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3 A Review of Methods for the Evaluation of Handwashing Efficacy
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Abstract

Handwashing is relied upon in numerous fields as a primary means to prevent transmission of harmful pathogens. While handwashing is a key step in disease prevention, the factors controlling its effectiveness are not always well understood, and there are extensive variations in the methodology used to assess each of these factors. This review summarizes the various factors that can impact handwashing effectiveness as well as the methods and results of studies evaluating each of these factors related to handwashing. Numerous methods are available to inoculate hands as well as to recover microorganisms from hands, and for a given method, experimental variables can be changed between researchers. These variations amongst methods as well as variations in reporting experimental results can make it difficult to compare studies as well as challenging to accurately interpret the results between studies. Standardization of methods and reporting requirements are necessary to allow for comparison of studies so that more accurate conclusions about the handwashing process can be made. Therefore, the need for 1) the development of more standardized handwashing test methods and 2) the formation of guidelines on the minimal information required for publication of handwashing experiments are considered and discussed.

KEY WORDS: handwashing, food safety, inoculation, microbial recovery

1. Introduction

Handwashing is widely accepted as a primary means to limit the spread of pathogens and aid in the prevention of infectious disease (Larson et al. 2000; Sickbert-Bennett et al. 2005).

Numerous fields from medicine to the food industry rely on proper and consistent handwashing to promote a safe and hygienic environment for both employees and clients. While much of the world's population relies on handwashing as a daily method to maintain hygiene and prevent disease transmission, the importance of hand hygiene has not always been understood.

In the food industry, strict guidelines are provided to minimize contamination of food and aid in the production or preparation of a quality and safe product for consumers. The United States Food and Drug Administration's (FDA) Food Code describes in detail the appropriate manner in which hands should be washed as well as the recommended times to wash hands when preparing food, stating that food workers must wash hands immediately before handling: food, clean equipment and utensils, and unwrapped single-service and single-use articles (USFDA 2013). Additionally the Food Code further describes particular situations where handwashing should occur before handling food, including: after using the restroom, switching between raw and ready-to-eat food, after touching bare human body parts, and before putting on gloves when handling food (USFDA 2013).

Despite the vast focus of the food industry on proper handwashing, compliance with proper handwashing often fails, and numerous foodborne disease outbreaks occur each year due to improper handwashing (Green et al. 2006). Understanding and correcting this lack of compliance is key to reduce foodborne illnesses caused by food handlers. In addition, understanding the various factors involved in the handwashing process, and optimizing that handwashing process are also beneficial in minimizing foodborne illness. Therefore, the

objectives of this review are to i) conduct a search of peer-reviewed publications available in the field of handwashing; ii) summarize the different methodologies used in the evaluation of handwashing efficacy; and iii) discuss the need for standardized methodologies and reporting requirements to allow for comparison and consistency between handwashing studies. While a previous meta-analysis by Montville and Schaffner (2011) focused on the effectiveness of antimicrobial soaps along with the factors that may impact study results—including methodologies—the primary focus of the present review is to discuss the experimental steps used to determine the effectiveness of any given handwashing agent and highlight the need for standard approaches and reporting requirements.

2. Background

2.1. Handwashing and Impact on the Food Industry

Proper hygiene and effective handwashing are essential to food safety. It is estimated that foodborne pathogens, both major known pathogens as well as unspecified agents, cause 47.8 million illnesses, 127,830 hospitalizations, and 3,037 deaths in the U.S. each year (Scallan et al. 2011). While eliminating all foodborne disease is unrealistic, certain food safety practices such as handwashing are an effective tool to reduce disease incidence. Section 2-301.12 of the FDA Food Code states that proper handwashing can result in a 2 to 3 log reduction in transient bacteria as well as a 2-log reduction in transient viruses and protozoa (USFDA 2013). Transfer of pathogens from the hands of food workers to food significantly contributes to the spread of foodborne illness, and the improvement of handwashing in food workers is critical to decrease the amount of foodborne illness outbreaks (Edmonds et al. 2012; Green et al. 2006; Michaels et al. 2004; Pragle et al. 2007; Todd et al. 2010a).

Although handwashing is heavily relied on in the food industry to limit microbial contamination of food, and although clear guidelines on proper handwashing are provided, the transmission of harmful microorganisms from food workers' hands to food remains a significant factor in transmission of foodborne illness (Green et al. 2006). Michaels et al. (2004) conducted a study of 308 outbreaks attributed to ill or asymptomatic food handlers, and 59% of the outbreaks were due to contamination of the food product through hand contact. Not surprisingly, the majority of these outbreaks were due to foods that required a great deal of handling, such as potato salad, salad mixed by hand, and guacamole. While these tasks involving more abundant quantities of food are a significant contributor to foodborne illness, Michaels et al. (2004) also found that much smaller tasks such as handling a slice of tomato or garnishing a dish before serving can have a significant impact on foodborne illness.

One common strategy to aid in safe preparation of food is to utilize gloves when handling foods. While gloves can be a great solution, they must be used properly and not as a substitute for handwashing (Green and Selman 2005; Guzewich and Ross 1999; Michaels et al. 2004). Green et al. (2006) conducted an observational study on the handwashing practices of 321 food workers and found that appropriate handwashing rates decreased at a significant rate when gloves were worn. The use of gloves in food production can present a false sense of security, causing food handlers to practice unsafe food handling techniques such as washing their hands less frequently or less often after high-risk tasks (i.e. handling raw meat) leading to potential microbial contamination of foods (Todd et al. 2010b). Moreover, Montville et al. (2001) conducted a study on glove usage and cross-contamination in food and demonstrated that bacteria can transfer from food to the hands and from hands to food through the glove. The

results of this study further emphasize that gloves are not an ideal solution and can actually cause a false sense of security.

While it has been established that food workers do contribute significantly to foodborne illness outbreaks through improper handling of food, it is important to understand why proper and consistent handwashing is not occurring. Pragle et al. (2007) conducted a study directly asking food handlers about their knowledge, practices and barriers to handwashing in the restaurant environment. The most significant barriers to handwashing included the availability of supplies, accessibility of sinks, time pressure (i.e. not enough time to wash hands between tasks), high volume of business, stress, lack of accountability, type of restaurant, insufficient training at the restaurant, and inadequate food handler training (Pragle et al. 2007).

Even though handwashing is commonly relied on as one of the foremost methods to prevent transmission of pathogens to food, it is clear that food is still frequently contaminated by poor handwashing practices of food handlers. Having a clear understanding of how to make handwashing optimally effective is essential for training employees and preventing contamination of food from food handlers.

2.2. Primary Factors Influencing Effective Handwashing

While handwashing is a beneficial method to aid in disease control, there are a few key variables that can impact the effectiveness of handwashing. Frequency, agent (e.g., soap or sanitizer), appropriateness (i.e. whether the hands were washed when needed to be washed), duration, and technique are all variables that determine the effectiveness of handwashing (Larson et al. 2006). The 2013 FDA Food Code emphasizes the importance of every step in the cleansing of hands, including scrubbing, rinsing, and drying, noting that failure to perform any of these

steps in the handwashing process can have a negative effect on the handwashing episode (USFDA 2013). To improve handwashing, it is essential to understand the factors that make handwashing effective.

2.2.1. Soap Type: Antimicrobial vs. Non-antimicrobial

There are two primary types of hand soap available: antimicrobial and non-antimicrobial hand soap. Both types of hand soap lead to a reduction in microorganisms found on the hands, but reports vary as to the overall effectiveness of each type of soap. Sickbert-Bennett et al. (2005) conducted a study on the efficacy of hand hygiene agents using tap water, non-antimicrobial soap, alcohol solutions, and various antimicrobial agents. Sickbert-Bennett et al. (2005) and others concluded that while antimicrobial handwashing agents were the most effective in bacterial removal, no handwashing agents were significantly superior to non-antimicrobial soap or tap water alone. Edmonds et al. (2013) studied the effectiveness of hand hygiene for removal of *Clostridium difficile* spores from hands. Handwashing agents in this study included: tap water, 4% chlorhexidine gluconate (CHG) hand wash, non-antimicrobial hand soap, 0.3% triclosan hand wash, and peracetic acid wipes which would not be used routinely as they are too harsh on hands. Results of the study found a 0.76 log₁₀ colony forming units (CFU)/mL reduction in *C. difficile* spores with tap water alone. The non-antimicrobial hand soap and the 4% chlorhexidine gluconate hand wash had similar reductions in *C. difficile* when compared to tap water alone. The 0.3% triclosan hand wash had a slightly higher increase in *C. difficile* removal at a 0.99 log₁₀ CFU/mL reduction while the harsh peracetic acid wipes provided the most significant reduction in *C. difficile* spores at a 1.1 log₁₀ CFU/mL reduction.

Another study by Fuls et al. (2008) investigated the bacterial reduction associated with antimicrobial and non-antimicrobial hand soaps as well as the change in bacterial reduction when other variables such as wash time, product type, and soap volume were included. The authors indicated that a greater reduction of bacteria occurred with the use of antimicrobial soap. Additionally, an increase in wash time led to an increase in bacterial reduction with antimicrobial soap while the same was not true for non-antimicrobial soap (Fuls et al. 2008). This lack of correlation between wash time and increased microbial reduction may be explained since non-antimicrobial hand soap relies only on the physical removal of bacteria from hands while antimicrobial hand soap combines physical removal as well as antimicrobial inactivation of bacteria.

Soap type can also impact viruses and bacteria differently. While most hand soaps will aid in the physical removal of microorganisms, antimicrobial soap can inactivate pathogens given a sufficient contact time as discussed previously. However, the majority of antimicrobial soaps do not include compounds that are able to inactivate viruses, most notably human norovirus—the primary cause of foodborne illness in the U.S. (Liu et al. 2009). In addition, some antimicrobials such as CHG, are more effective against bacteria (especially vegetative forms) than viruses and protozoa (McDonnell and Russell 1999). One reason that antimicrobials can have a different effect on viruses, bacteria, and protozoa is microbial structure. Protozoa can develop into their protective cyst forms under certain types of stress such as the presence of antimicrobial compounds thus allowing the protozoa to remain dormant and unaffected by chemicals (Nester et al. 2009). Some bacteria (*Bacillus* and *Clostridium*) are also capable of forming spores that are extremely stable and resistant to stressful conditions such as heat and toxic chemicals (Nester et al. 2009). Viruses are obligate intracellular infectious agents

composed of nucleic acid (either DNA or RNA) surrounded by a protein coat (capsid). Some viruses have a lipid membrane (envelope) which surrounds the capsid, while other viruses—most notably enteric viruses (e.g., human norovirus) transferred by the fecal-oral route—do not have this membrane and are thus naked (Nester et al. 2009). These differences in structure lead to a difference in how each type of microorganism is affected by antimicrobials and handwashing in general.

Sickbert-Bennett et al. (2005) investigated the efficacy of hand hygiene agents against viruses and bacteria. The results indicate that physical removal of non-enveloped viruses either through use of water or a non-antimicrobial soap was more effective than using a hand soap with an antimicrobial agent. Triclosan—one of the most common active ingredients in antimicrobial soap—actually functions by slowing down or inhibiting the growth of bacteria, fungi, and mildew (USEPA 2010). The effectiveness of triclosan against viruses—especially non-enveloped viruses (i.e. enteric viruses)—remains somewhat unclear (Mbithi et al. 1993). Contreras et al. (1999) echoed the findings of Sickbert-Bennett et al. (2005) with findings indicating that liquid hand dishwashing detergents were 100 times more effective than antibacterial soaps in reducing respiratory syncytial virus.

While individual studies have led to a somewhat inconclusive stance with respect to the differences in effectiveness between antimicrobial and non-antimicrobial handsoap, a recent meta-analysis conducted by Montville and Schaffner (2011) reported that antimicrobial soap consistently resulted in a significantly greater reduction of microorganisms on hands than non-antimicrobial handsoap. Although this difference does appear to be small (approximately a 0.5 log CFU reduction difference), this difference does exist and cannot be ignored (Montville and Schaffner 2011). Moreover, Schaffner et al. (2014) showed possible reduction in incidence of

foodborne illness when antibacterial soaps are used; however, this is based on a risk simulation and focused on reduction of bacterial pathogens and not viruses. It is important to take into consideration that the active compounds in antibacterial and antimicrobial soaps are not necessarily antiviral and thus may not have the same effect on viruses—or other pathogens such as protozoa—as they do on bacteria.

2.2.2. Soap Volume

While the type of hand soap used can impact effectiveness, the volume of the handwashing agent applied can also change the effectiveness of the handwashing episode. Larson et al. (1987) conducted a study focused on the volume of soap as a variable in handwashing. At that time, many studies were using a standard volume of 5 mL of handwashing agent, and Larson et al. (1987) stressed the need to investigate the effect of volume on handwashing effectiveness. The authors used two quantities of soap (1 and 3 mL) as well as multiple handwashing agents (4% CHG antiseptic agent, two alcohol-based hand-rinses with emollients, and a liquid non-antimicrobial hand soap). The authors found that a larger volume of 3 to 5 mL was beneficial for the antiseptic soap while a volume no greater than 1 mL was more appropriate for the nonantiseptic, liquid soap (Larson et al. 1987). Fuls et al. (2008) reported similar findings with respect to soap volume indicating an increase in bacterial reduction with larger volumes of antimicrobial soap, while the same did not hold true for non-antimicrobial hand soap. The meta-analysis by Montville and Schaffner (2011) found that while a strong correlation does not exist between soap volume and effectiveness of soap (antimicrobial or non-antimicrobial), there does seem to be an indication that it might be beneficial to use more than 1

mL of antimicrobial soap and that abnormally large volumes of soap (> 5 mL) are potentially less effective.

As discussed previously (Section 2.2.1), non-antimicrobial soaps use physical removal to reduce the level of bacteria on hands. This physical removal of bacteria occurs through the use of surfactants, and because of this, there is a maximum amount of bacteria that are capable of being removed. An increase in soap amount and wash time will not lead to increased bacterial removal after the maximum removal from the use of surfactants is achieved (Fuls et al. 2008). Antimicrobial soap however will benefit from increased volumes of soap as it combines the surfactant abilities of the non-antimicrobial soap with the inactivation of microorganisms, specifically bacteria (Fuls et al. 2008).

Relationships have also been linked between soap volumes, time spent washing, and overall bacterial reduction of handwashing. Miller et al. (2011) completed a study on the use of time, hand-to-hand friction, and the use of non-antimicrobial hand soap for hand decontamination and found that adding soap actually caused an initial delay of bacterial reduction within the first 10 seconds of handwashing. After the initial 10 seconds however, the delay in bacterial reduction was no longer present (Miller et al. 2011). The authors discussed a possible conclusion that the delayed bacterial reduction occurred as a result of a sort of lubricant effect of the soap in the handwashing process, leading to an initial delay in the amount of microorganisms removed from hands.

2.2.3. Handwashing Time

Time spent washing hands is another key variable. Generally, a 20 second hand wash is considered sufficient to reduce microorganisms on hands. The 2013 Food Code (section 2-

301.12) requires food employees to wash hands as well as exposed portions of the arm for 20 seconds, designating 10 to 15 seconds of this handwashing to vigorous rubbing of the hands. If performed properly, this handwashing regimen can result in a 2 to 3 log reduction in transient bacteria as well as a 2-log reduction in transient viruses and protozoa (Food Code 2013).

Although many organizations including the World Health Organization, the Mayo Clinic, and U.S. Centers for Disease Control and Prevention, recommend a handwashing time of a minimum of 20 seconds for optimal removal of microbes, people in public restrooms as well as in hospitals often wash their hands for 15 seconds or less (Soap and Detergent Association 2007). Munger and Harris (1989) conducted a study testing the social influence on handwashing behavior in a public restroom and found that observed participants washed their hands for an average of 5.2 seconds, while participants who were not observed washed their hands for an average of 4.7 seconds.

Sickbert-Bennett et al. (2005) utilized a 10 second handwash based on the assumption that this amount of time was more representative of what people were actually practicing. The authors of the study concluded that a significant reduction occurred in transient hand flora, and therefore more focus should be placed on increasing handwashing compliance, as a shortened handwashing time could aid in increasing handwashing compliance (Sickbert-Bennett et al. 2005). However, Montville and Schaffner (2011) showed that with a wash time of 30 seconds, a significant difference occurred in reduction of microorganisms with antimicrobial and non-antimicrobial soap. The authors reported that antimicrobial soap resulted in a 2.42 ± 0.88 log reduction while non-antimicrobial soap had a reduction of 1.91 ± 0.75 log. Regardless, there is still a need for additional studies looking at wash time and overall handwashing effectiveness, as

the research focusing on this particular factor is somewhat limited (Montville and Schaffner 2011).

Stroehbehn et al. (2008) conducted a study analyzing the handwashing practices of food service employees in operations that serve ready-to-eat food to immunocompromised individuals. The authors found that during food production in schools, of the 69 times out of the 300 times employees should have washed hands, soap was only used 62 times, and actual lathering of soap in hands only occurred 37 times. When compared to employees in the food production area, the employees were found to be more compliant with handwashing when first entering the work area with 12 of 19 participants washing hands (Stroehbehn et al. 2008). However, soap was only used 11 of 12 times, and lathering for 10 seconds only occurred nine times (Stroehbehn et al. 2008). In restaurants, only six of 83 participants washed hands after handling soiled equipment, and while all six participants used soap, only two actually lathered for the 10 seconds recommended by the Food Code (Stroehbehn et al. 2008).

Poor compliance with proper handwashing time also occurs frequently in the medical industry. Graham (1990) conducted a study on the frequency and duration of handwashing in an intensive care unit and found that for observed handwashing episodes, the average handwash duration time was 10 seconds (range of 3 to 45.2 seconds). Meengs et al. (1993) conducted a study on handwashing frequency in an emergency department and found that for the 126 times handwashing occurred with soap and water, the average duration was only 9.5 seconds.

The authors of the present review conducted a study to observe behavioral changes in 12 subjects when using foaming or liquid hand soap. The average wash time for individuals using foaming hand soap was 13.6 seconds with a standard deviation of 6.1 seconds while the average wash time for liquid hand soap was 15.3 seconds with a standard deviation of 6.6 seconds

(unpublished data). No significant difference in wash time occurred between foaming and liquid hand soap. However, there was a large wash time range between the two soaps, with the shortest time spent washing at 6 seconds and the longest at 26 seconds. With research studies indicating that handwashing time is often well below the recommended 20 seconds, it seems that using a more realistic amount of time in studies will produce data that will be more applicable to reductions occurring in everyday handwashing.

2.2.4. Drying

As previously stated, all steps in the handwashing process are critical, and the last step of drying is no less important. Bacteria transfer more efficiently from a wet surface than a dry surface (CDC 2009; Fuls et al. 2008; Patrick et al. 1997). Patrick et al. (1997) observed that drying hands after washing can decrease microbial transfer to skin, tools, and food by as much as 99.8%, or nearly 3-logs.

Various methods are available for hand drying including paper towels, cloth towels on rotary dispensers, mechanical air dryers utilizing heated air, and simple air drying (Gustafson et al. 2000). However, the answer to which drying method is the most effective is not entirely clear. Gustafson et al. (2000) had participants wash hands with a non-antimicrobial hand soap after inoculating one hand of each participant with 1×10^7 bacterial cells of *Micrococcus luteus* and then evaluated four different methods to dry hands including cloth towels on a rotation dispenser, paper towels, a hot air dryer, and spontaneous air evaporation. The authors reported no significant difference in overall bacterial reduction between the four drying methods evaluated. However, Jensen (2015) mentions that Gustafson et al. (2000) reported the data in CFU rather than in log CFU, and that if reported in log CFU, a 0.5 log CFU greater reduction occurred with

paper towel drying over air drying or drying with warm air. Yamamoto et al. (2005) evaluated the effectiveness of paper towels and warm air drying and reported that a warm air dryer combined with a 4 W ultraviolet light without rubbing hands together was the most effective. The authors hypothesized that rubbing hands together could actually allow for an increase in bacteria as bacteria are brought to the surface of skin from the hair follicles (Yamamoto et al. 2005). Conversely, Huang et al. (2012) reported results contrary to Yamamoto et al. (2005) indicating that paper towels are superior to electric air dryers. Despite the fact that there does not seem to be a conclusive answer to which hand drying method is optimum, removing residual moisture from hands is essential to allow for optimum handwashing effectiveness and to prevent unwanted transfer of microorganisms (Jumaa 2004).

3. Methods for Evaluation of Handwashing Efficacy

Since the 1980s, numerous studies have been conducted on handwashing. The focus of these studies spans a wide range of variables from looking at different microorganisms (viruses and bacteria) to handwashing time, soap volume, soap type, etc. While a common goal to optimize handwashing effectiveness is the underlying premise in each study, the methods to evaluate and achieve this goal are inconsistent across studies. Numerous methods to inoculate and recover bacteria from hands are utilized, and different microorganisms are selected for evaluations in each study. Table 1 summarizes the various studies (starting from 1985) along with the inoculation and recovery methods and the microorganisms used. It is important to note here that the authors did not include studies focused on cross contamination as these were out of the scope of this review. In the following sections, the different inoculation and recovery

techniques will be discussed as well as the impact of selection of microorganisms on the reported results.

3.1. Hand Inoculation Techniques

Numerous methods are used to inoculate the hands of participants (Table 1). Inoculation methods include: hand contact with inoculated blotting paper, contact with public environmental surfaces (i.e. natural inoculation), pouring of microbial suspension in cupped hands, direct contamination of microbial suspension on fingertips, palmar-surface techniques of contamination, and immersion of hands in microbial suspension.

3.1.1. Palmar Surface Methods

The palmar surface method (PSM) is one of the most common methods used to inoculate hands. There are different variations of this method as highlighted in Table 1. American Society for Testing and Materials (ASTM) Standard Test Method E2870-13 prescribes palmar surface contamination to evaluate effectiveness of antimicrobial handwashing formulations (ASTM 2013a). The standard protocol for this procedure (ASTM E2870-13) is to inoculate each palm with 100 μ L of approximately 8 log CFU/mL *Escherichia coli* suspension. Subjects then spread the inoculum across their palms and fingertips for 15 ± 1 seconds, and then hands are air-dried for 30 ± 5 seconds. Bettin et al. (1994) used 100 μ L of $6.7 \log_{10}$ CFU/mL *C. difficile* suspension pipetted onto the right palm and then gently rubbed onto the palmar surface of both hands for 10 seconds Edmonds et al. (2013) completed a similar procedure to Bettin et al. (1994) in which the palms of participants' hands were inoculated with 150 μ L of *C. difficile* spore suspension followed by rubbing the palms together for 15 seconds. Fuls et al. (2008) also used a PSM

though with significant modifications. Briefly, sterile paper towels were contaminated with 30 mL of a bacterial suspension (6 log CFU/mL), and hands were then inoculated by pressing on the towels for five seconds. Based on the sterile bag technique for recovery of microorganisms from hands (Section 3.2.1), the calculated transfer of bacteria to hands was approximately 5.8 to 6.4 log CFU total depending on the bacteria (Fuls et al. 2008).

Although each individual method is still a variation of the PSM for inoculation of hands, each method leads to different levels of inoculation. While Ansari et al. (1989), Bettin et al. (1994), and Edmonds et al. (2013) used a more direct method of inoculation (Table 1), Fuls et al. (2008) used a more indirect method of the palmar surface inoculation technique. More specifically, the paper towels were directly contaminated with the inoculum while the hands were the secondary recipient of this inoculum. When interpreting the data in these studies, it will be important to understand the actual number of microorganisms transferred to hands during the inoculation procedure, so that accurate conclusions on handwashing effectiveness can be determined.

3.1.2. Natural Inoculation

Another common method of inoculation is indirect inoculation (i.e. natural inoculation) or inoculation through contact with everyday surfaces present in either public or controlled environments. Larson et al. (1987) conducted a study that used this particular method (Table 1). In this study hands were initially washed with a control soap, and a baseline hand culture was obtained from each subject. Subjects washed their hands 15 times a day for five days, and hand cultures were taken after the first and last handwash of days one and five to observe the effects of initial and long-term use of a particular soap (Larson 1987). Burton et al. (2011) also used

natural inoculation in which participants were either taken to a large, frequently visited museum or were instructed to travel on public transportation (e.g., bus or the “underground”). Participants were instructed to intentionally wipe hands across commonly touched surfaces such as handrails, door handles, and seats to obtain as much bacteria on their hands from the environment as possible (Burton et al. 2011). The researchers found that washing with water reduced total bacteria on hands from 44% to 23%, while washing with soap and water reduced the total from 44% to 8%. Another study by Amin et al. (2014) also used a type of natural inoculation technique and focused on comparing the removal efficacy of water with barsoap or plain water. Here, the authors did not artificially inoculate hands, but rather recovered microbes from one hand of each participant prior to handwashing (Amin et al. 2014). The participants were then instructed to perform either the wash with barsoap or the wash with water, and microorganisms were recovered from the hand that was not initially sampled to determine the reduction in initial bacterial levels achieved with each soap type (Amin et al. 2014).

Natural inoculation of hands can be beneficial in providing a realistic view of the diversity and concentration of microorganisms that subjects can accumulate on their hands in daily life. However, there are a number of limitations to this method of inoculation if the primary goal is to test the effectiveness of a particular handwashing variable (i.e. handwashing time, soap type, soap volume). For instance, there was an assumption in the study by Amin et al. (2014) that microorganisms would be homogenously distributed between both hands without consideration of possible bias in contamination of the participant’s dominant hand. Moreover, depending on the surfaces that subjects are coming in contact with, the types and levels of microorganisms they are coming in contact with have the potential to vary from day to day and even between the hands of a single participant.

In the meta-analysis by Montville and Schaffner (2011), the authors observed that studies using resident microflora on hands as opposed to inoculated transient bacteria (either gram-positive or gram-negative bacteria) as an indicator of hand washing efficacy resulted in significantly different reductions (i.e. 0.31 log CFU resident microflora and approximately 1.9 log CFU transient bacteria) on hands. Initially, these results may indicate that natural inoculation methods would be preferable; however, there is no indication of the number of studies included in each category of microorganism (resident vs. transient) or whether the recovery efficiencies of each method applied were considered when reporting actual log reduction. Overall, if the goal of the research is to understand how one particular variable can impact handwashing, using a more direct and consistent method of contamination such as the PSM or the pouring methods (Section 3.1.3) will likely be more beneficial as they are more controlled and consistent methods.

3.1.3. Pouring Methods

Handwashing studies also utilize the pouring method of inoculation (PMI) which involves pouring a microbial suspension into the participants' hands and instructing the participant to spread the suspension over their hands. Unlike the PSM, the PMI involves dispersing the microorganism suspension over the entire hand (both ventral and dorsal surfaces) rather than just the palmar (ventral) region. Sickbert-Bennett et al. (2005) poured a microbial suspension (volume not specified) containing 8.5 log CFU/mL *Serratia marcescens* and 9.5 log plaque forming units (PFU)/mL MS2 bacteriophage into the subjects' cupped hands and subjects spread the suspension over their hands for 45 seconds. Here, it is interesting to note that while Sickbert-Bennett et al. (2005) referenced ASTM E-1174, stating the only modifications to the procedure included the addition of MS2 bacteriophage and the adjustment to handwash time,

additional modification to the ASTM E-1174 occurred. In ASTM E-1174, the procedure states that three 1.5 mL aliquots of the test organisms are to be poured into the subjects cupped hands, with a 20 second spread onto the hands, and a 30 second air dry between each application (ASTM 2013b); however, the PMI utilized by the authors clearly deviates from this standard method (Sickbert-Bennett et al. 2005).

Edmonds et al. (2013) used two different inoculation methods—depending on the microorganism—similar to Sickbert-Bennett et al. (2005). The authors inoculated the hands of participants with 6 log₁₀ spores of either *Bacillus atrophaeus* or *C. sporogenes* by placing a total of 5 mL of the spore suspension onto the hands in three separate aliquots (1.5 mL, 1.5 mL, and 2.0 mL) with the participants spreading each aliquot over the entire hand surface for 45 seconds (Edmonds et al. 2013). Edmonds et al. (2013) also used a PSM of contamination (Section 3.1.1). In this instance, it appears that the different volumes were used to achieve the same inoculum level as opposed to simply diluting the *C. difficile* spore stock to match the concentration of the *B. atrophaeus* and *C. sporogenes*. However, one could speculate that inoculating the hands with two different volumes would likely impact dispersion of the bacteria on the hands. Sasahara et al. (2014) followed a similar procedure to Edmonds et al. (2013) spreading only 2.5 mL of *B. cereus* suspension over the entire surface of each hand. Chamberlain et al. (1996) applied the pouring method as well with slight modifications (Table 1).

3.1.4. Fingerpad Methods

Another technique frequently employed is limitation of contamination to the fingers. Snyder, Jr. (2007) placed 10 µL of 11 log₁₀ CFU/mL *E. coli* on the tips of the second and middle fingers of the hand. Stowell et al. (2014) also completed a similar inoculation procedure,

466 pipetting 400 μ L of cytomegalovirus suspension onto the ventral surface of participants' fingers.
467 Ansari et al. (1989) also used a fingerpad method in which the five fingerpads of one hand were
468 each inoculated with 10 μ L of rotavirus at approximately 4 log PFU or *E. coli* at approximately 6
469 log CFU, followed by the drying of hands for 20 minutes. Meanwhile, Miller et al. (2011)
470 adapted a variation on the FPM by inoculating a 25 $\times$ 6 cm section of blotting paper with a 6 mL
471 aliquot of *E. coli* and allowed the fingers and thumbs of the participants' hands to contact the
472 contaminated blotting paper for 10 seconds. Stiles and Sheena (1985) performed an additional
473 variation on the FPM. Here, the finger and thumb tips were pushed into ground beef containing
474 naturally present coliforms, and then the hands were rubbed together until dry. Based on Table 1,
475 it is evident that the FPM of inoculation is one method featuring a large amount of variation
476 throughout the published literature.

478 3.1.5. Additional Methods

479 In addition to the methods described in Section 3.1, Jensen et al. (2014) chose two
480 different methods of inoculation not previously described. The first method was selected to
481 compare the efficacy of soap versus no soap (water only) in microbial removal on soiled hands.
482 To inoculate hands, study participants picked up and spread 5 g of ground beef inoculated with 6
483 log CFU/5 g *Enterobacter aerogenes* over their hands. Here, the researchers also evaluated
484 nonsoiled hands by inoculating the participants' hands with two 0.5 mL aliquots of 6 log
485 CFU/mL *E. aerogenes* followed by dispersion when the subjects rubbed their hands together—a
486 possible variation of PMI though it is unclear. Stiles and Sheena (1985) also used ground beef to
487 inoculate hands. In this particular study, finger and thumb tips were pushed into ground beef
488 (inoculated at 6 to 8 and 7 to 9 log CFU/g ground beef of *E. coli* and *Pseudomonas fluorescens*,

respectively) for 5 seconds, and then hands were rubbed together up to the wrists until hands were dry (Stiles and Sheena 1985). Toshima et al. (2001) also used ground beef to contaminate hands, with participants working 200 grams of a ground meat blend in the palms of the hands at two separate times for 30 seconds each time.

3.1.6 Inoculum Level

As evidenced in the preceding sections as well as in Table 1, it is clear that many different techniques are used to inoculate hands with microorganisms in order to test numerous variables and their impact on handwashing. Within these different inoculation methods are many inconsistencies related to reporting inoculum concentrations. While some studies such as Bartzokas et al. (1987), Nicoletti et al. (1990), and Bettin et al. (1994) clearly list the level of microorganisms on inoculated hands, other studies do not make this as clear. For instance, Miller et al. (2011) did not clearly state amount of *E. coli* but simply explained the process of preparing the *E. coli* for suspension in a saline solution and inoculating blotting paper with the bacterial suspension. Even though the baseline level of *E. coli* on participants' is indicated, there is no information provided in relation to the efficiency of the inoculation method used in transferring *E. coli* to fingerpads—how much *E. coli* needs to be added to the blotting paper to result in the baseline levels reported?

In another example, Toshima et al. (2001) inoculated hands by having participants work 200 grams of ground meat in their palms twice for 30 seconds each time. The authors do not mention inoculation of the ground meat, but are studying the effect of handwashing on total coliforms transferred from ground meat. In this particular case, if the ground meat was not artificially inoculated, it is unlikely that coliform contamination in the ground meat will be

homogenous or consistent between subjects and replicates, making the full potential for coliform reduction on hands due to handwashing difficult to determine. In Chamberlain et al. (1997) the inoculation process is discussed, and the authors state that early stationary phase cultures of *Micrococcus* sp. were diluted (1:50) into fresh broth which subjects then immersed their hands into for one minute. Once again, the true effectiveness of the handwashing procedure in this study is difficult to determine because the initial inoculum level was not reported. Not clearly stating inoculum level is a common occurrence in the literature, and makes it difficult to draw accurate conclusions about effectiveness of handwashing methods.

3.2. Methods to Recover Microorganisms from Hands

Numerous methods have been described for recovery of microorganisms from hands to determine handwashing effectiveness. There are five primary methods that have been reported in the published literature including the glove juice method (GJM), sterile bag technique (SBT), swabbing of hands or fingertips (SWT), the glass bead method (GBM), handling surrogate surfaces, and contact on a RODAC™ (Replicate Organism Detection and Counting) plate.

3.2.1. Sterile Bag Technique

Of the recovery methods listed, SBT is one of the most common—applied by five studies evaluating handwashing efficacy (Table 1). Although there is a standard ASTM protocol (ASTM E2870-13) for application of the SBT (ASTM 2013a), this method is often modified in studies reported in the peer-reviewed literature. Larson et al. (1987) utilized the SBT to recover microorganisms from hands. Here, the authors filled a sterile bag with 50 mL of sampling solution and massaged participants' hands for 3 minutes. Fuls et al. (2008) also utilized the SBT,

but chose to use 75 mL of a sampling solution in the sterile bag with a massage time of 1 minute. Meanwhile, Amin et al. (2014) applied the SBT for recovery of microbes from hands and utilized 200 mL of sterile Ringer's solution as well as a 30 second massage—participants rubbed their fingers against their palm for 15 seconds and then their hands were massaged for 15 seconds. While all of these studies had slightly different objectives, the overall goal of each study was focused on handwashing, and the effect of a given handwashing variable on microbial reduction. The slight modification of the SBT between studies makes it difficult to compare the results to one another as differences in buffer type as well as hand massage type have the potential to impact the bacterial recovery; however, to our knowledge, no systematic comparison of these variables has occurred.

Buffer type and volume is a variable that can potentially have a significant impact on the recovery efficiency of the microorganisms. Based on the studies listed in Table 1, numerous sampling solutions are used to recover the microorganisms from hands of participants. Moreover, the addition of a neutralizing solution was included in various studies as well—specifically those evaluating antimicrobial soaps (Benton et al. 1990). Studies that employed the SBT selected a wide range of buffer volumes (50 to 200 mL). This range of buffer volume potentially impacts the ability to remove bacteria from the hands of the participants in order to determine an accurate representation of the efficacy of the handwashing process being evaluated. For instance, the higher the volume of elution buffer, the more dilute the microorganisms will be thus the sensitivity of the chosen detection method is critical. Additionally, massage time ranges from 30 seconds to 3 minutes. While a massage time of 30 seconds may not be enough to remove microorganisms from the hands of participants, a massage time of 3 minutes is likely more time

than what is needed. Standardization of this method is essential to provide consistency and allow for more accurate comparison between studies.

3.2.2. Glove Juice Method

The GJM for recovery of microorganisms from hands is another method that varies significantly between studies (Table 1). Sickbert-Bennett et al. (2005) utilized 75 mL of sampling solution per glove and then massaged hands in nonsterile latex gloves for 30 seconds. Edmonds et al. (2013) also used the GJM and followed ASTM standard method E1174-13 (previously ASTM E1174-06) for evaluating the effectiveness of health care personnel and consumer handwash formulations. The ASTM protocol E1174-13 lists procedures for using *S. marcescens* and recommends *E. coli* (American Type Culture Collection [ATCC] 11229) as an alternative challenge organism (ASTM 2013c). After hands are washed and inoculated, the GJM is applied. For this method, gloves (loose-fitting, powder-free, no antimicrobial properties) are put on hands and filled with 75 mL of the ASTM E1174-13 sampling solution with validated neutralizers. Hands are then massaged for 1 min $\pm$ 5 seconds, flipping the hands after 30 seconds to ensure the palm as well as the back of the hand are massaged. After massaging, 3 to 5 mL of the fluid in the glove is aseptically retrieved, diluted, and plated within 30 minutes of sampling. Another study by Jensen et al. (2014) utilized the GJM. Here, the authors used 20 mL of phosphate buffered saline (PBS) per glove with a 1 minute massage. Last, Sasahara et al. (2014) used the GJM with 50 mL of sampling solution (0.04% KH₂PO₄, 1.01% Na₂HPO₄, and 0.10% Triton X-100) per glove with a 1 minute massage.

Similar to the SBT, there are several variables that have been modified within the studies applying the GJM. These variations in procedure can impact the resulting data as well as the

manner in which the data are analyzed. For instance, the authors of this review experimented with various volumes of recovery solution while evaluating the GJM for use in an ongoing study. It was observed that too low of a volume in the glove (20 to 30 mL) was not sufficient as the volume did not cover the entire surface of the hand (especially a concern on larger hands) and would not be able to adequately recover microorganisms from the entire surface of the hand (unpublished data). Additionally, a larger volume in each glove (more than 40 to 50 mL) was too high of a volume resulting in leakage of the sampling solution from the opening of the glove (a concern for safety as well as for determining an accurate recovery of microorganisms). Based on this, there is a need to optimize these variables to create consistency and assist with comparison of data between various studies.

3.2.3. Swabbing Techniques

While the GJM and SBT are the two most popular methods used in handwashing research, swabbing techniques (SWT) are also employed. For the SWT methods, various swab types are used as well as swabbing protocols (Table 1). For instance, Chamberlain et al. (1996) used a single, moistened, cotton-wool swab and passed the swab over the skin area (wrists, dorsal and palmar surfaces, fingertips, and the interdigital spaces) in a single continuous movement five times. Meanwhile Burton et al. (2011) used a sodium chloride soaked charcoal swab that was passed only over the fingers of the dominant hand of participants. Another study by Stowell et al. (2014) uniformly swabbed the entire contaminated finger surfaces with cotton-tipped swabs that were premoistened with 100 μ L of PBS. While these studies use a variety of swab types, one consistent trend is that the swab is always pre-moistened. In a study investigating the factors that influence recovery of microorganisms from surfaces, Moore and

Griffith (2002) found that a pre-moistened swab when used on a wet surface, increased efficiency of the swabbing procedure when compared to a dry swab. Although swabbing is sometimes used in handwashing studies, it is more commonly used in other areas of research such as evaluation of surface sanitation practices and is a valuable tool in the food industry (Davidson et al. 1998; Moore and Griffith 2002).

There are numerous variables involved in optimizing the effectiveness of the SWT, and for this reason, it is possible that it may not be the best method for recovery of microorganisms from hands in handwashing research. While swabbing is often selected based on the ease of sampling, its accuracy depends on the ability of the swab (i.e. buffer and swab composition) to pick up microorganisms from the sampling surface as well as the ability of the swab to release those microorganisms (Moore and Griffith 2002). Moore and Griffith (2002) conducted a study investigating the factors influencing the recovery of microorganisms from fomite surfaces by use of traditional hygiene swabbing. In this study, stainless-steel squares were inoculated with *Salmonella* spp. and then swabbed with sterile cotton swabs, Dacron swabs, or calcium alginate swabs. Inoculated surfaces were sampled both wet and dry with swabs that were either wet or dry and the results indicated that swabbing efficiency increased when a wet surface was sampled. However, the authors noticed a general trend that if a swab was able to remove a large number of microorganisms from a sampling surface, it was often difficult to then remove those microorganisms from the swab surface (Moore and Griffith 2002). Overall, the many variables present in ensuring efficiency and accuracy of the SWT caused Moore and Griffith (2002) to question the reliability of swabbing as a method to monitor surface cleanliness. If the use of the SWT as a tool for detecting microorganisms in small areas is questioned, then its reliability in swabbing a larger area such as the surface of an entire hand should be considered as well.

3.2.4. Glass Bead Method

Another method less frequently utilized within the past decade is the GBM (Table 1). The GBM typically involves pouring a sampling solution over fingers, and then massaging fingers on glass beads to recover any remaining microorganisms from hands. Ayliffe et al. (1988) used the GBM to compare various handwashing agents in hospital laboratories and wards. In this study, after treatment with the specified handwashing agent, the fingers and thumbs were immersed in a bowl containing 100 mL of nutrient broth with neutralizer and rubbed vigorously over glass beads (3-5mm diameter) for 1 minute (Ayliffe et al. 1988). Nicoletti et al. (1990) utilized the GBM for comparison of CHG detergents and soap. In this study, after treatment with the specified soap samples, hands were sampled by rubbing fingertips for 2 minutes on 35 g of 3mm glass beads in a bowl with 50 mL of saline (concentration not provided) with 3% Tween 80. The most recent published study using a glass tube variation of the GBM was reported by Guilhermetti et al. (2001) to investigate the effectiveness of hand-cleansing agents for removing methicillin-resistant *Staphylococcus aureus* from contaminated hands. Here, eight fingers were sampled from participants by rubbing for 3 minutes in short, flat-bottomed glass tubes containing 5 mL of 0.1% peptone water with neutralizer to prevent carryover inactivation.

3.2.5. Additional Techniques

In addition to the more commonly used recovery techniques, a few less commonly used techniques are also mentioned in the handwashing literature and have been included in Table 1 as well. Bettin et al. (1994) used TCCFA (tau-rocholate-cycloserine-cefoxitin-fructose agar) RODAC™ plates after hands were inoculated and treated with the particular handwashing

method followed by sampling in three locations on the TCCFA RODAC™ plates including the fingertips and thumbtips, the palmar surfaces of the fingers, and the palms. Toshima et al. (2001) used an agar stamping method in which participants stamped the fingers (including thumb and all fingers except the little finger) from the fingertip up to the first phalangeal joint on various selective media. Snyder, Jr. (2007) studied the removal of bacteria from fingertips and the residual amount remaining on a handwashing nailbrush. To recover microorganisms from hands, a rinse of 10 mL of letheen broth was applied to the fingertips in a 1-pint ziplock bag and then participants rubbed the thumb against the second and middle fingers for 20 seconds (Snyder, Jr. 2007). Ansari et al. (1989) eluted microorganisms from the fingertips by inverting fingertips in a vial containing Earle balanced salt solution with 20% tryptose phosphate broth (EBSS-TPB). In this particular method, fingertips were inverted in the vial for 40 seconds with 40 full inversions. In the same study, the authors also applied a technique in which 20 mL of the EBSS-TPB was poured on the hands over a funnel while the hands were rubbed together. Miller et al. (2011) used touch-transfer quantification to recover microorganisms from hands by choosing two items for the participants to handle—licorice to represent a food item and soft synthetic chamois to represent skin. The surrogate surfaces were picked up by the participant and handled firmly for 5 seconds followed by transferring the surrogate surface to sterile 20 mL glass containers containing 9 mL of saline and vortexed for 10 seconds to recover microorganisms (Miller et al. 2011).

3.2.6. Recovery Efficiency and Baseline Inoculum

Although each recovery method has its own set of benefits, there is a need to standardize 1) how long the method should be applied (e.g., how long should hands be massaged in the GJM?) and 2) the appropriate steps and reagents to be used for each method. **Standardization of**

the procedures for the various methods commonly used in handwashing research would allow for easier comparison between studies as well as for more clear and accurate conclusions to be made when comparing handwashing efficacy. Moreover, not only is methodological standardization important, but handwashing researchers could benefit from the formation of guidelines on the minimal information required for publication of handwashing experiments—these types of guidelines have been established for common methods plagued by lack of standardization such as real time, quantitative PCR (Bustin et al. 2008).

For instance, a major concern with handwashing studies is that the recovery efficiency of the microbial recovery methods applied to hands are often not provided. Of the 13 studies covered in Section 3.2, no researchers directly reported the recovery efficiency of the applied method. However, two studies (Fuls et. al, 2008 and Sickbert-Bennett et al.2005) provided enough information and data to allow for the recovery efficiency to be determined by the authors of the present review. Knowing the recovery efficiency of a particular method is a key detail to determine the true effectiveness of a particular handwashing variable. Variation in a particular method from lab to lab, or even within one particular lab, can be significant, and thus the recovery efficiency of a particular method is vital for determining the true effectiveness of a particular handwashing regimen. Jensen (2015) stressed the importance of taking the sampling method into consideration when designing experiments and comparing results as different techniques can result in different observed log CFU or PFU reductions for similar initial microbial concentrations.

Edmonds et al. (2013) did not detail the recovery efficiency of their method, but they do mention that the baseline population was determined through the GJM prescribed by ASTM E1174-13 (ASTM 2013b). Some methods such as ASTM E2870-13, which uses the PSM for

inoculation, do not require a baseline sampling of the hands because the method is designed to determine the difference between handwashing products, rather than an overall reduction (ASTM 2013a). Additionally, the inoculum volume at such a low level (i.e. 100 μ L), and the distribution of the small volume of inoculum is controlled and limited to the palms and fingers, thus there is limited opportunity for spillage of inoculum solution. While recovery efficiency is vital to report, the baseline level is also an important detail that needs to be reported. Although baseline sampling is not essential when a small, controlled volume is applied to hands (e.g. PSM of inoculation), it is of more concern in inoculation methods such as the blotting paper method used by Miller et al. (2011) to contaminate hands and the PMI used by other researchers (Table 1). In the blotting paper technique, the hands are not directly inoculated with the microorganisms, rather blotting paper is contaminated and hands then come in contact with the blotting paper, leading to a lower inoculum level on the hands than on the blotting paper. The baseline level of microorganisms here would be essential to know in order to calculate the recovery efficiency for accurate determination of microbial reduction due to handwashing. Similarly, PMI would result in comparable challenges for calculation of recovery efficiency.

4. Selection of Microorganisms

Numerous types of microorganisms (i.e. species, strains, bacteria [vegetative and spores], viruses) are used in handwashing studies. As shown in Table 1, 14 different microorganisms were used to inoculate hands with most being bacteria (n = 11) followed by one bacteriophage and two viruses. Of the bacteria, *E. coli* was used most frequently (n = 8). The only other organisms used in more than one study were *C. difficile*, *Micrococcus*, *S. aureus*, and *S. marcescens* (Table 1). Eight of the studies did not artificially inoculate hands with microorganisms, but rather used natural inoculation through environmental microbes which may

720 have included bacteria, viruses, and protozoa though analyses were only chosen for detection of
721 specific types of bacteria.

722 Some standard methods such as ASTM E1174-13 state the microorganisms that are to be
723 used for the particular procedure—in this case, *S. marcescens* (ATCC 14756) or *E. coli* (ATCC
724 11229) (ASTM 2013b). Other standard procedures, such as ASTM E2870-13 are more general.
725 ASTM Standard Test Method E2870-13 simply states that the microorganism should have
726 characteristics that allow it to be readily identified (ASTM 2013a). Although some standard
727 methods do list the microorganisms to be used for that particular method, numerous adjustments
728 to the standard protocol are typically made by researchers, and details such as the type of
729 microorganism to be used in a particular method are not always followed. However, this may not
730 necessarily be problematic. For instance, Edmonds et al. (2013) use ASTM E1174-06 which
731 states that either *S. marcescens* or *E. coli* are the microorganisms to be used in this method;
732 however, the authors used *C. difficile* spores as the test organism to focus on the dynamics of
733 removing spores during handwashing rather than vegetative bacteria. One possible option to
734 allow for consistency between studies would be to create a standardization of microorganisms,
735 where certain surrogate microbes could be chosen as standards to use to represent pathogenic
736 bacteria, viruses, and protozoa of human health concern. Consistency between microorganism
737 types used is not necessarily key, but rather reporting details such as recovery efficiency (which
738 could vary depending on the type of microorganism used) to ensure accuracy when determining
739 reduction of microorganisms on hands in the handwashing process is critical.

741 **5. Conclusions and Recommendations**

742 As evidenced in this review, handwashing research utilizes many different methods to
743 inoculate hands as well as to recover microorganisms from washed hands. In addition to this,

744 numerous microorganisms are used to inoculate hands. While there is a lot of variation in
745 different methods used to test handwashing efficacy, there is also extensive variation within the
746 various methods leading to some confusion in interpretation of published results as well as in
747 study reproducibility to determine handwashing effectiveness. Additionally, the vast amount of
748 variation that is occurring within the numerous studies makes it difficult to compare results of
749 studies and draw definitive conclusions from the data. For this reason, there is a need to create
750 standardized methods that can be routinely used by researchers. These standardized methods
751 will provide consistency and comparability between studies allowing for more transparent
752 conclusions to be drawn. Moreover, the formation of guidelines for the minimal information
753 required for publication of handwashing experiments could be an essential step in allowing
754 accurate comparisons of future published studies.

755 In preliminary, unpublished data from the authors of this review, the PSM was an ideal
756 method for inoculation. In addition, several commonly used recovery methods were evaluated
757 including the GJM, SWT, and SBT. From both a recovery efficiency standpoint and an ease of
758 use standpoint, the GJM with 35 mL of sampling solution and a 1 minute massage seemed to be
759 the optimum choice (unpublished data). Conversely, the SBT required a much higher volume of
760 sampling solution (around 200 mL), and as the sampling solution was in a larger area (i.e. a
761 plastic bag), it did not seem to thoroughly cover the hands. One issue observed with both the
762 SBT and the GJM was that the sampling solution would often leak and those recovered
763 microorganisms would be lost. Because the opening of the glove or the sterile bag was not
764 entirely closed around the participants' lower arm, a slight change in angle, or an increase in
765 sampling solution volume often led to leakage at the opening of the glove or bag (both a concern
766 for subject safety and accuracy of recovery of microorganisms). For this reason, keeping the

sampling solution to a minimum volume of no more than about 50 mL is also optimal. With respect to the SWT, the recovery efficiency of *E. coli* from hands was much lower at 0.47% when compared to the GJM at 2.3% (unpublished data). As discussed previously, swabbing the entire hand is not an ideal recovery efficiency method as the area is far too great compared to the surface area of the swab.

Although the appropriate inoculation and recovery methods as well as the microorganism(s) chosen will vary based on the objectives of a particular study, in the future, consistency within an experimental method should occur to allow for consistency and assist in ease of study comparisons. Additionally, details such as efficiency of the recovery method are essential to report in the future as these types of details allow for a clear understanding of the true reduction in microorganisms that is occurring due to handwashing. Reporting of essential details such as recovery efficiency by researchers will aid in the determination of accurate conclusions about the handwashing process to be made. Our conclusions support Jensen (2015) who stated that although the factors that influence handwashing have been studied, comparing these studies is difficult because of both methodological differences as well as statistical flaws in studies. It is clear that inconsistencies between methods and between researchers is causing confusion in interpreting data and is making it difficult to develop accurate conclusions about what details and methods will make handwashing most effective. Creating standardization in handwashing research methodology as well as reporting guidelines is essential to allow for comparison between studies so that researchers can accurately draw conclusions about the handwashing process. Advancements in handwashing research will then allow for optimization of handwashing and proper training of food-handlers so that handwashing effectiveness can be

maximized, and foodborne illness caused by improper handling due to food-handlers can be minimized.

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Table 1. Review of methods selected for evaluating handwashing efficacy.

Reference	Purpose of the Study	Inoculation Method	Recovery Method	Microorganisms Used (Concentration or Total)
Stiles and Sheena (1985)	To compare the efficacy of iodophor products, CHG detergents, and non-germicidal soap for reducing microorganisms on hands	1) NI 2) FPM: Finger and thumb tips pushed into ground beef inoculated with bacteria; hands rubbed together until dry	GBM: one hand immersed in 100 mL letheen broth in plastic bag containing 35 g of 4 mm sterile glass beads; rubbed glass beads 20 times over palms	Bacteria naturally present; <i>Escherichia coli</i> (6 and 8 log CFU/g beef), <i>Pseudomonas fluorescens</i> (7 and 9 log CFU/g beef)
Bartzokas et al. (1987)	To evaluate initial and cumulative efficacy of two antiseptic HW preparations in eliminating enterobacteria from hands	PMI: 5 mL of bacterial suspension poured into cupped hands, rubbed over hands, and air-dried for 60 s	GJM: 75 mL of sterile tryptone water and 0.075 M phosphate buffer in each glove; hands massaged 60 s	<i>Serratia marcescens</i> (9 log CFU/mL in suspension; 6.6 log CFU total baseline)
Larson et al. (1987)	To determine effect of soap volume on microbial reduction during HW	NI	SBT: 50 mL of SSN in in bag, hands massaged for 3 min	Bacteria naturally present
Ayliffe et al. (1988)	To test the efficacy and residual activity of 14 HW or disinfectant preparations on hands	FPM: 0.02 mL of bacteria on fingers and opposing fingers; thumbs rubbed 40 s and air dried 80 s	GBM: fingers and thumbs immersed in 100 mL of nutrient broth + neutralizer and rubbed over glass beads for 60 s	<i>E. coli</i> (concentration not provided)
Ansari et al. (1989)	To evaluate a protocol using finger pads for testing the microbial-eliminating efficacy of HW agents	1) FPM: fingerpads on one hand each inoculated with 10 µL of rotavirus or <i>E. coli</i> and air dried 20 min 2) PSM: 0.5 mL rotavirus or <i>E. coli</i> suspension placed on palm of one hand and palms rubbed together; 20 min air dry	Microorganisms eluted with 1 mL EBSS + 20% tryptose phosphate broth for 40 s with 40 full inversions; 20 mL of eluate poured on hands while rubbed together over a plastic funnel	Rotavirus (4.2 log PFU total), <i>E. coli</i> (5 log CFU total)
Larson et al. (1989)	To compare the antimicrobial effects of three products containing CHG, PCMX, or TRI on normal hand flora after HW at different	NI	SBT: dominant hand inserted into a sterile bag with 50 mL SSN; hand massaged for 60 s	Bacteria naturally present

frequencies over 5 days

Nicoletti et al. (1990)	To compare two CHG HW detergents and liquid soap for removal of bacteria contaminated fingers	FPM: 10 µL applied to each of 5 fingertips and rubbed in 10 s per finger with the thumb and then dried	GBM: fingertips rubbed for 2 min on 35 g of 3 mm glass beads in a bowl of 50 mL of saline with 3% Tween 80	<i>S. marcescens</i> (9.3 log CFU total on 5 fingertips), <i>Micrococcus</i> sp. (8.6 log CFU total on 5 fingertips)
Bettin et al. (1994)	To compare liquid soap versus 4% CHG in 4% alcohol for the decontamination of bare or gloved hands	PSM: 100 µL of bacterial suspension placed on right palm; palms rubbed together for 10 s	RODAC™ plate with TCCFA imprinted by finger/thumb tips, palmar surfaces of fingers, and palm	<i>Clostridium difficile</i> (6.7 log ₁₀ CFU/mL)
Miller et al. (1994)	To compare the bacterial reduction of plain and various antimicrobial handsoaps and instant hand sanitizers	NI: determination of transient flora prior to washing; determination of resident flora after washing	RODAC™ plate with Difco D/E Neutralizing imprinted by fingertips	Bacteria naturally present
Paulson (1994)	To evaluate the immediate, persistent, and residual efficacy of five surgical hand scrub products	NI: baseline samples of hands obtained after condition wash to remove transient microflora	GJM: 75 mL 0.1M PBS with 0.1% Triton X-100; 60 s massage	Resident microflora
Chamberlain et al. (1997)	To investigate the effectiveness of a HW procedure	PMI: Hands immersed in bacterial suspension for 60 s	SWT: premoistened, cotton-wool swab passed over skin five times)	<i>Micrococcus</i> sp. (concentration not provided)
Paulson et al. (1999)	To examine four HW regimens for reduction of transient microorganisms on hands	PMI: 5 mL of <i>E. coli</i> poured into cupped hands in two 2.5 mL aliquots and spread over both hands for 45 s; 2 min air dry	GJM: sterile latex gloves on each hand with 75 mL of SSN in each glove, wrists secured and hands massaged for 60 s	<i>E. coli</i> (8 log CFU/mL)
Guilhermetti et al. (2001)	To investigate the effectiveness of hand-cleansing agents for removal of bacteria from hands	FPM: 0.02 mL of bacterial suspension on 4 fingertips of left hand and opposing fingertips rubbed together for 40 s and air dried 80 s	GBM: 8 fingers rubbed 3 min against 10 g of 3-5 mm sterile glass beads in 5 mL of 0.1% peptone + neutralizer	methicillin-resistant <i>Staphylococcus aureus</i> (3.8 and 6.8 log CFU/fingertip)
Toshima et al. (2001)	To investigate the efficacy of a commercial antibacterial soap	NI: Ground meat (200 g) massaged in the palm, two times, 30 s each time	GJM: 30 mL SSN poured into gloved hand and hand massaged, covering each side of the hand 10 times	Total coliforms naturally present in ground meat

Sickbert-Bennett et al. (2005)	To evaluate efficacy of antimicrobial agents in soap.	PMI: Microbial suspension (volume not listed) poured into the subject's cupped hands and spread over hands for 45 s	GJM: 75mL of SSN in one nonsterile glove per hand; massaged for 30 s	<i>S. marcescens</i> (8.5 log CFU/mL), MS2 bacteriophage (9.5 log PFU/mL)
Snyder, Jr. (2007)	To determine efficacy of a double HW procedure for removal of bacteria from fingertips	FPM: 10 µL of <i>E. coli</i> spread on the tips of the second and middle fingers	Modified SBT: Fingertips rinsed in 10 mL letheen broth in a plastic bag; thumb rubbed against second and middle fingers for 20 s	<i>E. coli</i> (11 log CFU/mL)
Fuls et al. (2008)	To optimize ASTM E1174 method of hand contamination technique and to measure effect of time and soap volume on antimicrobial and non-antimicrobial soap effectiveness	PSM: sterile paper towels inoculated with 30 mL of bacterial suspension and hands pressed on towels for 5 s	SBT: 75 mL of SSN in bag, hands massaged vigorously for 60 s	<i>S. marcescens</i> , <i>S. aureus</i> , <i>E. coli</i> , <i>Shigella flexneri</i> (variable: 6 to 9 log CFU/mL)
Burton et al. (2011)	To determine whether non-antibacterial soap reduces enteric bacteria better than water alone	NI	SWT: NaCl soaked charcoal swab wiped across fingers of dominant hand	Bacteria naturally present
Miller et al. (2011)	To evaluate HW time, friction, and soap for reduction in bacterial translocation to skin and food surfaces	Modified FPM: Fingertip and thumb contact with blotting paper inoculated with 6 mL of <i>E. coli</i>	Handling Surrogate Surfaces: surrogate placed in sterile 20 mL glass containers with 9 mL of saline; vortexed for 10 s to elute <i>E. coli</i>	<i>E. coli</i> (concentration not provided)
Edmonds et al. (2013)	To compare the efficacy of hand hygiene agents against <i>C. difficile</i>	1) PMI: 5 mL of microbial suspensions in 3 aliquots (1.5, 1.5, and 2.0 mL) placed in hands and rubbed all over for 45 s 2) PSM: 150 µL of spore suspension rubbed into palms of hands for 15 s	GJM: 75 mL of SSN in each glove, hands massaged uniformly 1 min ± 5 s, flipping hands after 30 s	<i>C. difficile</i> (7 log CFU/mL), <i>Bacillus atrophaeus</i> (6 log CFU/mL), <i>C. sporogenes</i> (6 log CFU/mL)
Amin et al. (2014)	To compare microbial efficacy of	NI	SBT: 200 mL sterile Ringer's	Naturally present

	soapy water with bar soap and water alone		solution; participant rubbed fingers against palm for 15 s; hands massaged for 15 s	thermotolerant coliforms and <i>C. perfringens</i>
Jensen et al. (2014)	To determine effectiveness of a minimal 5 s wash and a longer U.S. FDA Model Food Code compliant hand wash (20 s) with and without food debris	1) Spread inoculated ground beef on hands 2) Two 0.5 mL aliquots of bacteria placed in each hand and evenly dispersed by rubbing hands together	GJM: 20 mL of PBS per glove; 60 s hand massage	<i>Enterobacter aerogenes</i> (6 log CFU/5 g beef or 6 log CFU/mL)
Sasahara et al. (2014)	To determine the appropriate HW procedure for removing <i>B. cereus</i> spores	PMI: 2.5 mL of microbial suspension spread on each hand	GJM: 50 mL of sampling solution in each glove; hands massaged for 60 s	<i>B. cereus</i> (6 log CFU/mL), <i>E. coli</i> (6 log CFU/mL)
Stowell et al. (2014)	To evaluate the removal of cytomegalovirus (CMV) through HW	Modified FPM: 400 µL of virus suspension applied to ventral surface of fingers	SWT: ventral surface of fingers swabbed with cotton-tipped swabs premoistened with 100 µL of PBS	CMV (5 log PFU/mL)

ASTM = American Society for Testing and Materials; CFU = colony forming unit; CHG = chlorhexidine gluconate; EBSS = Earle's balanced salt solution; FPM = fingerpad method; GBM = glass bead method; GJM = glove juice method; HW = handwashing; NI = natural inoculation via contact with daily surfaces; PBS = phosphate buffered saline; PCMX = parachlorometaxlenol; PFU = plaque forming unit; PMI = pouring method of inoculation (i.e. microorganisms poured into cupped hands and distributed over the entire hand surface) ; PSM = palmar surface method of inoculation (i.e. microorganisms placed in palm of hand and spread of microorganisms limited to the palmar surface of the hand) ; RODAC™ = Replicate Organism Detection and Counting plate; RTE = Ready to eat; SBT = sterile bag technique; SSN = sampling solution and neutralizer; SWT = swabbing technique; TCCFA = tau-rocholate-cycloserine-cefoxitin-fructose agar; TRI = triclosan; US FDA = United States Food and Drug Administration;