JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1986, p. 343–348
0095-1137/86/090343-06$02.00/0

Vol. 24, No. 3

Pig Skin as Test Substrate for Evaluating Topical Antimicrobial Activity

LEE W. BUSH,* LESLEE M. BENSON, AND JOHN H. WHITE
Calgon Corporation, St. Louis, Missouri 63133

Received 4 February 1986/Accepted 5 June 1986

The feasibility of using pathogen-contaminated pig skin as a model substrate for evaluating skin disinfectants was demonstrated. A test methodology is described that is safe, convenient to use, and adaptable to a variety of hand-washing conditions. The treatment protocol, pathogen contamination conditions, and application technique variables can all be carefully controlled to simulate clinical use conditions. The number of organisms transferred by contact was compared with the total organism count on the pig skin. The quantity of organisms transferred ranged from 10 to 60% of the total organisms, depending on the nature of the contamination conditions. The cumulative results of multiple imprint and stripping measurements were consistent with the concentration of inoculated organisms. Tests with alcohol solutions validated the methodology and clearly showed the dependence of topical antimicrobial activity on both the concentration and structure of the alcohol. Activity increased with increasing alcohol concentration and in the following order: ethanol, isopropanol, and *n*-propanol. All of the alcohols became less active as the severity of the test conditions was increased, i.e., higher inoculum levels for a longer incubation time before treatment. The contact imprint and stripping methods used to evaluate bacterial growth on the skin clearly showed that the alcohol treatments reduced but did not eliminate the inoculated pathogens. It was found that long lifetimes (several hours) for pathogens on the skin are possible under some environmental conditions. This observation strongly suggests that frequent hand washing is a necessary infection control practice even when opportunities for repeated pathogen contamination have not occurred.

Hand washing by health care personnel is generally accepted as the most effective action that can be taken to prevent nosocomial infections (7). Despite this acceptance, an effective and convenient technique for evaluating the antimicrobial efficacy of hand-washing agents in controlling the contact transfer of pathogens is not available. We developed a new test methodology that meets these requirements.

Data to support the antimicrobial claims of hand-washing products are typically provided by the product supplier and oftentimes include both in vitro and in vivo tests (8). A variety of microbiological tests can be run in vitro, but only data on formulated products that show antimicrobial activity in a hand-washing time frame are relevant in product evaluation. However, even good in vitro data with suitable controls are limited in value because the tests are not carried out on a skin substrate.

A variety of in vivo hand-washing tests have been reported (3, 4, 9, 11, 14–16) that can best be described by considering whether they address a reduction in transient or resident organisms (15). The healthcare personnel handwash type of evaluation typically involves inoculating the hands of qualified human subjects with transient organisms, following a hand-washing protocol, and stripping (washing the skin with a buffered surfactant solution) the hands to determine the degree of reduction of the inoculated organism population. The glove juice or surgical hand scrub test is similar but does not use inoculated organisms, so that only a reduction in the resident organism population is measured.

Although both of these tests can be of value when run with valid controls, they have three major deficiencies. First, they have severely limited value for testing many of the most common and dangerous pathogens because of the use of human subjects. Second, the use of a stripping technique that measures total organisms, as opposed to contact-transmitted organisms, can be misleading. And finally, the results obtained are frequently more related to hand-washing technique and practices than to product efficacy.

The test method we developed using pig skin as a model substrate overcomes these limitations and offers the benefit of being applicable to a wide variety of hand-washing conditions. This paper presents the methodology and discusses some initial data on the antimicrobial efficacy of alcohol solutions that support the validity of the test method.

MATERIALS AND METHODS

Preparation of pig skin. Hides from freshly killed adult pigs were obtained from a local slaughterhouse. The hides were washed with cold water and dehaired with a large animal clipper. They were then cut into smaller sections with a scalpel, rinsed in cold water, placed in plastic bags, sealed, and frozen. Before use in a test, a section of pig skin was thawed, destubbled with disposable razors, and de-fatted with a scalpel. The skin section was then rinsed in cold tap water and cut into pieces (3 by 3 cm) with a scalpel. The pieces were glued onto individual mounting holders (plastic caps; diameter, 4 to 5 cm) with 5-min epoxy such that the skin surface was exposed (Fig. 1A). Two pieces of mounted skin were needed for each sample being tested. The mounted skins were placed into petri dishes (100 by 20 mm) containing a 7.0-cm filter paper disk moistened with 1 ml of water to prevent drying. The prepared skins were placed in a refrigerator overnight.

Before use, the pig skin was tested for the presence of residual antibiotics. Randomly cut plugs (cut with a no. 7 cork borer) from the hide were placed skin side down in individual agar plates seeded with the test organisms. If a

* Corresponding author.

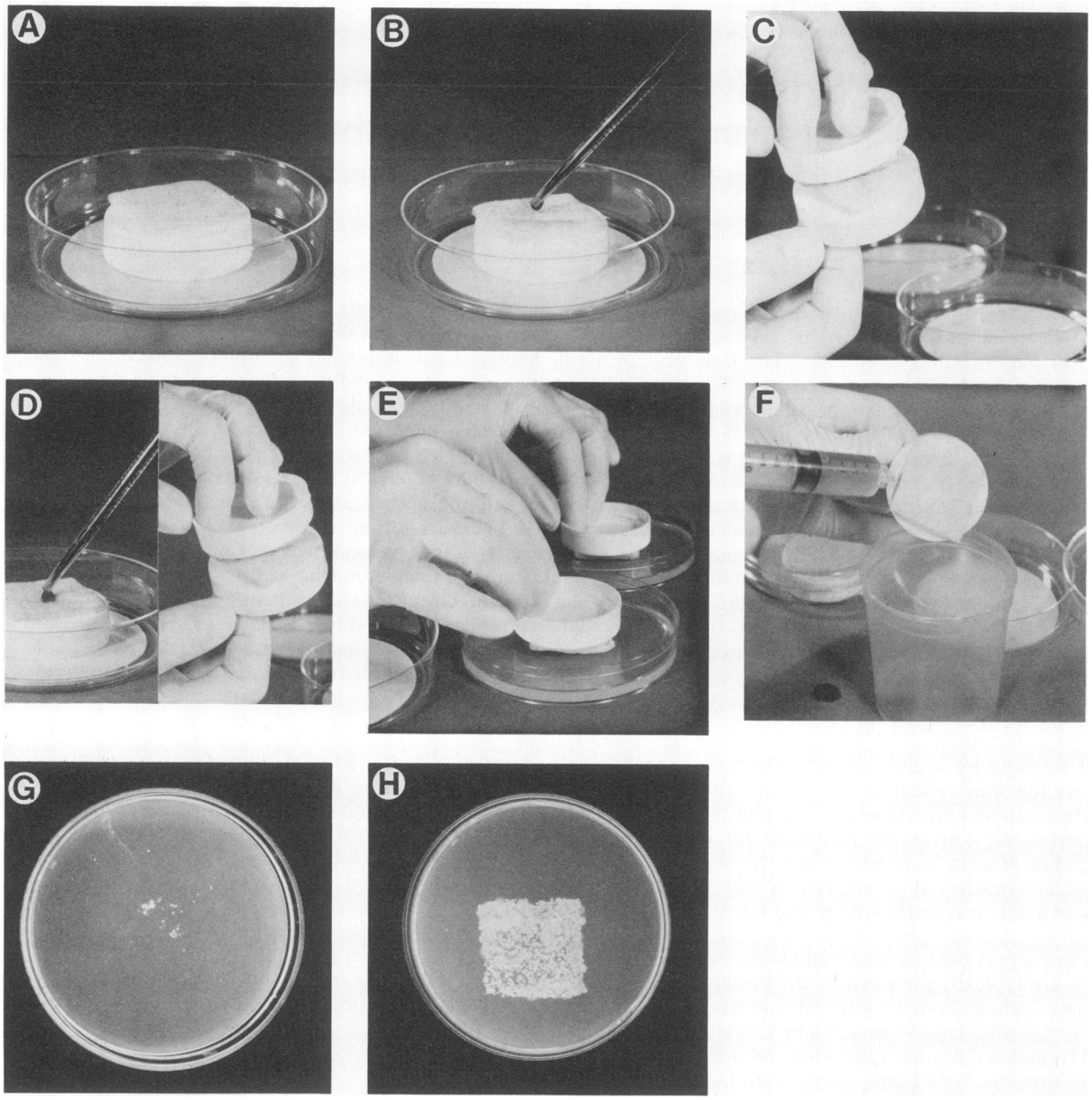

FIG. 1. (A) Mounted pig skin; (B) inoculation of skin; (C) rubbing pieces together to simulate actual hand contamination; (D) after incubation, pig skins are treated with a measured volume of test agent, rubbed together, and allowed to dry for 3 min; (E) mounted pig skins being imprinted onto a neutralizing growth agar; (F) stripping can also be performed. Plates are graded to indicate the extent of organism growth. Ratings of 0 to 10 are given to denote coverage, where 1 = 10% of the imprint surface covered (G) and 10 = 100% of the imprint surface covered (H).

zone of inhibition surrounding the plug occurred, the skin contained residual antibiotics and was not used.

Test organisms. *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Serratia marcescens* ATCC 990, *Candida albicans* ATCC 10231, and *Escherichia coli* ATCC 8739 were maintained on tryptone glucose agar slants (Difco Laboratories).

Test media and solutions. Butterfield buffer (GIBCO Laboratories), plate count agar (Difco), tryptone glucose extract agar (Difco), standard methods agar with lecithin and polysorbate 80 (BBL Microbiology Systems), and letheen thioglycolate broth (GIBCO) were used throughout the procedure.

Application of test organisms. Suspensions of the various organisms were made by overlaying overnight agar slant cultures with 10 ml of Butterfield buffer and gently rubbing the agar surface with a sterile pipette. These suspensions were mixed together to give a mixed inoculum of 10^9 microorganisms per ml. The mixed inoculum was further diluted to give either 10^7 or 10^5 organisms per ml, depending on the test conditions desired. One of two pieces of skin was inoculated with 0.1 ml of the diluted inoculum (Fig. 1B). The two pieces of skin were then rubbed together for 15 s and incubated at 30°C for either 15 min or 2 h, depending on the test conditions desired (Fig. 1C).

Imprints were made by inverting the mounting holder and pressing the treated skin onto the agar surface (Fig. 1E). Imprints could have been made at any desired time intervals.

TABLE 1. Quantitation of imprint measurements

Size of mixed inoculum (CFU/ml)[a]	No. of organisms stripped (CFU/skin pair)	% Growth coverage of imprint area	No. of organisms imprinted (CFU/skin pair)	% of inoculum transferred
10^7	9.0×10^5	100	3.6×10^5	40
10^6	1.0×10^5	90	4.7×10^4	47
10^5	1.0×10^4	80	5.0×10^3	50
10^4	1.0×10^3	60	6.2×10^2	62
10^3	3.0×10^2	30	5.0×10^1	17
No inoculum,[b] low resident population on pig skin	3.0×10^1	10	<10	
No inoculum,[b] high resident population on pig skin	8.5×10^3	60	1.3×10^3	15
10^4[c]	2.0×10^5	90	5.0×10^4	25

[a] Inoculum was 0.1 ml of the indicated concentrations of test organisms per skin pair. Except where noted, incubation was for 1 h at 30°C.
[b] Pig skin evaluated with no inoculum applied, representing only the resident organism population.
[c] Incubated for 6 h.

In these tests, imprints with the same skins were made at 3, 10, and 30 min and 1, 2, and 4 h. The imprinted plates were incubated for 24 h at 30°C. The plates were graded to indicate the extent of organism growth in the imprint. All of the samples were coded and graded blindly to eliminate operator bias. Ratings of 0 to 10 were given to denote coverage, where 0 = no visible growth, 1 = 10% of the imprint surface covered, 5 = 50% of the imprint surface covered, etc. (Fig. 1G and H).

The total number of organisms present at any time in the treatment sequence could be obtained by replacing the imprint procedure with a standard skin stripping procedure by using 10 ml of letheen thioglycolate broth as a neutralizing stripping fluid and counting the organisms removed by a standard plate count technique. At the desired time, 0.2 ml of letheen thioglycolate broth was applied to the surface of one of two pieces of treated skin, the two pieces were rubbed together for 15 s, and each piece was washed with 4.9 ml of the letheen thioglycolate neutralizing stripping fluid (a sterile 10-ml syringe works well for this washing technique) (Fig. 1F). The 10-ml wash from both pieces of skin was collected in an appropriate sterile container and used for the determination of the microbial population. A 1-ml sample of this fluid was removed immediately after stripping and plated by using standard methods agar with lecithin and polysorbate 80. Further dilutions (to 10^{-5}) were made in letheen thioglycolate broth and plated as described above. Microbial populations were determined after incubation at 30°C for 48 h.

Quantitation of imprint measurements. The imprint measurements were compared with the actual numbers of CFU by inoculating three sets of skins with 0.1 ml of test organisms ($\sim 10^9$ organisms per ml) diluted in Butterfield buffer to 10^7, 10^6, 10^5, 10^4, and 10^3 organisms per ml. After 1 h of incubation at 30°C one set of skins was imprinted and another set was stripped, both as described above. The third set was imprinted onto plate count agar, and the imprinted area was cut out with a sterile scalpel and vortexed in 10 ml of Butterfield buffer in test tubes (25 by 150 mm). Standard counts were made with Butterfield buffer dilution blanks and plate count agar.

Multiple measurements were taken per skin pair by both the imprint method and the stripping technique. The skins used in these studies had a low resident population due to pretreatment of the intact hides with low concentrations of alcohol. No treatment took place after inoculation and incubation.

Application of alcohol preparations. Three alcohols, ethanol, isopropanol, and *n*-propanol, were prepared as 40, 60, 80, and 90% solutions (vol/vol) with sterile distilled water. One of two pieces of inoculated skin was treated with 0.1 ml of the desired alcohol preparation (Fig. 1D). The two pieces of skin were then rubbed together for 15 s and allowed to air dry for 3 min to simulate normal evaporation before they were imprinted onto the surface of a neutralizing growth medium (standard methods agar with lecithin and polysorbate 80).

RESULTS

The pig skin used in the test method was handled by techniques intended to maintain the resident organism population. The organism composition was investigated briefly and found to be in agreement with published information (17), i.e., the organisms were predominantly staphylococci. Although some variance among skin sources was observed, the range of the numbers of resident CFU on the skin squares was fairly consistent (5×10^4 to 2×10^5). The resident organism population appeared very close to that on human skin in both the type and concentration of bacteria.

Quantitation of imprint measurements. The imprint measurements were compared with the actual numbers of CFU stripped from the skin pairs. Contamination conditions were determined by the combination of organisms present (resident or inoculated) and incubation time. The data (Table 1) indicate that only 40 to 60% of the total number of organisms present were transferred on the initial imprint after 1 h of incubation. The data (Table 1) also indicate that the number of pathogens transferred by imprinting depends on the contamination conditions of the skin, i.e., at higher resident populations and at longer incubation times fewer organisms were transferred on the initial imprint.

A reproducible correlation between imprint grading and the actual numbers of organisms transferred to the agar surface during imprinting was demonstrated by performing plate counts on agar sections cut from imprinted plates. The correlation between the percent growth coverage of the imprinted area and the number of CFU transferred by imprinting is shown in Table 2.

Multiple measurements. Multiple measurements were taken per skin pair by both the imprint method and the stripping technique. The data (Fig. 2) indicate that a significant number of pathogenic organisms can be transferred by repetitive contact and that these numbers remain relatively high even after 10 transfers. This suggests that skin can act as a reservoir for contact transfer of pathogens. The cumulative data, represented by the solid line for both the imprint

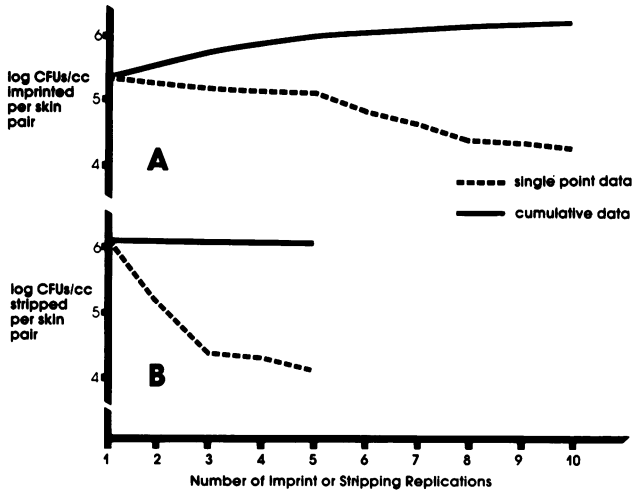

FIG. 2. Multiple measurements (per skin pair) with imprint method and stripping technique. Inoculum was 0.1 ml (10^7 CFU/ml) of a mixture of organisms; incubation was for 1 h.

and stripping measurements, reached the same total organism count and were consistent with the concentration of the inoculated organisms. This apparent agreement may have been fortuitous, however, since the colony growth being compared was from different assay procedures.

Disinfection of pig skin by alcohol solutions. Comparisons of the efficacy of alcohol solutions under three different test conditions are presented as follows: uninoculated skin (Fig. 3), inoculation at 10^5 CFU/ml with a 15-min incubation (Fig. 4), and inoculation at 10^7 CFU/ml with a 2-h incubation (Fig. 5). It is important that in all three cases the controls (no alcohol treatment) gave consistent imprint values in the 80 to 100% range, indicating that the organisms remained viable on the humidified skin in the absence of any treatment (to simplify the datum presentation, the control bars are omitted from the figures). Complete kill was not observed under any treatment condition since 100% regrowth was observed in all cases after 24 h of incubation. Microscopic evaluation of the organism regrowth imprints indicated that selective killing of the organisms did not occur. Each of the species of inoculated bacteria grew back under all test conditions.

The three test conditions differed in the severity of the contamination, as controlled by the type and quantity of microorganisms, as well as by their penetration into the skin. The results show two consistent trends for both test conditions: an increase in antimicrobial activity with increasing alcohol concentration and variability in activity with alcohol structure. The effectiveness changed with these changing conditions, but the order of activity (*n*-propanol, isopropanol, ethanol) was maintained.

TABLE 2. Imprint rating correlation

% Growth coverage of imprint area	No. of CFU transferred on imprint
10	10
20	10–25
30	25–50
40	50–75
50	75–100
60	100–500
70	500–1,000
80	1,000–5,000
90	5,000–50,000
100	50,000

DISCUSSION

Pig skin has been previously proposed as a good test model for human skin based on many similarities in behavior and composition (5, 12). The value of such a test model for hand-washing studies is its ability to simulate a wide variety of skin contamination and treatment conditions. Considerations of health, safety, and expense preclude conducting the type of testing presented here on human volunteers.

The test methodology is sufficiently flexible to produce conditions of contamination and treatment that are very relevant to actual hand-washing practices. Parameters that influence hand-washing results include the type and concentration of microorganisms, contamination conditions, and hand-washing technique relating to time, the volume of product used, and the degree of mechanical action. All of these parameters are controlled in the pig-skin hand-washing test model.

A mixture of pathogens commonly encountered in the hospital environment was used in this study in combination with the resident microbial population of the pig skin. The three test conditions represented variations in contamination conditions produced by changes in inoculum level and the time allowed for penetration into the skin. The increase in

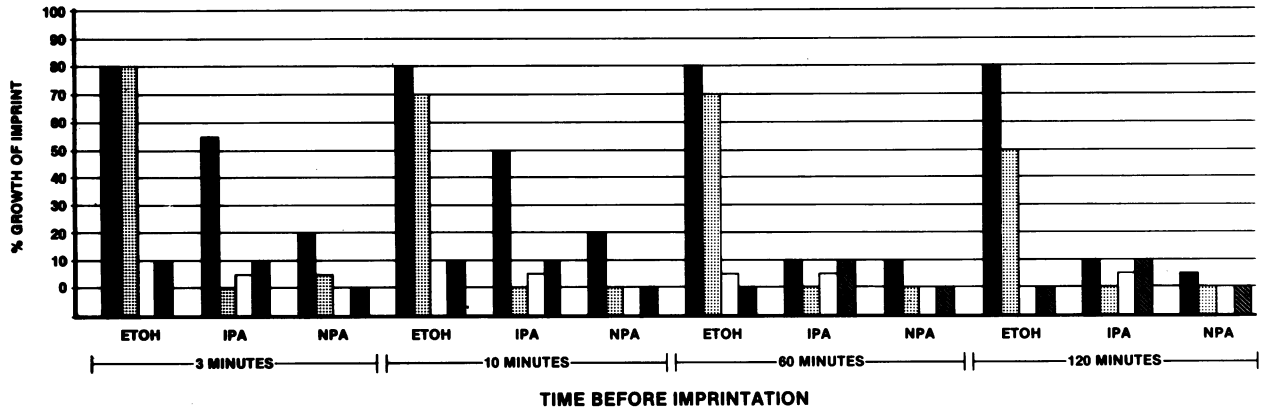

FIG. 3. Disinfection of uninoculated skin by alcohol solutions. ■, 40% alcohol; ▨, 60% alcohol; □, 80% alcohol; ▧, 90% alcohol; ETOH, ethyl alcohol; IPA, isopropanol; NPA, *n*-propanol.

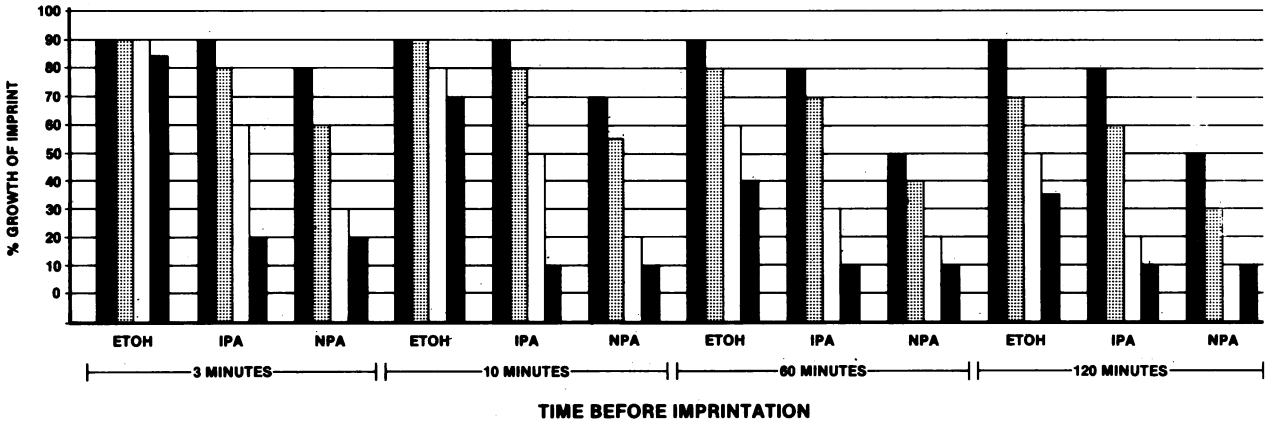

FIG. 4. Disinfection of skin inoculated with 10^5 CFU/ml and incubated for 15 min. Bars and abbreviations are defined in the legend to Fig. 3.

the difficulty of microorganism removal that was observed is in agreement with the work of Lilly and Lowbury (10) in which rubbed-in inoculum was more difficult to remove than dried-on microorganisms.

The test conditions were designed to be relevant to the time frame of a hand disinfection procedure. The 15-s application time was selected on this basis and was held constant throughout the tests. The 3-min delay between alcohol application and the initial imprint was necessary to allow evaporation to occur. Shorter times resulted in the transfer of alcohol to the imprint media, and since the alcohol was not neutralized, these data were poorly reproducible.

The time between alcohol application and imprinting was a variable and was representative of the rate of initial kill and regrowth. The maximum antimicrobial effectiveness of a solution was generally reached quickly and maintained for several hours before regrowth occurred.

The dependence of the topical antimicrobial activity of alcohols on both concentration and structure has been reported (15), and the trends are in general agreement with the results reported here. This agreement is good support for the validity of the pig-skin test methodology.

The value of the pig-skin test methodology lies not only in the flexibility of the method in controlling test conditions but also in the use of an imprint technique to measure the results. Hand washing is an effective technique for controlling the spread of infection because good practices can reduce contact transmission of microorganisms. Therefore, a measurement procedure for evaluating the efficacy of skin disinfection techniques should be most concerned with the number of organisms transferred by contact and less concerned with a total organism count. The imprint procedure focuses on measuring this contact transmission, as opposed to a skin-stripping procedure that measures the total number of organisms.

A skin-stripping procedure can be used with the pig-skin test model but is a much more time-consuming measurement technique. Data were collected that demonstrated a correlation with the imprint technique and the quantity of inoculated organisms. The same trends were observed with both measurement methods, but the stripping data clearly show that significant levels of microorganisms were present on the skin even when only a fraction of this quantity was being transferred on contact. This suggests that at least some transient organisms are capable of deep penetration into the skin, where they can remain viable and grow back after several hours. This strongly indicates the necessity for repeated hand washing even when only a single opportunity for transient organism contamination has occurred.

The observation of prolonged viability of inoculated pathogens on the skin also challenges the current definition

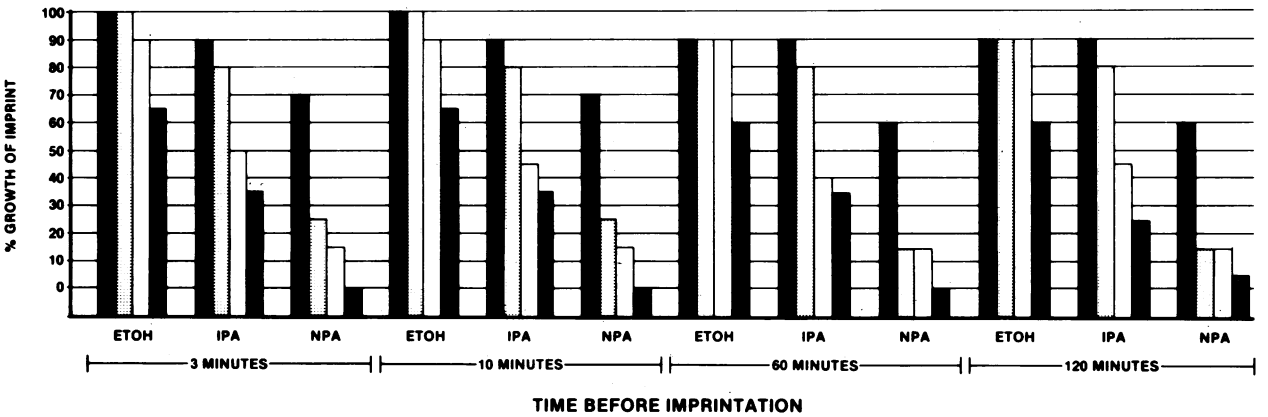

FIG. 5. Disinfection of skin inoculated with 10^7 CFU/ml and incubated for 2 h. Bars and abbreviations are defined in the legend to Fig. 3.

of transient organisms as short-lived, easily removed bacteria (3, 7). The observed regrowth of inoculated organisms suggests that resident and transient organisms may be changing categories, depending on the organism, the skin environment, and time. The suggestion that transient organisms may become a part of the resident population is in agreement with previous work (1, 2, 6) relating to the isolation of a low level of gram-negative rods from samplings of resident populations on hands.

A new test methodology based on the use of pig skin was developed and appears to have high applicability for evaluating the antimicrobial efficacy of skin disinfection agents. The method is adaptable enough to simulate a variety of hand-washing conditions and can safely evaluate activity against common hospital pathogens. Additional work is in progress on the use of this method to evaluate such hand-washing-related unknowns as the influence of the resident organism population on transient organism viability on the skin, pathogen-skin disinfectant activity profiles, hand-washing protocols, organism regrowth rates after hand washing, and product efficacy comparisons.

The applicability of using an imprint technique to measure contact-transmitted pathogens is discussed. Quantitation of the organisms imprinted showed a good correlation between the grading technique and the number of CFU transferred on contact. Repetitive imprints and colony regrowth on the skin showed that viable populations of organisms can be sustained on the skin for several hours. These results suggest the desirability of repeated hand washing even when an opportunity for recontamination with transient organisms has not occurred.

The dependence of the topical antimicrobial activity of alcohols on both structure and concentration was demonstrated. These observations are in agreement with previous reports, giving support to the validity of the pig-skin test model. The high evaporation rate of alcohol is suggested as a limit to the antimicrobial efficacy of simple alcohol solutions in topical applications.

LITERATURE CITED

1. **Adams, B. G., and T. J. Marrie.** 1982. Hand carriage of aerobic gram-negative rods by health care personnel. J. Hyg. **89**:23–31.
2. **Adams, B. G., and T. J. Marrie.** 1982. Hand carriage of aerobic gram negative rods may not be transient. J. Hyg. **89**:33–46.
3. **Ayliffe, G. A. J.** 1984. Surgical scrub and skin disinfection. Infect. Control **5**:23–27.
4. **Ayliffe, G. A. J., J. R. Babb, and A. H. Quoraishi.** 1978. A test for "hygienic" hand disinfection. J. Clin. Pathol. **31**:923–928.
5. **Bissett, D. L., and J. F. McBride.** 1983. The use of the domestic pig as an animal model of human dry skin and for comparison of dry and normal skin properties. J. Soc. Cosmet. Chem. **34**:317–326.
6. **Casewell, M.** 1981. The role of hands in nosocomial gram-negative infection, p. 192–202. *In* H. Maibach and R. Aly (ed.), Skin microbiology. Springer-Verlag, New York.
7. **Centers for Disease Control.** 1982. Centers for Disease Control antiseptics and handwashing. Guideline for prevention and control of nosocomial infections. Centers for Disease Control, Atlanta.
8. **Federal Register.** 1978. The tentative final monograph for OTC topical antimicrobial products. Fed. Regist. **43**:1210–1249.
9. **Larson, E.** 1984. Current handwashing issues. Infect. Control **5**:15–17.
10. **Lilly, H. A., and E. J. L. Lowbury.** 1978. Transient skin flora. Their removal by cleaning or disinfection in relation to their mode of diposition. J. Clin. Pathol. **31**:919–922.
11. **Lowbury, E. J. L.** 1981. Topical antimicrobials: perspectives and issues, p. 158–168. *In* H. Maibach and R. Aly (ed.), Skin microbiology. Springer-Verlag, New York.
12. **Meyer, W., R. Schwarz, and K. Neurand.** 1978. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. Curr. Probl. Dermatol. **7**:39–52.
13. **Price, P. B.** 1938. The bacteriology of normal skin: a new quantitative test applied to a study of the bacterial flora and the disinfectant action of mechanical cleaning. J. Infect. Dis. **63**:301–318.
14. **Rotter, M., G. Wewalka, and W. Koller.** 1981. Influence of some variables on the results of evaluation of procedures for hygienic hand disinfection. Hyg. Med. **7**:157–166.
15. **Rotter, M. L.** 1984. Hygienic hand disinfection. Infect. Control **5**:18–22.
16. **Sheena, A. Z., and M. E. Stiles.** 1982. Efficacy of germicidal handwash agents in hygienic hand disinfection. J. Food Prot. **45**:713–720.
17. **Smith, R. F., and B. L. Evans.** 1972. Bacteria of porcine skin, xenografts, and treatment with neomycin sulfate. Appl. Microbiol. **23**:293–297.