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Susceptibility to desferrioxamine: a new test for the identification of *Staphylococcus epidermidis*

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Summary. The ability to identify *Staphylococcus epidermidis* quickly and accurately has become increasingly important in clinical microbiology. Susceptibility to desferrioxamine, an iron-chelating agent, was investigated as a new test for the identification of *S. epidermidis*. All strains of *S. epidermidis* and *S. hominis* tested were susceptible to a 1000-μg disk of desferrioxamine when grown on brain heart infusion agar. All other strains of coagulase-negative staphylococci, *S. aureus* and micrococci were resistant. As a single test, susceptibility to desferrioxamine was 96·4% efficient in identifying *S. epidermidis*; when combined with additional tests such as alkaline phosphatase production and fermentation of trehalose, the efficiency improved to 100%. Desferrioxamine disks were easy to prepare, stable and inexpensive. The test was simple to perform and interpret and should readily find application in clinical microbiology laboratories.

Introduction

Coagulase-negative staphylococci (CNS) have become recognised increasingly as a cause of nosocomial infections, particularly bacteraemia associated with indwelling central venous catheters[1,2] and peritonitis associated with continuous ambulatory peritoneal dialysis (CAPD).[3,4] The species of CNS most commonly isolated from human clinical specimens has been identified as *Staphylococcus epidermidis*, with a frequency of 72[5]–92%.[1] Conventional methods of identifying *S. epidermidis*, such as that of Kloos and Schleifer,[6] are considered to be too slow and unsuitable for routine clinical microbiology laboratories.[7] Many commercially available miniaturised systems are also slow, have a limited database, lack specificity[7] and are expensive.

Desferrioxamine is a siderophore, synthesised by *Streptomyces pilosus* and used clinically in the treatment of acute and chronic iron overload.[8] Studies of the effects of desferrioxamine on bacterial growth *in vitro* and *in vivo* have produced conflicting results. With some *Neisseria* spp. and *Listeria monocytogenes*, for example, desferrioxamine inhibited growth, whereas growth of *Salmonella typhimurium*, *Klebsiella* spp. and *Yersinia enterocolitica* was stimulated.[9] When we investigated the effects of desferrioxamine on CNS, only the growth of *S. epidermidis* and *S. hominis* was inhibited, suggesting that inhibition of growth by desferrioxamine may form the basis of a simple test for the identification of these organisms.

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Materials and methods

Bacterial strains

The 95 isolates of CNS studied were from blood samples submitted for culture to the Clinical Microbiology Department, Sir Charles Gairdner Hospital, Perth, Western Australia. They were identified by either the API-Staph strip (API System, Montalieu Vercieu, France) or the Vitek GPI Card (Vitek Systems, Hazelwood, MO, USA) and comprised 57 *S. epidermidis* isolates, 13 *S. haemolyticus*, six *S. warneri*, five *S. simulans*, four *S. hominis*, four *S. capitis*, three *S. saprophyticus*, two *S. cohnii* and one *S. auricularis*. Isolates were stored at -70°C in brain heart infusion broth with glycerol 20% added.

For comparative purposes 10 clinical isolates of *S. aureus* and six reference cultures—*S. epidermidis* NCTC 14990, *Micrococcus luteus* NCTC nos. 7743 and 2665, *M. varians* NCTC nos. 8340 and 7944 and *M. roseus* NCTC 7520 were also investigated.

Reagents

Desferrioxamine B mesylate (Desferal; Ciba-Geigy Ltd, Lane Cove, Australia) was obtained from the Pharmacy Department, Sir Charles Gairdner Hospital. A 0·5 *g* vial was reconstituted with 5 ml of sterile distilled water to give a final concentration of 100 mg/ml.

Minimum inhibitory concentrations (MICs)

MICs of desferrioxamine were determined by an agar dilution method. Brain Heart Infusion Agar

(BHIA; Oxoid) plates were prepared containing 20 ml of agar. Doubling dilutions of desferrioxamine up to 1 in 64 were prepared in sterile distilled water and 0·1 ml of each dilution was spread over a BHIA plate and allowed to absorb into the medium and equilibrate, giving a maximum final concentration of 500 μg/ml.

The inoculum was prepared by diluting an overnight culture to the turbidity of a 0·5 McFarland standard with sterile saline 0·85%. The agar plates were inoculated with a ten-fold dilution of this suspension by means of a multipoint inoculator (Mast Laboratories Ltd, Liverpool) giving a final inoculum of 10^4 bacteria per spot. The plates were incubated aerobically at 37°C for 18 h. After incubation, the MIC was determined as the lowest concentration of desferrioxamine that caused maximum reduction in growth. Susceptible strains grew as a fine haze only, similar to that seen when testing for sulphonamide susceptibility,[10] whereas resistant strains produced growth of a nature similar to that seen on a control plate containing no desferrioxamine.

Disk-diffusion susceptibility testing

As an agar dilution method of testing might be unsuitable for a routine laboratory, the test was modified to use 6-mm diameter filter-paper disks. In preliminary experiments, disks were prepared that contained 1 μg, 10 μg, 100 μg and 1000 μg of desferrioxamine and allowed to dry. BHIA, Blood Agar (Oxoid), Mueller-Hinton Agar (Oxoid) and Heart Infusion Agar (Gibco, Madison, USA) plates were spread-inoculated to produce an even lawn with a cotton swab and an inoculum of test organism prepared as described above. The disks containing different amounts of desferrioxamine were placed on each agar plate; the plates were incubated at 37°C for 18 h and the diameters of zones of inhibition were recorded. After these preliminary experiments, BHIA plates and 1000-μg desferrioxamine disks were chosen for tests with all 95 CNS isolates, the 10 clinical isolates of *S. aureus* and the reference strains of *S. epidermidis* and *Micrococcus* spp.

The stability of the prepared disks at room temperature (approximately 25°C) and at 5°C was assessed by repeated testing of the representative group of CNS over a period of weeks and recording any variation in zone size.

Additional tests

The ability of *S. epidermidis* and *S. hominis* to produce alkaline phosphatase was assessed with a commercially available rapid test (Rosco Diagnostica, Taastrup, Denmark) according to the manufacturers' instructions. Briefly, a bacterial suspension (equivalent in turbidity to a 2 McFarland standard) was prepared in 0·25 ml of saline 0·85% in an Eppendorf tube. One alkaline phosphatase tablet was added to the tube which was incubated at 37°C for 4 h. A yellow colour was recorded as indicating a positive result, and a colourless result was recorded as negative.

The fermentation of trehalose was assessed by the purple agar plate method and the criteria of Kloos and Schleifer.[6]

Results

The susceptibility of 95 strains of CNS to desferrioxamine as assessed by the agar dilution method is shown in table I. None of the strains of *S. haemolyticus*, *S. warneri*, *S. simulans*, *S. capitis*, *S. saprophyticus*, *S. cohnii* and *S. auricularis* were inhibited by the highest concentration of desferrioxamine tested, 500 μg/ml. All strains of *S. epidermidis* and *S. hominis* were inhibited at a concentration of 62·5 μg/ml.

Table I. In-vitro susceptibility of CNS to desferrioxamine

Species (number tested)	Number of strains inhibited at the concentration (μg/ml) indicated						
	7·8	15·6	31·2	62·5	125	250	500
S. epidermidis (57)	8	22	54	57	57	57	57
S. haemolyticus (13)	0	0	0	0	0	0	0
S. warneri (6)	0	0	0	0	0	0	0
S. simulans (5)	0	0	0	0	0	0	0
S. hominis (4)	1	3	4	4	4	4	4
S. capitis (4)	0	0	0	0	0	0	0
S. saprophyticus (3)	0	0	0	0	0	0	0
S. cohnii (2)	0	0	0	0	0	0	0
S. auricularis (1)	0	0	0	0	0	0	0

The results of experiments to determine the suitability of a disk-diffusion susceptibility testing method are shown in table II. The three strains of *S. epidermidis* used were chosen because they represented the spectrum of MICs obtained for this species. The 1000-μg disks gave zones >20 mm for susceptible strains and no zone for resistant strains, and were chosen for further study.

Table II. Diameters of zones of inhibition of five CNS strains around disks containing various amounts of desferrioxamine

Strain no.	MIC (μg/ml)	Zone size (mm) around disk			
		1 μg	10 μg	100 μg	1000 μg
S. epidermidis 4415	7·8	0	7·0	16·9	21·9
S. epidermidis 4416	15·6	0	6·7	15·6	21·5
S. epidermidis 4434	62·5	0	8·5	18·9	25·2
S. simulans 4417	>500	0	0	0	0
S. capitis 4413	>500	0	0	0	0

All 95 CNS, 10 *S. aureus* and reference strains were tested for susceptibility by the disk diffusion method with the 1000-μg desferrioxamine disks. All strains of

S. epidermidis (including NCTC 14990) and *S. hominis* gave readable zones of inhibition whereas all other strains tested produced no zones of inhibition (table III).

Table III. Susceptibility of various staphylococci and micrococci to a 1000-μg desferrioxamine disk

Species (number tested)	Zone diameters (mm)	
	Mean	Range
*S. epidermidis** (58)	24·8	20·5–29·6
S. hominis (4)	27·3	25·8–28·9
Other CNS (32)	0†	0
S. aureus (10)	0	0
Micrococcus spp. (5)	0	0

* Includes *S. epidermidis* NCTC 14990.
† No zones of inhibition observed.

If desferrioxamine susceptibility alone was used for the identification of *S. epidermidis*, the sensitivity of the test was 100%, with specificity 92·5%, a predictive value of a positive result of 93·1% and a predictive value of a negative result of 100%. The overall efficiency of the desferrioxamine susceptibility test was 96·4%.

To differentiate *S. epidermidis* from *S. hominis*, additional tests were performed (table IV): 50 of 57 *S. epidermidis* isolates produced alkaline phosphatase and all *S. hominis* isolates fermented trehalose, as did one strain of *S. epidermidis*.

Table IV. Additional tests for the identification of *S. epidermidis* and *S. hominis*

Species (number tested)	Alkaline phosphatase production		Trehalose fermentation	
	Positive	Negative	Positive	Negative
S. epidermidis (57)	50	7	1	56
S. hominis (4)	0	4	4	0

The stability of the 1000-μg desferrioxamine disks over a 5-week period is shown in table V. There was no significant variation in zone diameters during the test period.

Table V. Diameters of zones of inhibition of five CNS strains around 1000-μg desferrioxamine disks after various weeks of storage

Strain no.	Temperature (°C) of storage	Zone size (mm) at week				
		0	1	2	3	5
S. epidermidis 4415	RT	21·9	19·6	22·9	21·8	25·6
	5°	...	21·6	23·6	22·1	23·8
S. epidermidis 4416	RT	21·5	20·0	21·4	21·8	25.4
	5°	...	20·0	20·5	22·5	25·0
S. epidermidis 4434	RT	25·2	19·6	20·9	20·4	25·5
	5°	...	20·5	18·9	19·6	22·9
S. simulans 4417	RT	0	0	0	0	0
	5°	...	0	0	0	0
S. capitis 4413	RT	0	0	0	0	0
	5°	...	0	0	0	0

RT, room temperature (*c.* 25°C).

Discussion

The identification of CNS has assumed greater importance in recent years because of the increasing number of infections caused by these organisms. This is particularly relevant with blood cultures, in which CNS are also common contaminants, as the identification of *S. epidermidis* may be a guide to the clinical significance of an isolate. A simple, rapid, reliable and inexpensive test for the separation of *S. epidermidis* from other CNS is required.

Inhibition of growth by desferrioxamine offers such a test. In the present study, all strains of *S. epidermidis* were susceptible to a 1000-μg desferrioxamine disk and all *S. aureus*, micrococci, and non-*S. epidermidis* strains, with the exception of *S. hominis* strains, were resistant. The test gave a predictive value for a positive result of 93·1% and a predictive value for a negative result of 100%. If additional tests, such as alkaline phosphatase production and, more particularly, trehalose fermentation, were performed, the efficiency of the three tests in identifying *S. epidermidis* improved to 100%.

Although only four strains of *S. hominis* were included in our series, previous investigations[11] have successfully used trehalose fermentation to separate *S. epidermidis* from other CNS. However, the utility of phosphatase activity for this purpose has been more variable. In our study, 88% of the 57 *S. epidermidis* strains produced alkaline phosphatase as detected by the Rosco Diagnostica 4-h tablet technique. However, five of the seven strains that gave negative results by this method had previously given a positive alkaline phosphatase result by the API-Staph strip, indicating that the detection of alkaline phosphatase in *S. epidermidis* depends on the method employed. This may explain the discrepancies between various studies with regard to phosphatase activity in *S. epidermidis*, from 7% negative in one study[5] to 30% negative in another.[11]

Various media were investigated for their suitability in the desferrioxamine disk test. Media that did not contain blood were superior to those containing blood and BHIA consistently gave good results. The unsuitability of media containing blood gives an indication of how the desferrioxamine disk test works. Unsupplemented bacteriological media contains up to 4 μM iron[12] and this is usually adequate for the intrinsic iron requirement of most organisms. Once desferrioxamine is added to the medium it binds the iron to form ferrioxamine. It would appear that, as for some *Neisseria* spp. and *L. monocytogenes*,[9] *S. epidermidis* cannot utilise the iron-containing form of desferrioxamine. However, in media supplemented with blood, either the iron-containing haemoglobin molecule is usable by *S. epidermidis* or the desferrioxamine is saturated and there is an excess of iron. Therefore, it is important that the desferrioxamine disk test be performed on culture media lacking blood or any other form of iron supplementation.

It is interesting to note that there was no correlation between zone size, at any disk concentration, and MIC of desferrioxamine for the three representative strains of *S. epidermidis* tested. This lack of correlation has been consistent for all isolates of *S. epidermidis* tested so far (results not shown) but cannot be explained.

A number of factors would suggest that the desferrioxamine disk test will prove to be extremely useful in the clinical laboratory. It can be performed easily with other disk tests for the rapid identification of staphylococci and micrococci from both blood cultures and urine samples. Blood cultures containing gram-positive cocci with appropriate morphology could be subcultured on to BHIA and both a Taxo A bacitracin disk added to separate micrococci from staphylococci[13] and a desferrioxamine disk added to separate *S. epidermidis* from other staphylococci. Staphylococci from urine samples could be separated into *S. epidermidis*, *S. saprophyticus* and other CNS by the use of a 1000-μg desferrioxamine disk and a 5-μg novobiocin disk.[14]

Desferrioxamine disks were readily prepared in the laboratory and showed no decrease in activity at 5°C or room temperature over a 5-week period.

In summary, susceptibility to a 1000-μg desferrioxamine disk will reliably separate *S. epidermidis* and *S. hominis* from other staphylococci and micrococci. When used with one or two additional tests, *S. epidermidis* alone may be identified with 100% efficiency. The test is cheap and simple to perform and should find application in the clinical diagnostic microbiology laboratory.

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