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Crystal violet reactions of fresh clinical isolates of *Staphylococcus aureus* from two British hospitals

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SUMMARY

When 168 fresh clinical isolates of *Staphylococcus aureus* were examined for their reactions on a medium containing 1 part in 100000 crystal violet 50·6% of strains produced a purple appearance, 39·3% produced a white appearance and 10·1% produced a yellow appearance. Purple-reacting isolates were significantly associated with both invasive infections ($P < 0{\cdot}01$) and hospital origin ($P < 0{\cdot}001$). There were no significant associations between the crystal violet reactions and either animal contact or other properties previously reported to be characteristic of white and yellow-reacting strains (beta haemolysin and bovine coagulase production). The results of phage typing showed associations between susceptibility to group III phages and purple-reacting strains and between phage group II susceptibility and white and yellow-reacting strains. There was also a highly significant association between white reactions on crystal violet agar and susceptibility to lysis by a combination of all three groups (that is, I + II + III) and white-reacting strains were significantly more susceptible to lysis by phages 94 and/or 96, whether as a restricted pattern or as part of a broader pattern. The purple reaction on crystal violet medium may be a reliable marker of the 'hospital staphylococcus'.

INTRODUCTION

In 1966 Meyer [1] proposed that the species *Staphylococcus aureus* be subdivided into three varieties, *S. aureus humanis*, *S. aureus bovis* and *S. aureus canis*. Several criteria were used to make these distinctions but a major factor was the differential appearances produced on medium containing crystal violet at a concentration of 1 part in 100000. The test does not consist of plating organisms onto the medium but entails the transfer by a loop of material from a mature colony in quantities sufficient to form a recognizable circle 5–6 mm in diameter. After overnight incubation the deposits are found to be either purple (*humanis*), white (*canis*) or yellow (*bovis*). Meyer's observations were repeated and added to by Marsalek and Hajek [2] who developed a classification of 689 pathogenic staphylococci, embracing strains from nine animal species as well as strains of human origin. They examined the usefulness of several criteria, including fibrinolysin, pigment production, coagulases, haemolysins and phage susceptibility as well as the crystal violet reaction, and were able to define distinct

biotypes which largely correlated with species origin. In Marsalek, Hajek's study most of the human strains were found to produce a purple reaction on crystal violet medium.

In 1987 Narasimha Rao, and coworkers [3], reported a study on 102 cases of staphylococcal pyoderma in an Indian hospital. Using the crystal violet reaction they showed that 30·8% of the *S. aureus* isolates from pyoderma in their series were either *bovis* or *canis*. Enquiry of the patients showed that only 35% of those in whom the isolate was of a 'non-human' variety had close and regular contact with animals but it was postulated that staphylococcal pyoderma was frequently a zoonotic infection and that infection with non-human varieties in patients without close contact with animals represented indirect spread of the zoonotic strains within a community where animals were common (a 'saprozoonosis').

Because of these observations and because of other studies linking the different crystal violet reactions with other properties of *S. aureus* [4, 5] we thought it important to examine the crystal violet reactions of *S. aureus* isolates from British hospitals. We now report our initial findings.

MATERIALS AND METHODS

One hundred and sixty-eight isolates of *S. aureus* (coagulase-positive) were collected from Freeman Hospital, Newcastle upon Tyne. Isolates from asymptomatic carriage sites (such as preoperative nasal screening swabs) were excluded but otherwise the collection represented all clinically significant isolates over a 9-week period. Isolates were stored on nutrient agar (Lab M) slopes until required for further testing. At the time of collection the following details were noted: 1. The age and sex of the patient and the interval (where appropriate) between hospital admission and the taking of the specimen. 2. The site of the specimen. These were categorized into (a) superficial (minor skin sepsis, including infected eczema, pyoderma and ulcer swabs) and (b) invasive (infections of surgical wounds, pus, sputum and blood cultures). 3. An attempt was made to designate the isolates as being of either community or hospital origin. Isolates from patients within the community and any isolates obtained from a site infected at admission and sampled within three days of admission to hospital were designated 'Community'. Isolates from infected lesions present at admission in patients transferred from another hospital were regarded as being of 'Hospital' origin.

All isolates were then examined for their reaction on crystal violet medium, beta-haemolysin production and for their ability to coagulate bovine plasma. Phage typing results were also noted.

Crystal violet reaction

This was performed after the technique described by Meyer [1]. Briefly, after overnight culture on nutrient agar plates (Lab M), growth from separated colonies was transferred by loop and inoculated in circles of 5–6 mm diameter onto a medium consisting of nutrient agar (Lab M) containing 1 part in 100000 crystal violet. Following overnight incubation at 37 °C the resultant growth was examined and classified as purple, white or yellow. On several occasions, individual strains were serially subcultured and re-tested in order to confirm that the reactions

obtained were reproducible and that an individual isolate would not produce different reactions at different times.

Beta haemolysin production

Staphylococci were inoculated into nutrient broth (Lab M) and incubated at 37 °C overnight. The broth was centrifuged at 3000 rpm for 15 min and the supernatant was harvested. The presence of beta haemolysin was detected by adding 0·5 ml of the supernatant to 0·5 ml of a 2% suspension of sheep red blood cells in saline. The resultant mixture was incubated at 37 °C for 1 h and the presence or absence of haemolysis was noted. Tubes were then placed at 4 °C overnight and further inspected for haemolysis by naked eye appearance in comparison to a control tube containing 0·5 ml of uninoculated broth and 0·5 ml of sheep red blood cell suspension which had been similarly processed. Tubes in which haemolysis was not seen after the first stage at 37 °C but which then developed visible haemolysis after being held at 4 °C overnight were recorded as positive for the presence of haemolysin.

Bovine coagulase production

Five drops of an overnight broth culture of the staphylococcus were added to tubes containing 0·5 ml of bovine plasma diluted 1 in 10 in isotonic saline. The tubes were then incubated at 37 °C and examined after 1 h and at intervals up to 24 h for the formation of a visible clot. Control tests were included using a known positive strain and a negative control tube contained only plasma and saline. All isolates were shown to be positive in an identical test using human plasma.

In a separate parallel study at the Queen Elizabeth Hospital, Gateshead, 110 hospitalized patients from whom *S. aureus* was isolated and characterized in the crystal violet reaction were questioned for details of their contact with animals, especially cats and dogs.

Phage typing

Phage typing had been performed on 125 of the 168 isolates. Typing was performed by standard methods [6] at routine test dilution (RTD) and at 100 RTD. Strains which did not react at either RTD or 100 RTD were designated as non-typable. In addition to the International Basic Set of phages, phages 94, 96, 81 and 95 were tested. Isolates were allocated to phage groups I, II and III. Where strains were lysed by phages of more than one group the isolate was designated 'I+II', 'I+II+III', and so on. Finally, particular note was made of susceptibility to the unclassified phages, especially 94 and 96, and whether or not susceptibility to these phages was restricted or was part of a broader lytic pattern.

RESULTS

Of the 168 isolates 85 (50·6%) were found to produce a purple reaction on the crystal violet medium, 66 (39·3%) produced a white reaction and the remaining 17 (10·1%) produced a yellow reaction. Only one isolate coagulated bovine plasma and although 46 (27·3%) of the isolates produced detectable beta haemolysin there was no correlation between beta haemolysin production and any particular

Table 1. *Observed relationship between superficial or invasive nature of the site sampled and the crystal violet reaction of 168* Staphylococcus aureus *isolates*

Category of isolate	Reaction obtained on crystal violet medium (numbers of isolates)		
	Purple	White	Yellow
Superficial	20 (35·1%)	29	8
Invasive	65 (58.5%)	37	9

Statistical note: In a direct comparison of the incidence of purple-reacting isolates between superficial and invasive sites $\chi^2 = 7{\cdot}38$ ($P < 0{\cdot}01$).

Table 2. *Observed relationship between origins of strains (community or hospital) and reactions obtained on crystal violet medium for 168 isolates of* Staphylococcus aureus

Origin of isolates	Reaction obtained on crystal violet medium (numbers in each category)		
	Purple	White	Yellow
Community (45 isolates)	10 (22·2%)	28	7
Hospital (123 isolates)	75 (60·9%)	38	10

Statistical note: In a direct comparison of the incidence of purple-reacting isolates between hospital and community origins $\chi^2 = 18{\cdot}3$ ($P < 0{\cdot}001$).

Table 3. *Observed association between a history of regular contact with animals and the crystal violet reaction obtained with* S. aureus *isolates from 110 patients*

Contact with dogs or cats	Reaction obtained on crystal violet medium (numbers in category)	
	Purple	White or yellow
Regular contact	7	10
No contact	64	29

Statistical note: Comparing the incidence of white or yellow-reacting isolates in the two groups $\chi^2 = 3{\cdot}66$ ($P > 0{\cdot}05$).

crystal violet reaction. The crystal violet reactions of individual strains were found to be stable and reproducible. There was no significant association with age or age groups or with the sex of the patient.

Table 1 shows the observed relationship between the crystal violet reaction of the 168 staphylococci and their isolation from superficial or invasive sites. Analysis shows that purple-reacting strains were isolated significantly more commonly from invasive situations (wounds, pus and blood) than from superficial sites (nose, throat, sputum, skin and ulcers). Table 2 shows the observed relationship between the hospital or community origin of the isolates and the crystal violet reaction. Purple-reacting strains are seen to be statistically highly significantly more common in the hospital isolates and the white- and yellow-reacting strains are correspondingly more common in community isolates.

Table 3 shows the relationship between the reaction obtained on crystal violet medium with the isolates from the 110 patients in Gateshead subsequently

questioned about animal contact. Overall 17 patients (15·5%) admitted to regular and frequent contact with animals. Although such a history was more common in patients from whom white- and yellow- reacting strains of *S. aureus* were isolated (25·6% of patients with such isolates versus 9·8% of patients with purple-reacting isolates) the association falls short of statistical significance.

Table 4 shows the results of phage typing and the correlations between the three appearances on crystal violet medium and the phage groups and types of the isolates. It is seen that susceptibility to group II phages is significantly commoner in strains producing a white or yellow reaction on crystal violet medium than in purple-reacting strains. Conversely, susceptibility to group III phages is commoner in the purple-reacting strains than in the white and yellow strains. A striking association between shared susceptibility to phages of all three groups (that is, a pattern of 'I+II+III') and the production of a white reaction on crystal violet medium is seen. Finally, the most highly significant association is that between susceptibility to lysis by phages 94 and/or 96 and a white reaction on crystal violet medium. This association is seen whether or not the pattern is that of susceptibility to phages 94 and 96 as a restricted pattern or as part of a broader susceptibility including other phages as well.

DISCUSSION

Meyer's suggestion that the different appearances on crystal violet medium are characteristic of the human or animal sources of the strains is not supported by our findings. Our results do show that white- and yellow-reacting strains are commoner in patients with animal contact, but not significantly so. We were also unable to find any correlation with production of beta haemolysin or bovine coagulase and the various crystal violet reactions. Both these properties were associated with white and yellow reactions on crystal violet medium in the original classification suggested by Meyer [1] and in the subsequent study by Marsalek and Hajek [2]. The finding is that 105 out of 125 of our isolates were susceptible to the International Basic Set of phages, with the addition of phages 94, 96, 95 and 81, does not exclude the possibility that our strains are of animal origin since many animal strains are lysed by these phages [2] and we were unable to test for the characteristic additional susceptibility to bovine and canine phages.

A larger study should be undertaken to explore the possibility that a substantial proportion of routine isolates of *S. aureus* from human clinical material may be zoonotic since there will be considerable implications for prevention and treatment of human staphylococcal infections if this is so. In the meantime we have avoided the terms *humanis*, *bovis* and *canis* and preferred simply to describe our isolates as purple-, white- and yellow-reacting.

An alternative explanation of our findings is that some isolates of *S. aureus* from human material produce white and yellow reactions in the crystal violet test and that, although such white and yellow reactions are also typical of *S. aureus* of canine and bovine origins, the two phenomena are not essentially related.

We have presently simply shown that the crystal violet reaction test described by Meyer results in three different appearances when applied to fresh isolates of *S. aureus* from two British routine hospital laboratories and that these

Table 4. *Associations between phage groups and types and crystal violet reaction in 125 strains of* S. aureus

Phage group patterns	Number of strains in each crystal violet reaction category		
	Purple	White	Yellow
I	7	3	1
II	0	11	4
III	12	7	1
I+II	0	0	1
I+III	6	3	1
II+III	0	0	0
I+II+III	5	24	3
Non-typable	11	2	7
Lysis by individual phages			
94 and/or 96 in any pattern	4 (1)*	41 (11)	2 (0)
81	7	14	1
95	4	3	1

* Indicates number which typed as 94/96 alone, i.e. no other reactions.

Statistical notes:

1. Phage group II pattern is significantly commoner in white-reacting strains and yellow-reacting strains than in purple-reacting strains ($\chi^2 = 6{\cdot}3$; $P < 0{\cdot}02$ in both cases).
2. Phage group III pattern is significantly commoner in purple-reacting strains than in white-reacting strains ($\chi^2 = 4{\cdot}2$; $P < 0{\cdot}05 > 0{\cdot}02$).
3. A pattern of I+II+III is highly significantly commoner in white-reacting strains than in purple-reacting strains ($\chi^2 = 7{\cdot}12$; $P < 0{\cdot}01$).
4. Reaction with phages 94 and/or 96, including such reactions as part of a broader pattern, is very highly significantly commoner in white-reacting strains than in purple-reacting strains ($\chi^2 = 28{\cdot}68$; $P < 0{\cdot}001$) and this association remains with the restricted pattern of 94/96 alone ($\chi^2 = 4{\cdot}16$; $P < 0{\cdot}05 > 0{\cdot}02$).

appearances seem to be stable and reproducible. Purple-reacting strains were significantly associated with invasive staphylococcal disease, whereas the other two reactions were associated with staphylococcal infections of a much more superficial nature. Purple-reacting strains were also significantly more likely to have been isolated from hospital-acquired infections whilst white- and yellow-reacting strains were predominantly found in community-acquired infections. The convention which was adopted to distinguish between hospital-acquired and community-acquired infections is somewhat arbitrary, but it is based upon previous studies [7].

The purple reaction in the crystal violet test may, therefore, be a reproducible and consistent marker for the 'hospital staphylococcus'. In this light the results of the phage typing of the strains are of considerable interest. The finding that purple-reacting strains are more likely to react with group III phages is compatible with their being also associated with hospital origin and invasive disease. The converse association with group II susceptibility and white and yellow-reacting strains is also consistent with such strains being typically found in the community and superficial infections. There were, however, two other phage typing associations which were notable. Firstly, the highly significant association between white-reacting isolates and lysis by the very broad pattern of groups I+II+III. *S. aureus* strains with this pattern and with other patterns reflecting

reactions with phages of more than one group have been observed to evolve in hospitals over long periods [8]. There is a relative scarcity of other broad patterns in our strains, however, and this association remains to be explained.

The second, and even more significant, association is between white-reacting strains and susceptibility to lysis with phages 94 and/or 96. Susceptibility to phages 94 and/or 96 alone or susceptibility to these phages as part of a broader susceptibility pattern is a property highly significantly associated with white-reacting strains on crystal violet medium. Phage type 94/96 has been observed to increase in incidence in recent years [9] and it is of interest that the same report claimed that 504 strains of this phage type were uniformly of crystal violet type A (white-reacting). The characteristic absence of beta haemolysin and presence of fibrinolysin in 94/96 strains [9, 10] also supports our suggestion that white-reacting strains of *S. aureus* in the crystal violet test may often be of human origin and not necessarily related to an animal source.

Because of previously described associations between the crystal violet reactions of *S. aureus* and specific pathogenic properties, notably enterotoxin production [11] and adhesiveness [12], we intend to extend our studies. A further important aspect concerns the nature of the crystal violet reaction itself. The test is empirical and its basis has not been determined. Susceptibility of staphylococci to crystal violet and similar dyes has been investigated in the past [13, 14] and it is likely that the various appearances seen on crystal violet medium imply important metabolic and physiological differences between the isolates tested. Such associations with a reliable marker of the 'hospital staphylococcus' might further our understanding of the ability of some staphylococci to thrive within the hospital environment.

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