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손 위생 제품에 대한 *in vitro, ex vivo, in vivo* 항균 시험법 비교

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Comparison of *In Vitro*, *Ex Vivo*, and *In Vivo* Antibacterial Activity Test Methods for Hand Hygiene Products

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ABSTRACT - Numerous methods have been applied to assess the antibacterial effectiveness of hand hygiene products. However, the different results obtained through various evaluation methods have complicated our understanding of the real efficacy of the products. Few studies have compared test methods for assessing the efficacy of hand hygiene products. In particular, reports on *ex vivo* pig skin testing are limited. This study aimed to compare and characterize the methodologies applied for evaluating hand hygiene products, involving *in vitro*, *ex vivo*, and *in vivo* approaches, applicable to both leave-on sanitizers and wash-off products. Our further aim was to enhance the reliability of *ex vivo* test protocols by identifying influential factors. We performed an *in vitro* method (EN1276) and an *in vivo* test (EN1499 and ASTM2755) with at least 20 participants, against *Serratia marcescens* or *Escherichia coli* and *Staphylococcus aureus*. For the *ex vivo* experiment, we used pig skin squares prepared in the same way as those used in the *in vivo* test method and determined the optimal treated sample volumes for sanitizers and the amount of water required to wash off the product. The hand sanitizers showed at least a 5-log reduction in bacterial load in the *in vitro* test, while they showed little antibacterial activity in the *in vivo* and *ex vivo* tests, particularly those with a low alcohol content. For the hand wash products, the *in vitro* test was limited because of bubble formation or the high viscosity of the products and it showed low antibacterial activity of less than a 1-log reduction against *E. coli*. In contrast, significantly higher log reductions were observed in *ex vivo* and *in vivo* tests, consistently demonstrating these results across the two methods. Our findings revealed that the *ex vivo* and *in vivo* tests reflect the two different antibacterial mechanisms of leave-on and wash-off products. Our proposed optimized *ex vivo* test was more rapid and more precise than the *in vitro* test to evaluate antibacterial results.

Key words: Antibacterial activity, *ex vivo*, Hand hygiene product, Sanitizer, Hand washing

Infections caused by pathogenic bacteria often occur by transfer via the hands; thus, hand hygiene is crucial for personal health. The previous pandemics have driven the development of hand hygiene products, resulting in a significant demand increase. Today, these products come in various forms, including gel-, paste-, liquid-, and stick types. National organizations such as European Standards (EN) and American Society for Testing and Materials (ASTM) provide the evaluation method of antibacterial efficacy for these products and their antibacterial efficacy has been demonstrated given that they meet specific standards based on *in vitro* (EN 1040, Europe; or ASTM E 2315, USA and Canada) or *in vivo* (EN 1499 and EN 1500, Europe; or ASTM E 1174, USA and Canada) tests[1,2]. However, meeting the standard of *in vitro* tests is easy given that these tests tend to overestimate the actual efficacy of disinfectants to kill bacteria[3,4]. Furthermore, the suspension-based time kill test, a typical *in vitro* test, is not suitable for evaluating

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some products such as wash-off or stick-type hygiene products[7]. Conversely, the *in vivo* test method can offer the most accurate actual antimicrobial activity of disinfectants. However, this method is time-consuming and costly owing to the necessity to recruit research subjects and ensure infection prevention for participants' safety as they directly handle pathogens. Therefore, it is challenging to perform *in vivo* tests during the product development stage, i.e., when new products are tested to confirm whether their efficacy is greater than those of marketed products. The pig skin model offers the possibility to overcome the limitations associated with *in vitro* and *in vivo* tests. Specifically, Herron[5] reported that pig skin, with structure and composition similar to those of human skin, is readily available, and its use is time-saving and cost-effective. Although this model also could not thoroughly reflect a living person's skin mechanisms such as self-sterilization[6], *ex vivo* tests can be applied to a wider range of hand washing conditions that cannot be simulated in *in vitro* test[4]. Several researchers have used pig skin to evaluate the antibacterial effects of hand rubs[7-9]. However, studies with a focus on the differences between the *ex vivo* and the *in vivo* method are limited. The *ex vivo* tests performed without identifying these gaps may lead to results that are different from those observed in practice, as is the case with *in vitro* tests[10].

The category of hand hygiene products contains leave-on type of rubs (sanitizers) and wash-off type of wash products. Notably, there exists a distinct mechanism employed in each of the leave-on and wash-off products to reduce bacteria. The efficacy of hand washing in eliminating bacteria and viruses may be attributed to the role of surface-active agents, disrupting interactions that hold the cell walls of bacteria and viruses and bind to human skin, and facilitating their removal from the skin. On the other hand, leave-on type of sanitizer contains the antibacterial agent and works through chemical antibacterial mechanisms to reduce the levels of bacteria and viruses on the hands[1,2,5]. Recognizing the difference between these two mechanisms is essential to select the appropriate test method of hand hygiene products. For wash-off products, the common *in vitro* test protocol for assessing antibacterial activity has some inadequate parts because it could not fully simulate the real-action of hand wash. This method overlooks the role of mechanical action in microbe removal, focusing on the product's chemical effects, which might not accurately represent the overall efficacy of the wash-off products in real usage[7].

We propose and validate each of the *ex vivo* antibacterial activity test methods that mimics the *in vivo* test for hand hygiene products, including sanitizer and washing products. We also comprehensively compared the antibacterial activity results of *in vitro, ex vivo* and *in vivo* test methods. The findings of this study could be useful in developing new hand hygiene products by using a reliable evaluation method at the laboratory stage before conducting *in vivo* studies.

Materials and Methods

Test microorganisms, culture media, and sampling fluid

Serratia marcescens (*S. marcescens,* ATCC 13880), *Escherichia coli* (*E. coli,* ATCC 8739), and *Staphylococcus aureus* (*S. aureus,* ATCC 6538) were grown in tryptic soy broth (BD Difco, Sparks, MD, USA) at 32±2°C for 18-24 h in an incubator. The bacterial concentration was determined by spreading the suspension on tryptic soy agar (TSA, BD Difco). Furthermore, the sampling and dilution fluid was sterile Eugon LT 100 broth (Oxoid, Basingstoke, England) containing Tween 80, lecithin, and Triton X-100.

In vitro test to detect the efficacy of hand rub and wash products

The suspension-based time kill test, which is similar to the previously described method based on the British Standard EN 1276, was used as an *in vitro* test[3]. *S. marcescens* or *S. aureus* were used for the evaluation of hand rub products, and *E. coli* and *S. aureus* were used for the evaluation of hand wash products. Briefly, the test material was added to the bacterial inoculum ($1\text{-}5\times10^7$ CFU mL^{-1}) at a ratio of 1:10, and the contact time was set to 1 min. Subsequently, the inoculum suspension was diluted with the appropriate neutralizer broth, and a serial dilution was conducted in Eppendorf tubes containing saline. To recover viable bacteria, the suspensions were plated on TSA and bacteria were counted after incubation at 32±2°C for 18–24 h. All the *in vitro* tests were conducted in triplicate.

Ex vivo test method to detect the efficacy of hand rub and wash products

The *ex vivo* tests for assessing the antibacterial activity of hand rubs were conducted according to the previously reported method, with modifications[1,7-9]. Fresh pig skin was obtained from a local butcher shop (Seongnam, Korea) immediately after slaughtering and was cut into small pieces (3×3 cm). Each piece was then attached to a plastic plate for a rubbing step during the *ex vivo* test and then disinfected with alcohol. At least ten pieces of skin were used for testing one formulation by pairing the two pieces. Therefore, all the *ex vivo* tests were conducted with at least five repetitions.

For the skin contamination step, the pair of pieces was inoculated with 60 μL of *S. marcescens* or *S. aureus* (10^8–10^9 CFU mL^{-1}) by rubbing for 15 s. After drying for 5 min, 30 μL of the tested hand sanitizer (or saline for control) was placed on each piece and rubbed for 30 s. To inactivate the

disinfectant, 0.2 mL of the sampling fluid was applied on the skin pieces and rubbed for 15 s. Thereafter, the pig skin samples were washed with 9.8 mL of sampling fluid and the wash-off suspension samples were collected in a petri-dish, after which they were serially diluted and plated on TSA to determine bacterial count. Four alcohol-based hand sanitizers made for the experiment were used in this study: Hand Rub A (HR A), gel containing 70% ethanol with a small amount of isopropanol; Hand Rub B (HR B), liquid containing 85% ethanol; Hand Rub C (HR C), gel containing 54.7% ethanol; and Hand Rub D (HR D), gel containing 45% ethanol.

The *ex vivo* method for testing the antibacterial activities of the Hand Wash products (HW) was similar process to that used for the hand sanitizers, except for the bacterial strains used and the treatment process. Specifically, *E. coli* and *S. aureus* (10^8–10^9 CFU mL^{-1}) were used for the hand wash product tests. The contaminated skin pieces were treated with 100 μL of the hand wash products, and a minimum of 30 μL of water was added to the lather followed by rubbing for 30 s. Thereafter, the residue and foam on the skin pieces were immediately rinsed with tap water at 40°C and, after 30 s, the samples were lightly wiped with a paper towel and washed with 10 mL of the sampling fluid. The surviving bacteria in the collected suspension were then counted using the same process as described above for the hand sanitizer tests.

In vivo test method to detect the efficacy of hand rub and wash products

The *in vivo* test were conducted on 22 healthy adults (aged 20-55 years) who voluntarily applied and signed a consent form after receiving explanations about information and objectives of the study. Participants have short fingernails and there were no abnormalities such as skin disease, wounds, or scars. Specifically, subjects washed their hands with tap water at 40°C and non-antibacterial soap to remove oil and dirt, after which their hands were air-dried. Then hand sanitizer containing 70% ethanol and 0.02% chlorhexidine aqueous solution were used to sterilize so that there were no naturally contaminated bacteria on the hands. Using *S. aureus* solution (0.5 mL, 2.0×10^3 CFU mL^{-1}), both hands of the subject were contaminated by spreading for 1 min using a sterile spreader, and this was followed by drying for 3 min. One hand served as the test group for spreading the test formulation (0.5 mL), whereas the other was considered the control group (0.5 mL of phosphate-buffered saline). After 1 min, the hands of the subjects were washed with the test products and 50 mL of the sampling fluids was collected, after which the surviving bacteria in the sampling fluid were counted via plating on TSA.

For *in vivo* testing of six hand wash products, 20 participants were recruited for each formulation. Participants cleaned their hands as described above. Thereafter, the hands were contaminated by placing a total of 4.5 mL of *E. coli* and *S. aureus* inoculum (10^8-10^9 CFU mL^{-1}) on the hands. The baseline was obtained by washing the contaminated hands immediately after contamination with 50 mL of the sampling solution. For the test group, after placing 2 mL of each product on their hands, the subjects passed their hands rapidly through tap water to protect them from becoming too dry, lathered the product thoroughly over the front and back of their hands for 30 s, and thereafter, washed their hands with tap water at 40°C for 30 s. To collect bacteria, the washed hands were lightly patted with a paper towel and exposed to 50 mL of the sampling solution for 1 min. The bacteria in the solution were then counted on TSA, and the number of bacterial colonies was calculated and presented as log reduction values compared with the control treatments in figures and tables.

All individuals participating in this study have provided written informed consent attesting their willful participation. The study design was approved by the ethics committee and the experiments involving human subjects were conducted in accordance with the principles embodied in IRB regulation at the Korea Testing & Research Institute and the Global Medical Research Center (TEK-2020-249).

Results

Sampling method for *ex vivo* test

Ex vivo antibacterial activity tests using pig skin can involve different sampling methods. According to guidelines provided by ASTM International, a cup scrub sampling technique was used to collect the remaining bacteria on pig skin. Specifically, the pig skin area was scrubbed for 1 min using a sterile scraper, and the specified volume of the sampling solution was transferred into a sterile cylinder. We observed that the antibacterial activity of the hand rub or wash products did not change depending on the sampling method (Table 1). In this study, we used the wash sampling method to be similar process to *in vivo* test.

In vivo, ex vivo, and *in vitro* evaluation of antibacterial activity for hand rub

To compare the antibacterial activity of the hand hygiene products, four commercial ethanol-based hand rub products were assessed via *in vivo, ex vivo,* and *in vitro* tests (Table 2). The significant differences ($P<0.05$) among three test methods were evaluated by on-way ANOVA using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and the different letters in Table 2 indicate significant difference following by Duncan's multiple-range test. The results of the *in vivo* tests

Table 1. Mean $\log_{10}$ reduction of bacteria after commercial hand hygiene products treatment in the *ex vivo* test according to sampling methods

Sampling method	*Serratia marcescens*[1)]	*Escherichia coli*[2)]	*Staphylococcus aureus*[2)]
Washing	2.87±0.50	2.17±0.59	2.00±0.43
Cup scrub	2.89±0.62	2.18±0.23	1.93±0.07

[1)] Tested for the hand rub product.
[2)] Tested for the hand wash product.

Table 2. Mean $\log_{10}$ reduction of bacteria after commercial ethanol-based hand rub products treatment in the *in vivo*, *ex vivo*, and *in vitro* test

Hand Rub (HR)[1)]	*in vivo*[2)]	*ex vivo*	*in vitro*
HR A	3.33±1.16[a]	2.87±0.59[a]	> 5.00±0.00[b]
HR B	2.90±0.30[a]	2.23±0.69[a]	> 5.00±0.00[b]
HR C	-	2.00±0.43[a]	> 5.00±0.00[b]
HR D	0.26±0.16[a]	0.14±0.18[a]	> 5.00±0.00[b]

[1)] HR A, B and C were tested against *Serratia marcescens* and HR D was tested against *Staphylococcus aureus*. HR A; 70% ethaol and isopropanol (gel), HR B; 85% ethanol (liquid), HR C; 54.7% ethanol (gel), HR D; 45% ethanol (gel).
[2)] The results of HR A and B were quoted from reference 9 and 10, respectively.

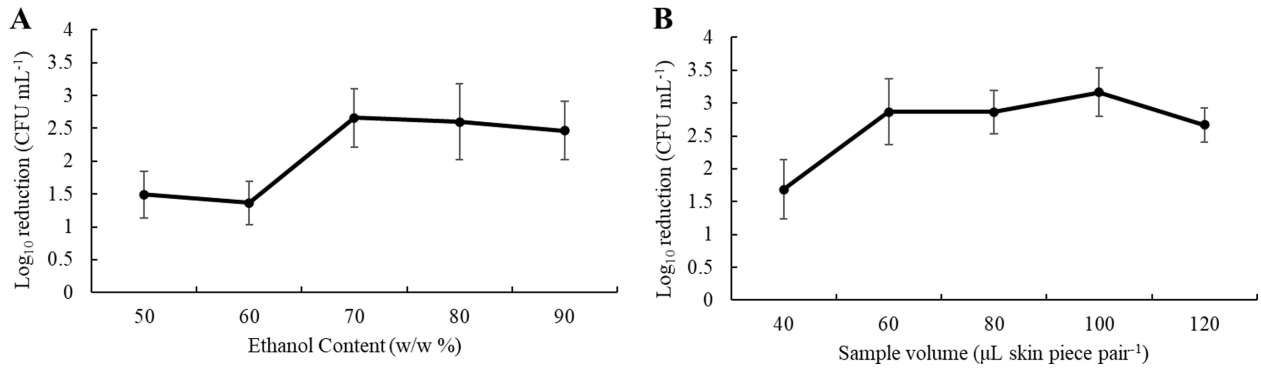

Fig. 1. Antibacterial activity according to ethanol content (A) or treatment volume of hand rub product A (B) against *Serratia marcescens* in the *ex vivo* test.

corresponding to HR A and B were reported in previous studies using the same commercial hand rub products[11,12)]. Furthermore, the two skin model methods, *in vivo* and *ex vivo*, showed similar bacterial load log reduction results, which were significantly differed from the results of the *in vitro* suspension test, exhibiting notably high log reduction values. While the *in vivo* test yielded overall higher log reductions compared to the *ex vivo* test, the difference in values was relatively modest, ranging from 0.12 to 0.46. Notably, HR D, containing 45% ethanol, yielded a substantial 5-log reduction in bacterial load during the *in vitro* test. However, the *in vivo* and *ex vivo* tests showed more conservative results, with reductions of 0.26 and 0.14 logs, respectively.

We performed a supplementary test to evaluate the suitability of the *ex vivo* test for determining the antibacterial activity of hand hygiene products. The results of the changes in antibacterial activity according to ethanol content (%) and treatment volume of the hand rub product (HR A) based on the *ex vivo* tests are shown in Fig. 1. The *ex vivo* test using pig skin was an appropriate alternative to *in vivo* testing. Additionally, the bacterial load log reduction corresponding to 70% ethanol was significantly higher than that corresponding to 60% ethanol. However, that corresponding to 90% ethanol was not higher than that corresponding to 70-80% ethanol. When the treatment volume of the hand rub increased, the log reduction value also increased, except for the 120 μL per skin piece pair treatment volume. Specifically, when 120 μL of hand rub was applied to a 9 cm^2 skin piece, it did not get dry within the allocated time (30 s).

In vivo, ex vivo, and *in vitro* evaluation of the antibacterial activity of hand wash products

The antibacterial activities of the hand wash products were compared to examine the differences among the three test

Table 3. Mean $\log_{10}$ reduction of bacteria after commercial hand wash products treatment in the *in vivo*, *ex vivo*, and *in vitro* test

Hand Wash (HW) product [1)]	*in vivo*		*ex vivo*		*in vitro*	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
HW A	> 5.00±0.00 [a]	4.54±0.62 [a]	3.07±0.60 [b]	2.24±0.11 [b]	1.05±0.10 [c]	> 3.00±0.00 [c]
HW B	> 5.00±0.00 [a]	2.68±1.30 [a]	2.17±0.59 [b]	2.00±0.43 [b]	0.95±1.87 [c]	1.86±0.17 [b]
HW C	1.94±1.32 [a]	2.70±1.02 [ab]	1.36±0.71 [b]	1.62±0.10 [bc]	0.87±0.78 [c]	1.36±0.14 [c]
HW D	3.34±0.56 [a]	2.64±0.61 [a]	2.40±0.37 [b]	1.65±0.33 [b]	0.07±0.07 [c]	1.36±0.24 [b]
HW E	3.99±0.87 [a]	3.47±0.81 [a]	2.16±0.46 [b]	1.85±0.32 [b]	0.52±0.03 [c]	2.59±0.37 [a]
HW F	4.04±0.63 [a]	3.41±0.84 [a]	2.42±0.51 [b]	1.58±0.26 [b]	0.13±0.07 [c]	1.14±0.01 [b]

[1)] HW A-C; Liquid-type product, HW D; Gel-type product, HW E-F; Paste-type prodcut.

methods (Table 3). Significant differences (P<0.05) between these three assays were also evaluated and different letters marked in Table 3 for all products and bacteria indicated significant differences. Among the three experimental methods, the *in vivo* test of the six hand wash products showed the highest antibacterial activity. Even though the bacterial load log reduction corresponding to liquid hand soap (HW A-C) in the *ex vivo* test was lower than that observed in the *in vivo* test, its *ex vivo* results showed a similar pattern to that obtained in the *in vivo* test. The antibacterial activity of all hand wash products except HW C against *E. coli* was better than that against *S. aureus* in the *in vivo* and *ex vivo* tests; however, the reduction of *E. coli* in the *in vitro* test was generally less than 1-log. The *in vitro* suspension test was applicable for evaluating liquid hand wash products; but it encountered challenges with gel and paste products due to their high viscosity, leading to the formation of bubbles and difficulty in mixing. This phenomenon explains the significantly low bacterial load log reduction observed for gel and paste products in the *in vitro* test, highlighting the inappropriateness of this test method. For the *in vivo* and *ex vivo* tests, the difference between these values for the gel-type product (HW D) was approximately 0.94-0.99, and that for the paste-type product (HW E-F) was 1.62-1.83.

Table 4. Mean $\log_{10}$ reduction of bacteria according to concentration of total surfactants contained wash product in *ex vivo* test using pig skin

Concentration of total surfactants (%)	*E. coli*	*S. aureus*
20	2.58±0.09	1.72±0.15
10	2.29±0.38	1.78±0.30
5	1.96±0.08	1.71±0.10
0	1.67±0.26	1.04±0.17

We also confirmed that experimental factors, such as the amount of surfactant in the product or volume of water added, influenced the results of the *ex vivo* test. Specifically, we observed that the concentration of surfactants in hand wash products played an important role in the bacterial removal efficacy of the products. Antibacterial activity tests were conducted according to the surfactant content of the products (Table 4). The log reduction of bacteria was proportional to the surfactant concentration. In contrast, we found that the amount of 30 to 150 μL added water generally did not significantly affect the antibacterial activity of the wash products (Fig. 2).

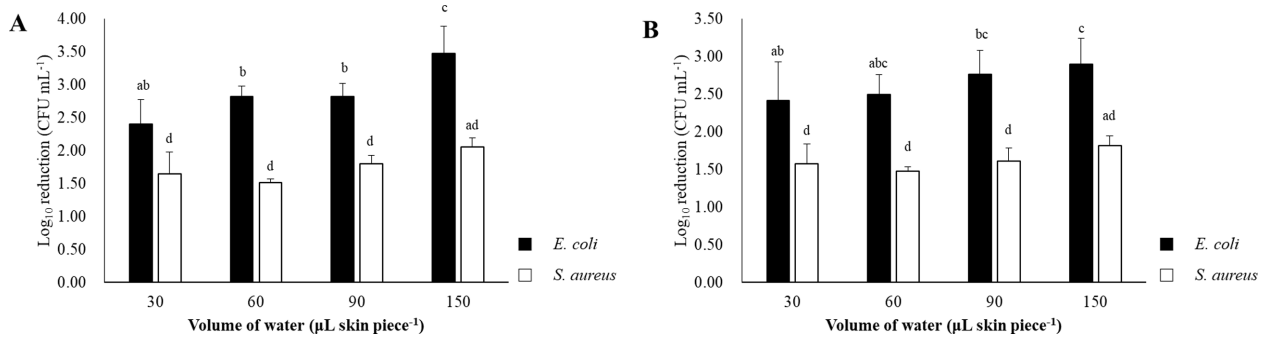

Fig. 2. Antibacterial activity according to treatment volume of water for gel-type, HW D (A) and paste-type, HW F (B) against *Escherichia coli* (■) and *Staphylococcus aureus* (□) in the *ex vivo* test. The significant differences were tested by analysis of variance (ANOVA) and Duncan's multiple range tests using SPSS 18.0 (SPSS Inc.).

Discussion

We conducted a comparative analysis of the antibacterial activity of commercial hand hygiene products using *in vitro, ex vivo,* and *in vivo* methods to identify differences in their efficacy. For accurate comparison, the results corresponding to these three methods were calculated in the same manner and presented as bacterial load log reductions.

Sampling method for *ex vivo* test

The sampling method is an important factor that could yield variable results. The American Society for Testing and Materials (ASTM), an international standards organization, has suggested the cup scrub technique as a sampling method. Although this technique, which used an open-ended cylinder and blunt rod to scrub, showed consistent results, it was challenging to manipulate the instrument on the pig skin. Compared to cup scrubs that require more tools and steps, our proposed washing method mimics the process of sampling using the human hand in the *in vivo* test and makes it easier to collect samples. We suggested this washing method that was convenient to operate because there was no difference between the results of two sampling methods.

Advantages and limitations of the *ex vivo* method for hand hygiene product efficacy assessment

To minimize the differences between the *ex vivo* and *in vivo* tests, we standardized the sample amount relative to applied skin area. The antibacterial activity of each hand sanitizer showed a distinct log reduction value according to the test methods. For instance, the bacterial load log reduction attributed to hand rub sanitizers significantly diverged between *in vivo* or *in vitro* tests. This explains why the evaluation criteria for antibacterial activity generally vary depending on the test method[13,14]. Hand rub sanitizers that yielded log reductions ≥5 based on *in vitro* tests or ≥2 based on *in vivo* tests were considered to have high antibacterial activity. Notably, HR D did not show bactericidal activity in the *in vivo* test; however, it showed log reduction > 5 in the *in vitro* test. Thus, the results of the *in vitro* tests did not reflect the actual bactericidal activity of the product. Additionally, these results showed that the *in vitro* test could cause confusion by overestimating the bactericidal efficacy of hand sanitizers. The suspension test has previously raised the problem of over measurement. Furthermore, another *in vitro* test method, the glass carrier method, may also lead to the inactivation of bacterial inoculum owing to a relatively long drying process, which makes it difficult to obtain reliable results[3,4]. There are also several limitations to testing hand wash products using *in vitro* suspension test. First, the foam produced by the surfactants makes it difficult to obtain an accurate volume in the suspension test sample. Second, the suspension test cannot reflect the antibacterial mechanism, such as is the case resulting from the rubbing effect of hand wash products. The ingredients of hand sanitizers destroy bacterial components to reduce the abundance of pathogens; conversely, hand wash products remove pathogens by forming an oil-water complex on the hand surface, owing to the high content of anionic surfactants, and washing them off with water. Because these processes could not be applied in *in vitro* tests, the suspension test is not suitable for measuring the antibacterial activity of hand wash products.

The animal species from which skin pieces are obtained for *ex vivo* testing could influence bacteria recovery[4]. Pig skin has structure and physiological activity that is most similar that of the human skin; thus, using it to minimize the gap between the results of *ex vivo* and *in vivo* tests. Our study results confirmed that the *ex vivo* test using pig skin showed more similarity to the *in vivo* test results than the *in vitro* test results, owing to skin conditions, such as wrinkles and sebum. Although the pig skin used no longer had any biological activity (such as gland and vascularization in the basal layer), this had little effect on bactericidal activity given that hand sanitizers work on the skin surface, i.e., the stratum corneum, which is a non-living tissue[3]. In a previous study, inactive skin samples were also shown to be as adequate as active skin samples for *ex vivo* antibacterial efficacy tests[4].

Overall, our results showed greater similarity between the *in vivo* and *ex vivo* tests than between the *in vitro* and *in vivo* tests for hand rub products. Unfortunately, unlike results of hand rub products, the results of the two test methods, *in vivo* and *ex vivo,* were not significantly identical for hand wash products. We suggest it is a limitation of *ex vivo* test that the washing process (rubbing) using pig skin does not mimic the dynamic finger movements like the hand washing process in the *in vivo* test. The rubbing using pieces of pig skin on plastic plates could only remove the contaminated bacteria through one-dimensional friction, which would have caused much less reduction of bacteria than *in vivo* test results. Messager et al.[6] also attributed the difference in log reduction between the *in vivo* and *ex vivo* tests to the “self-sterilizing” effect of the human skin, wherein sweat secreted on the skin surface, metabolites, and peptides of resident microflora could cause pathogens to remain on the skin surface for a long time. Pig skin does not exactly reflect human skin mechanisms, such as self-sterilization; thus, this is another limitation of this protocol. Nevertheless, the *ex vivo* test results showed overall better reproducibility than the *in vivo* test results owing to the advantage of being able to fully control the test process.

Verification of the *ex vivo* method for the assessment of hand rub antibacterial activity

To verify the validity of *ex vivo* methods, we measured the decrease in viable bacteria count in ethanol depending on its concentration in the hand rub product. Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) recommend using sanitizers with 60-95% alcohol, and the World Health Organization (WHO) reported that an alcohol content in the range 60-80% is the most effective. In this study, the sample containing 70% ethanol showed a higher bacteria load log reduction than those containing 80% and 90% ethanol. However, the differences were not significant (Fig. 1). This result aligns with prior findings indicating that bacterial activity decreases at ethanol concentrations above 80%[15-17]. Furthermore, the presence of water in alcohol disinfectants plays an important role with respect to its penetration of cells and completely coagulating the membrane and the associated proteins[18,19].

In the *in vivo* test, the antibacterial effect was affected by the amount of hand rub applied, which is one of the main factors affecting the antibacterial activity of hand hygiene products. To confirm a similar pattern in the *ex vivo* test, we adjusted the treatment volume of the sample on a pair of pig skin samples (18 cm^2) in proportion to the volumes used on the hand area (800–1,000 cm^2) in clinical studies (Fig. 1A). In accordance with the EN 1500, a standard dose of 3 mL was used as the experimental volume. Furthermore, the FDA recommends the use of an experimental volume of 5 mL[1,20]. Proportionately, the appropriate sample treatment amount in pig skins is about 60 μL according to the EN 1500 guide and about 100 μL according to the FDA guide. When the sample volume was 40 μL, the antibacterial effect was significantly lower than that observed when the volume was ≥60 μL (Fig. 1B). This implies that *in vivo* test using approximately over 3 mL of hand rub showed higher microbial load reduction values than test involving the use of 2 mL of the product. In clinical studies, hand rub products reportedly have a lower antibacterial effect when 1 mL is used than when 3 mL is used, and when using 2.4 mL rather than 3.6 mL[20,21]. Additionally, we confirmed that drying the hand rub product completely is an important factor in its sterilization effect. When the sample volume was 120 μL, the skin was not completely dried within a rubbing time of 30 s, and this resulted in the inhibition of antibacterial activity. The efficacy of hand sanitizers according to drying time has also been shown in a previous *in vivo* study[22]. It shows that the antibacterial mechanism of the *ex vivo* test method is very similar to that of the *in vivo* test method.

Verification of the *ex vivo* method for assessing the antibacterial activity of hand wash products

Once the critical micelle concentration is exceeded, a positive correlation between surfactant concentration and bacterial log reduction is observed[23,24]. In this study, our results showed a gradual increase in bacterial load log reduction with increasing total surfactant concentration, demonstrating the validity of the *ex vivo* method using pig skin (Table 4). Even with the rubbing action using only water, the products showed a bacterial removal effect ≥ log 1.0. This result is consistent with that of a previous study, which showed that physical removal of bacteria is caused by rubbing action as well as the inherent surfactant properties of the products[23]. This feature was more definite for *E. coli* than for *S. aureus*.

Given that the excessive use of water dilutes the sample, it is necessary to adjust the amount of water according to the characteristics of the product. Conversely, too little water causes insufficient micelle formation. We observed that mixing a liquid product with 30 μL of water was most suitable for lather formation; however, the gel and paste wash products still tended to be somewhat dry. In case of the gel type product, significantly enhanced antibacterial efficacy was shown at a sample-to-water ratio was 2:3 against *E. coli*. However, there are no significant differences in antibacterial performance were observed for gel products against *S. aureus* and paste products against both *E. coli* and *S. aureus*. For practical guidance, we recommend not exceeding a water volume of 150 μL, as excessive water may over-dilute the product. This result means that the required amount of water differs based on the type of wash product used in the *ex vivo* tests, however it can be adjusted within a range prevents excessive dryness, 30-150 μL, aligning with the conditions of *in vivo* testing.

To facilitate more accurate predictions of the antimicrobial activity of hand hygiene products, we conducted a comprehensive examination of commercial hand rub sanitizers and hand wash products by *ex vivo*, *in vivo*, and *in vitro* tests. We suspected that the test protocol would differ depending on whether the product is a rub or a wash-off type, as they have different antibacterial mechanisms against bacteria. Our study also investigated the conditions that could influence *ex vivo* test results. Significantly, we discovered that the antibacterial efficacy of hand sanitizers was influenced by the volume of treated samples when applied to pig skin pieces. Our study suggests that an optimal sample volume of 60-100 μL per skin piece pair (18 cm^2) is suitable. In contrast, for wash products, the addition of water did not significantly impact antibacterial activity.

This study clarified the strengths and limitations of three methods: *in vitro, ex vivo*, and *in vivo*. The *ex vivo* test with closely resembling *in vivo* conditions, complements the time-consuming and potential microbial risk of *in vivo* test and *in vitro* test, which has a low accuracy and limited sample

diversity. In this study, we proved the reliability of the *ex vivo* test result using pig skin, which may help in easily evaluating the efficacy of hand hygiene products. Our findings may enable the *ex vivo* tests to be applied as a reference for determining the antibacterial activity of hand hygiene products, offering a more practical and reliable approach.

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국문요약

손 위생 제품이 다양화됨과 동시에 각 활용 방법에 따라 그 효능을 평가하는 여러 시험 방법들이 보고되고 있다. 하지만 평가 방법에 따라 각 제품의 항균 효능은 다르게 나타나며, 이로 인해 제품의 실제적인 효능을 확인하는 데에 어려움이 있을 수 있다. 손 위생 제품의 효능 평가방법 비교에 초점을 둔 연구는 매우 제한적이며, 특히 돼지피부를 이용한 *ex vivo*에 대한 연구는 극히 드물다. 이에 본 연구는 손 위생 제품 중 리브온 소독제와 워시오프 세정제에 대해 각각의 항균 평가 방법을 종합적으로 비교했고, *ex vivo* 시험에 영향을 미칠 수 있는 요인을 파악하여 연구 단계에서 효율적인 *ex vivo* 시험의 신뢰성을 향상시키고자 하였다. *in vitro* 시험으로써 액체 현탁을 기반으로 하는 time-kill 시험을 진행했고, *in vivo* 시험은 최소 20명의 참여자를 대상으로 진행되었다. *ex vivo* 시험은 규격화된 돼지 피부를 이용하여 *in vivo* 시험과 동일한 방법으로 진행하면서 소독제의 최적 처리량과 세정제 사용 시 첨가되는 물의 양을 제안했다. 시험에 사용된 손 소독제는 *in vitro* 시험에서 모두 5 log 이상의 세균 감소율을 보인 반면, *ex vivo*와 *in vivo*에서는 훨씬 낮은 살균 활성을 보였으며, 특히 알코올 함량이 낮은 손 소독제에서는 1 log 미만의 살균 활성을 나타냈다. 반면에 손 세정제의 *in vitro* 시험 결과, 대장균에 대해서는 1 log 이하의 낮은 항균력을 보였으나, *ex vivo* 와 *in vivo* 시험 결과에서는 이보다 높은 항균력을 유사하게 나타냈다. 본 연구에서는 *ex vivo* 와 *in vivo* 시험 방법이 리브온과 워시오프 타입 제품의 두가지 다른 항균 메커니즘을 반영할 수 있음을 확인했다. 이로 인해 최적의 조건으로 설정된 *ex vivo* 시험은 빠르고 정확한 항균 평가법이 될 수 있음을 제시한다.

Conflict of Interest

The authors declare no conflict of interest.

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References

1. Herruzo, R., Vizcaino, M., Herruzo, I., *In vitro–in vivo* sequence studies as a method of selecting the most efficacious alcohol-based solution for hygienic hand disinfection. *Clin. Microbiol. Infect,* **16**, 518-523 (2010).
2. Word Health Organization (WHO), 2009. WHO guidelines on hand hygiene in health care: first global patient safety challenge clean care is safer care, WHO, Geneva, Switzerland, pp. 157-173.
3. Messager, S., Goddard, P.A., Dettmar, P.W., Maillard, J.Y., Determination of the antibacterial efficacy of several antiseptics tested on skin by an ‘ex-vivo’ test. *J. Med. Microbiol.,* **50**, 284-292 (2001).
4. Maillard, J.Y., Messager, S., Veillon, R., Antimicrobial efficacy of biocides tested on skin using an ex-vivo test. *J. Hosp. Infect.,* **40**, 313-323 (1998).
5. Herron, A.J., 2009. Pigs as dermatologic models of human skin disease. In 60th annual meeting of the American college of veterinary pathologists, Monterey, CA, USA.
6. Messager, S., Goddard, P.A., Dettmar, P.W., Maillard, J.Y., Comparison of two *in vivo* and two *ex* vivo tests to assess the antibacterial activity of several antiseptics. *J. Hosp. Infect.,* **58**, 115-121 (2004).
7. Bush, L.W., Benson, L.M., White, J.H., Pig skin as test substrate for evaluating topical antimicrobial activity. *J. Clin. Microbiol.,* **24**, 343-348 (1986).
8. Shintre, M.S., Gaonkar, T.A., Modak, S.M., Evaluation of an alcohol-based surgical hand disinfectant containing a synergistic combination of farnesol and benzethonium chloride for immediate and persistent activity against resident hand flora of volunteers and with a novel *in vitro* pig skin model. *Infect. Control Hosp. Epidemiol,* **28**, 191-197 (2007).
9. Gaonkar, T.A., Geraldo, I., Caraos, L., Modak, S.M., An alcohol hand rub containing a synergistic combination of an emollient and preservatives: prolonged activity against transient pathogens. *J. Hosp. Infect.,* **59**, 12-18 (2005).
10. O. Y., Song, J.S., Park, H.S., Lee, Y.H., Shin, J.S., Park, D.S., NamGung, E., Cho, T.J., Improvement of the Efficacy Test Methods for Hand Sanitizers (Gel, Liquid, and Wipes): Emerging Trends from *in vivo*/*ex vivo* Test Strategies for Application in the Hand Microbiome. *J. Food Hyg. Saf.,* **38**, 1-11 (2023).
11. Edmonds-Wilson, S., Campbell, E., Fox, K., Macinga, D., Comparison of 3 *in vivo* methods for assessment of alcohol-based hand rubs. *Am. J. Infect. Control.,* **43**, 506-509 (2015).
12. Kampf, G., Ruselack, S., Eggerstedt, S., Nowak, N., Bashir, M., Less and less–influence of volume on hand coverage and

bactericidal efficacy in hand disinfection. *BMC Infect. Dis.,* **13**, 1-7 (2013).
13. American Society for Testing and Materials (ASTM), 2021. Standard test method for evaluation of the effectiveness of health care personnel or consumer handwash formulations, vol. 100, ASTM, West Conshohocken, PA, USA.
14. Rotter, M., 1999. Hand washing and hand disinfection (Chapter 87). Hospital epidemiology and infection control, Lippincott Williams & Wilkins, Philadelphia, PA, USA.
15. Edmonds, S.L., Macinga, D.R., Mays-Suko, P., Duley, C., Rutter, J., Jarvis, W.R., Arbogast, J.W., Comparative efficacy of commercially available alcohol-based hand rubs and World Health Organization-recommended hand rubs: formulation matters. *Am. J. Infect. Control.,* **40**, 521-525 (2012).
16. Kampf, G., Efficacy of ethanol against viruses in hand disinfection. *J. Hosp. Infect.,* **98**, 331-338 (2018).
17. Dharan, S., Hugonnet, S., Sax, H., Pittet, D., Comparison of waterless hand antisepsis agents at short application times: raising the flag of concern. *Infect. Control Hosp. Epidemiol*, **24**, 160-164 (2003).
18. Harrington, C., Walker, H., The germicidal action of alcohol. *Boston Med. Surg. J.*, **148**, 548-552 (1903).
19. Price, P.B., Ethyl alcohol as a germicide. *Arch. Surg.,* **38**, 528-542 (1939).
20. Kampf, G., How effective are hand antiseptics for the post contamination treatment of hands when used as recommended. *Am. J. Infect. Control.,* **36**, 356-360 (2008).
21. Larson, E.L., Strom, M.S., Evans, C.A., Analysis of three variables in sampling solutions used to assay bacteria of hands: type of solution, use of antiseptic neutralizers, and solution temperature. *J. Clin. Microbiol.,* **12**, 355-360 (1980).
22. Ji, Y.J., Jeong, J.S., Comparison of antimicrobial effect of alcohol gel according to the amount and drying time in health personnel hand hygiene. *J. Korean Acad. Nurs.,* **43**, 305-311 (2013).
23. Jensen, D.A., Rogers, M.A., Schaffner, D.W., Surfactant concentration and type affects the removal of Escherichia coli from pig skin during a simulated hand wash. *Lett. Appl. Microbiol.,* **65**, 292-297 (2017).
24. Krawczyk, J., Surface free energy of the human skin and its critical surface tension of wetting in the skin/surfactant aqueous solution/air system. *Skin. Res. Technol.,* **21**, 214-223 (2015).