiser formulations are usually measured through standardised quantitative suspension tests and fingerpad tests; these cannot evaluate long-lasting formulations or are impractical due to biological risks, costs or time required for testing. With increased numbers of long-lasting microbicidal activity claims of commercially available hand sanitisers, alternative testing strategies are required.

Aim

To explore the use of a standardised ex-vivo pig skin model to reproducibly measure long-lasting efficacy of an alcohol-free hand sanitiser formulation.

Methods

The microbicidal efficacy of an alcohol-free hand sanitiser was tested against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, and the enveloped virus SARS-CoV-2 with quantitative suspension tests (EN13727 and EN14476) with a contact time of 5 min. The product was then tested over a 6-hour period using an ex-vivo pig skin model with a modified version of PAS 2424 to simulate the impact of skin abrasion.

Findings

Quantitative suspension tests yielded a $> 5\text{-log}_{10}$ reduction for all organisms tested within a 5 min contact time. Pig skin tests showed reduced but consistent efficacy at all time points and indicated no significant impact of abrasion on efficacy.

Conclusion

The use of the ex-vivo pig skin model provides a potentially viable and convenient model system to test long-lasting hand sanitisers formulations, providing a path for sustainable hand sanitiser formulation claims of activity on skin.

Keywords: hand sanitiser, lasting activity, ex-vivo test,

Introduction

The antimicrobial activity of hand sanitisers and similar disinfectant products is determined through the use of standard efficacy test protocols that define conditions under which products should be tested. These tests enable manufacturers to make a product claim on label. Quantitative suspension tests are often used as a primary means of determining hand sanitiser efficacy, although standards are also available for hand wash products that are based on fingerpad testing to determine transient transfer of microbes (Table I). While straight forward to perform, suspension tests may result in an inflated demonstration of product efficacy compared to tests conducted either directly or indirectly on skin [1–3]. Undoubtedly, there are a number of advantages to using the fingerpad testing approach on human volunteers, including the use of intact “live” skin, and human behaviour which includes multiple surfaces touching that cannot be perfectly mimicked in ex vivo models. However, testing for virucidal activity on human volunteers is limited due to associated infection risks and it can only be performed by trained virologists in facilities equipped for infectious work at biosafety level-2 (BSL-2; see ASTM E1838-17 and pr EN17430). The use of bacteriophages, such as MS-2, as surrogates for mammalian viruses [4–6] may overcome the need for dedicated virology facilities in testing, however, they cannot replace the full range of required viral test strains.

The COVID-19 pandemic, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), resulted in increased usage of hand sanitisers [7] and led to product claims of long-lasting microbicidal formulations that reportedly remain active over several hours. Determining the long-lasting efficacy of such formulations requires a more enhanced testing procedures than afforded by suspension tests in order to determine that formulations remain active on skin over prolonged time periods. Fingerpad tests are impractical in this situation due to the length of time volunteers would need to remain sequestered in the testing facility, in addition to the health risks associated with using viruses. In addition, testing approaches using human volunteers require participants to refrain from using medicated soaps and lotions for considerable periods before the test period and risk volunteers drop-out before test completion.

Ex-vivo tests based on the use of skin as a substratum have been used to determine the efficacy of skin disinfectants against bacterial pathogens [8–11] and the flexibility

of the test enables the effect of repeated biocide exposures to be measured, as well as understanding bacterial regrowth post-application [12,13]. The use of the ex-vivo test also correlated well with fingerpad-based tests [14]. Due to its similarities to human skin, sterile pig skin [7,8,12] offers an alternative for in-use testing of hand sanitisers, without the need of human volunteers or sourcing of human skin samples. In addition, the use of the ex vivo pig skin model presents a practical and a much safer alternative to using pathogenic viruses on volunteer's hands [3] which may cause ethical concerns. It should however be noted that pig skin has a thicker stratum corneum than human skin which in this case may contribute favourably to the stringency of the test. Artificial skin models could be considered to performing such tests, but the associated costs and lack of variability in surface topography make them a less attractive substitute.

The feasibility of pig skin as a substrate to test disinfectants has been demonstrated under standard test procedures using bacteria, where disinfectant is applied to skin pieces previously inoculated with the test microorganism. However, the ex vivo test has not been used to look at the long-term efficacy of products on skin nor has it been used with viruses [12,14,15].

In response to a problem identified by a manufacturer of a hand sanitiser product, where a suitable protocol to test residual activity could not be found, we evaluated the suitability of using viruses with the ex-vivo pig skin model and determined its suitability for substantiating long-lasting activity claims on skin against diverse pathogens.

Materials and Methods

Bacterial Test Strains

A selection of clinically important bacterial species that can be transmitted by hands were selected: *Staphylococcus aureus* ATCC 6538; *Escherichia coli* ATCC 10538; *Klebsiella pneumoniae* NCTC 13368. Bacteria were sub-cultured from frozen stocks onto tryptone soya agar (TSA) and incubated at 37°C for 24h. An inoculum was prepared by transferring 3 colonies into tryptone soya broth (TSB) and incubating with agitation at 37°C for 16h. Bacteria in the overnight inoculum were resuspended in sterile tryptone sodium chloride (TSC; peptone, pancreatic digest of casein: 1 g;

NaCl: 8.5 g; water 1L; pH 7.0 ± 0.2) and optical density (OD_{600nm}) adjusted to get a test inoculum concentration of 1.5 to 5.0×10^8 cfu/mL.

Cells and Virus

This study used SARS-CoV-2 rather than other enveloped viruses and non-enveloped viruses since, at the time this work was conducted, there were still uncertainties in the importance of secondary transmission of SARS-CoV-2 from surfaces to hands.

VeroE6 cells expressing ACE2 and TMPRSS2 (Vero A/T) were maintained at 37°C , 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma). The England-2 strain of SARS-CoV-2 was obtained from Public Health England and grown in DMEM, supplemented with 2% FBS.

SARS-CoV-2 was quantified by plaque assay on Vero A/T cells seeded at 1×10^5 /well in 12-well plates. Samples were 10-fold serially diluted in 2%-DMEM and cells infected in duplicate for 1 h at 37°C , 5% CO_2 . Virus inoculum was removed and cells overlaid with a 1:1 mix of 2.4% Avicel® and 2X MEM (20% 10X MEM; 2% L-Glutamine; 4% FBS; 5.4% sodium bicarbonate (7.5% soln.)). Cells were incubated for 72 h at 37°C , 5% CO_2 at which point overlay was removed, cells were washed once with PBS and fixed with 1 mL/well methanol for 5 min. Cells were stained with 0.1% (w/v) crystal violet, and plaques counted. All SARS-CoV-2 work was carried out in an approved Category 3 facility.

Product

An alcohol-free hand sanitiser formulation based on didecyl ammonium chloride and benzalkonium chloride was kindly provided by a manufacturer. The product was ready to use. The contact times and volume of product used were as per the manufacturer's instructions.

Suspension Test

Suspension tests were used as an initial screening tool to demonstrate bactericidal efficacy of the product against bacteria of clinical interest. Suspension tests were conducted according to EN13727 for bactericidal testing [16], and EN14476 for virucidal testing [17] in the presence or absence of an interfering substance (Bovine

serum albumin; BSA). However, the full range of bacterial species listed in EN13727 were not tested and some bacteria were substituted for more clinically relevant ones. Briefly 9 mL of product was inoculated with 1 mL of washed bacterial suspension ($1.5\text{--}5.0 \times 10^8$ cfu/mL) and 1 mL of organic load (final concentration of 0.3 g/L BSA) for 5 min before neutralisation which was achieved by adding 1 mL samples to a 200-fold excess of neutralising solution (Lecithin 10 g/L, Tween80 30 g/L, Sodium Thiosulphate 20 g/L, L-Histidine 1 g/L, Saponin 30 g/L, Sodium Dodecyl Sulphate 5 g/L in deionised water). Samples were 10-fold serially diluted in tryptone sodium chloride (TSC) and enumerated by pour-plate plating on tryptone soy agar (TSA) and overnight incubation at 37°C.

For SARS-CoV-2, 50 µL of virus suspension at 4×10^7 pfu/mL was added to 450 µL of product, and BSA at a final concentration of 0.3 g/L for a 1- or 5 min contact time. For neutralisation, an equal volume of DMEM was added upon completion of the contact time and 100 µL was added to a Microspin S-400 HR column (Cytiva) and centrifuged for 2 min at 700 x g. Eluates were 10-fold serially diluted and titrated by plaque assay as above.

Ex-vivo Test

Freshly obtained pig ears were washed in sterile deionised water, shaved, and 2 x 2 cm sections were excised with a scalpel. Pig skin sections were sterilised each side by 3 x 10 min cycles of exposure to UV-C (254 nm), with 10 min rest between cycles. One hundred µL of product was applied to 2 cm² pieces of sterile pig skin and left to dry for 1 h in a Biosafety level 2 cabinet. Test skins with 100 µL TSC (bacterial tests) or 100 µL DMEM (virucidal tests) were included as controls. After drying, triplicate pieces of skin were subjected to 1 of 3 wiping conditions using a modified form of abrasions tests outlined in BS PAS 2424:2014 [18] (1) no wiping, (2) skin wiping and (3) wet cloth wiping. For skin wiping, the protocol was based on Messenger et al. [12] with the difference that an untreated skin piece was attached to the lid of a 50 mL centrifuge tube containing 210 (± 2) g of metal weights. Using only the pressure exerted by the weights, the untreated skin piece was wiped backward and forward twice over the test skin. For wet cloth wiping, a double layer of 4 x 4 cm² piece of J-Cloth previously dampened with sterile water was attached to the lid of a 50 mL centrifuge tube containing 210 (± 2) g of metal weights. Using only the pressure exerted by the weights, the J-Cloth was wiped backward and forward twice over the

test skin. Skin samples were kept in an environment maintained at 60% relative humidity at 21°C post-wiping. At 1-, 2-, or 6-hours post-wiping, test skins were inoculated with a bacterial (100 µL of $1.5\text{--}5.0 \times 10^8$ cfu/mL) or viral (100 µL of 4×10^7 pfu/mL) suspension for a contact time of 5 min. For bacterial tests, each piece of skin was transferred to a 50 mL falcon tube containing 5 g of glass beads and 20 mL of neutralising solution. The tubes were vortexed for 5 min to remove adhered bacterial cells and 10-fold serial dilutions were prepared in TSC and enumerated as per suspension tests.

To recover residual SARS-CoV-2, 1 mL of DMEM was washed over the surface of each skin piece 5X and transferred to 1.5 mL tubes. Residual product was neutralised by applying 100 µL of wash medium to a Microspin S-400 HR column (Cytiva) as per suspension test, and virus enumerated by plaque assay.

Statistical analysis was determined by 2-Way ANOVA with Tukey's multiple comparisons tests using GraphPad Prism (v9.5.1).

Results and Discussion

The alcohol-free hand sanitiser demonstrated bactericidal and virucidal efficacy ($>5 \log_{10}$ microbial reduction) within a 5 min contact time in the presence of soiling in the suspension test (Table II). Against SARS-CoV-2, no residual virus was detected after 5 min. Due to the limits of assay sensitivity this equated to a minimum $\log_{10}$ reduction of 5.08. We repeated the SARS-CoV-2 suspension test with a 1 min contact time and again no residual virus was detected (Table II).

This study aimed to investigate an appropriate protocol to sustain skin disinfectant claims of long-lasting activity. To validate claims of long-lasting antimicrobial activity, it has been suggested that tests must be conducted in which the disinfectant is applied first, followed by inoculation with the test microorganism [19]. Following this principle and combining the ex-vivo test with some principles from the PAS2424 [18] test we mimicked different skin abrasions using skin to skin contact or wet wiping, albeit only one abrasion event took place following product deposition and drying on skin. In comparison to the suspension tests (Table I), we observed reduced efficacy against all organisms tested, with no tests reaching the required mean $4\text{-}\log_{10}$

reduction (Figure 1). When we considered the simplest test condition (unwiped and inoculation 1h post treatment), *K. pneumoniae* was the least susceptible with <1 $\log_{10}$ reduction, followed by *E. coli*, *S. aureus* and SARS-CoV-2 with approx. 2 $\log_{10}$ reduction. Longer product incubation time on skin did not result in a significant change in product activity (Figure 1) demonstrating a residual activity of the product on skin when left untouched. Our results correlate with previous studies of short-term virus disinfection on human hands [3], and suggest that suspension tests may overestimate hand sanitiser efficacy. Skin models are inherently more complex than suspension tests, and factors such as differences in the organism/disinfectant ratio, penetration of product into the dermis, and evaporation/dehydration of product from skin, will contribute to reduced efficacy. However, these factors are present during real-world use and therefore should also be reflected in testing procedures.

We observed no statistically significant difference in product activity following different residual contact times, with any of the wiping conditions tested for *E. coli*, *K. pneumoniae*, or SARS-CoV-2 (Figure 1). We did observe a significant reduction in efficacy against *S. aureus* for skin-skin abrasion at 6 hr compared to 1 hr ($P \leq 0.05$), and for skin-skin abrasion compared to unwiped at 6 hr ($P \leq 0.01$; Figure 1A). Apart from *S. aureus*, the addition, of one abrasion event following product deposition did not impact on the observed bactericidal or virucidal efficacy over time for these microorganisms. This may be counter intuitive as one might have expected product to be transferred to the skin or cloth (2nd or 3rd test conditions) during abrasion. Although we did not quantify the amount of active (didecyl ammonium chloride and benzalkonium chloride) deposited or left on skin following initial deposition or after abrasion, there was enough active to maintain a microbicidal effect against the test microorganisms even 6 h following initial deposition with or without abrasion. Our experimental approach was simple and differed from the PAS2424 [12] in that it did not use wet and dry regular abrasions as well as skin reinoculation with microorganisms over a period of time and with that in mind, it was less stringent than the PAS2424 [12]. In addition, we used a 5 min contact time, as recommended by the manufacturer, which might not reflect the reality [20]. Other shortcomings of this work should be noted. The use of SARS-CoV-2 only, which at the time was the virus of concern with uncertainties as to its secondary mode of transmission from surfaces to hands; the use of more relevant viruses known to be transmitted by hands such as norovirus (surrogate) and another enveloped virus surrogate (e.g. vaccinia virus)

would now be more appropriate. The use of other products including an alcohol-based one with no residual activity, and possibly a chlorhexidine-based one would have strengthened our results.

Overall, this study showed that relying on suspension tests alone may not provide an accurate measure of product performance and in-use tests should be included where appropriate. The ex-vivo pig skin model offers a potential option for in-use testing, that may better mimic real-world results, particularly for long-lasting formulations where the use of fingerpad tests is impractical. Whilst the use of ex vivo test as described in this study would be suitable to help the industry to investigate residual activity of products for hygienic hand washing, it would not necessarily be appropriate at this stage, for investigating products for surgical handwash, since the wearing of surgical gloves would create a different environment on skin, likely impacting product efficacy and biocidal longevity.

Conclusions

Long-lasting hand sanitiser formulations need to be tested under in-use conditions to ascertain efficacy over long time periods. Whilst the fingerpad testing approach certainly offers testing under in-use conditions, the use of human volunteers presents many challenges, including volunteer dropout, unfeasible time periods and biological risks when using pathogenic microorganisms. On the other hand, the PAS2424 test is conducted using stainless steel surfaces and cannot truly simulate the residual activity that would occur on skin. The use of the ex-vivo skin model as described here provides a potential alternative that merit to be further explored. Although this paper presents a test method to measure the residual efficacy of for hand sanitisers on skin, ultimately the protocol would need to be validated by other laboratories before it can be widely recognised and used.

Conflict of Interest

None to declare

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Table I. Standard efficacy protocols to evaluate the efficacy of skin disinfectants

Product Type	Standard	Test Type	Test Microorganism
Disinfectant	EN1276	Quantitative suspension test	Bactericidal in non-medical settings
	EN13727	Quantitative suspension test	Bactericidal in medical settings
	EN13624	Quantitative suspension test	Fungicidal/yeastcidal in medical settings
	EN14476	Quantitative suspension test	Virucidal in medical setting
Hand Wash	ASTM E1838-17	Fingerpad	Virucidal in non-medical settings
Hand Wash	EN1499	Fingerpad	Bactericidal in medical settings
Hand Rub	EN1500	Fingerpad	Bactericidal in medical settings
Surgical Hand Wash	EN12791	Fingerpad	Bactericidal in medical settings

Table II: Antimicrobial efficacy evaluated by quantitative suspension test

Test microorganisms	Contact time (min)	Log ₁₀ reduction [#]
<i>S. aureus</i>	5	5.86 ± 0.12
<i>E. coli</i>	5	5.95 ± 0.21
<i>K. pneumoniae</i>	5	6.15 ± 0.05
SARS-CoV-2	1 and 5	*5.08

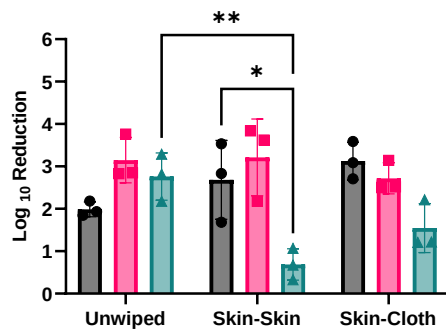
* no viruses recovered

[#] bacteria measured in cfu/ml, viruses pfu/ml

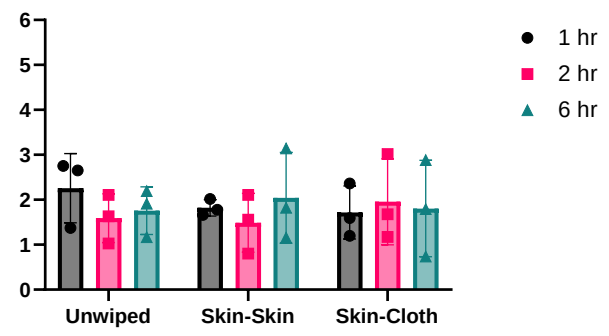
Figure 1. Antimicrobial efficacy evaluated on an ex-vivo pig skin model.

Product efficacy was calculated as the log₁₀ reduction in organism count compared to a media-only control under three conditions: unwiped, skin wiped, or cloth-wiped at 1- (black bars), 2- (pink bars), or 6-hr (green bars) post-wipe treatment. Error bars represent the standard deviation of triplicate tests. Statistical significance: * P≤0.05; ** P≤0.01.

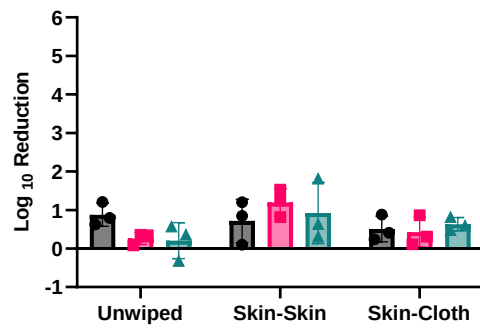
A *S. aureus*



B *E. coli*



C *K. pneumoniae*



D SARS-CoV-2

