

Crystal violet reactions of coagulase negative staphylococci

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Abstract

Twenty four reference strains and 112 clinical isolates of coagulase negative staphylococci (CNS) were examined for their reactions in the crystal violet test. Some species gave a white reaction and others a purple reaction. Results were consistent and reproducible and each species gave only one pattern of crystal violet reaction. Within the limited variety of species represented in the clinical isolates, *Staphylococcus saprophyticus* and *S haemolyticus* gave crystal violet purple reactions, in contrast to *S epidermidis*, which always gave a white reaction.

Investigations suggested that the mechanism of the crystal violet test in *S haemolyticus* may be similar to that previously described in *S aureus*. Further work is needed to characterise the ability of crystal violet to modify *S epidermidis* and other central nervous system species. The crystal violet reaction, which has strong associations with invasiveness, phage group susceptibilities, colonisation persistence abilities, and nosocomial origin in *S aureus* may also be useful in studies of CNS disease.

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The different appearances produced on incubation by different strains of *Staphylococcus aureus*, following transfer of colonial material on to nutrient agar containing 10 µg/ml of crystal violet, form the basis of the crystal violet reaction. Meyer suggested that purple-reacting strains were of human origin (*S aureus humanis*), white-reacting strains were from dogs (*canis*) and yellow-reacting strains were of bovine origin (*bovis*).¹ We have subsequently shown that *S aureus* strains of undoubted human origin can produce all three crystal violet appearances and that the yellow reaction is due to good pigment production in an otherwise white-reacting strain.² Hence we have recognised only two categories—purple and white reactors.

We have also shown that the crystal violet purple reaction is associated with hospital origin, susceptibility to phage group III phages, invasiveness, ability to colonise infants, and thereafter to persist in the colonised site. Conversely, we found that non purple-reacting strains (whether white or yellow) were significantly more likely to be of community origin, to belong to phage group II, to be asso-

ciated with superficial infections and to be significantly less able to persist in sites of colonisation. The crystal violet white reaction was also significantly associated with lysis by phages 94 and 96.^{3,4}

One of the earliest reports on the behaviour of staphylococci on crystal violet-containing nutrient agar was that of Chapman and Berens in 1934,⁵ who clearly examined coagulase negative staphylococci (CNS) as well as *S aureus* and observed differing crystal violet reactions within the CNS group.

Because CNS are increasingly involved in serious nosocomial infections, we examined some recent isolates and some reference strains in the crystal violet reaction.

Methods

Twenty four reference strains and 112 clinical isolates of CNS were collected. The clinical isolates were obtained from blood cultures and venous catheter tips from open heart surgery patients and from peritoneal dialysis effluents from patients in the nephrology unit undergoing continuous ambulatory peritoneal dialysis (CAPD). None of the isolates was from urine specimens. All the clinical isolates had been identified by a commercial technique (API-STAPH, API Laboratories, Basingstoke, England) and were tested for slime production by a standard method.⁶

Organisms were stored in glycerol broth and held at -20°C until required for study. Crystal violet reactions were assessed after initial recovery from the glycerol broth and a single subculture to check purity.

Crystal violet agar consisted of nutrient agar (Lab M, Bury, England) to which was added 10 µg/ml crystal violet (CI 2555, Sigma)—that is, a dilution of 1 part in 100 000.

Following overnight incubation at 37°C on nutrient agar (Lab M), a visible quantity of colonial growth from individual colonies of each organism was transferred by loop and inoculated in circles of 5-6 mm diameter on to crystal violet agar. After overnight incubation at 37°C the resultant growth was examined and classified as purple or white (no examples of yellow reactions were seen). Several different strains were serially subcultured on nutrient agar and re-examined to confirm that individual reactions were reproducible.

Finally, two strains of *S epidermidis* and two strains of *S haemolyticus* were examined to determine the results of the crystal violet

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reaction under varying conditions, as previously described for *S aureus*.² Briefly, the crystal violet reaction was ascertained: (i) on a series of nutrient agar plates in which the crystal violet content varied from 1 in 10 000 to 1 in 1 000 000 and (ii) on standard crystal violet medium (content of crystal violet 1 in 100 000) with, sequentially, one, two, and three sterile cellulose acetate filters interposed between the medium and the transferred inocula. Inocula were as described for the standard crystal violet reaction.

Results

The crystal violet reaction results of all 24 reference strains and 112 clinical isolates are seen in table 1 which shows a consistent association between the crystal violet reaction and the speciation of the organisms. In the two species which were particularly well represented in the clinical collection *S epidermidis* always gave a white reaction and *S haemolyticus* always gave a purple reaction. The modest numbers of other species also behaved consistently and all these results agreed with the results obtained on the corresponding reference strains.

The results seen in table 2 show that varying the conditions of the crystal violet reaction, either by altering the crystal violet content of the medium or by interposing increasing numbers of cellulose acetate filters between the standard medium and the inoculum, had no effect on the crystal violet reaction of two strains of *S epidermidis*.

In contrast, both strains of *S haemolyticus* tested were affected by these variations in that as the crystal violet concentration was decreased or as filters were interposed, the crystal violet reaction of *S haemolyticus* changed from purple to white.

There was no correlation between the crystal violet reactions of any of the clinical isolates and their known origin, whether this was from peritoneal dialysis effluent, blood culture, or catheter tip (data not shown).

Table 1 Crystal violet reactions of 24 reference strains and 112 clinical isolates of coagulase negative staphylococci

Strain identification	Number tested	Crystal violet reaction	
		Purple	White
<i>Clinical isolates</i>			
<i>S capitis</i>	2 (0)	0	2
<i>S cohnii</i>	1 (0)	0	1
<i>S epidermidis</i>	62 (54)	0	62
<i>S haemolyticus</i>	28 (13)	28	0
<i>S hominis</i>	6 (1)	0	6
<i>S saprophyticus</i>	6 (4)	6	0
<i>S simulans</i>	3 (0)	0	3
<i>S warneri</i>	2 (0)	2	0
<i>S xyloso</i>	2 (0)	0	2
<i>Reference strains</i>			
<i>Purple</i>		<i>White</i>	
<i>S arlettae</i>	<i>S auricularis</i>	<i>S capitis</i>	<i>S caprae</i>
<i>S chromogenes</i>	<i>S gallinarum</i>	<i>S carnosus</i>	<i>S caseolyticus</i>
<i>S intermedius</i>	<i>S haemolyticus</i>	<i>S cohnii</i>	<i>S epidermidis</i>
<i>S klooseri</i>	<i>S lentus</i>	<i>S equorum</i>	<i>S hominis</i>
<i>S lugdenensis</i>	<i>S saprophyticus</i>	<i>S hyicus</i>	<i>S simulans</i>
<i>S schleiferi</i>	<i>S sciuri</i>	<i>S xyloso</i>	
<i>S warneri</i>			

() indicates number of isolates for each species showing slime production.

Table 2 Effects of variations in crystal violet content and filter interposition on crystal violet reaction of *S epidermidis* and *S haemolyticus*

Conditions	Observed reaction	
	<i>S epidermidis</i>	<i>S haemolyticus</i>
<i>Standard CV agar</i>	White	Purple
<i>Varying CV content of medium</i>		
CV 1 in 10 000	White	Purple
CV 1 in 50 000	White	Purple
CV 1 in 500 000	White	White
CV 1 in 1 000 000	White	White
<i>Interposing filters on to standard (CV 1 in 100 000) medium</i>		
1 filter added	White	White
2 filters added	White	White
3 filters added	White	White

Discussion

Our results show that CNS vary in their crystal violet reaction, both purple and white reactions being obtained. No yellow reactions were seen, but unlike *S aureus*, the standard crystal violet reaction does not apparently vary within the same species. In CNS it varies between species. Thus, for instance, all strains of *S epidermidis* gave a white reaction whereas all strains of *S haemolyticus* gave a purple reaction.

Our previous work on *S aureus* showed that the crystal violet white reaction was a positive character and due to a modification of the dye, possibly by an as yet unidentified nicotinamide adenine dinucleotide (NAD) dependent enzyme system separate from the respiratory chain.² The purple and non-purple (that is, white and yellow) biotypes of *S aureus* represented discrete populations on a spectrum of the capacity of the species to convert crystal violet to less coloured products. Strains belonging to the purple biotype modified the crystal violet dye but only at a very slow rate. The result of the crystal violet reaction could be varied by manipulation of the inoculum or the dye content of the medium or by interposing filters between the inoculum and the crystal violet medium.

Strong associations were found in *S aureus* between phage groups II and V and the crystal violet white-reacting phenotype, and between phage group III and the crystal violet purple-reacting phenotype, but sufficient exceptions occurred to exclude an absolute dependent linkage between the phage group of individual strains of *S aureus* and their crystal violet reaction phenotype. The picture was one of co-selection of two or more independent determinants.²

In this study it was possible to alter the crystal violet purple reaction of two strains of *S haemolyticus* in the same way as previously described for purple-reacting strains of *S aureus*. This suggests that *S haemolyticus*, like the purple phenotype of *S aureus*, has the ability to modify crystal violet, but only very slowly. Representatives of other purple-reacting species, especially *S saprophyticus*, will need to be tested to determine if this pattern is a general one.

In contrast, it proved impossible to produce conditions in which *S epidermidis* gave a purple reaction in the crystal violet test,

despite concentrations of crystal violet up to 1 part in 10 000 being used. Under similar circumstances white-reacting strains of *S aureus* give a purple crystal violet reaction. This suggests that the ability of *S epidermidis* to modify crystal violet either is the same mechanism but present more abundantly than that in the white (and yellow) reacting biotype of *S aureus* and is unable to be overwhelmed by the higher dosage of substrate, or that the crystal violet reaction of *S epidermidis* (and possibly of the other white-reacting species of CNS) represents a different phenomenon to that of *S aureus* and *S haemolyticus* (and possibly of the other purple-reacting species of CNS). Further investigations along these lines may yield information of taxonomic use.

Conventionally, statements regarding the crystal violet reaction ability of strains of *S aureus* refer to results obtained on the empirically standardised medium containing 10 µg/ml crystal violet. In this context our previous studies on *S aureus* showed strong correlations within that species between crystal violet purple reactivity and strains showing evidence of invasiveness, nosocomial origin, and an ability to persist at colonised sites.^{3,4} In this study with CNS no correlation was seen between the standard crystal violet reaction and the clinical site of isolation or with the ability to produce extracellular slime. Our results show that the crystal violet reaction varies between species rather than strains, at least in those four species which were well represented in the clinical isolates (*S epidermidis*, *S haemolyticus*, *S saprophyticus* and *S hominis*).

Previous work on CNS isolates from catheter tips from open heart surgery patients has suggested that *S saprophyticus* (crystal violet purple) may be able to colonise and persist in such sites better than *S epidermidis* (crystal violet white),⁷ and a recent survey showed that *S saprophyticus* and *S haemolyticus* (both crystal violet purple) were more likely to be associated with pyuria when isolated from

urine.⁸ *S haemolyticus* also binds to human serum proteins much more actively than *S epidermidis* or *S hominis*.⁹ As with *S aureus*, therefore, the picture which emerges is one suggestive of the crystal violet purple reaction being a general marker for those organisms (in this case, those species of CNS) which, each through different mechanisms, are associated with colonisation and persistence, rather than being a marker for a single virulence associated property.

Further investigations into the associations between the crystal violet reaction, species of CNS, and the disease producing roles of these organisms are necessary. It will also be important to characterise the crystal violet-modifying mechanisms of CNS and determine if they are the same in the different species of CNS and as those of *S aureus*. This study suggests, however, that the crystal violet reaction may be an interesting tool in studies of CNS.

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