Use of a new, simple, laboratory method for screening the antimicrobial and antiviral properties of hand sanitizers

BABAK BABAN, PhD, JUN YAO LIU, BS, FRANKLIN R. TAY, BDSc (Hons), PhD & DAVID H. PASHLEY, DMD, PhD

ABSTRACT: Purpose: To develop a simple, laboratory method for screening the antimicrobial/antiviral activity of hand sanitizers, to replace the more time consuming use of human volunteers. Methods: A Rapid Agar Plate Assay (RAPA) was developed that uses sterile agar plates to simulate skin surfaces. After treating the agar plates with putative hand sanitizers, the plates were inoculated with gram-positive S. aureus or gram-negative E. coli. Untreated agar plates served as controls. After incubation for 48 hours, the bacteria were recovered and stained with fluorescent dyes. The number of live/dead bacteria was quantitated by flow cytometry. For anti-viral activity, mammalian cell lines were grown to confluence and infected with noroviruses (murine norovirus or feline calicivirus), and the number of dead cells was quantitated as the log₁₀ of number of cells killed. A liquid hand soap without any antibacterial activity (LHS) was used as the control. A popular ethanol-based hand sanitizer (GHS) was compared to a new quaternary ammonium-containing bactericidal hand cream (ABC). Results: The liquid soap was not effective against either gram-positive or gram-negative bacteria, or viruses. Both GHS and ABC were very effective against S. aureus, but much less so against E. coli. Both GHS and ABC were even more effective against the two noroviruses that cause gastrointestinal diseases, than they were against gram-positive bacteria. These results support the use of RAPA as an effective laboratory screening test to evaluate the antibacterial/antiviral activity of hand sanitizers or other antimicrobial products. (Am J Dent 2012;25:327-331).

Clinical significance: This laboratory study showed that some no-rinse anti-bacterial hand sanitizers can inactivate viruses better than they can kill bacteria. Hand sanitizers can contribute to universal precautions used in dental offices.

Introduction

Proper hand hygiene in dental offices can significantly reduce the risk and spread of infection.¹ A recent survey of hand hygiene procedures of dental practitioners concluded that there is lack of knowledge among dentists regarding proper hand hygiene.²

When selecting hand hygiene techniques, one must determine the type of procedures to be performed (whether blood will contaminate gloves or skin), the persistence of the decontamination (whether the antimicrobial in the hand disinfectant adhere to the skin or easily rinse off), and the potential risk of spreading infection (if the patient is likely to carry hepatitis or HIV viruses).³

Examination gloves contain microscopic imperfections but are adequate for routine, noninvasive procedures. Surgical sterile gloves are preferred in high risk patients or procedures. Wearing gloves reduces the risk of contamination 70-80%.³ However, wearing gloves creates a warm, moist environment where bacteria can multiply. This increases the number of microflora on skin under gloves. Thus, hand hygiene is essential before donning new gloves.

Although 69-93% of general dentists use soap and water for hand hygiene,¹ most do not wash for at least 15 seconds.³ Other studies have shown that alcohol-based hand disinfectants are more effective than soap and water,⁴ especially those that contain agents with persistent anti-microbial activity.⁵ For invasive surgery in high risk patients, clinicians are advised to wash hands with soap and water, followed by an alcohol-based hand sanitizer with persistent activity, before donning sterile gloves.

Dentists who come into hand contact with many patients each day can inadvertently become contaminated with pathogens from one patient that can be transferred to the next patient. Between patient hand washing or hand sanitizer use is known to significantly lower the risk of such cross-contamination.⁵-⁸ It is not always possible to wash hands if a sink and running water is unavailable. In such cases, good disinfection may be accomplished by using no-rinse hand sanitizers such as ethanol-based gels.⁹ Alcohol-based hand sanitizers are popular and have been reported to be an effective means for increasing hand hygiene compliance and reducing infection rates.¹⁰ Although alcohols used in hand sanitizers are generally regarded as nonallergic,¹¹ there is concern that using 20-30 applications of ethanol-based hand sanitizers each day may extract important lipids from the skin that serve as a protective chemical barrier to water-borne pathogens¹² and cause microabrasions in skin.¹³ There is also concern that inadvertent or intentional ingestion of ethanol-based hand sanitizers may result in elevation of blood alcohol levels, leading to acute toxicity.¹⁴

If hands were treated with an antibacterial/antiviral cream that was hydrophobic, the cream could coat the hands with antimicrobial agents such as chlorhexidine or quaternary ammonium compounds. These would not only disinfect skin but increase its barrier properties, using an external hydrophobic layer of silicones, for instance. Such products would not extract lipids from skin, would be nonirritating, and would offer persistent protection.¹⁵,¹⁶

Dentists and dental staff must protect themselves and their patients from office-acquired infections.¹,²,¹⁷ While all dental instruments, burs and handpieces are sterilized and patients are protected by fresh examination gloves and face masks, the disinfection of hands and surfaces remains an important part of taking “universal precautions”.⁵

Prior to marketing, antimicrobial and antiviral hand sanitiz-
Antibacterial evaluation

The agar surface of the TSA plate was briefly rinsed in running sterile water, to ensure that the agar surface was hydrated. Then, with a sterile gloved hand, ABC cream, GHS and LHS (ca. 45 µg/cm²) were directly applied on the entire agar surface for 10-15 seconds. The plates were air dried under a level 2 safety cabinet in the inverted position without lid for 45 minutes. Subsequently, 100 µL of bacterial challenge dose (containing 200-300 CFU) was applied and evenly distributed using a sterile metal spreader. Plates were incubated at 37°C for 24 hours in 5% CO₂ air. The bacterial colonies were counted and analyzed using appropriate statistical tools. Each product was tested in replicates of 3-6 TSA plates.

Antibacterial susceptibility testing

To assess bacterial membrane integrity as one of the mechanisms by which test products in this study might act against bacterial growth, the LIVE/DEAD membrane permeability method² was performed according to the manufacturer’s guidelines and our previously described flow cytometry protocols.²⁸ In brief, bacterial fluorescent stain dyes (usually red and green) were used for detecting and quantitating dead vs. live bacteria, respectively. Bacteria stained with these non-nucleic acid labeling stains exhibit bright fluorescence, and can be resolved using the appropriate flow cytometric channels. These fluorescent stains efficiently label a variety of different bacteria species. The intensity of the staining appears to depend on several factors, including gram character, outer membrane composition and overall membrane integrity. In the species tested, gram-positive bacteria generally exhibited brighter fluorescence than gram-negative bacteria, and cells with compromised membranes accumulated more dye than intact cells.

Bacterial stain experimental protocol

All procedures were done according to the manufacturer’s instructions, and are summarized as follows:

Preparation of stock solution of the fluorescent bacterial stains
- A vial of the bacterial stain was warmed to room temperature before opening. A 1 mM stock solution of dye was prepared by dissolving the vial contents in 74 µL DMSO for the green bacterial stain or 69 µL for the red bacterial stain.

Preparation of working solution of the fluorescent bacterial stains
- A 100 mM working solution of the fluorescent bacterial stains was prepared by adding 2 µL of each of the 1 mM stock solutions prepared above to 18 µL of DMSO in a microcentrifuge tube and mixed well.

Staining protocol - Bacterial colonies were harvested from culture plates as described above for RAPA, centrifuged and diluted in PBS. Then, bacterial cells were stained by adding 2 µL of the working dye solution as prepared above to 1 mL of bacterial samples, that were incubated for 15 minutes at room temperature (25°C).

Flow cytometry measurements - These measurements were performed on a flow cytometer (BD FACS Calibur)¹ according to our previously described flow cytometry protocols.²⁸ Briefly, red fluorescence was measured above 630 nm (FL2) and green fluorescence was measured at 488 nm (FL1). The trigger was set for the green fluorescence channel FL1. Cells were gated based on forward and side scatter properties of total bacterial cell population stained with bacterial fluorochromes as described above. Dead bacteria stained red and live bacteria stained green.²⁹ All experiments were repeated 3-4 times.
Antiviral activity

Both viruses used in these studies, feline calicivirus (FCV) and murine norovirus (MNV), 30-33 were propagated in their allocated cell lines. The FCV was used to infect CrFK cells (ATCC CCL-94) and MNV was used with RAW 267.4 cells (ATCC TIB-71). Cells were grown in Dulbecco’s Modified Eagle’s Medium, complemented with 10% heat-inactivated fetal calf serum, 2% penicillin (5000 U/ml) and streptomycin (5000 mg/ml), and 1% HEPES buffer. Monolayers of the respective cells were prepared in 25 cm² flasks (5 x 10⁶ cells) at an m.o.i. (multiplicity of infection) of 10.

Infectivity and plaque assays - In order to determine if cells were infected by MNV or FCV, titers of each virus production were determined by conventional plaque assay, as previously described. 33,34 Briefly, the appropriate mammalian cells were dispensed in culture plates at a density of 2 x 10⁶ cells per plate, and grown to 80 to 90% confluency in 5 mL of complete minimum essential medium at 37°C. Then, the mammalian cells were infected with MNV or FCV for 1 hour, with gentle 15-second shaking every 15 minutes to allow viral absorption. After 1 hour, the inoculum was removed, the cells were overlaid with 5 mL of medium containing 0.5% agarose, and incubated for 48 hours to allow for viral replication and cell death. A second agarose overlay, which included 0.75% neutral red solution to identify living cells, was then added (3 mL). Plaques of dead cells were counted 8 hours later. Plates with 5 to 50 plaques were used to determine the virus titer in plaque forming units (PFU). Plaques are areas in a cell monolayer which appear as white spots when visualized by eye, caused by virus-induced lysis.

Suspension viral assays - Suspension tests for antiviral activity were performed according to the protocol previously described by Macinga el al, 21 with the following modification. Briefly, 4.5 mL of test substances were dispensed into a sterile 15 mL falcon tube, and mixed with 0.5 mL of the virus suspension. The mixture was vortexed for 10 seconds, and held for the remainder of the 45-second exposure time. Immediately following the exposure period, a 0.1 mL aliquot was removed from the tubes, and tested for the presence of viable virus by infectivity assays and the plaque assay described above.

Statistical analyses

Because the LHS controls gave reduction factors (mean log_{10}) that were always lower than the GHS and ABC values, the data were not normally distributed. Thus, the data were analyzed using Kruskal-Wallis ANOVA. Where the corresponding omnibus test indicated statistically significant results, Dunn’s multiple comparisons post-hoc analyses were used for comparing the different formulations. Statistical significance was present at α= 0.05.

Results

Quantitation of the efficacy of antimicrobial agents is generally done by expressing the results in a reduction factor (RF) 15 i.e., how well a test product decreases the amount of bacteria or viruses on an agar plate. It is calculated as the control or baseline count in log_{10} minus the post-treatment number using a logarithmic scale to the base of 10. That is, if a treatment reduced the number of microbes 302-fold, the mean log_{10} reduction of those bacteria would be 2.48 (Table 1). Any treatment that yields a reduction of more than 2 (i.e. reduced microbi-

<table>
<thead>
<tr>
<th>Test products</th>
<th>Log_{10} reduction (Log_{10} CFU/mL; mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHS (non-antibacterial)</td>
<td>0.06 ± 0.03 a</td>
</tr>
<tr>
<td>GHS (antibacterial ethanol)</td>
<td>2.48 ± 0.47 b</td>
</tr>
<tr>
<td>ABC (antibacterial QACs)</td>
<td>2.12 ± 0.55 b</td>
</tr>
<tr>
<td>LHS – liquid soap hand sanitizer containing no antibacterial activity; GHS – ethanol-based antibacterial hand sanitizer; ABC – antibacterial hand cream containing 0.1% benzalkonium chloride and 0.5 wt% octadecyltrimethyl-trimethoxysilylpropyl ammonium chloride, a quaternary ammonium compound (QAC). For S. aureus (first data column), groups with the same upper case letter superscripts are not statistically significant (P&gt; 0.05). For E. coli (second data column), groups with the same lower case letter superscripts are not statistically significant (P&gt; 0.05).</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Laboratory method for screening hand sanitizers
reduced the viral count of feline calcivirus more than 1585-fold. It was even more effective against murine norovirus, reducing the virus count 70,795-fold. The ABC hand sanitizer reduced the feline calcivirus 224-fold and that of murine norovirus by 1513-fold. For each virus, the difference between the GHS and ABS hand sanitizers is statistically significant (P< 0.05). Similar results were obtained from the plaque assays, although the difference between the GHS and ABS hand sanitizers are not statistically significant (P> 0.05). The results of the two hand sanitizers and the LHS control in inhibiting plaque formation by murine norovirus are summarized in Fig. 2. A similar tendency could be seen for the feline calcivirus (not shown). In Fig. 2, three plates of mammalian cells infected with murine norovirus are illustrated. The plate treated with LHS exhibits multiple clear areas (virus-induced lytic zones called plaques). The bar graph below indicates the cumulative area of cell lysis in the LHS-treated plate. Both ABC- and GHS-treated plates exhibited significantly fewer plaques than the LHS-treated plates (P< 0.05), indicating that ABC and GHS treatments were very effective in inactivating noroviruses. No difference could be detected between the two hand sanitizers.

Discussion

Since the liquid soap had no antibacterial or antiviral activity, while GHS and ABC hand sanitizers had good antiviral and antibacterial activity against gram-positive bacteria, the results of this study require rejection of both test null hypotheses that there are no differences in the antibacterial or antiviral activities of the three tested hand sanitizers.
agents were after multiple hand washings.

This method uses equipment and techniques that are commonly found in microbiological laboratories. It saves time and money and is more precise than the "glove-contamination" method used with human volunteers.

The RAPA technique easily identified the lack of efficacy of liquid soap hand sanitizer (LHS) and the good efficacy of both the ethanol-based GHS and the hydrophobic quaternary ammonium-based AMC. Preliminary results using ABC indicate that its residual antimicrobial activity survives far more water washings than either LSH or GSH. This is because the latter are water-soluble, while the quaternary ammonium compounds in ABC have limited water solubility.

Within the limitations of such laboratory studies, the RAPA method looks promising for evaluating the antimicrobial properties of hand sanitizer activity against various microorganisms. Future studies should be done using oral microorganisms.

Acknowledgement: To Mrs. Michelle Barnes for her outstanding secretarial support.

Disclosure statement: Drs. Baban, Tay and Pushley and Mrs. Liu had no commercial interest in any of the products used in this study. This study was not supported by any manufacturer.

Dr. Baban is an Assistant Professor of Oral Biology, Mr. Liu is a Research Assistant in Oral Biology, Dr. Tay is a Professor of Endodontics and Dr. Pushley is an Emeritus Regents’ Professor of Oral Biology, College of Dental Medicine, Georgia Health Sciences University, Augusta, Georgia, USA.

References