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Crystal violet reactions of *Staphylococcus aureus* strains colonizing infants in the first six weeks

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SUMMARY

Nasal colonization with *Staphylococcus aureus* occurred in 18% of babies leaving a maternity unit and had risen to 40% by 6 weeks after birth. *S. aureus* was first acquired by 34·5% of babies after discharge. Female infants were more likely to be colonized than males. Colonization was not significantly different between babies receiving standard postnatal care and those nursed on the Special Care Baby Unit.

Crystal violet (CV) tests showed that purple-reacting isolates accounted for approximately 60% of strains, whether first detected at hospital discharge or subsequently acquired. Purple-reacting strains, once acquired, were significantly better able to persist than non purple-reacting strains and formed a cumulatively higher proportion of the strains isolated at 6 weeks after birth than at hospital discharge. CV purple-reactions were significantly associated with lysis by phages of groups III and I and non-purple-reactions were significantly associated with lysis by phages of group II and/or 94/96.

Maternity units remain a significant route whereby strains of *S. aureus* with some characteristics associated with a hospital origin gain access to the community.

INTRODUCTION

Staphylococcus aureus colonization of infants remains a common finding and within hospital the organism is the second commonest isolate from neonatal septicaemias [1]. After discharge from hospital, skin and other superficial staphylococcal infections occur. Recent reports of outbreaks of neonatal pustulosis [2] and staphylococcal scalded skin syndrome in infants [3] emphasize the importance of understanding the epidemiology of this organism within maternity units and then beyond, into the community.

We have investigated *S. aureus* nasal colonization of neonates in a modern large district general hospital maternity unit with follow-up at 6 weeks after birth.

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Recent observations [4] have shown that strains of *S. aureus* giving a purple reaction in the crystal violet (CV) test are significantly associated with invasive disease. CV purple-reactors were found to account for the majority of hospital isolates whereas non-purple-reactors formed the majority of community isolates. In the same report purple-reacting strains were also shown to be significantly associated with phage group III, whereas non-purple (white- or yellow-reacting) strains were significantly more susceptible to lysis by group II phages and by phages 94 and/or 96.

We therefore determined the CV reaction of the infant isolates and examined the correlations between the CV reaction and other results, including phage typing, to assess the information provided by the CV reaction in this new context of infant colonization.

PATIENTS, MATERIALS AND METHODS

The nasal colonization rate with *S. aureus* at birth, at discharge from hospital and at the routine postnatal examination (usually 6 weeks after birth) was studied in 517 consecutive babies, delivered over a 3-month period in one maternity unit. The maternity unit comprised 52 maternal beds, of which 36 were used for postnatal care, and a Special Care Baby Unit (SCBU) with 11 cots.

Standard post-natal care was unsupervised and performed by the mother after initial demonstration by the nursing staff. Infant bathing was carried out on alternate days, using a liquid soap preparation (Infacare; Proctor and Gamble Ltd.) which contained no active antiseptic ingredient. Routine cleansing of eyes, face and perineum ('topping and tailing') was with sterile saline. Cord care consisted of a daily application of a hexachlorophene-containing powder (Sterzac; Hough, Hoseason and Company Ltd.). A chlorhexidine-based antiseptic soap (Hibiscrub soap; ICI Pharmaceuticals Ltd.) was provided for maternal hand-washing. Babies transferred to the SCBU were mostly cared for in incubators. Alternate day bathing with Infacare was carried out, eye and perineal care was with sterile saline and cord care was again with daily applications of hexachlorophene-containing powder. In addition, hexachlorophene-containing powder was applied to the perineum at each nappy change. On the SCBU all these procedures were carried out by either nursing staff or by mothers under direct nursing supervision.

Approximately 40% of babies initially admitted to the SCBU were thereafter returned to the routine postnatal ward prior to discharge and the rest remained on the SCBU until discharged from hospital.

Isolation and identification of organisms

Nasal swabs were obtained using a serum-coated swab applied to both nares and placed into Amies transport medium. Swabs were inoculated onto blood agar plates incubated overnight at 37 °C. The following day all putative *S. aureus* colonies were tested for coagulase production (Staphylase; Oxoid Ltd.). Coagulase-positive staphylococci were stored on agar slopes prior to further investigation. *Staphylococcus aureus* isolates from any clinical infections in the babies over the study period were also stored and included in the investigations.

Crystal violet tests

The crystal violet reaction was determined as previously described [4], using nutrient agar (Oxoid Ltd.) containing crystal violet at a concentration of 1:100000. Isolates were designated as 'purple' and 'non-purple'. The second category included both white- and yellow-reacting strains. In a minority of instances cultures produced more than one reaction. In such cases the result was recorded as 'mixed' and kept as a separate category.

Phage typing was performed by standard methods [5], using the International Standard Set of phages.

RESULTS

Infant nasal colonization with S. aureus

Of the 517 consecutive deliveries occurring over the study period, swabs were obtained at birth in 460 instances. At discharge from hospital further samples were obtained from 454 of these infants. Three hundred and eighty had received standard postnatal care with a mean duration of stay of 3·78 days and a range of 1–9 days and 74 had been admitted to the SCBU. Transfer to postnatal wards prior to discharge occurred for 32 of these 74 babies with a mean duration of stay of 8·7 days and a range of 2–13 days. The other 42 infants remaining on the SCBU until discharge had a mean duration of stay of 10·8 days with a range of 2–48 days.

Further swabs were obtained at 6 weeks after birth in 363 of the 454 babies swabbed at hospital discharge. The isolation rates of *S. aureus* at these three stages is shown in Table 1, together with the crystal violet reactions of these isolates. The difference in the colonization rate between those babies nursed in the SCBU throughout their stay (4 isolates from 42 babies; 10%) and those receiving standard postnatal care (72 isolates from 380 babies; 19%) was not significant, despite the longer duration of hospital stay for the SCBU babies.

Female infants were more commonly colonized at both stages. At the time of hospital discharge *S. aureus* was isolated from 43 of 198 female infants (21·7%) and from 39 of 256 male infants (15·2%). This difference is not statistically significant. However, at 6 weeks 75 of 156 samples (48%) from female infants yielded *S. aureus* compared to 71 of 207 samples (34·2%) from male infants, which is a statistically significant difference ($P < 0{\cdot}02$). Of 217 infants shown to be free of nasal colonization at hospital discharge, 75 (34·5%) were found to have become colonized 6 weeks after birth. This occurred in 38·4% of female infants (40 out of 104) and 30·9% of male infants (35 out of 113), a difference which is not statistically significant ($P > 0{\cdot}05$).

The crystal violet test showed that the proportion of purple-reacting strains, which accounted for 47 of the 82 (57·3%) isolates at hospital discharge, increased to 68·5% (100 of 146) at 6 weeks after birth, although this difference falls short of statistical significance. The proportion of purple-reactors (47 of 82; 57·3%) in the isolates acquired between birth and hospital discharge and in those isolates acquired between hospital discharge and 6 weeks after birth (47 out of 75; 62·6%) did not differ significantly ($P > 0{\cdot}05$).

Follow-up swabs, taken at 6 weeks after birth, were available from 52 of the 72 babies colonized at hospital discharge with strains of *S. aureus* which were

Table 1. *Isolation rates and crystal violet test reactions of* S. aureus *strains isolated from nasal swabs taken at discharge from hospital and at 6 weeks after birth in a cohort of 460 babies shown to be uncolonized at birth*

	Number swabbed	Number positive	Crystal violet reaction		
			Purple	Non-purple	Mixed
At discharge from hospital					
Standard postnatal care throughout	380	72 (19%)	42	23	7
SCBU + subsequent postnatal care	32	6 (18.75%)	4	1	1
SCBU throughout	42	4 (9·5%)	1	1	2
All babies at discharge	454	82 (18%)	47	25	10
At 6 weeks after birth					
Babies negative at discharge swabbing	217	75 (34·5%)	47	24	4
All babies at 6 weeks after birth	363	146 (40%)	100	31	15

unequivocally purple- or non-purple reacting in the CV test (Table 1). This allowed a direct comparison in individual infants of the results at discharge and 6 weeks. In 35 instances in which the discharge isolate was purple-reacting an isolate of identical crystal violet and phage type was isolated at 6 weeks in 31, a non-purple-reacting isolate of distinct phage type in 2 and no growth was obtained in the remaining 2. In 17 instances in which the original (hospital discharge) isolate was non-purple-reacting, isolates of identical crystal violet and phage type were isolated in 6 instances, a purple-reacting isolate of distinct phage type was isolated in 2 instances and no growth was obtained in the remaining 9.

Thus, of 35 instances in which a purple-reacting strain was isolated at hospital discharge an indistinguishable strain was still present at 6 weeks after birth in 31 cases, whereas persistence of a non-purple-reacting strain of identical phage type 6 weeks after birth was only documented in 6 of 17 instances. This difference is statistically highly significant ($P < 0{\cdot}01$).

Clinical infections of infants

Thirteen of the 454 infants (2·8%) swabbed at hospital discharge had developed minor superficial infections from which *S. aureus* had been isolated. In 10 instances the organism was isolated from an inflamed eye and in 3 from an inflamed umbilical stump. The mean duration of stay of these infants was 4·2 days, with a range of 2–10 days, and this did not differ significantly from that of the uninfected majority. These infections occurred in 8 of 42 (19%) infants nursed on SCBU throughout their stay compared to 5 of 380 (1·3%) undergoing standard postnatal care, a difference which is statistically highly significant ($P < 0{\cdot}001$). Eleven of the 13 clinical isolates of *S. aureus* were purple-reacting in the CV test. Phage typing of all the 13 clinical isolates together with any isolates recovered from the affected babies at the times of routine sampling showed that *S. aureus* of an identical phage type had been present in the nose at discharge in two infants and that a strain of different phage type to the clinical isolate had been present in the discharge nose swab in another three. The remaining 8 of these 13 infected

Table 2. *Observed associations between crystal violet test reaction and reactions with phages of the International Standard Set in 152 isolates of* S. aureus *from infant nasal colonization*

CV test reaction result	Phage non-typable	Reaction with phages			
		Group I	Group II	Group III	94/96
Purple ($n = 90$)	21	14 (15·5%)	5 (5·5%)	50 (55·5%)	0
Non-purple ($n = 62$)	20	2 (3·2%)	24 (38·7%)	5 (8·1%)	11

infants had been uncolonized at hospital discharge. There was no evidence of cross-infection.

Association of phage types and crystal violet reactions

In total, 152 isolates were submitted for phage typing. The associations between phage typing results and the crystal violet reaction of these organisms are seen in Table 2.

These results show statistically highly significant associations between purple reaction and phage group III ($P < 0{\cdot}001$), non-purple reaction and phage group II ($P < 0{\cdot}001$) and non-purple reaction and susceptibility to phages 94 and 96 ($P < 0{\cdot}001$). There is a weaker, but still significant, association between purple reaction and phage group I ($0{\cdot}05 > P > 0{\cdot}01$).

DISCUSSION

Our results showed that 18% of babies born on a single maternity unit had acquired *S. aureus* in the anterior nares by the time they were discharged from hospital after a mean stay of 3·8 days. The rate was lower (10%) but not significantly so, in the subset of babies nursed on the SCBU for much longer periods. Our study also showed that 34·5% of babies found to be free of *S. aureus* nasal colonization at the time of hospital discharge had acquired the organism in this site by 6 weeks after birth. By the time of the routine postnatal follow-up appointment at 6 weeks after birth, the nasal colonization rate was 40%.

The colonization rate at the time of hospital discharge is similar to that reported by Hargiss and Larson in 1978 [6], or at least, to the rate reported by them when hexachlorophene bathing had been discontinued. Hexachlorophene bathing was not used on the unit which we studied, but it is of interest that the nasal colonization rate was lower (10%) in the babies nursed on the SCBU where hexachlorophene-containing preparations were more frequently used and always under nursing supervision than in the majority of babies receiving standard postnatal care (19%), in which any hexachlorophene usage was unsupervised.

We found that female infants were more commonly colonized than males. It should be noted that although the difference was demonstrated at hospital discharge (21·7% of females versus 15·2% of males) it became more pronounced (48% of females versus 34·2% of males) and of statistical significance 6 weeks after birth. Comparison of the male and female post-hospital acquisition rates in

those not colonized at hospital discharge showed no significant difference. In many previous studies both colonization and infection were found to be substantially commoner in male infants. In one large survey of over 5000 infants [7] the sex of the infant was found to be the strongest determining factor amongst several examined. We are unable to explain the difference between our findings and these previous reports.

There are few recent data on colonization rates after hospital discharge. Our finding that nasal colonization continues to increase between discharge and 6 weeks after birth, cumulatively reaching 40% at the latter sampling time, is not easily compared with the standard observations from the 1950s and 1960s when the duration of hospital stay was considerably longer. One possibility is that umbilical and/or axillary colonization precedes nasal colonization [8]. Since these sites were not sampled in this study it is possible that the observed post-discharge increase in nasal colonization simply reflects the completion of a sequential colonization process begun prior to discharge. However, other studies have shown that strains recovered from the umbilicus are rarely of the same phage type as those subsequently recovered from the nose in the same infant [9].

It is also of interest that Wolinsky and co-workers [10] showed in 1962 that 38% of babies became colonized between 3 and 28 days after hospital discharge with a *S. aureus* strain of identical phage type to that found in the mother's nose although strains acquired within hospital and prior to discharge were only rarely of maternal origin. Community midwifery staff are another possible source, considered, but discounted, by Williams in 1961 [11]. Further surveys involving umbilical and axillary swabs from the infants and maternal nasal swabs at both admission and discharge will be necessary to investigate these possibilities formally. However, the crystal violet test results and the associations observed between them and those of phage typing suggest that the properties of the staphylococci as well as their origin may be of importance in explaining infant colonization. We have previously shown that *S. aureus* strains of hospital origin are significantly more likely to be CV purple-reactors and that CV purple-reactors typically comprise approximately 60% of hospital isolates in contrast to a significantly lower proportion (approximately 20–25%) in strains from the community [4].

In that earlier study, CV purple-reactions were found to be significantly associated with susceptibility to group III phages whereas non-purple-reactors were significantly likely to belong to phage group II or be susceptible to phages 94 and/or 96. Although the earlier study was concerned exclusively with strains from clinical infections, the phage group associations have been confirmed in this present study of strains from colonization. The finding that 57·3% of the *S. aureus* isolates colonizing infants' noses at hospital discharge were CV purple-reactors is consistent with the previous work and with their known hospital origin.

At 6 weeks after birth there was an increase (to 68·4%) in the proportion of CV purple-reactors as well as an increase in the overall colonization rate, although the CV purple-reactors formed only the expected proportion of those strains newly acquired between hospital discharge and 6 weeks. These results raise the possibility that CV purple-reacting *S. aureus* strains were better able to persist in the colonized site over the study period than CV non-purple-reactors.

This explanation is supported in the comparisons made of sequential swabs from individual infants, in which it was clearly shown that the apparently persistent CV purple-reactors detected at 6 weeks were of identical phage types to those found in the same site at discharge. Thus, 60% of the *S. aureus* strains which had colonized the infant nose by the time of discharge from hospital were CV purple-reactors; a feature associated with susceptibility to group III phages and an ability to persist better than the remaining 40% of colonizing strains which were non-purple-reactors and had the phage associations of such strains. These results suggest a dynamic colonization process, whereby CV purple-reactors colonize and persist whilst non-purple-reacting strains colonize more transiently, are lost and replaced, sometimes by CV purple-reactors which then persist.

Continued acquisition after hospital discharge of similar proportions of CV purple- and non-purple-reacting staphylococci (from a source or sources yet to be determined) explains the observed increase in the overall nasal colonization rate and in the cumulative proportion of strains which are CV purple-reactors.

The incidence of clinical infection within hospital (2·8%) was higher than that reported by Hargiss and Larson in 1978 [6] for their period when hexachlorophene bathing was not in use (1·1%). However, if the small number of SCBU babies, in whom a significantly higher incidence of infection occurred, are omitted to make the comparison fairer, our figure becomes 1·3%. It is also of interest that 11 of 13 isolates of *S. aureus* from clinical infections were purple-reacting in the CV test.

The characterization of some *S. aureus* strains which are significantly better able to persist after having colonized the infant nose than other strains is consistent with earlier studies. Both Hurst in 1958 [12] and Rycroft and Williams in 1960 [13] showed that strains with some of the characteristics of 'hospital staphylococci' acquired by infants could persist for at least 1 and possibly up to 4 years. This was thought to be an important mechanism whereby hospital strains gain continued access to the community. The *S. aureus* strains identified in the present study are characterized by a CV purple reaction and susceptibility to group III phages. Both of these properties have been associated with 'hospital staphylococci' [4].

On the evidence presented here, maternity units remain an important conduit between the hospital and the community in the complex epidemiology of *S. aureus*. The CV test may be a promising marker for those strains deserving further study. The lengthening list of significant associations between crystal violet reactions and other important properties of human strains of *S. aureus*, including now the apparent ability to persist in a common site of colonization, again emphasizes the need to discover the basis of this reaction.

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