THE JOURNAL OF INVESTIGATIVE DERMATOLOGY VOL. 45, NO. 6

Printed in U.S.A.

A NEW METHOD FOR THE QUANTITATIVE INVESTIGATION OF CUTANEOUS BACTERIA*

PETER WILLIAMSON, B.A. AND ALBERT M. KLIGMAN, M.D., PH.D.

Over the past 25 years, many different methods have been used for sampling the bacterial flora of the human skin (1–3). The results obtained have for the most part lacked reproducibility, and have been extremely divergent. We have embarked upon an intensive study of the bacterial ecology of normal and abnormal skin. The development of a better quantitative sampling technic was an indispensable step. The method to be described is a modification of the cup scrub technic of Pachtman *et al* (4). The principal refinements which have resulted from our study have to do with the composition of the wash fluid which is placed in the cup. This study deals exclusively with the aerobic microflora.

MATERIALS AND METHODS

Subjects.—Subjects were healthy adult Negro men, inmates of Holmesburg Prison, Philadelphia, Pa.

Bacteriological technics.—Bacteria were counted in pour plates of appropriately diluted samples using 15–20 ml of Tryptic Soy Agar (Difco) per plate. After 36–48 hours of aerobic incubation at 37° C., the colonies were counted on a Quebec counter.

Selection of surfactants.—Of the three classes of surfactants, the cationics, anionics and nonionics, the first was dismissed because it is made up largely of quaternary ammonium compounds; these are potent bactericides *in vitro*. Anionic surfactants are among the best known and most effective detergents in use today; for instance, the alkyl benzene sulfonates, the alkyl sulfates, and the soaps (salts of long chain fatty acids). Ten representative compounds of this type were tested and rejected as too toxic to bacteria; *viz.* alkyl benzene sulfonates and soaps.

Non-ionic detergents seem suited for skin sampling because of low irritancy, low bacterial toxicity, high stability, relative purity of commercial products and great effectiveness as detergents and dispersants. In the present study sixteen non-ionic detergents were chosen to give a representative sample of the various chemical types (Table I). The usefulness of a detergent was estimated in two different ways:

(1) *In vitro screening.*—Both in culture and especially on the skin, bacteria grow in clusters, not as single entities. Unless effectively dispersed, colony counts reflect aggregates, not individual cells, leading to underestimations. To study dispersing ability, aqueous solutions of surfactants were added to water suspensions of an 18 hr. broth culture of *Staphylococcus albus* having a bacterial density of 5–30 $\times$ 10^4 organisms/ml to make final surfactant concentrations of 0.1%. These were briefly shaken mechanically (Vortex Jr. Mixer) and incubated for 10 minutes at room temperature. After re-shaking, aliquots were removed, diluted in water in 10-fold steps, and plated as described above. Water controls were included and treated in the same manner. The effectiveness of a detergent was estimated by taking the ratio of the test count to the water control count, times 100. We have termed this the 'dispersal efficiency'.

(2) *In vivo* testing.—Those surfactants having the highest dispersal efficiency were tested for their ability to remove bacteria from the glabrous region of the forearm. One ml of 0.1% detergent solution was pipetted into the sterile glass cylinder circumscribing an area of 3.8 cm sq and the surface rubbed as evenly as possible with a blunted Teflon 'policeman' (Arthur H. Thomas) for two minutes. Pilot studies made it clear that hard rubbing or mechanical disruption of the horny layer were superfluous. The wash solution was aspirated, an aliquot diluted, and pour plates prepared; after 48 hour aerobic incubation, the colonies were counted. Each test was done in triplicate.

Effect of pH.—Arnold's (5) observations that strongly basic solutions removed far more bacteria from the skin than did acidic solutions were confirmed later by Blank and Coolidge (6). The latter suggested that bacteria were held to keratinized cells by electrostatic charges that were strongest at low pH and could be negated at highly alkaline pH. Preliminary studies with aqueous salts, acids and bases in the pH range 4–10 indicated a preferential removal of bacteria in the strongly alkaline range, but in no case was aqueous alkali alone more effective than aqueous non-ionic detergent in the slightly acid range. Consequently, 0.1% solutions of the most effective non-ionic detergents were made in 0.067 M (physiologic) phosphate buffer ranging in pH from 6–10, and examined for optimal efficiency using the *in vivo* method described above.

Efficacy of surface sampling.—The scrub technic is valuable only if it can be shown that the great

* From the Department of Dermatology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104.

This study was conducted under the sponsorship of the Commission on Cutaneous Diseases of the Armed Forces Epidemiological Board and was supported by The Surgeon General, Department of the Army.

Presented at the Twenty-sixth Annual Meeting of The Society for Investigative Dermatology, Inc., New York, N.Y., June 22, 1965.

majority of organisms are actually located on or near the surface and are hence removable. Past teaching emphasizes a follicular situation. Histologic studies now in progress in this laboratory indicate that aerobic bacteria are found neither in the follicles nor deeply within the horny layer; only anaerobic bacteria of the *C. acnes* type are regularly found in deep follicular recesses of certain areas.

Four quantitative methods were utilized to demonstrate the superficial location of the aerobic microflora. (1) Cellophane tape stripping: Röckl and Muller (7) and Updegraff (2) have previously shown by qualitative methods that the majority of bacteria are present in the outer layers of the stratum corneum; these investigators relied on culturing bacteria directly from the tape strips. In the present study, bacterial counts were made on adjacent areas scrubbed before and after firm serial stripping with 'Scotch' brand cellophane tape (Minnesota Mining and Manufacturing Co.). (2) A comparison of scrubbing to soaking: Two minutes of normal manual scrubbing was compared on adjacent sites to gentle agitation of the wash fluid alone, with no manipulation of the skin surface. (3) Normal cleansing: Subjects washed their own foreheads and forearms with Ivory soap and warm tap water for approximately 15 seconds following a control sample and then were resampled. (4) Serial scrubbing: Successive one minute washes were taken at the same site with as short an interval as possible between each scrub. Each sample was then worked up separately. Preliminary studies were done on the forearms of five men, three with high average bacterial counts and two with low. Similar experiments were then performed on areas of high bacterial population such as forehead and axilla.

RESULTS

1. Dispersion.—The concept of dispersal efficiency has been of considerable assistance in the selection of detergents. As can be seen in Table I, the list of 16 non-ionic detergents can be narrowed to four worthy of further study, those having a dispersal efficiency of 170 or greater. This value indicates that these particular detergents disperse the test bacteria 1.7 times greater than water, thereby nearly doubling the count. When the most effective substances were buffered to pH 7.9, Plurafac A-26 and Triton X-100 gave dispersal efficiency values ranging from 205 to 220 which were consistently higher than those of Surfonic N-95 and Triton N-101. In general, *in vivo* studies with these compounds paralleled *in vitro* results; Triton X-100 however appeared to be more satisfactory in regard to reproducibility and to maximal removal of microorganisms.

TABLE I

Nonionic detergents

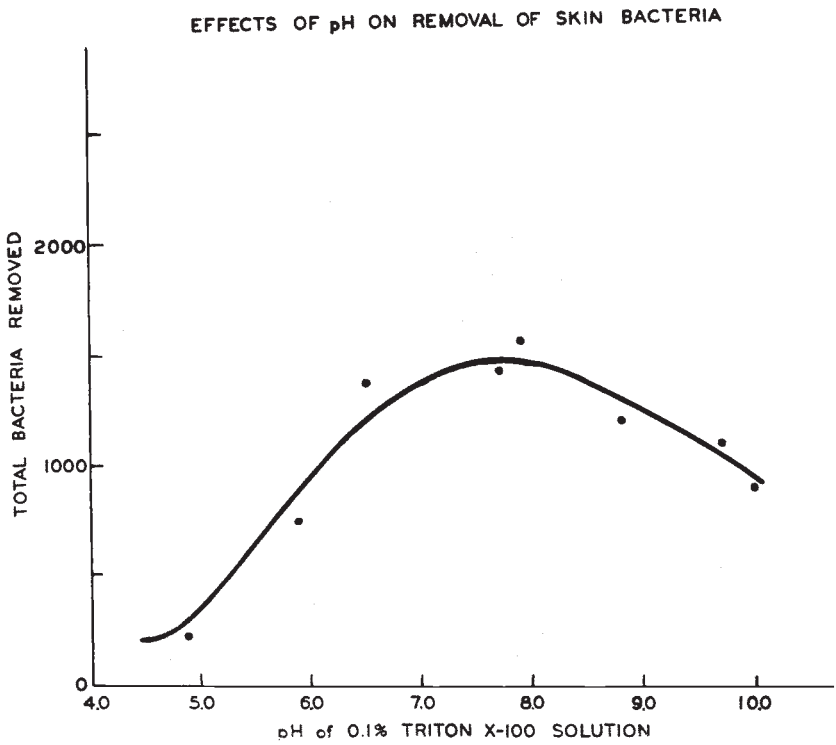
Chemical Type	Trade Name	Manufacturer[1]	Dispersal Efficiency[2]
Polyoxyethylene aliphatic ethers	Plurafac A-16	(1)	139
	Plurafac A-26	(1)	182
	Poly-Tergent J-400	(2)	147
	Brij-35	(3)	80
	Sterox AJ-100	(4)	120
	Surfonic LF-7	(5)	150
Polyoxyethylene sorbitan fatty acid esters	Tween 20	(3)	139
	Tween 40	(3)	122
	Tween 60	(3)	126
	Tween 80	(3)	127
	Tween 81	(3)	119
Alkyl phenoxy polyoxyethylene ethers	Poly-Tergent B-300	(2)	156
	Surfonic N-95	(5)	186
	Triton X-100	(6)	180
	Triton N-101	(6)	173
Polyoxyalkalene block copolymer	Plurafac RA-10	(1)	138

[1] Manufacturers listed according to the following scheme: (1) Wyandotte Chemical; (2) Olin Matheison; (3) Atlas Chemical; (4) Monsanto; (5) Jefferson Chemical; (6) Rohm and Haas.

[2] Dispersal Efficiency

$$= \frac{\text{Bacterial count, detergent-treated sample}}{\text{Bacterial count, water control}} \times 100$$

2. pH.—The effect of pH on the efficiency of a skin sampling solution is apparently related to some phenomenon of the cutaneous surface, since pH changes in the range of 4–10 have no effect on bacteria in suspension when examined by the method described above. *In vivo* studies, on the other hand, show a small but definite increase in the number of organisms recovered with aqueous alkali over neutral or acid solutions, but it must be emphasized that alkali alone does not approach a slightly acidic aqueous detergent in its ability to recover bacteria from the skin. Examination of buffered solutions of effective detergents has shown in the case of Triton X-100 a definite increase in bacterial removal as the pH increases from 6 to 8, followed by a slight decline which may be due to a



deleterious effect of the alkali alone. The peak efficiency from the five experiments summarized in Fig. 1 is at approximately 7.9. Because of these findings, it was decided that the standard wash fluid be 0.1% detergent (Triton X-100) in 0.075 M phosphate buffer, pH 7.9. The deliberately high buffer concentration was chosen to prevent any lowering of the pH during sampling by the strongly acid buffered skin. Thus, the change in pH of the wash solution after a two-minute scrub was less than 0.05 pH units.

3. Superficial location of aerobic organisms.—

(a) Scotch Tape. Regardless of area, 95% of the organisms were removed after four strips. Ten strips were no more effective. This suggests that the bacteria are in the very outermost horny layer. The almost complete removal by four strips holds whether a normal area having 1,000 bacteria per sq cm is studied or an experimentally hydrated one having 50×10^6 organisms per sq. cm. These results are in accord with recent findings (8) which indicate that the major portion of the horny layer is a coherent membrane which does not provide crevices for the enlodgement of bacteria. The sloughing of the horny layer is a surface phenomenon restricted to the outer three to four layers.

(b) If the wash fluid is left in contact with the skin of the forearm and merely agitated, it was found, in ten different cases, that the percentage recovery was 42.6% of that obtained by manual scrubbing. The aerobic organisms are not only superficial, but also rather weakly adherent.

(c) The same conclusions can be drawn from examining the effect of simple cleansing of the skin with soap. An average of several experiments show a 60–80% reduction in the bacterial flora of the forearm, and a 65–95% reduction on the forehead.

(d) The results of nine serial scrub experiments on three individuals of widely varying bacterial counts are given in Table II; 85% of the organisms are removed in a single one minute scrub

TABLE II

Serial sampling

Subject	% Organisms removed during each wash			Average
	A[1]	B[2]	C[3]	
1st Wash	81.6	86.5	87.4	85.1
2nd Wash	18.4	8.7	10.6	12.7
3rd Wash	0	4.8	2.0	2.2
Average total organisms recovered per experiment	69	21,665	229	

[1] Average of four experiments.
[2] Average of three experiments.
[3] Average of two experiments.

and 97–98% in two. This emphasizes the necessity of utilizing two scrubs to provide near complete removal.

The efficiency of a detergent-based sampling solution can be appreciated by considering the great ease with which bacteria are removed from keratinized cells not attached to the cutaneous surface. Specimens taken in buffered detergent which have heavy tissue sediments, *e.g.* from the axilla, were found microscopically to contain only disaggregated organisms; subsequent homogenation of the sediment in a glass tissue homogenizer frees no additional bacteria. This is most easily shown with scurf samples from the scalp. Table III summarizes an experiment in which weighed scurf samples were exposed to buffered Triton X-100 for a short period of time and the dispersed bacteria counted. The counts per mg scurf were very much greater than those reported in a similar experiment by VanderWyk and Roia (9), the essential difference between the two studies being the presence of a surfactant. The increase in counts points out the effectiveness of the buffered Triton sampling without the use of severe mechanical methods.

DESCRIPTION OF METHOD AND REPRESENTATIVE RESULTS

The method routinely used in our laboratory is as follows:

(1) The area to be scrubbed (3.8 sq cm) is delineated by a sterile glass cylinder held firmly to the skin by two attached handles.
(2) One ml of wash solution—0.1% Triton X-100 in 0.075 M phosphate buffer, pH 7.9—is pipetted in and the area scrubbed with moderate pressure for one minute using a sterile Teflon 'policeman.'
(3) The wash fluid is aspirated, replaced with a fresh 1 ml, and the scrub repeated.
(4) The two washes are then pooled and an aliquot diluted in 10-fold steps using as diluent 0.05% Triton X-100 in 0.0375 M phosphate buffer to prevent any reaggregation of organisms.
(5) The appropriate dilutions (usually 10^0, 10^{-1}, 10^{-2} for normal skin; 10^{-3} and 10^{-4} for areas of high bacterial density) are plated in 15–20 ml Tryptic Soy Agar per plate.
(6) After 48 hour aerobic incubation at 37° C, colonies are counted and viable cells in the original sample calculated by standard methods.

It must be noted however that in spite of the improved sampling method, variation in bacterial density is still encountered. Table IV lists results obtained with multiple scrubs of adjacent sites on the back of one man and the forearm of another. The similarity of multiple counts on a given day is obvious, as is the fact that day-to-day variations in a single subject are pronounced. These data emphasize the necessity for a minimum of triplicate samples at a given site on a given day.

The possibility of standardizing test conditions of the skin by pretreatment has been considered, in the hope of reducing the variability between counts. Certain treatments most assuredly change the bacterial count; for instance, the use of occlusive dressings or a sustained rise in temperature and humidity, but these serve to increase the population above so-called normal levels, with no decrease in variability between

TABLE III

Dispersion of bacteria from scurf samples

Technique	Number of Subjects	Average bacterial counts per milligram scurf
10 minute soak; one minute mechanical shake in detergent	2	530,000
30 minute mechanical shake in 0.85% saline[1]	9	7558 (Range: 1340–22,970)

[1] For reference, see text.

TABLE IV

Representative counts from multiple skin samples on adjacent sites

Area Sampled	Day	Bacterial Counts per sq cm		Average
Back	1	110	120	181
		233	253	
		95	276	
Forearm				
	1	1937		2621
		3521		
		2404		
	2	7974		6640
		7263		
		4684		
	3	3947		3273
		2599		
	4	6200		6287
		6354		

samples. Soap and water washes 5 to 24 hours before sampling, quick scrubbing with 95% ethanol 20 hours before sampling, normal cleansing of the skin followed by non-occlusive covering with sterile gauze for as much as 50 hours before sampling, all had no effect on the total number of bacteria obtained or on the variability between samples if triplicate samples were taken. One cause of innocent error, resulting in low counts, is the use of antibacterial soaps by the subject.

TABLE V

Representative aerobic bacterial counts obtained using buffered Triton X-100 scrub technic

Area	Number of Samples	Counts/sq cm	Range
Scalp	24	1.46×10^6	0.22×10^6–14.3×10^6
Axilla	31	2.41×10^6	6300–16.7×10^6
Forearm[1]	17	4500	400–19,000
	15	105	25–300
Back	19	314	50–1450
Forehead	19	0.20×10^6	0.03×10^6–0.55×10^6

[1] Individual forearm results could be conveniently grouped into high and low counts.

As reported by many other investigators (3, 10, cf. 1), there are great regional variations in the quantity of organisms. Table V shows some representative data obtained using the Triton scrub technic described here. As expected, the moist intertrigenous areas and hairy sebaceous areas have the highest counts, the normal glabrous areas of back and forearm having counts lower by a factor of 10^3 to 10^4. The range in counts is given in the last column of Table V to indicate the large differences in bacterial numbers between different individuals. It should be noted that the ranges listed include the extreme values which make up only a minority of the subjects for all test sites. For instance, the axilla figures include out of 31 subjects only two with counts in the 6–7 $\times$ 10^3 range and two in the 12–17 $\times$ 10^6 range.

SUMMARY

A simple, reproducible procedure has been described for accurate sampling of the cutaneous microflora. This method utilizes a buffered non-ionic detergent as sampling fluid in order to assure complete removal of bacteria from the skin and to disperse the removed microorganisms so that subsequent colony counts reflect single bacterial cells rather than aggregates. Supporting data from various areas of the body are also given to show the efficiency of a detergent-based sampling fluid.

REFERENCES

1. Marples, M. J.: The Ecology of the Human Skin. Springfield, Ill., Charles C Thomas, 1965.
2. Updegraff, D. M.: A cultural method of quantitatively studying the microorganisms in the skin. J. Invest. Derm., *43:* 129, 1964.
3. Evans, C. A., Smith, W. M., Johnston, E. A. and Giblett, E. R.: Bacterial flora of the normal human skin. J. Invest. Derm., *15:* 305, 1950.
4. Pachtman, E. A., Vicher, E. E. and Brunner, M. J.: The bacteriologic flora in seborrheic dermatitis. J. Invest. Derm., *22:* 389, 1954.
5. Arnold, L.: Relationship between certain physio-chemical changes in the cornified layer and the endogenous bacterial flora of the skin. J. Invest. Derm., *5:* 207, 1942.
6. Blank, I. H. and Coolidge, M. H.: Degerming the cutaneous surface. I. Quaternary ammonium compounds. J. Invest. Derm., *15:* 249, 1950.
7. Röckl, H. and Muller, E.: Studies on the localization of bacteria in the skin. Arch. Klin. Exp. Derm., *209:* 13, 1959.
8. Kligman, A. M.: *in* The Epidermis, p. 387. W. Montagna and W. C. Lobitz, Jr., ed. New York, Academic Press, 1964.
9. VanderWyk, R. W. and Roia, F. C.: The re-

lationship between dandruff and the microbial flora of the human scalp. J. Soc. Cosmet. Chem., *16:* 761, 1964.

10. Pillsbury, D. M. and Rebell, G.: The bacterial flora of the skin. J. Invest. Derm., *18:* 173, 1952.

DISCUSSION

DR. IRVIN H. BLANK, Boston, Mass.: Dr. Williamson, in your curve relating pH to the number of organisms removed with a non-ionic solution, as I remember it, somewhere above a pH of 8, the curve tends to fall slightly. Do you feel that this fall is real and if so, do you think that it is due actually to a decrease in the number of viable organisms removed or is it due possibly to death, because of the high pH, of some of those which are removed.

PETER WILLIAMSON (in closing): We have done a number of these pH curves, and the decrease at high pH seems to be real. What it is due to, we don't really know, but I am inclined to believe that it is due to some sort of selective killing because these studies were done on an arm with a series of different types of organisms, some of which may be more susceptible to the effects of alkali than others.