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The action of three antiseptics/disinfectants against enveloped and non-enveloped viruses

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Summary: The antiviral action of chloroxylenol, benzalkonium chloride and cetrimide/chlorhexidine was assessed against a range of enveloped and non-enveloped human viruses using a suspension test method. Viral suspensions of 10^6 – 10^7 pfu/TCID₅₀ or sfu were prepared in each of the antiseptic/disinfectant solutions in the presence of a bovine serum/yeast extract mixture to simulate 'dirty conditions'. During incubation, aliquots were removed at predetermined timepoints up to 10 min to assess the kinetics of inactivation. Results indicate that all products were effective in inactivating the enveloped viruses herpes simplex virus type 1 and human immunodeficiency virus type 1, whilst being ineffective in inactivating human coronavirus, also enveloped, and the non-enveloped viruses. The exception to this was the benzalkonium chloride-based product (Dettol Hospital Concentrate) which was active against the non-enveloped human coxsackie virus. Four antiseptic/disinfectant solutions with chloroxylenol, benzalkonium chloride, cetrimide/chlorhexidine and povidone–iodine were also assessed for antiviral effect against human immunodeficiency virus in the presence of whole human blood. All four solutions proved to be effective within 1 min despite the cytotoxic nature of the compounds to the detection system.

Keywords: Antiviral; antiseptic/disinfectant; chloroxylenol benzalkonium chloride; cetrimide/chlorhexidine enveloped; non-enveloped viruses.

Introduction

There are specific guidelines for testing the efficiency of antimicrobial agents against bacteria but there are no generally accepted guidelines for testing their efficiency against viruses. Some guidance is issued by the German Federal Health Office for testing the effectiveness of chemical disinfectants against viruses.¹ There are two commonly used methods for validating the antiviral activity of antimicrobial agents; these are the suspension and surface tests. The suspension test involves exposure of a known virus titre to the agent over time, then determining the amount of infective virus remaining during, and at the end, of incubation. Normal

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conditions include incubation times of up to 10 min at room temperature.²⁻⁵ Surface tests involve drying a viral suspension of known titre on a hard or porous surface and exposing the surface to the germicide for the required length of time.

In order to accurately test the effectiveness of an antiseptic/disinfectant in the 'real' situation, 'dirty' conditions can be simulated by the use of serum or yeast extract.^{3,4} The suspension test has been utilized for several studies, in particular where comparison of antimicrobial agents against herpes simplex virus (HSV) and human immunodeficiency virus (HIV) has been assessed.⁶⁻¹⁰ Our aim in this study was to assess the antiviral nature of a group of antiseptic/disinfectants against a range of enveloped and non-enveloped human viruses. The methodology employed was based on the protocol of the European disinfectant tests for bactericidal activity from the CEN Technical Committee TC216, and specifically on the so called Phase 2 Step 1 test for disinfectant products for use on surfaces in the medical area. The active ingredient(s) of the products tested were chloroxynol, benzalkonium chloride and cetrimide/chlorhexidine, all of which are used throughout the world for skin antiseptics.

As blood spillages can pose an infection risk with HIV, this study also investigated the antiviral effect of the agents already mentioned together with the iodophore povidone-iodine against HIV in the presence of whole human blood.

Materials and Methods

Tissue culture cells and media

All media were supplied by Life Technologies and were supplemented with fetal calf serum, penicillin/streptomycin (50 µl/mL/50 µg/mL) and 1% non-essential amino acids. Vero (African green monkey kidney) cells were grown in M199 with Glutamax I and 5-10% fetal calf serum and HeLa (human epithelioid carcinoma) cells, BSC-1 (African green monkey kidney) cells and MRC-5 (human embryonic lung) cells were grown in EMEM with 10% fetal calf serum. C8166 (human T-lymphoblastoid) cells were grown in RPMI-1640 with Glutamax I and 10% fetal calf serum. Reduced serum concentrations of 1-2% were used in viral and cell maintenance medium except for C8166 where the 10% serum concentration was maintained. The cells were obtained from the American Type Culture Collection, Maryland, USA, and European Collection of Animal Cell Cultures, Porton Down, UK, except C8166 cells which were obtained from the Medical Research Council Collaborative Centre, London, UK.

Virus stocks

Human coxsackie virus (strain A11) ATCC VR-169, a non-enveloped RNA virus and human adenovirus (type 25) ATCC VR-223, a non-enveloped

DNA virus, were grown in HeLa cells. Herpes simplex virus type 1 ATCC VR-539, an enveloped DNA virus, was grown in Vero cells and poliovirus type 1 (Sabin strain) ATCC VR-192, a non-enveloped RNA virus, was grown in MRC-5 cells. Human coronavirus (strain OC43) ATCC VR-759, an enveloped RNA virus, was grown in BSC-1 cells. Cell monolayers showing advanced cytopathic effect were disrupted aseptically by freeze-thawing, centrifuged to remove cell debris and the supernatant harvested and stored at below -70°C until required. Human immunodeficiency virus type 1 (strain RF), an enveloped human retrovirus, was prepared by infecting C8166 cell suspensions with virus. Three to five days post-infection the supernatant was harvested, aliquoted and stored at below -70°C until required. Virus titres were determined by TCID₅₀ 50% (tissue culture infectious dose) assay for poliovirus type 1, human adenovirus, human coxsackie virus and human coronavirus, by plaque assay for herpes simplex virus type 1, and by syncytia-forming assay for human immunodeficiency virus type 1.

Solutions

Five antiseptic/disinfectant solutions were tested; their active constituents and final test concentrations are shown in Table I. The neutralization medium contained 3 g lecithin (ICN Biochemicals), 30 mL Polysorbate 80, USP XX (Tween, ICI), 5 g sodium thiosulphate GP11 (Analar) 1 g, L-Histidine (Sigma Chemicals), 10 mL 0.25 N KH_2PO_4 pH 7.2 (Analar) and 990 mL purified water. The completed medium was sterilized by autoclaving at 121°C for 15 min. Heat inactivated (56°C , 30 min) horse serum (Life Technologies) was added to a final concentration of 10%.

Hard water for dilution of products

Solution A contained 19.84 g anhydrous MgCl_2 and 46.24 g anhydrous CaCl_2 diluted in purified water; then diluted to 1 L in deionized water and sterilized at 121°C for 20 mins.

Solution B contained 35.2 g NaHCO_3 diluted to 1 L in deionized water and sterilized by $0.45\ \mu\text{m}$ membrane filtration. Purified water 600 mL, 6 mL Solution A and 80 mL of Solution B were mixed then diluted to 1 L with deionized water. The final water hardness in test was 300 ppm as CaCO_3 . The pH at 25°C was 7.0 ± 0.2 .

Bovine serum albumin/yeast extract

Bovine serum albumin (10% w/v; Sigma Chemicals) was prepared in deionized water and sterilized by $0.45\ \mu\text{m}$ membrane filtration. Yeast extract (10% w/v; Oxoid) was prepared in deionized water, autoclaved at 121°C for 20 min then pH adjusted to 7.0 ± 0.2 . Equal quantities of the bovine serum albumin and yeast extract solutions were then mixed to provide a combined organic soil load of 10% w/v.

Table 1. *Antiseptic/disinfectant solutions*

| Product (manufacturer) | Active ingredients (% in undiluted product) | Final in test product concentration % v/v | Final in test concentration of active ingredients |
|---|---|---|---|
| Dettol (Reckitt & Colman) | Chloroxylenol (4.8 w/v) | 5 | 0.24% w/v Chloroxylenol |
| Dettol Hospital Concentrate (Reckitt & Colman) | Benzalkonium chloride (BKC) (20 w/v) | 1 | 0.2% w/v BKC |
| Savlon* (Zyma Healthcare) | Cetrimide (2.25 w/v) Chlorhexidine gluconate (0.225 w/v) | 5 | 0.1125% w/v Cetrimide 0.01125% w/v Chlorhexidine |
| Hibicet Hospital Concentrate† (Zeneca) | Cetrimide (15 w/v) Chlorhexidine gluconate (1.5 w/v) | 1 | 0.15% w/v Cetrimide 0.015% w/v Chlorhexidine |
| Betadine Antiseptic Solution† (Seton Healthcare Products) | Povidine-iodine (10 w/v) | 80 40 | 8% w/v Povidine iodine 4% w/v Povidine iodine |

* Not tested versus human immunodeficiency virus type 1 in the presence of whole human blood.

† Only tested in the presence of whole human blood versus human immunodeficiency virus type 1.

Cytotoxicity of the antiseptic/disinfectants and neutralization system

The test antiseptic/disinfectant solutions and the reaction neutralization system was assessed for cytotoxic effects on the detector cell lines (except C8166 cells). Aliquots of the solutions in serial 10-fold dilutions were inoculated onto confluent monolayers of cells in sterile Linbro 24 well cell culture plates, 0.5 mL per well and incubated at 37°C/5% CO₂ for 90 mins. The inocula were removed, the cells washed with phosphate-buffered saline and 1 mL cell maintenance medium added per well. The cells were observed over a period of seven days for general condition, rounding, granularity and cell death. It was noted that the solutions were all cytotoxic, but this cytotoxicity was mostly confined to the undiluted products. The neutralization medium was not cytotoxic to the detector cells.

Four of the antiseptic/disinfectant solutions (Dettol, Dettol Hospital Concentrate, Betadine Antiseptic solution and Hibicet Hospital Concentrate) were tested against the C8166 cell line in the presence of whole human blood at 10 and 50% concentrations. The products were not cytotoxic to the detector cell line, but it was observed that Hibicet Hospital Concentrate lysed the red blood cells up to 1 in 10 dilution.

Evaluation of neutralization system on virus activity

In order to ensure that the neutralization system itself was not antiviral to the viruses employed in the study, controls were performed where the neutralization medium was challenged separately with each virus by incubating over the 5 min \pm 10 s neutralization time at 20°C \pm 1°C. At the end of the incubation period, samples were assayed for virus infectivity. In addition, the neutralization medium was challenged with human immunodeficiency virus type 1 in the presence of 10 and 50% whole blood and treated as described previously.

The neutralization system was also checked to ensure that it neutralized the antiseptic/disinfectant products. The test materials were prepared to 1.25 \times concentration in water of standard hardness to allow for further dilution that occurred with addition of inorganic soil. One millilitre of the bovine serum albumin/yeast extract mixture was added to 8 mL diluted test material or distilled water and 1 mL distilled water and incubated at 20°C \pm 1°C for 5 min. One millilitre aliquots were removed, added to 8 mL neutralization medium and incubated for 5 min \pm 10 s at 20°C \pm 1°C to effect neutralization. One millilitre virus challenge (diluted to give a final concentration of approximately 10³ TCID₅₀/mL, pfu/mL or sfu/mL) (pfu = plaque-forming units; sfu = syncytia-forming units) was added and after 5 min \pm 10 s at 20°C \pm 1°C, the samples were assayed for virus infectivity on the appropriate detector cells by TCID₅₀, plaque or syncytia-forming assay.

For 10% human blood experiments, test materials were prepared to 1.25 \times concentration in water of standard hardness (except Betadine). One millilitre of whole human blood was added to 8 ml diluted test material or

distilled water and 1 mL distilled water and incubated at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 5 min. One millilitre aliquots were removed and added to 8 ml neutralization medium and incubated for $5 \text{ min} \pm 10 \text{ s}$ at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ to effect neutralization. One millilitre of human immunodeficiency virus type 1 (diluted to give a final concentration of approximately 10^3 sfu/mL) was added and after $5 \text{ min} \pm 10 \text{ s}$ at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ the samples were assessed for virus infectivity by syncytia-forming assay.

For 50% whole human blood experiments, the test materials were prepared to $2.5 \times$ concentration in water of standard hardness (except Betadine). Five millilitres of whole human blood was added to 4 mL diluted test material or distilled water and 1 mL distilled water and incubated at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 5 min. The samples were then treated as described previously for the 10% whole human blood experiments.

Virucidal activity of the antiseptic/disinfectants in the presence of albumin/yeast extract

The virucidal activity of Dettol, Dettol Hospital Concentrate and Savlon was compared to that of water of standard hardness against all the test viruses. The test products were diluted in water of standard hardness, initially at $1.25 \times$ concentration to allow for the further dilution that occurred as the inorganic soil and virus challenge were added. One millilitre bovine serum albumin/yeast extract mixture was added to 8 mL diluted test product, or hard water, and mixed at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$, after which 1 mL virus challenge was added. One millilitre aliquots were removed at 0, 1, 5 and 10 min and added to 8 mL neutralization medium and 1 mL water of standard hardness, and then neutralized for $5 \text{ min} \pm 10 \text{ s}$ at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The neutralized samples were then assayed for virus infectivity on the appropriate detector cells by TCID₅₀, plaque or syncytia-forming assay.

Virucidal activity of antiseptic/disinfectants in the presence of whole human blood

Dettol, Dettol Hospital Concentrate, Hibicet Hospital Concentrate and Betadine Antiseptic solutions were assessed for their virucidal action on human immunodeficiency virus type 1 in the presence of 10 and 50% whole human blood. When tested in the presence of 10% blood, the solutions (except Betadine) were diluted in water of standard hardness initially to $1.25 \times$ concentration. Eight millilitre of this was then mixed with 1 mL whole human blood and challenged with 1 mL human immunodeficiency virus type 1 at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. As Betadine is supplied as a ready-to-use product, 8 mL undiluted product was used giving a final concentration of 80% (v/v). When tested in the presence of 50% whole human blood, all the solutions (except Betadine) were diluted in water of standard hardness initially at $2.5 \times$ concentration. Four millilitres of this was then mixed with 5 mL whole human blood and challenged with 1 mL human immunodeficiency virus type 1 at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. With Betadine, 4 mL undiluted

Table II. *Effect of neutralization system on virus challenge*

| Virus | Titre of virus challenge (TCID ₅₀ log ₁₀) | Titre of virus recovered after neutralization (TCID ₅₀ log ₁₀) |
|-------------------------------------|--|---|
| Poliovirus type 1 | 6·80 | 5·46 |
| Human coxsackie virus | 7·05 | 6·71 |
| Human coronavirus | 7·80 | 6·71 |
| Human adenovirus | 7·30 | 6·21 |
| Herpes simplex virus type 1 | 6·87 (A) | 6·53 (A) |
| Human immunodeficiency virus type 1 | 6·05 (B) | 5·55 (B) |

TCID₅₀ = 50% tissue culture infectious dose endpoint.

(A) pfu = plaque forming units.

(B) sfu = syncytia forming units.

product was used giving a final concentration of 40%. One millilitre aliquots of each reaction mixture were removed at 0, 1, 5 and 10 min and added to 8 mL neutralization medium and 1 mL water of standard hardness for 5 min \pm 10 s at 20°C \pm 1°C. The neutralized samples were then assayed for virus infectivity on the C8166 detector cell line by syncytia-forming assay. Water of standard hardness replaced the antiseptic/disinfectants for both whole human blood concentrations to act as a control.

Results

Evaluation of effectiveness of neutralization system to inactivate the products and assessment of the system on virus activity

The neutralization system was not significantly antiviral to the viruses used for challenge in this study (Table II). The neutralization system was determined to be effective in the inactivation of all the products. The difference in virus titres recovered were within 0·50 log₁₀ of the water control for all products in all conditions against all challenge viruses (Tables III and IV).

Activity in dirty conditions

All the antiseptic products were effective against the enveloped viruses herpes simplex virus type 1 and human immunodeficiency virus type 1 within 1 min, except for Savlon versus human immunodeficiency virus type 1 which was inactive at 5 min but active at 10 min (Table V). The reduction factors obtained where no virus was detected are an underestimate of the inactivating potential of the disinfectants/antiseptics. This is due to their cytotoxic effects decreasing the sensitivity level of the detection system. Reduction factors of >4 log indicate a good antiviral system. Reduction of >2–3 logs suggests a more moderate antiviral effect but consideration must

Table III. *Evaluation of neutralization system (in presence of 10% bovine serum albumin/yeast extract)*

| Product | Titre of virus recovered following neutralization | | | | | |
|-----------------------------|---|---|--|--|---|---|
| | Herpes simplex virus (pfu log ₁₀) | Human immunodeficiency virus (sfu log ₁₀) | Poliovirus (TCID ₅₀ log ₁₀) | Human adenovirus (TCID ₅₀ log ₁₀) | Human coxsackie virus (TCID ₅₀ log ₁₀) | Human coronavirus (TCID ₅₀ log ₁₀) |
| Dettol | 4.66 | 4.80 | 4.80 | 4.80 | 4.80 | 4.80 |
| Savlon | 4.74 | 4.80 | 4.80 | 4.80 | 4.80 | 4.80 |
| Dettol Hospital Concentrate | 4.66 | 4.80 | 4.55 | 4.80 | 4.80 | 4.80 |
| Water control | 4.67 | 4.80 | 4.80 | 4.80 | 4.55 | 4.55 |

Virus challenge: Herpes simplex virus = 4.70; Human immunodeficiency virus type 1 = 4.80; Poliovirus = 4.55; Human adenovirus = 4.80; Human coxsackie virus = 4.80; Human coronavirus = 4.80.

Table IV. *Evaluation of neutralization system (human immunodeficiency virus type 1 in presence of whole human blood)*

| Sample | Titre of virus recovered following neutralization (sfu log ₁₀) | | | | |
|-----------------------|--|-----------------------------|---|------------------------------|---------------|
| | Dettol | Dettol Hospital Concentrate | Betadine Antiseptic/disinfectant Solution | Hibicet Hospital Concentrate | Water control |
| 10% Whole human blood | 4.55 | 4.80 | 4.55 | 4.30 | 4.80 |
| 50% Whole human blood | 4.80 | 4.55 | 4.55 | 4.55 | 4.80 |

Virus challenge (in presence of whole human blood) = 4.60 log₁₀.

Virus challenge (positive control, no blood) = 4.80 log₁₀.

be made of any cytotoxic effects of the products on the detection system. These results are consistent with other investigations on the inactivation of such viruses with detergents.^{6,7,8} The antiseptics were not effective in the inactivation of the more resistant non-enveloped poliovirus Sabin type 1, human adenovirus and coxsackie virus and the enveloped human coronavirus even at the 10 min timepoint. The exception was Dettol Hospital Concentrate (active agent benzalkonium chloride) which was effective in the inactivation of the non-enveloped human coxsackie virus with a reduction of >5 log₁₀ after the 1 min timepoint.

Activity in whole human blood

The antiseptic/disinfectants Dettol, Dettol Hospital Concentrate, Betadine Antiseptic solution and Hibicet Hospital Concentrate were all effective in inactivating human immunodeficiency virus in the presence of whole human blood within 1 min (Table VI). Hibicet Hospital concentrate was tested in the presence of blood as it is a hospital product whereas Savlon, which delivers similar levels of active ingredients at the recommended dilution, is for domestic usage. Hibicet Hospital Concentrate inactivated human immunodeficiency virus type 1 within 1 min compared with 10 min inactivation time for Savlon in the presence of albumin/yeast mixture. The faster inactivation with Hibicet Hospital Concentrate may be due to the slightly higher concentration of active ingredient present in comparison to Savlon, or the lack of protective effect from the blood which the product lysed. The albumin/yeast mixture in the Savlon experiment provided a protective effect towards the virus particles preventing rapid inactivation.

Discussion

The limited effect of phenolic disinfectants on virus inactivation and the non-virucidal effect of cetrimide/chlorhexidine antiseptic on non-enveloped

Table V. *Reduction in virus infectivity by the antiseptic/disinfectants*

| Product | Reduction factor (\log_{10}) after 1 min | | | | | |
|--------------------------------|--|--|--|--|---|---|
| | Herpes simplex virus ($\text{pfu}\log_{10}$) | Human immunodeficiency virus ($\text{sfu}\log_{10}$) | Poliovirus ($\text{TCID}_{50}\log_{10}$) | Human adenovirus ($\text{TCID}_{50}\log_{10}$) | Human coxsackie virus ($\text{TCID}_{50}\log_{10}$) | Human coronavirus ($\text{TCID}_{50}\log_{10}$) |
| Dettol 5% | >4.60 | >2.37 | 0.62* | 0.37* | 1.00* | 0.00* |
| Savlon 5% | >4.37 | >0.50† | 0.12* | 1.50* | 0.37* | 0.00* |
| Dettol Hospital Concentrate 1% | >4.51 | >1.87 | 0.00* | 0.25* | >5.12 | 0.00* |
| Water of standard hardness | 0.13 | 0.77 | 0.75 | 1.12 | 0.25 | 0.50 |

Figures presented are the mean of the duplicate experiments.

* Products inactive against virus at 1 min were also inactive at 10 min.

† Savlon inactivated HIV at 10 min sampling. (RF >3.35 $\text{sfu}\log_{10}$) but not at 5 min (RF = 0.8 $\text{sfu}\log_{10}$).

RF = reduction factor.

Virus challenge herpes simplex virus = 7.55–7.78 \log_{10} ; poliovirus = 7.05–7.80 \log_{10} ; human immunodeficiency virus = 6.05–6.55 \log_{10} ; human adenovirus = 7.30–7.80 \log_{10} ; human coxsackie virus = 7.30–8.05 \log_{10} ; human coronavirus = 6.80–8.05 \log_{10} .

Table VI. Reduction in human immunodeficiency virus type 1 infectivity by the antiseptic/disinfectants in presence of whole human blood

| Sample | Titre of virus challenge (sfu _{log10}) | Reduction factor (log ₁₀) after 1 min | | | |
|-----------------------|--|---|-----------------------------|---|------------------------------|
| | | Dettol | Dettol Hospital Concentrate | Betadine Antiseptic/disinfectant Solution | Hibicet Hospital Concentrate |
| 10% Whole human blood | 6.58 (6.85) | >3.62 | >3.40 | >2.40* | >3.40 |
| 50% Whole human blood | 6.27 (7.10) | >3.62 | >3.09 | >2.09† | >3.09 |

Figures presented are the mean of the duplicate experiments.

() = For Dettol; * 80% Betadine; † 40% Betadine.

viruses was also demonstrated by Narang and Codd.¹¹ An extensive study by Grossgebauer¹² indicates that quaternary ammonium products are adsorbed on proteins with subsequent inactivation. Whilst they are active against lipophilic viruses (e.g., herpes simplex, vaccinia, influenza and adenovirus), they have less effect against hydrophilic viruses (enterovirus e.g., poliovirus, coxsackie and ECHO).¹² We have demonstrated that a quaternary ammonium product can rapidly inactivate a strain of coxsackie virus at room temperature in the presence of organic soil. As virucidal activity depends upon concentration of active ingredient and pH, the conditions within our reaction mixture appear to have allowed this inactivation to occur. As quaternary ammonium compounds are surface acting agents, inactivation may have been due to alterations in the surface components at, or adjacent to the attachment site, which interacts with the receptor on the surface of the host cell.

In conclusion, the solutions tested at their recommended concentrations for antiseptic use were very effective in inactivating the non-enveloped viruses, human immunodeficiency virus type 1 and herpes simplex virus type 1 in the presence of significant levels of organic soil.

All these products could be useful in infection control programmes, both in hospital and community, particularly in developing countries. Many patients in the latter, including those who are human immunodeficiency virus positive, are cared for in the community by their own families. Antiseptics, with a combination of good bacterial activity against enteric organisms and some antiviral activity, could prevent the spread of infection in these areas. Products such as Dettol and Savlon, which are widely available all over the world, have low toxicity and a good safety record and can be recommended for use by non-health professionals.

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