Br. J. exp. Path. (1972) **53**, 550

A QUANTITATIVE METHOD FOR INVESTIGATING THE BACTERIOLOGY OF SKIN: ITS APPLICATION TO BURNS

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Received for publication June 22, 1972

Summary.—A technique is described for homogenizing biopsies of skin and determining their total viable bacterial content. The method has been carefully checked to ensure that homogenization did not alter the bacterial content of skin and the technique was shown to give reproducible results. The bacterial content of normal and burned human and guinea-pig skin has been determined. Possible applications of the method are discussed.

THE bacteriological flora of normal or injured skin is commonly investigated by taking surface swabs; this method is simple but does not permit detailed quantitative investigation. The use of impression plates or other similar sampling procedures (Eade, 1958) possibly yields better results but these techniques only sample the external surface of a wound which, if dry, may carry few bacteria even when there is pus under the slough, and may not reflect the complete bacteriological situation (Lowbury, Crockett and Jackson, 1954).

Recent studies have suggested that homografts or heterografts can be used to cleanse and debride contaminated wounds (Burke and Bondoc, 1968) before conventional autografting. The tangential excision and grafting of burns involve placing skin grafts on an atypical and possibly non-viable graft bed which might contain sufficient bacteria to cause infection (Jackson, 1969). To attempt evaluation of the role of split thickness skin grafted on tangentially excised and infected burns (Lawrence, Stone and Lilly, 1972), it was necessary to develop a technique which would give a representative quantitative measure of the bacterial content of burned and of normal skin.

Homogenization of burn eschar to obtain a measure of the number of infecting bacteria has been mentioned by various workers (*e.g.* Teplitz, Mason and Moncrieff, 1964; Lindberg *et al.*, 1965) but these authors do not describe a technique. This paper describes a procedure for homogenizing biopsies and shows how it can be applied to study the bacteriology of normal and burned mammalian skin.

MATERIALS AND METHODS

The skin samples were weighed rapidly using an electric balance and shredded with scissors, a strict aseptic procedure being observed. The chopped tissue was transferred to a sterile glass homogenizer flask and Ringer solution (quarter strength) added, 1 ml per g of tissue. The flask was attached to a top-drive homogenizer (Edmund Bühler, Tubingen); this was homogenized for 2 minutes, after which a further aliquot of Ringer solution was added, 2 ml per g of tissue. The mixture was then homogenized for a further 10 minutes. If the homogenate was too viscous to pipette, a further measured amount of diluent was added and the mixture homogenized again for a further minute. The pipettes used in this study were the untipped version of the disposable 1 ml pipette. Viable counts of bacteria in the homogenate were determined by the method of Miles, Misra and Irwin (1938).

The exterior of the homogenizer flask was cooled with equal parts of crushed ice and water. Before each experiment the cutting blade and spindle of the homogenizer were sterilized by autoclaving; between runs within an experiment the spindle, blade and cover plate were washed with saline and disinfected by wiping with 70% ethanol.

The following tests were made to see if the procedure allowed survival and full recovery of bacteria. A previously calibrated copper-constantan thermocouple was placed just above the bottom of the glass homogenizer flask and the temperature monitored during homogenization, the output being measured with a chart recorder (Telsec Model 700). Different methods of cooling were tested. Previously determined numbers of bacteria were added to the tissue before homogenization to see what effect the process had on viable organisms. Sometimes it was necessary to store skin samples for several days before determining their bacterial content. The samples were kept at $-20°$; to check that the numbers of organisms did not alter significantly over the storage period several comparable skin samples were divided equally; one portion was homogenized immediately and its total viable bacterial count determined; the remaining portion was stored at $-20°$ for a week before its total viable bacterial count was determined.

Experiments were than made to see whether the method could be used to investigate the sequential bacteriology of small experimental burns in the guinea-pig. Red-eyed white animals (Dunkin–Hartley) aged 4–6 months were anaesthetized with ether and an area shaved on one flank (about 40×60 mm). Two burns, each 3·6 cm^2, were made on this area by applying a brass block ($1{\cdot}9 \times 1{\cdot}9 \times 3$ cm, weight 120 g), which had been heated to the required temperature in a water bath, for 10 seconds. Groups of 6 animals were burned at 50°, 60°, 70°, 80° and 100° respectively. These burns were left exposed. Biopsies were made from each group by killing the animal with chloroform and excising the burns and an equal area of normal shaved skin from the unburned flank; these biopsies were taken 3 hours, 1, 3, 5, 7 and 9 days after injury and excision was to the panniculus carnosus. A strict aseptic collection procedure was observed. Before the biopsies were excised moist swabs were taken from the surface as described by Cason and co-workers (1966).

Specimens of normal and burned human skin were also examined. Some samples of split thickness skin were obtained from tissue to be stored in a skin bank. Other samples of full thickness skin were obtained from cadavers within a few hours of death, and samples of burned skin were obtained when the injured area was excised for grafting.

For the cultivation of homogenized material horse blood agar was used containing 4% New Zealand Agar. The medium was incubated at 37° for 18–24 hours and colonies were picked for Gram stain morphology and necessary confirmation tests (Cason *et al.*, 1966).

RESULTS

The temperature of the skin suspension during homogenization under various conditions is shown in Fig. 1. As might be expected, the ice-water mixture provided the most efficient cooling; the temperature of the homogenate did not rise above 15° during the course of the run. Ice-cold water was less efficient, the temperature rising from 6° to 25° during the first few minutes and to 32° by the time the run had been completed. With no cooling the temperature of the homogenate rose rapidly, 60° being reached after 7 minutes, at which time the run was terminated. These tests were made on larger volumes of homogenate than would normally be used in practice, the final volume being 35 ml, whereas most biopsies yielded a final volume of 7–15 ml.

The viable count of various bacteria added to skin biopsies before homogenization, on completion of a run and after storage at $-20°$ for 7 days, are shown in Table I. There appeared to be no substantial alteration in the bacterial count after homogenization or after storage at $-20°$ for 7 days.

Homogenates of normal skin from 25 guinea-pigs showed a mean viable count 2454 ± 650 organisms per g, the range being from 800 to 11,000 organisms per g. Table II shows the type, incidence and numbers of organisms isolated.

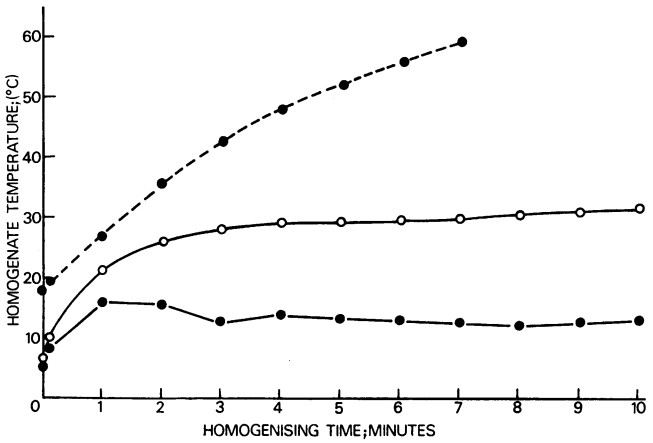

FIG. 1.—The effect of various methods of cooling on the temperature of skin homogenates during homogenization. ●- - - -● no cooling; ○——○ ice-cold water; ●——● crushed ice in water.

TABLE I.—*The Effect of Homogenizing and Storage at −20° on Various Bacteria Added to Skin Biopsies*

Organism		Bacterial count added to skin biopsies	Bacterial count after homogenizing	Bacterial count from skin after storage at −20° for 7 days and homogenizing
Staph. aureus	1	12·46	12·78	12·52
	2	0·85	0·78	0·72
E. coli	1	3·25	3·17	3·34
	2	0·02	0·017	0·021
Micrococci	1	8·70	8·56	8·63
	2	0·095	0·088	0·10
Ps. aeruginosa	1	3·94	3·78	3·82
	2	0·004	0·005	0·0048

All counts × 10^6 and expressed per g of tissue.

TABLE II.—*The Type, Incidence and Number of Bacteria Found in Homogenized Normal Guinea-pig Skin*

Organism	Incidence	% incidence	Viable count per g: Animals with organism	Viable count per g: All animals
Micrococci	17/25	68	2070	1408
Skin diphtheroids	11/25	44	2272	978
Staph. aureus	1/25	4	91	4
" Coliform " bacilli	2/25	8	804	64

The bacterial flora of these skin samples was also investigated by taking surface swabs from the skin before the tissue was excised. The results are shown in Table III.

Skin biopsies from animals burned at various temperatures were analysed similarly. For convenience the burns were divided into 2 groups, those with full skin thickness burns (80° and 100°) which only healed from the edge of the wound

TABLE III.—*The Bacteriological Flora of Normal Guinea-pig as Detected by Surface Swabbing*

Organism	Incidence	Growth on blood agar	Percentage incidence
Aerobic sporing bacilli	27/30	Usually +	90
Skin diphtheroids	15/30	Usually ±	50
Staph. aureus	18/30	Variable	60
" Coliform " bacilli	2/30	±	7
Micrococci	8/30	++	27
Strep. faecalis	5/30	±	17
Strep. viridans	2/30	±	7
Fungi	2/30	—	7

± very slight; + moderate; ++ heavy; +++ very heavy.

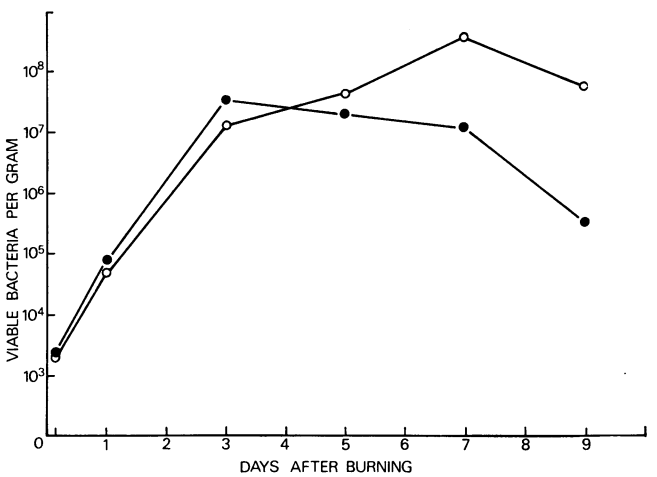

FIG. 2.—The variation of the viable bacterial count in partial and full thickness burns of guinea-pig skin with the passage of time. ●——● partial skin thickness burns; ○——○ full skin thickness burns.

and partial skin thickness burns (50°, 60° and 70°) which healed from the edge of the wound and from epithelial elements remaining in the deeper layers of the dermis. As might be expected, the viable bacterial count in these wounds increased with the passage of time following injury (Fig. 2). The viable count 3 hours after burning was similar to that of normal skin, 3236 organisms per g for the partial skin thickness burns and 2213 organisms per g for burns involving the full thickness of the skin. The viable count reached a maximum 3 days after injury in partial skin thickness burns; after this time the count gradually declined. The maximum count in full skin thickness burns occurred 7 days after injury; the number of organisms detected, $4{\cdot}9 \times 10^8$, was greater than that found in the biopsies from partial skin thickness burns, $3{\cdot}5 \times 10^7$.

The type and incidence of organisms isolated from homogenates of burned skin were similar irrespective of the time elapsed between injury and sampling although the actual number of organisms varied considerably. Table IV shows the type and number of organisms isolated from partial and full skin thickness burns of the guinea-pig 5 days after injury.

TABLE IV.—*The Type, Incidence and Number of Bacteria Found in Homogenized Burned Guinea-pig Skin 5 Days after Injury*

Organism	Partial skin thickness burns	Percentage of total no. of organisms	Full skin thickness burns	Percentage of total no. of organisms
Micrococci	0·28	0·13	5·95	22·1
Skin diphtheroids	18·15	86·4	20·0	74·2
Staph. aureus	2·57	12·23	0·97	3·6
" Coliform " bacilli	0·00063	—	0·041	0·2
Aerobic sporing bacilli	0·0003	—	0·0004	—
Total	21·0	100	26·96	100

All counts $\times 10^6$.

The bacterial flora of these burns was also investigated by taking surface swabs; the results are shown in Table V. The incidence of isolation of particular micro-organisms from partial and full skin thickness burns did not significantly differ; for *Staphylococcus aureus* $\chi^2 = 1{\cdot}65$, $0{\cdot}1 < P > 0{\cdot}2$; for skin diphtheroids $\chi^2 = 0{\cdot}009$, $0{\cdot}95 < P > 0{\cdot}98$ and for micrococci $\chi^2 = 3{\cdot}28$, $0{\cdot}02 < P > 0{\cdot}05$. However, the incidence of micrococci and diphtheroids was significantly higher in burned skin compared to normal skin, $\chi^2 = 11{\cdot}07$ and $14{\cdot}5$ respectively, $P < 0{\cdot}001$ in both instances. Aerobic sporing bacilli, on the other hand, were isolated more frequently from normal skin, $\chi^2 = 16{\cdot}1$, $P < 0{\cdot}001$. As this organism is commonly found in the bedding material of animal cages, it is reasonable to assume that this was only a small surface contaminant.

TABLE V.—*The Bacterial Flora of Burned Guinea-pig Skin as Detected by Surface Swabbing*

Organism	Incidence	Growth on blood agar	Percentage incidence
(a) Partial skin thickness burns			
Micrococci	8/12	±	67
Skin diphtheroids	12/12	+ usually	100
Staph. aureus	5/12	± usually	42
Aerobic sporing bacilli	8/12	+ usually	67
" Coliform " bacilli	3/12	±	25
Strep. faecalis	2/12	c.m.	17
(b) Full skin thickness burns			
Micrococci	9/10	+ usually	90
Skin diphtheroids	9/10	+ usually	90
Staph. aureus	6/10	+ usually	60
Aerobic sporing bacilli	6/10	+ usually	60
" Coliform " bacilli	2/10	c.m.	20
Strep. faecalis	1/10	c.m.	10

c.m. cooked meat; ± very slight; + moderate.

Biopsies of normal human skin (full thickness) showed a mean viable count of 19,000 organisms per g, with a coefficient of variation of 10%. The coefficient of variation was based on measurements from 3 adjacent samples of skin obtained from 5 separate sources; the means ranged from 1200 to 120,000 organisms per g. Table VI shows the type, incidence as shown by swabbing, and numbers of these organisms isolated; Table VII shows the comparison of viable counts measured in homogenates of excised burns with the growth noted from swabs spread on surface

TABLE VI.—*The Bacterial Flora of Human Cadaver Skin as Detected by Surface Swabbing and the Type, Incidence and Number of Organisms Detected in Homogenates of this Skin*

	Surface swabbing		Homogenized skin	
Organism	Incidence	Percentage incidence	Viable counts per g (specimens with organism)	Percentage incidence
Micrococci	11/14	79	18,205	100
Skin diphtheroids	3/14	21	812	29
Staph. aureus	1/14	7	0	0
Aerobic sporing bacilli	1/14	7	0	0
Total			19,017	—

TABLE VII.—*Comparison of Viable Counts from Homogenates and Density of Growth from Swabs of Same Burns*

	Density of growth on culture plates from surface swabs						Burns with organism	Total burns
Organism	+++	++	+	±	CM	NG		
Staph. aureus	81·91*	—	18·16	0·37	—	—	10	14
Micrococci	54·13	—	2·93	—	—	0·14	9	14
Ps. aeruginosa	20·00	—	—	—	28·54	0·04	4	14
Proteus spp.	1·16	—	2·93	—	—	0·05	5	14
Skin diphtheroids	—	—	—	0·08	—	0·13	3	14
" Coliform " bacilli	7·09	—	5·87	0·86	0·65	0·21	7	14
Strep. faecalis	7·79	—	5·87	—	—	3·98	5	14

* Mean viable counts expressed as 10^6.

plates. The viable counts determined in biopsies of burned human skin were usually higher than those from normal skin except when specimens were obtained shortly after injury. There appeared to be a very imperfect correlation between the growth obtained from a surface swab and the total number of organisms present in the tissue; a heavy growth on agar was usually associated with a high viable count, but scant or no growth (even in cooked meat) was not an indication of a particularly low bacterial content. This series included both exposed and covered burns; in contrast, Lowbury *et al.* (1971) obtained a better correlation in a similar study of burns treated with creams and covered with a dressing.

Homogenates were also prepared from specimens of split thickness human skin (0·1–0·3 mm thick); before excision of these biopsies the sites were cleansed with

TABLE VIII.—*Bacteria Present on Human Skin Before and After Skin Disinfection with 0·5% Chlorhexidine in 70% Alcohol for 2 Minutes*

		Growth on culture of surface swabs							
		Pre-disinfection				Post-disinfection			
No. of donors	No. of sites	+	±	cm	No growth	+	±	cm	No growth
5	13	2	8	2	1	0	1	3	9

Viable bacteria per g:

Donor	
1	99
2	< 400
3	< 300
4	< 100
5	47

0·5% chlorhexidine in 70% ethanol. Surface swabs were taken before and after cleansing. As shown in Table VIII surface swabbing suggested that many of the bacteria on the surface of the skin were eliminated by the cleansing with alcoholic chlorhexidine; 12/13 swabs taken before cleansing yielded bacteria on culture; after cleansing bacteria were detected in only 4/13. This reduction was highly significant ($\chi^2 = 7{\cdot}96$; $0{\cdot}01 < P > 0{\cdot}001$). The total bacterial content of the skin was also low, less than 400 organisms per g.

DISCUSSION

The method of homogenizing skin was based on that described by Allgower and his colleagues (1963) and subsequently used in this unit to study the possible toxicity of burned skin to mice (Lawrence, 1969). This technique produces excellent homogenates fairly quickly; other methods have proved less satisfactory unless the biopsy is very small. For these a glass tissue grinder is excellent (Brooks, Lawrence and Ricketts, 1959). In order to determine the number of viable bacteria in a skin specimen the homogenizing must be efficient; Lawrence (1969) produced homogenates of mouse skin which would pass through a 21-gauge hypodermic needle. The guinea-pig skin homogenates prepared in the current series of experiments could be similarly manipulated, but most samples of human skin could not. This seemed to be attributable to long, thin fibres which tended to aggregate on standing.

The number of bacteria in the specimen should not alter during homogenizing or during any subsequent manipulation of the homogenate. The experiments in which a known inoculum was added to the tissue indicated that this requirement was met, though it is possible that some bacteria could be occluded in the fibrous material present in all the skin homogenates. To prevent destruction of bacteria it is important that the homogenizing vessel be adequately cooled; failure to provide sufficient cooling may result in relatively high temperatures being attained during homogenization. This can readily be prevented by ensuring that plenty of wet crushed ice surrounds the vessel. The experiments also showed that it was possible to store biopsies for at least a week at $-20°$ without apparently altering the bacterial count. This investigation was made because it was not always possible to process a large number of biopsies on the day that they were taken.

The number of viable bacteria found on normal guinea-pig skin was 2454 ± 650 organisms per g full thickness skin. This was equivalent to about 600 organisms per cm^2 as each biopsy measured 2×2 cm. This area was only approximate since the area excised was not measured accurately. These counts were, perhaps, surprisingly low if considered in relation to the animal's environment. However, the fact that the area providing the biopsy had been shaved 4 days before sampling may account at least in part for this low count. No information is available at the present time concerning the bacterial count of unshaved skin.

Bacterial counts of homogenized experimental burns showed that the number of organisms present increased rapidly during the first few days following injury. The burns were classified as either partial or total skin thickness in depth; in the former the bacterial count reached a maximum 3 days after injury, in the latter 7 days after injury. The ensuing decrease in the viable bacterial count appeared to be associated with the onset of healing and the elimination of the burn eschar. Groves and Lawrence (1971) reported that epithelialization of full skin thickness

burns of the guinea-pig commenced 7 days after injury and slough started to separate 2–3 days earlier. Partial skin thickness burns of the guinea-pig heal fairly quickly, depending on the severity of the burn; those made at 70° heal in 10–14 days (Lawrence and Stone, 1972). There was some evidence from the present study that the number of bacteria per g detected in biopsies from experimental burns varied with the burning temperature, the lowest count being found in burns made at 50°. Unfortunately there were insufficient data available from the current study to permit a detailed analysis of this question. On theoretical grounds it would be reasonable to expect the bacterial count in burns to depend on the thickness of the eschar and clearly the depth of the eschar is dependent to some extent, at least, on the temperature at which the burn was made.

The organisms isolated did not include *Ps. aeruginosa*, *Proteus* or other accepted pathogens of human burns. There was no evidence of bacterial invasion beyond the necrotic tissue of the burn and similar full thickness burns in other experiments were found to heal within 28 days (Groves and Lawrence, 1971). Half of the animals had *Staphylococcus aureus* in their burns, but the numbers were relatively small. The numbers of diphtheroid organisms and micrococci were high—$> 10^7$ per g—and remained above this level for several days after the maximum count had been reached. The bacteriology of these burns was not followed up for more than 9 days.

Full thickness human skin obtained from cadavers showed a mean viable count of 19,000 organisms per g; this was equivalent to about 4600 organisms per cm^2. The range of counts detected was from 1200 to 120,000 organisms per g; variation between skin samples taken from different sites on any particular cadaver was much less. Replication between 3 adjacent skin samples was within 10%; it was not possible to prepare larger numbers of replicates from any particular specimen. These numbers of viable bacteria are lower than those reported by Selwyn and Ellis (1972). The reason for the discrepancy is not clear though the treatment the cadavers had before removal of the skin biopsies is clearly relevant. Selwyn and Ellis (1972) do not mention the source of their specimens or state whether previous skin cleansing had been used. The cadavers in the present study were washed with a hexachlorophane bar soap. Furthermore, all but one of the 5 sets of skin specimens were taken from patients admitted at least 7 days before death and hexachlorophane soap had been used routinely daily. Consequently, there might have been a cumulative effect of hexachlorophane on the normal resident skin flora as reported by Lowbury and his co-workers (1964). In support of this hypothesis, the bacterial count determined in skin biopsies of a patient admitted the previous day was considerably higher than that measured in the other 4 patients who had been in hospital at least a week. This point merits further investigation.

Samples of split thickness skin showed a bacterial count of < 400 organisms per g (< 70 organisms per cm^2) after cleansing with 0·5% chlorhexidine in 70% alcohol. If it is assumed that 25% of the bacterial content of full thickness skin (4750 organisms per g) is in the outer layer, then treatment with alcoholic chlorhexidine reduces this number by at least 90%. Experiments are in progress to investigate this point further.

Investigation of burned human skin showed that the bacterial numbers were 10^2 counts per g less than those measured in burned guinea-pig skin. Clearly the actual count determined in any particular biopsy depends not only on the depth of

the burn but also on the method of treatment *e.g.* topical antibiotics or other therapeutic agents are likely to influence the bacterial flora of the wound. The method could be used to determine the extent of infection and the efficiency of topical antibiotic or chemotherapy. In this connection Lindberg *et al.* (1965) consider a bacterial count higher than 10^6 organisms per g evidence of severe infection. More evidence to support this view is needed.

This technique could also be applied to a variety of problems, *e.g.* the effect of skin autografts on infected recipient areas (Lawrence *et al.*, 1972). The technique is also being used to monitor the bacterial content of split thickness skin which is to be stored over liquid nitrogen. Current experiments are concerned with investigating the effect of various therapeutic procedures on the progressive pathology of burned skin; these include the use of possible substitutes for skin and it is hoped to report on these observations at a later date.

We wish to thank Dr S. Sevitt for the samples of cadaver skin and Mr D. M. Jackson for the samples of burn eschar.

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