Video Article

A Method to Test the Efficacy of Handwashing for the Removal of Emerging Infectious Pathogens

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Abstract

Handwashing is widely recommended to prevent infectious disease transmission. However, little comparable evidence exists on the efficacy of handwashing methods in general. Additionally, little evidence exists comparing handwashing methods to determine which are most efficacious at removing infectious pathogens. Research is needed to provide evidence for the different approaches to handwashing that may be employed during infectious disease outbreaks. Here, a laboratory method to assess the efficacy of handwashing methods at removing microorganisms from hands and their persistence in rinse water is described. Volunteers' hands are first spiked with the test organism and then washed with each handwashing method of interest. Generally, surrogate microorganisms are used to protect human subjects from disease. The number of organisms remaining on volunteers' hands after washing is tested using a modified "glove juice" method: the hands are placed in gloves with an eluent and are scrubbed to suspend the microorganisms and make them available for analysis by membrane filtration (bacteria) or plaque assay (viruses/bacteriophages). Rinse water produced from the handwashing is directly collected for analysis. Handwashing efficacy is quantified by comparing the log reduction value between samples taken after handwashing to samples with no handwashing. Rinse water persistence is quantified by comparing rinse water samples from various handwashing methods to samples collected after handwashing with just water. While this method is limited by the need to use surrogate organisms to preserve the safety of human volunteers, it captures aspects of handwashing that are difficult to replicate in an *in vitro* study and fills research gaps on handwashing efficacy and the persistence of infectious organisms in rinse water.

Video Link

The video component of this article can be found at https://www.jove.com/video/55604/

Introduction

Handwashing is widely recommended to prevent the spread of disease, particularly those transmitted by the fecal-oral or airborne route, including diarrheal and respiratory diseases[1]. Surprisingly, there is little comparable evidence on the efficacy of handwashing methods, such as handwashing with soap and water (HWWS) and with alcohol-based hand sanitizer (ABHS), on the removal of organisms from the hands. Initial research has found that the mechanical action of handwashing, as opposed to the handwashing method, may account for most organism removal[2,3]. Additionally, there is little comparative evidence on which handwashing method is most efficacious. In an informal literature review, 14 studies that compared the efficacy of soap and hand sanitizer on the removal of organisms were identified. Of these studies, five found ABHS to be more efficacious[4,5,6,7,8], seven found HWWS to be more efficacious[9,10,11,12,13,14,15], and two found no significant difference between the methods[16,17]. These findings are inconsistent and do not address the ongoing risk of disease from the persistence of organisms in the rinse water after handwashing. Overall, the evidence on the comparative efficacy of handwashing methods for the removal of infectious disease-causing pathogens is limited.

This limited evidence has led to uncertainty about which methods are most appropriate in outbreak settings. For example, during the Ebola Virus Disease (EVD) outbreak in West Africa from 2013 to 2016, several large international responders provided contradictory recommendations for HWWS, ABHS, or 0.05% chlorine solutions. Médecins Sans Frontières (MSF) recommends the use of 0.05% chlorine solution for handwashing, while the World Health Organization (WHO) recommends HWWS or ABHS (if the hands are not visibly soiled). The WHO goes so far as to state that chlorine should not be used unless no other options are available, because it is less effective than other methods due to the chlorine demand exerted by the skin[18,19,20,21,22]. Additionally, the chlorine solutions are commonly produced from four different chlorine compounds, including high-test hypochlorite (HTH), locally-generated and stabilized sodium hypochlorite (NaOCl), and sodium dichloroisocyanurate (NaDCC). A systematic review commissioned by the WHO in response to the EVD outbreak in West Africa recently found only four studies investigating the comparative efficacy of handwashing with chlorine[23]. These studies also produced conflicting results, and none of these studies used the recommended chlorine concentration of 0.05% for handwashing or investigated microorganisms similar to the Ebola virus[10,24,25,26,27]. Thus, the recommendations were not found to be evidence-based, and it was unclear which recommendations were most efficacious.

Additional research is needed to compare handwashing approaches to prevent the spread of infectious pathogens, as handwashing interventions are an important tool to prevent epidemic disease transmission. These handwashing recommendations must be based on

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evidence. Thus, a method for testing handwashing efficacy and rinse water persistence, performed with surrogates or non-infectious pathogens, was developed[2,28,29]. Sample results, using Phi6 as a surrogate for the Ebola virus and using *Escherichia coli* as a common indicator organism, are presented here. In this protocol, handwashing efficacy and rinse water persistence tests are presented.

Protocol

Ethics Statement: The study described here (on Phi6 and *E. coli* as surrogates for Ebola) was approved by the Institutional Review Board at Tufts Medical Center and Tufts University Health Sciences Campus (#12018); Harvard University ceded review to the Tufts Institutional Review Board.

NOTE: Prior to beginning this protocol, two steps must be completed. First, a Biosafety Level 1 (BSL-1) surrogate or non-infectious version of the pathogen to be studied that is safe to use on human subjects must be identified and selected[30]. A BSL-1 surrogate or non-infectious pathogen is necessary for this protocol, as the organism will be used to inoculate the bare hands of human volunteers. Second, approval from the local Institutional Review Board to conduct research with human subjects must be obtained before recruiting volunteers or beginning the experiment. Many aspects of this protocol can be adjusted to meet the specific needs of the research questions of interest.

1. Recruit Eligible Human Subjects

1. Recruit volunteers by posting paper flyers on public notice boards and sending emails to groups with members who may be interested in participating. These announcements should include the study purpose, contact information, and eligibility criteria.
2. Meet with the volunteers to evaluate eligibility. Confirm that the volunteers are healthy, between the ages of 18 and 65, and not currently pregnant or taking antibiotics and that they have no skin damage/disorders, known allergies to handwashing agents, or history of mental health issues related to hygiene.
3. Have eligible volunteers read consent forms. Answer any questions they pose and have the volunteer and investigator sign two copies of the form. Retain one form and provide one to the volunteer.
4. Administer a baseline survey, including questions on demographic information, personal history of skin conditions, and information on recent handwashing behavior. Examine hands for signs of dermatitis, skin injuries, or baseline skin abnormalities[31].
5. **Schedule the volunteers for two testing sessions for each organism of interest (one for testing with soil load and one for testing without). Instruct the volunteers to avoid antimicrobial products for a seven-day washout period prior to testing to avoid confounding from personal product use.**
    1. Provide volunteers with antimicrobial products (shampoo, conditioner, and soap) to use in place of their usual products. Provide heavy-duty vinyl gloves and instruct the subjects to wear them when using products such as house cleaning products.

2. Prepare Handwashing Solutions Commonly Used in Emergency Response (Soap, ABHS, 0.05% HTH, NaDCC, and NaOCl Solutions)

NOTE: Chlorine solutions can be prepared up to 12 h in advance of the experiment but will degrade if stored >12 h.

1. Choose and purchase a soap relevant to the context for which testing is being performed.
   NOTE: In most cases, for infectious disease emergencies in the developing world, this will be a bar of soap.
2. Choose and purchase an ABHS solution relevant to the context for which testing is being performed.
   NOTE: The chosen ABHS should have an ethyl alcohol context greater than or equal to 70% to ensure efficacy.
3. **Prepare a 0.05% calcium hypochlorite ($Ca(ClO)_2$) solution by adding granular $Ca(ClO)_2$ powder to ultrapure water. Determine the amount of solution needed based on the number of subjects to be tested.**
    1. Using the following equation, determine the amount of powder needed to prepare the desired amount of solution in a given volume of water using a given percent of available chlorine:

$$\frac{500\ {}^{mg\ Cl}/_{L}}{concentration\ of\ Ca(ClO)_2\ powder\ (decimal)} \times L\ water \times \frac{1\ g}{1000\ mg}\ g\ Ca(ClO)_2\ powder = g\ Ca(ClO)_2\ powder$$

   NOTE: $Ca(ClO)_2$ powder typically has 60-80% available chlorine.

4. **Prepare a 0.05% sodium dichloroisocyanurate (NaDCC) solution by adding a granular NaDCC powder to ultrapure water.**
    1. Using the following equation, determine the amount of powder needed to prepare the desired amount of solution in a given volume of water using a given percent of available chlorine:

$$\frac{500\ {}^{mg\ Cl}/_{L}}{concentration\ of\ NaDCC\ powder\ (decimal)} \times L\ water \times \frac{1\ g}{1000\ mg}\ g\ NaDCC\ powder = g\ NaDCC\ powder$$

   NOTE: NaDCC powder typically has about 50% available chlorine.

5. **Prepare stabilized 0.05% NaOCl solution by adding stock sodium hypochlorite solution to ultrapure water.**
    1. Confirm the concentration of the NaOCl stock solution (likely to be 5-8%) using a titration test method according to the manufacturer's instructions (*e.g.,* iodometric titration; see the Materials List for the suggested kit).
    2. Using the results of the test method, calculate the amount of solution to add to water using the following equation:

$$\frac{500\ {}^{mg\ Cl}/_{L}}{concentration\ of\ starting\ solution\ {}^{mg\ Cl}/_{L}} \times L\ water \times \frac{1000\ mL}{1\ L} = mL\ NaOCl\ stock$$

6. **Prepare stabilized 0.05% NaOCl solution by adding sodium hypochlorite solution produced using an electrochlorinator, ultrapure water, and laboratory-grade sodium chloride (NaCl) to ultrapure water.**
    1. Prepare a 1% chlorine solution with ultrapure water and NaCl, using an electrochlorinator according to the manufacturer's instructions.
    2. Use a titration test method (*e.g.,* iodometric titration) to confirm the concentration of the NaOCl stock solution[32].
    3. Using the results of the test, calculate the amount of solution to add to water using the following equation:

$$\frac{500\ {}^{mg\ Cl}/_{L}}{concentration\ of\ starting\ solution\ {}^{mg\ Cl}/_{L}} \times L\ water \times \frac{1000\ mL}{1\ L} = mL\ NaOCl\ stock$$

7. Confirm the concentration of each of the chlorine handwashing solutions using a titration method (*e.g.,* iodometric titration) and adjust the solutions by adding water or chlorine source powder/solution until they are within a 10% error of the target concentration (0.045-0.055%).

3. Prepare Organisms and Soil Load and Combine to Produce the Inoculate

NOTE: In the following sub-sections, *E. coli* and Phi6 are used as sample bacterial and viral organisms for the methods description.

1. **Prepare the organism to be used for testing at a concentration greater than 10 x 10_8 CFU/mL for bacteria and greater than 10 x 10^7 PFU/mL for viruses.**
    1. To prepare *E. coli*, streak a nonpathogenic strain of *E. coli* onto Luria-Bertani (LB) agar plates and incubate at 37 °C for 24 h to obtain single colonies. Store at 4 °C.
    NOTE: This can be done several days before experimentation.
        1. One day before the start of the experiment, pick a single colony from the plate and inoculate 10 mL of LB broth using a sterile loop. Incubate overnight at 37 °C with shaking.
        2. On the morning of the experiment, start a fresh culture by adding 1 mL of the overnight culture to 20 mL of fresh LB broth. Incubate for approximately 2.5 h to achieve a cell density greater than 10^8 CFU/mL.
        3. Use a spectrophotometer to estimate the concentration of the culture.
        NOTE: Use a previously established conversion factor from a standards curve for the *E. coli* strain used, ensuring a concentration greater than 10^8 CFU/mL[33]. If the cell density is not high enough, return the culture to the incubator and test again until ready.

    2. Confirm the concentration using membrane filtration[34].
    NOTE: Perform serial dilutions of the culture in phosphate-buffered saline (PBS) so that the solution filtered will produce a countable number of colonies on the plate (the exact number will depend on the medium used).
        1. Set up a flame and a filtration manifold with sterile filtration funnels and a vacuum connection. Sterilize forceps by flaming them with ethanol. Use them to place a 0.45-µm filter on the filtration manifold, with the grid facing up. Wet the filter with a small amount of sterile PBS.
        2. Place the funnel on the base and add the sample solution to be processed by pipetting or pouring directly onto the filter. Engage the vacuum until the entire sample has passed through the membrane. Rinse the sides of the funnel with sterile PBS and engage the vacuum again.
        NOTE: Samples should be at least 100 µL and up to 100 mL. If a sample is less than 10 mL, add approximately 20 mL of PBS to the filtration funnel prior to filtering to ensure the uniform filtration of the sample.
        3. Remove the funnel, flame-sterilize the forceps, and lift the filter from the base. Place the filter gently on the LB agar in a Petri dish, with the grid facing up, ensuring that the filter lies flush against the surface. Invert the plates and incubate for 24 h at 37 °C.
        4. After 24 h, remove the plates from the incubator and count the *E. coli* colonies. Use this data and the known dilution factor and volume of the solution to calculate the concentration of the filtered solution in CFU/mL.

    3. Propagate Phi6 in *Pseudomonas syringae* host using the double agar overlay method[35].
        1. Add 100 µL of Phi6 stock suspension and 100 µL of *P. syringae* overnight culture directly to 6 mL of Nutrient Broth Yeast (NBY) soft agar (0.3%). Pour it onto plates with NBY hard agar (1.5%) and incubate overnight at 26 °C. Prepare enough plates to produce enough inoculum for the experiment, estimating a yield of approximately 4 mL of viral suspension per plate.
        2. The next day, add 5 mL of PBS on top of the soft agar layer. Leave it at room temperature for 4 h, retrieve it with a pipette, and filter it using a 0.45-µm filter. Store at 4 °C.
        NOTE: This solution will serve as the viral inoculate.

    4. Use a plaque assay to confirm that the concentration is greater than 10^7 PFU/mL[35]. Perform serial dilutions of the viral suspension in PBS so that 100 µL produces a countable number of plaques on the plate.
        1. Pipette 100 µL of an appropriate diluted sample and 100 µL of overnight host culture directly into a tube containing 6 mL of NBY soft agar. Pour soft agar over NBY hard agar and incubate at 26 °C for 24 h.
        2. The next day, remove the plates from the incubators and count the number of plaques per plate. Use this data and the known dilution factor and volume of the solution to calculate the concentration of the filtered solution in PFU/mL.

2. **Prepare the tripartite soil load, intended to mimic human serum.**

1. Combine 7.80 mg/mL bovine serum albumin, 10.92 mg/mL tryptone, and 2.52 mg/mL bovine mucin to produce the required volume of soil load. After mixing the soil load, filter it through a 0.22-µm filter to sterilize. Store it at 4 °C until use. Do not heat sterilize, as the proteins will denature.

3. Prepare a 0.9% NaCl solution for mixing the inoculate for conditions without soil load.
4. Immediately prior to testing, prepare an inoculum composed of 68% bacterial or viral suspension and 32% soil load. For example, use 1.02 mL of bacterial or viral suspension from steps 3.1.1.2 or 3.1.3.2 and 0.48 mL of either soil load (step 3.2.1) or 0.9% NaCl solution (step 3.3). Swirl or vortex gently to mix.
   NOTE: 1.5 mL of this inoculum will be used for each volunteer under each condition, so ensure that the total volume of the inoculum prepared is sufficient for the intended number of tests.

4. Preparing Volunteers for the Experiment

NOTE: Determine the organism and soil load condition to be tested on that day. The same volunteers may be used for testing multiple conditions, but each volunteer should only be subjected to one round of testing within a 48 h period.

1. Before beginning testing, confirm that the volunteers remain eligible by verbally verifying that they adhered to the 7 day antimicrobial washout period and by visually confirming that they have not developed any breaks or abnormalities on their skin.
2. Using a random number generator, assign each volunteer to use either their right or left hand for sampling on this day of testing. Assign an order in which the handwashing conditions will be performed.
   NOTE: For example, ABHS may be assigned #3 and will be performed third.
3. **Perform a "cleansing wash" once at the start of testing to strip the skin of dirt and oils so that each subsequent test is conducted under equivalent conditions.**
   1. To do a cleansing wash, run through each step of the experiment (section 5, below), using a blank inoculate (LB broth or PBS only) and taking a sample without handwashing.

5. Experimental Procedure

1. To test the pH of the skin of each volunteer (to control for variation), place a flat-tipped skin pH probe on the palmar surface skin and the web space between the pointer and middle fingers. Ensure that the electrode is flat against the skin. Record the pH reading.
2. **Spike the hands.**
   1. Have the volunteers cup both hands together. Spike the hands with 1.5 mL of the inoculate by carefully pipetting 750 µL slowly into each palm.
   2. Have the volunteers gently rub their hands together until all surfaces of the hand are coated with the inoculate while subjecting the hands to as little friction as possible.
   3. Have the volunteers hold their hands still and away from their body for an additional 30 s to allow the inoculate to dry. The inoculate may not dry completely.

3. **Wash the hands.**
   1. For all following wash steps, capture the rinse water from the hands in a large sample collection bag. Add 4.5 mL of a 12% sodium thiosulfate solution to the bag to neutralize the chlorine on contact and process within 2 h.
      NOTE: Sodium thiosulfate should be added to all samples (even those without chlorine) to control for any impact that it may have on the organism.
   2. After inoculation (section 5.2), wash the hands with the next method in the designated order.
      1. For Control A, do not perform a handwashing step and move directly to step 5.5.
      2. For Control B, wash the hands with only ultrapure water at room temperature (approximately 21 °C) through a funnel with a known flow rate.
         NOTE: Here, a flow rate of 1.5 L/m and 500 mL of water were used.
      3. For handwashing with soap, wet the hands with 10 mL of ultrapure water. Have the volunteers lather their hands with soap and then rub their hands together for an additional 20 s. Rinse their hands by pouring 500 mL of ultrapure water at room temperature through a funnel at a flow rate of 1.5 L/m.
      4. For all chlorine solutions (*e.g.,* ABHS, HTH, NaDCC, and NaOCl), pour 200 mL of chlorine solution through a funnel at a flow rate of 1.5 L/m and have the volunteer rub their hands thoroughly.

4. **Hand rinse using a modified glove juice procedure.**
   1. After handwashing, immediately place each volunteer's hand (*i.e.,* the hand (right or left) selected for testing in step 4.2) into a sample bag containing 75 mL of eluent (*e.g.,* PBS) up to the wrist. Hold the top of the bag tightly around the wrist.
      NOTE: Use an eluent for sampling that contains sufficient sodium thiosulfate to neutralize any chlorine used for handwashing. PBS is a commonly used eluent that is appropriate for many organisms.
   2. Have the volunteers gently rub their hand in the solution for 30 s, taking care to reach in between the fingers and underneath the fingernails. Massage the hand from outside the bag gently for 30 s to ensure that the entire hand is rinsed thoroughly in the eluent, all the way up to the wrist.
   3. Seal the bag and process it according to the appropriate assay, described in section 6, within 2 h.

5. **Decontamination.**
   1. Before repeating the process with each handwashing method, have the volunteers wash their hands thoroughly in a sink with soap and warm water. Spray the volunteers' hands with 70% ethanol until they are coated on both sides. Allow them to dry.

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2. Repeat all steps in section 5 for each handwashing condition, only using the hand randomly selected in step 4.2 (**Figure 1**).

6. Quantification

1. Perform assays appropriate for the organism of choice (*e.g.,* membrane filtration for bacteria or plaque assay for viruses, described above in sections 3.1.2 and 3.1.4, respectively).
2. After counting the plates, record the estimated CFU/mL or PFU/mL for each test for the analyses (sections 3.1.2 and 3.1.4).

7. Analysis

1. **Using the results from step 6.2, calculate the log reduction value of organisms on the hands, for each organism and soil load status and for each subject and handwashing method.**
   1. For handwashing efficacy, compare the concentration of bacteria/virus in each handwashing sample to control A (no handwashing). For rinse water persistence, compare each rinse water sample to control B (washing with water only). Use the following standard formula:

      Log Reduction (handwashing) = $\log_{10}\left(\frac{\text{CFU or PFU/mL without handwashing}}{\text{CFU or PFU/mL selected handwashing method}}\right)$

      Log Reduction (rinse water) = $\log_{10}\left(\frac{CFU\ or\ PFU/mL\ \ handwashing\ with\ water\ only}{CFU\ or\ PFU/mL\ \ selected\ handwashing\ method}\right)$

      NOTE: Log reduction can also be expressed as $\log_{10}$(without handwashing) - $\log_{10}$(with handwashing)

2. **Use a one-way repeated measures analysis of variance (ANOVA) to assess the significant differences in the calculated log reduction values between handwashing methods and a post-hoc Tukey's HSD test for significant models to pairwise assess significant differences (p <0.05)[36].**
   1. Prior to running the ANOVA, assess each dataset for sphericity (*e.g.,* using Bartlett's test). Apply a correction (*e.g.,* the Greenhouse-Geisser correction) when the test indicates that sphericity was violated[37].

Representative Results

Here, the protocol (**Figure 1**) was completed with 18 volunteers, who were each tested using both *E. coli* and Phi6. Significant differences were found between handwashing results with *E. coli* both with and without soil load and Phi6 with soil load (**Figure 2** and **Figure 3**). For *E. coli* without soil load, handwashing with HTH, NaDCC, and stabilized NaOCl all resulted in significantly greater log reductions than handwashing with water only ($F(6,102) = 2.72$, $p = 0.034$). With soil load, HTH resulted in a significantly greater log reduction of *E. coli* than water only, HWWS, and ABHS ($F(6,102) = 3.94$, $p < 0.001$). There was no significant difference between methods for Phi6 without soil load ($F(6,66) = 2.04$, $p = 0.073$). However, for Phi6 with soil load ($F(6,102) = 7.01$, $p < 0.001$), water alone resulted in a greater log reduction than ABHS or stabilized NaOCl, and HWWS in a greater log reduction than ABHS, stabilized NaOCl, and generated NaOCl. HTH also had a greater log reduction than ABHS and stabilized NaOCl, and NaDCC resulted in a greater log reduction than stabilized NaOCl and ABHS. While HTH performed most consistently well across conditions, we would caution against over-interpretation of significant results, as many confidence intervals were large, ranging from less than 0.5 log to more than 1.5 log reduction in many cases.

In rinse water, chlorine resulted in a significantly greater log reduction of *E. coli* persisting in the rinse water than HWWS (without soil load, $F(4,68) = 331.7$, $p < 0.001$; with soil load, $F(4,68) = 162.44$, $p < 0.001$) (**Figure 4**). This same pattern was found in Phi6 without soil load (($F(4,43) = 8.95$, $P < 0.001$), with all chlorine solutions resulting in a significantly greater reduction of Phi6 in rinse water than HWWS. There were no significant differences in persistence in rinse water with Phi6 and soil load (($F(4,67) = 3.35$, $p = 0.071$) (**Figure 5**).

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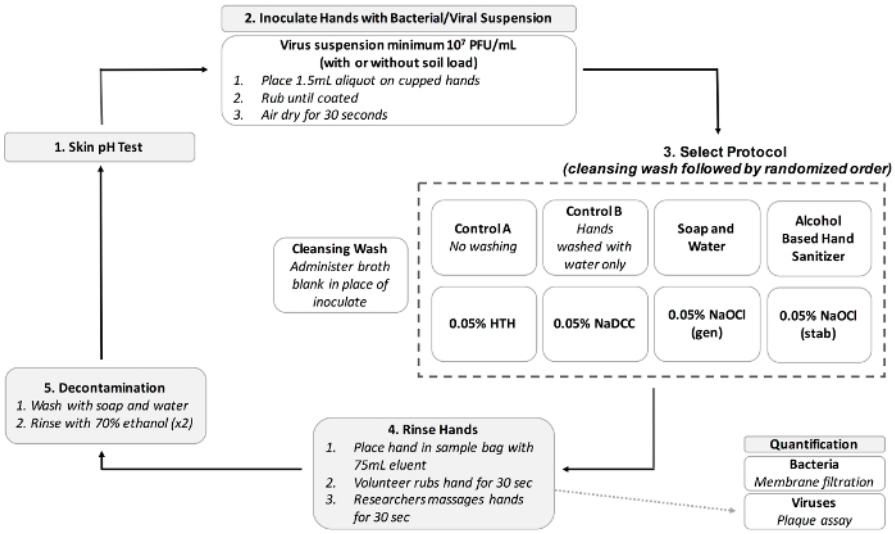

Figure 1: Experiment overview. The five steps undertaken for each round of handwashing include: 1) pH testing, 2) inoculating the hands, 3) handwashing, 4) rinsing the hands, and 5) decontaminating the hands for each of the eight conditions tested. Please click here to view a larger version of this figure.

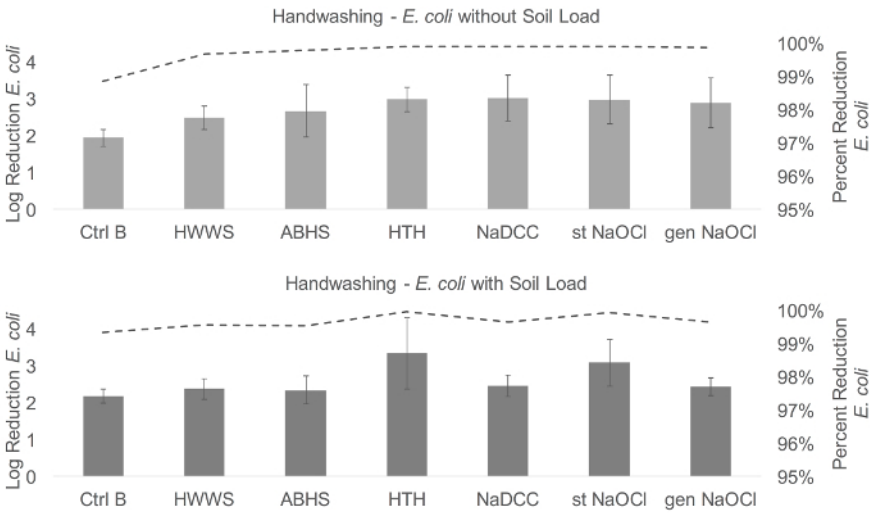

Figure 2: *E. coli* handwashing results. Compared to no handwashing, the handwashing methods tested resulted in an average log reduction in *E. coli* of 1.94-3.01 without soil load and 2.18-3.34 with soil load. Handwashing with water demonstrated the least reduction in *E. coli* in both conditions (1.94 and 2.18 log). Handwashing with NaDCC resulted in the greatest reduction without soil load (3.01), and HTH resulted in the greatest reduction with soil load (3.34). In the charts, the line represents the percent reduction in organisms, and the error bars represent the standard error of log reduction. Ctrl B, control B; HWWS, handwashing with soap; ABHS, alcohol-based hand sanitizer; HTH, high-test hypochlorite; NaDCC, sodium dichloroisocyanurate; st NaOCl, stabilized sodium hypochlorite; gen NaOCl, generated sodium hypochlorite. Please click here to view a larger version of this figure.

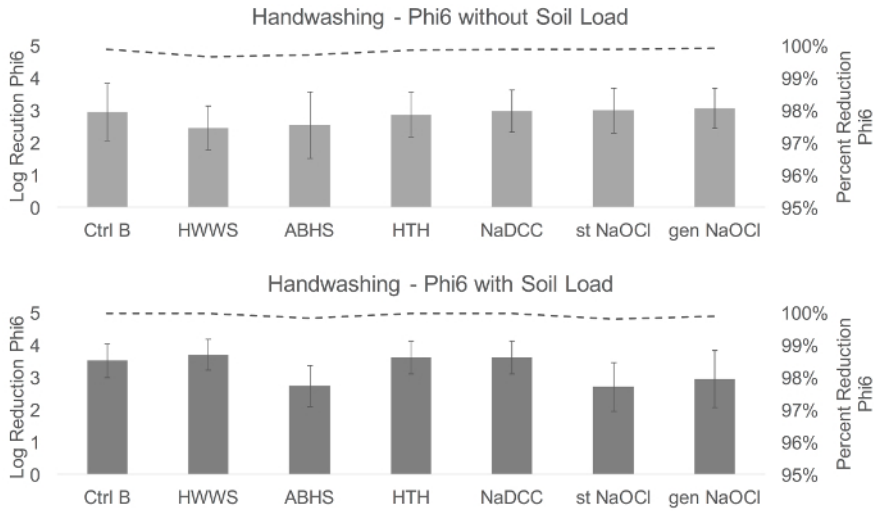

Figure 3: Phi6 handwashing results. Compared to no handwashing, the handwashing methods tested resulted in an average log reduction in Phi6 of 2.44-3.06 without soil load and 2.71-3.69 with soil load. Handwashing with soap demonstrated the least reduction in Phi6 without soil load (2.44), and handwashing with stabilized NaOCl resulted in the smallest reduction with soil load (2.71). Handwashing with generated NaOCl resulted in the greatest reduction without soil load (3.06), and handwashing with soap resulted in the greatest reduction with soil load (3.69). In the charts, the line represents the percent reduction in organisms, and the error bars represent the standard error of log reduction. Ctrl B, control B; HWWS, handwashing with soap; ABHS, alcohol-based hand sanitizer; HTH, high-test hypochlorite; NaDCC, sodium dichloroisocyanurate; st NaOCl, stabilized sodium hypochlorite; gen NaOCl, generated sodium hypochlorite. Please click here to view a larger version of this figure.

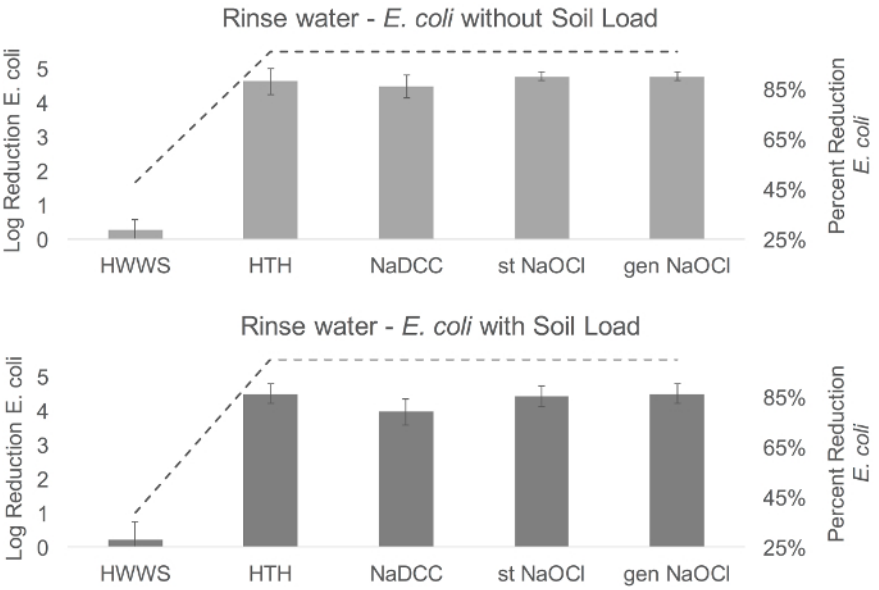

Figure 4: *E. coli* hand rinse results. Compared to hand washing with water only, the average log reduction of *E. coli* remaining in the rinse water was 0.28-4.77 without soil load and 0.21-4.49 with soil load. Both with and without soil load, the smallest reduction was found in handwashing with soap (0.28 and 0.21). The greatest reductions were observed with stabilized and generated NaOCl without soil load (both 4.77) and with HTH and generated NaOCl with soil load. In the charts, the line represents the percent reduction in organisms, and the error bars represent the standard error of log reduction. HWWS, handwashing with soap; ABHS, alcohol-based hand sanitizer; HTH, high-test hypochlorite; NaDCC, sodium dichloroisocyanurate; st NaOCl, stabilized sodium hypochlorite; gen NaOCl, generated sodium hypochlorite. Please click here to view a larger version of this figure.

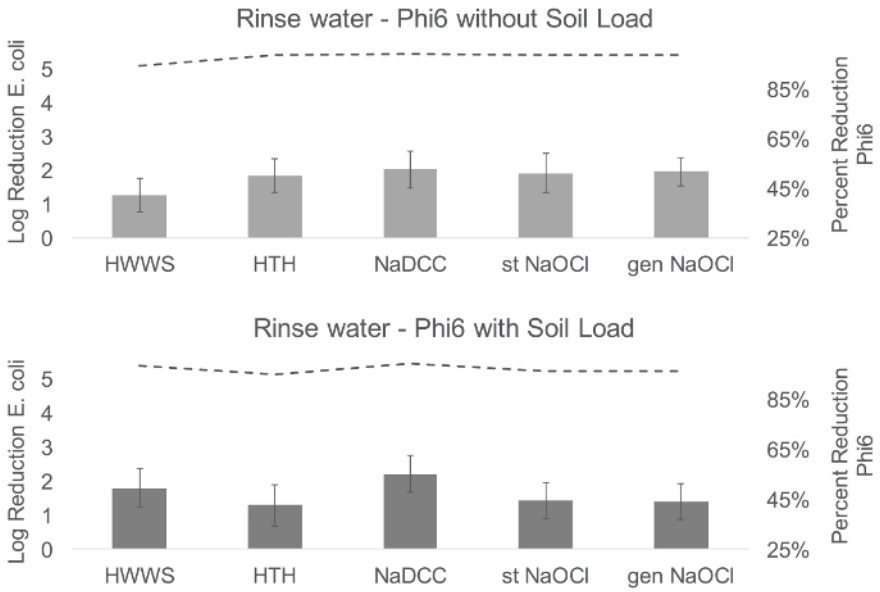

Figure 5: Phi6 hand rinse results. Compared to hand washing with water only, the average log reduction of Phi6 remaining in the rinse water was 1.26-2.02 without soil load and 1.30-2.20 with soil load. With soil load, the smallest reduction was found in handwashing with soap (1.26). Without soil load, HTH resulted in the smallest reduction (2.02). The greatest reductions were observed both with and without soil load with NaDCC (2.02 and 2.20). In the charts, the line represents the percent reduction in organisms, and the error bars represent the standard error of log reduction. HWWS, handwashing with soap; ABHS, alcohol-based hand sanitizer; HTH, high-test hypochlorite; NaDCC, sodium dichloroisocyanurate; st NaOCl, stabilized sodium hypochlorite; gen NaOCl, generated sodium hypochlorite. Please click here to view a larger version of this figure.

Discussion

The method described here provides an approach for testing handwashing efficacy in a controlled laboratory setting. This method highlights the use of human volunteers and surrogate, non-infectious organisms. Using the method, it was possible to demonstrate differences in: 1) the efficacy of handwashing methods and 2) organism persistence in rinse water. The purpose of presenting this protocol is to provide a general framework that can be adapted to test a wide range of surrogate organisms and handwashing methods relevant to infectious disease.

During the use of the method, two key data quality recommendations were noted as important. First, the inoculate must be applied both as similarly as possible across the rounds of testing and in a manner, that minimizes loss. This is to ensure that sufficient inoculate is applied to the hands to allow for statistically significant results. Second, be sure to complete the "cleansing wash" step, in which the protocol is performed without handwashing prior to testing, as previous work has shown that there are likely to be significant differences between a first wash and subsequent washes, but not between subsequent washes performed after a cleansing round[29]. Additionally, this step clears residual hand contamination, which would interfere with results.

The main limitation of this protocol is that it can be uncomfortable for volunteers. During each round of testing, which lasted about 2 h, volunteers' hands became cold. Some volunteers reported mild pain from their hands being constantly wet. Additionally, after a few rounds of testing, volunteers' hands became supersaturated, no longer fully drying between rounds. Although the randomization of the order of handwashing methods for each volunteer accounted for supersaturation, it is possible that the supersaturation could act as a confounding or modifying factor in this type of testing. To address this limitation, it is recommended that volunteers are appraised of this risk during consent disclosures and are reminded of their right to drop out of the study at any time. Volunteers should not undergo testing for more than 2 h per day to allow time for the hands to return to a baseline state and to minimize discomfort. A second limitation is the need to use a surrogate organism or non-infectious variant of a pathogenic organism to protect the health of volunteers. This might cause concern about the generalizability of results. However, for some pathogenic organisms (such as the Ebola virus), this limitation cannot be ethically overcome. Care must be taken during surrogate organism selection. Lastly, this is a laboratory study on efficacy. Results may only translate to effective disease prevention in real-word contexts where handwashing methods are made accessible to those in need and are used properly and consistently.

This protocol draws on previous work on handwashing efficacy but attempts to streamline methods and emphasizes the use of human hands (rather than surrogate surfaces) for testing. Additionally, rinse water is a transmission risk that had previously not been assessed. Existing studies on handwashing efficacy vary in methodology, leading to non-comparable data. We hope that standardized protocols for conducting handwashing method comparisons will encourage comparable and replicable results. Previous work has demonstrated that *in vitro* testing on surrogate surfaces such as pig skin, where, for example, the actual Ebola virus could be used, produced results that do not match those found after testing on human hands[38]. Therefore, a method using human hands and surrogate or non-infectious organisms is currently the best available approach to estimate handwashing efficacy and rinse water persistence for infectious microorganisms.

Handwashing is critical to prevent disease transmission. However, there is a lack of evidence on the comparative efficacy of handwashing methods that are commonly recommended. This protocol can be used to generate evidence about handwashing efficacy and rinse water persistence. This is especially important for infectious diseases with the potential to cause large outbreaks, such as the Ebola virus. We hope

that other researchers will find this protocol useful to generate much-needed additional evidence on handwashing method efficacy and rinse water persistence that will assist in developing recommendations to reduce the transmission of infectious diseases.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Kampf, G., & Kramer, A. Epidemiologic Background of Hand Hygiene and Evaluation of the Most Important Agents for Scrubs and Rubs. *Clin Microbiol Rev.* **17** (4), 863-893 (2004).
2. Miller, T., Patrick, D., & Ormrod, D. Hand decontamination: influence of common variables on hand-washing efficiency. *Healthc Infect.* **16** (1), 18 (2013).
3. Jensen, D. A., Danyluk, M. D., Harris, L. J., & Schaffner, D. W. Quantifying the effect of hand wash duration, soap use, ground beef debris, and drying methods on the removal of Enterobacter aerogenes on hands. *J Food Prot.* **78** (4), 685-690 (2015).
4. Girou, E., Loyeau, S., Legrand, P., Oppein, F., & Brun-Buisson, C. Efficacy of handrubbing with alcohol based solution versus standard handwashing with antiseptic soap: randomised clinical trial. *BMJ.* **325** (7360), 362 (2002).
5. Kac, G., Podglajen, I., Gueneret, M., Vaupré, S., Bissery, A., & Meyer, G. Microbiological evaluation of two hand hygiene procedures achieved by healthcare workers during routine patient care: a randomized study. *J Hosp Infect.* **60** (1), 32-39 (2005).
6. Lages, S. L. S., Ramakrishnan, M. A., & Goyal, S. M. In-vivo efficacy of hand sanitisers against feline calicivirus: a surrogate for norovirus. *J Hosp Infect.* **68** (2), 159-163 (2008).
7. Holton, R. H., Huber, M. A., & Terezhalmy, G. T. Antimicrobial efficacy of soap and water hand washing versus an alcohol-based hand cleanser. *Tex Dent J.* **126** (12), 1175-1180 Retrieved from http://www.tda.org/Publications/Texas-Dental-Journal (2009).
8. Salmon, S., Truong, A. T., Nguyen, V. H., Pittet, D., & McLaws, M.-L. Health care workers' hand contamination levels and antibacterial efficacy of different hand hygiene methods used in a Vietnamese hospital. *Am J Infect Control.* **42** (2), 178-181 (2014).
9. Steinmann, J., Nehrkorn, R., Meyer, A., & Becker, K. Two in-vivo protocols for testing virucidal efficacy of handwashing and hand disinfection. *Int J Hyg Environ Health.* **196** (5), 425-436 Retrived from https://www.journals.elsevier.com/international-journal-of-hygiene-and-environmental-health (1995).
10. Weber, D. J., Sickbert-Bennett, E., Gergen, M. F., & Rutala, W. A. Efficacy of selected hand hygiene agents used to remove Bacillus atrophaeus (a surrogate of Bacillus anthracis) from contaminated hands. *JAMA.* **289** (10), 1274-1277 (2003).
11. Grayson, M. L., Melvani, S., *et al.* Efficacy of Soap and Water and Alcohol-Based Hand-Rub Preparations against Live H1N1 Influenza Virus on the Hands of Human Volunteers. *Clin Infect Dis.* **48** (3), 285-291 (2009).
12. Oughton, M. T., Loo, V. G., Dendukuri, N., Fenn, S., & Libman, M. D. Hand hygiene with soap and water is superior to alcohol rub and antiseptic wipes for removal of Clostridium difficile. *Infect Control Hosp Epidemiol.* **30** (10), 939-944 (2009).
13. Liu, P., Yuen, Y., Hsiao, H.-M., Jaykus, L.-A., & Moe, C. Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands. *Appl Environ Micro.* **76** (2), 394-399 (2010).
14. Savolainen-Kopra, C., Korpela, T., *et al.* Single treatment with ethanol hand rub is ineffective against human rhinovirus--hand washing with soap and water removes the virus efficiently. *J Med Virol.* **84** (3), 543-547 (2012).
15. Tuladhar, E., Hazeleger, W. C., Koopmans, M., Zwietering, M. H., Duizer, E., & Beumer, R. R. Reducing viral contamination from finger pads: handwashing is more effective than alcohol-based hand disinfectants. *J Hosp Infect.* **90** (3), 226-234 (2015).
16. Steinmann, J., Paulmann, D., Becker, B., Bischoff, B., Steinmann, E., & Steinmann, J. Comparison of virucidal activity of alcohol-based hand sanitizers versus antimicrobial hand soaps in vitro and in vivo. *J Hosp Infect.* **82** (4), 277-280 (2012).
17. de Aceituno, A. F., Bartz, F. E., *et al.* Ability of Hand Hygiene Interventions Using Alcohol-Based Hand Sanitizers and Soap To Reduce Microbial Load on Farmworker Hands Soiled during Harvest. *J Food Protect.* **78** (11), 2024-2032 (2015).
18. Sterk, E. *Médecins Sans Frontières - Filovirus Haemorrhagic Fever Guideline.* <http://jid.oxfordjournals.org/content/204/suppl_3/S791.full (2008).
19. WHO Report. *Ebola Virus Disease (EVD) Key questions and answers concerning water, sanitation and hygiene.* 1-5 http://www.who.int/water_sanitation_health/WASH_and_Ebola.pdf , World Health Organization (2014).
20. Guidelines on Hand Hygiene in Health Care. *First Global Patient Safety Challenge, Clean Care is Safer Care.* http://whqlibdoc.who.int/publications/2009/9789241597906_eng.pdf , World Health Organization (2009).
21. Boyce, J. M., & Pittet, D. Guideline for Hand Hygiene in Health-Care Settings: Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. *Infect Control Hosp Epidemiol.* **23**(12 Suppl) Society for Healthcare Epidemiology of America. (2002).
22. Emmanuel, J., Ndoye, B., *UNDP Medical Waste ExpertsAssessment and Recommendations Regarding Management of Ebola-Contaminated Waste*. https://noharm-global.org/sites/default/files/documents-files/3127/Report%20to%20WHO%20WASH%20and%20Geneva%20on%20Ebola%20final.pdf , United Nations Development Programme (2015).
23. Hopman, J., Kubilay, Z., Allen, T., Edrees, H., Pittet, D., & Allegranzi, B. Efficacy of chlorine solutions used for hand hygiene and gloves disinfection in Ebola settings: a systematic review. *Antimicrob Resist Infect Control.* **4** (1), 1-1 (2015).
24. Lowbury, E. J. L., Lilly, H. A., & Bull, J. P. Disinfection of hands: removal of transient organisms. *BMJ.* **2** (5403), 230-233 (1964).
25. Edmonds, S. L., Zapka, C., *et al.* Effectiveness of Hand Hygiene for Removal of Clostridium difficile Spores from Hands. *Infect Control Hosp Epidemiol.* **34** (3), 302-305 (2013).
26. Rotter, M.L. 150 years of hand disinfection-Semmelweis' heritage. *Hyg Med.* (22), 332-9 (1997).

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27. Hitomi, S., Baba, S., Yano, H., Morisawa, Y., & Kimura, S. Antimicrobial effects of electrolytic products of sodium chloride--comparative evaluation with sodium hypochlorite solution and efficacy in handwashing. *Kansenshōgaku Zasshi.* **72** (11), 1176-1181 (1998).
28. ASTM International. *Standard E1174 - 13. Standard Test Method for Evaluation of the Effectiveness of Health Care Personnel Handwash Formulations.* <www.astm.org> (2013).
29. Casanova, L. M., & Weaver, S. R. Evaluation of eluents for the recovery of an enveloped virus from hands by whole-hand sampling. *J Appl Microbiol.* **118** (5), 1210-1216 (2015).
30. Sinclair, R. G., Rose, J. B., Hashsham, S. A., Gerba, C. P., & Haas, C. N. Criteria for Selection of Surrogates Used To Study the Fate and Control of Pathogens in the Environment. *Appl Environ Microbiol.* **78** (6), 1969-1977 (2012).
31. Held, E., Skoet, R., Johansen, J. D., & Agner, T. The hand eczema severity index (HECSI): A scoring system for clinical assessment of hand eczema. A study of inter- and intraobserver reliability. *Br J Dermatol.* **152** (2), 302-307 (2005).
32. *Chlorine Total, Iodometric Method Using Sodium Thiosulfate, Method 8209 Digital Titrator.* http://www.hach.com/asset-get.download-en.jsa?id=7639983937 (2016).
33. *GeneQuant 100 User Manual.* http://biochromspectros.com/media/wysiwyg/support_page/support-genequant-100/Genequant-100-UM.pdf (2016).
34. United States Environmental Protection Agency. *Method 1604: Total Coliforms and Escherichia coli in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium).* <https://www.epa.gov/sites/production/files/2015-08/documents/method_1604_2002.pdf (2002).
35. Adams, M. H., & Anderson, E. S. *Bacteriophages.* Interscience Publishers New York (1959).
36. Kao, L. S., & Green, C. E. Analysis of Variance: Is There a Difference in Means and What Does It Mean? *The Journal of surgical research.* **144** (1), 158-170 (2008).
37. Schutz, R. W., & Gessaroli, M. E. The Analysis of Repeated Measures Designs Involving Multiple Dependent Variables. *Research Quarterly for Exercise and Sport.* **58** (2), 132-149 (1987).
38. Woolwine, J. D., & Gerberding, J. L. Effect of testing method on apparent activities of antiviral disinfectants and antiseptics. *Antimicrob Agents Chemother.* **39** (4), 921-923 (1995).

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