The typing of *Staphylococcus epidermidis* by a lectin-binding assay

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Summary. A new typing method for *Staphylococcus epidermidis* was developed. Four biotinylated lectins—wheat germ agglutinin (WGA), soy bean agglutinin (SBA), lentil agglutinin (LCA) and Concanavalin A (ConA)—were added to immobilised whole cells of coagulase-negative staphylococci (CNS) in microtitration plates. The amount of bound lectin was measured by peroxidase-conjugated avidin followed by a peroxidase reaction. The method was compared to antibiotic-resistance analysis, phage typing, plasmid DNA profiles and slime production. A total of 113 isolates of CNS from 21 patients was investigated and 71 strains of CNS, including 64 strains of *S. epidermidis*, were detected if all typing methods were taken into consideration. If only one typing method was used the highest discriminatory power among the *S. epidermidis* isolates was obtained with the lectin-binding assay which allowed 49 different strains to be detected. If the lectin-binding assay was combined with plasmid-profile analysis, all 64 different strains could be identified. The typability of lectin-binding assay was 96.9% among the *S. epidermidis* isolates and 25 different lectin-binding patterns were established among the 64 strains. The highest number of strains belonging to one lectin-binding pattern was 13 (20.3%). The assay was reproducible, easy to perform, relatively inexpensive and therefore applicable to large scale typing of *S. epidermidis*.

Introduction

Coagulase-negative staphylococci (CNS), predominantly *Staphylococcus epidermidis*, are normal inhabitants of the skin and mucous membranes and these organisms may cause infections in patients with compromised immunity including neonates, those with prosthetic valves, cerebrospinal fluid (CSF) shunts and intravenous devices, and in patients undergoing chronic ambulatory peritoneal dialysis.^{1–6} When CNS are isolated from blood cultures, it may be difficult to differentiate between true infection and contamination.^{2,3,5,7,8} The isolation of the same CNS strain on several occasions from the same patient suggests the presence of a true infection, and, with this in mind, various typing systems to identify and type CNS have been described. Among these, species identification, biotyping, plasmid DNA analysis with or without the application of restriction endonucleases, antibiograms and phage typing are the most widely used.^{1,3–10} However, the typability of CNS by means of phage typing is low,^{3,6,11} antibiograms and plasmid-DNA profile analysis are not always repro-

ducible and conclusive,^{3–5,10} and the applicability of plasmid analysis is limited as the method is time-consuming and expensive.

Lectins are proteins or glycoproteins of non-immune origin, which bind specifically to carbohydrate residues.^{12–16} Interactions between lectin-like structures on bacterial surfaces and their role in attachment to host cells have been studied.^{14,17} Bacterial surface components such as peptidoglycan, teichoic acid and lipoteichoic acid^{18,19} contain many carbohydrate residues which might react with various lectins. Agglutination and gel-diffusion assays with lectins have been used to differentiate between *Neisseria gonorrhoeae* and other *Neisseria* species,²⁰ and between coagulase-positive and coagulase-negative staphylococci;²¹ lectins have also been used for epidemiological studies of *Haemophilus ducreyi* infections.²² Interactions between cell-wall components and lectins have been investigated by precipitation and gel-diffusion assays.^{23–25} However, these lectin-mediated agglutination and precipitation methods are probably not as sensitive as enzyme-linked assays (e.g., enzyme-linked immunosorbent assays, ELISA). The present study was undertaken to develop and evaluate a typing assay in which specific biotin-linked lectins were allowed to react with carbohydrates on the surface of CNS immobilised in microtitration plates.

Received 16 April 1991; revised version accepted 1 Dec. 1991.

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Table I. Biotinylated lectins used in the study

Lectin	Specificity	Inhibiting monosaccharide	Mol. wt	Biotinylation*
Wheat germ (WGA)	N-linked, bisected complex type glycans	N-acetyl-D-glucosamine	3.6×10^4	4.5
Soy bean (SBA)	O-linked glycans	N-acetyl-D-galactosamine	11×10^4	3
Lentil (LCA)	N-linked, biantennary complex type glycans	α -D-mannosyl	4.9×10^4	2-3
Concanavalin A (ConA)	N-linked, high mannose type glycans	α -D-mannosyl	10.2×10^4	4-8

* Moles of biotin/mole of lectin.

Materials and methods

Patients

In total, 113 CNS isolates were cultured from 91 specimens from 21 patients. Underlying conditions included haematological malignancies, prosthetic valve endocarditis, intravascular and intraventricular shunt infections and prosthetic hip sepsis. The isolates originated from blood, bone biopsies, intravenous devices, foci in the oral cavity, cardiac valves, CSF, lung biopsy, pleural effusion, an anal lesion and a cicatrice. The isolates were referred to the Staphylococcus Reference Laboratory, Statens Serum-institut, for further investigations to evaluate possible CNS infections.

Bacteria

CNS were identified initially by morphology, Gram's stain, and negative reactions for coagulase production and clumping factor and then speciated by a modified Kloos and Schleifer scheme.⁹ The isolates were identified as *S. epidermidis* (105), *S. hominis* (3) and *S. haemolyticus* (1). Four isolates could not be identified to species level and were designated "uncharacterised". All bacteria were stored in ox-bouillon with glycerol 10% at -80°C until further investigation.

Lectins

For preliminary investigations, biotinylated lectins were kindly provided by Kem-en-Tec, Copenhagen, Denmark. The following biotinylated lectins were used in later experiments and purchased from Sigma Chemicals (St Louis, MO, USA): wheat-germ agglutinin (WGA),^{15,16} soy bean agglutinin (SBA),^{13,15,16} *Lens culinaris* agglutinin (LCA)^{12,15,16} and Concanavalin A (ConA).^{12,15,26} The carbohydrate specificities and other characteristics of these lectins are shown in table I. Biotinylated lectins were used to increase the sensitivity.²⁷ All lectins were dissolved in normal saline supplemented with 5 mM NaN_3 to a concentration of 1 mg/ml and stored at 4°C until use.

Lectin-binding assay

Attachment of bacteria to the wells in microtitration plates was performed as previously described²⁸ with

the following modifications. Two or three colonies from a blood-agar plate were inoculated into 10 ml of Mueller-Hinton broth, incubated for 18 h at 35°C , harvested and washed three times (1600 g, 10 min) in phosphate-buffered saline (PBS), pH 7.38, and resuspended in PBS. Concentrations of bacteria from 2×10^5 cfu/ml to 10^9 cfu/ml were tested. A bacterial suspension of 5×10^7 cfu/ml was the highest concentration which gave a sufficiently low background level and was chosen for further studies. Bacterial suspensions (100 μl) were used to coat wells of 96-well, flat-bottomed microtitration plates (NUNC Immuno-plate, Denmark). Glutaraldehyde (200 μl ; 0.0625% w/v) was added to each well, and the plates were centrifuged at 2100 g for 15 min. After aspiration, free binding sites were blocked with 200 μl of blocking buffer (PBS, Tween 20 0.1%, 0.5 M NaCl, 0.5 mM MnCl_2 , 0.5 mM CaCl_2 and 0.5 mM MgCl_2) and plates were incubated at 22°C for 1 h. Plates were washed three times in washing solution (PBS, pH 7.2; Triton X 100 1% w/v) and stored at 4°C until used. Preliminary experiments demonstrated that plates could be stored for 1 week without altered activity.

Biotinylated lectins, WGA, SBA, LCA and ConA (100 μl) were added to the bacteria-coated microtitration plates. In preliminary studies, six concentrations were tested (range 0.5–30 $\mu\text{g}/\text{ml}$), except for WGA for which 10 concentrations were tested (range 0.05–30 $\mu\text{g}/\text{ml}$); blocking buffer was used as diluent. Incubation was performed for 0.5 h to 18 h at 22°C , and for 0.5 h to 2 h at 30°C and 35°C ; an incubation period of 2 h at 22°C was found to be optimal and was used in the final method. The plates were then washed five times and incubated with 100 μl of peroxidase-conjugated avidin (Dakopatts, Glostrup, Denmark) in blocking buffer for various periods (5–60 min) at six different concentrations (range 1 in 1000–1 in 20000); a concentration of 1 in 10000 incubated for 15 min at 22°C was judged to be optimal as this minimised background readings. The peroxidase reaction was performed with 1,2-phenylenediamine-dihydrochloride (Dakopatts) as chromogen, according to the manufacturers' instructions and the plates were then read at 492 nm (Intermed ImmunoReader NJ 200, Teknunc, Roskilde, Denmark). All tests were done in triplicate, and controls with and without bacteria and lectins were included in each plate. Intra-plate variation (i.e. variation of the same isolate after independent investigation in the same plate) and day-to-

day variation were determined by investigating the same strains in different plates and on different days (table II). As an additional control, three of the *S. epidermidis* isolates representing different lectin-binding patterns (table III) were examined in all plates to evaluate variations in lectin activity.

The results are expressed as $1000 \times (\text{mean OD value} - \text{background value})$. The ODs of the bacteria-free wells incubated with lectin-conjugated avidin were defined as the background. Results < 200 were considered as negative test values.

Inhibition studies

The specificity of the lectin binding to two *S. epidermidis* strains was investigated by testing the following carbohydrates for their ability to inhibit binding: N-acetyl-D-glucosamine, D-glucose, N-acetyl-D-galactosamine, D-galactose, D-mannose and methyl-gluco-pyranoside, each at three concentrations (0.01 %, 0.1 % and 1 % w/v).

Antibiotic susceptibility testing

Susceptibility tests were performed by the tablet-diffusion test with Rosco Neo-Sensitabs (Rosco Diagnostica, Taastrup, Denmark)²⁹ on Danish blood-agar plates²⁹ at 35°C, except for methicillin which was examined on salt-agar 7.5 % at 30°C. The following antibiotics were tested: penicillin-G (P), streptomycin (S), tetracycline (T), gentamicin (G), erythromycin (E) and methicillin (M).

Phage typing

Phage typing was performed according to the method of Rosdahl *et al.*¹¹ with the following phages: 15, 27, 28, 28A, 37, 48, 71, 82, 155, 157A, 165, 275, 275A, 456, 459, 471A, A6C, A9C, R6 and B1. More than two strong reaction differences between two isolates was regarded as indicating separate strains.

Plasmid-DNA analysis

Plasmid DNA analysis was performed as described by Rosdahl³⁰ with the modifications reported by Møller.³¹

Slime production

Slime-producing CNS strains were identified by the tube method described by Christensen *et al.*² and graded as follows: a strong reaction with a confluent slime layer, grade 3; a positive reaction without a confluent layer, grade 2; a weak reaction, grade 1; and a negative reaction, grade 0.

Statistical analysis

The analytical variation (intra-plate and day-to-day

variation) of the lectin-binding assay was determined by the double determinations of strains. The following formula was used

$$SD = \sqrt{\frac{\sum d^2}{2n}}$$

where SD is the standard deviation, $\sum d^2$ is the sum of squared differences of double determinations of the same strain and n is the number of strains tested ($n = 7-18$). The strains selected for these determinations had responses that covered the range of responses for the individual lectins.

Isolates were considered to be different if their lectin values deviated more than the maximal variation with 95 % confidence limits as calculated for the intraplate variations (table II); thus, variation $\times (\sqrt{2} \times t_{95\%})$, gave ratios of 1.22, 1.66, 1.52 and 1.37 between values for WGA, SBA, LCA and ConA, respectively; ratios greater than these indicate non-identity between isolates examined in the same plates.

Results

Optimisation and analytical variation of the lectin-binding assay

Optimal concentrations of each of the lectins incubated at 22°C for 2 h, and day-to-day and intra-plate variation are shown in table II. Variation in binding was observed for SBA and ConA with different supplies of the product. The stability of ConA in solution was rather low, particularly if stored for > 2 months. Repeated subculturing up to 10 times did not alter the lectin-binding reactions of two strains of *S. epidermidis*.

Inhibition studies

The lectin-binding reactions were highly specific except for LCA. WGA was inhibited 90 % by N-acetyl-D-glucosamine 1 % w/v. N-acetyl-D-galactosamine 0.1 % w/v and D-glucose 1 % w/v completely inhibited SBA. ConA was totally inhibited by D-mannose 0.1 % w/v and methyl-gluco-pyranoside 0.1 % w/v and D-glucose 1 % w/v. Methyl-gluco-

Table II. Optimal lectin concentration and analytical variation for the lectin-binding assay determined by double determination of strains

Lectin	Intra-plate variation (%)	N*	Day-to-day variation (%)	N*	Optimal concentration ($\mu\text{g/ml}$)
WGA	7.4	18	18.7	14	0.1
SBA	22.4	18	29.6	11	10
LCA	17.0	18	32.0	7	6
ConA	12.4	18	31.8	14	6

* N, number of strains included in the calculations. For the intra-plate variations all examined strains were considered; for the day-to-day variations only strains with OD values > 100 were included.

Table III. Lectin-binding patterns of 64 strains of *S. epidermidis* (primarily classified as "strains" by antibiograms, phage typing, plasmid profiles and lectin analysis)

Lectin-binding pattern* (WGA/SBA/LCA/ConA)	Number of strains
-/-/-/-	2
-/-/-/200	1
-/-/-/393	3
314/-/-/-	1
493/-/-/-	2
1908/-/-/-	6
200/-/-/-/393	1
314/-/-/772	1
774/-/-/772	1
1215/-/-/200	1
1215/-/-/393	1
1215/-/-/772	2
1908/-/-/200	5
1908/-/-/393	13
1908/-/-/772	4
2995/-/-/200	1
2995/-/-/393	7
2995/-/-/772	5
-/739/-/-	1
-/739/-/200	1
774/384/-/-	1
1215/200/-/393	1
774/200/-/1516	1
2995/200/414/772	1
2995/739/414/200	1
1908/-/-/200	5

* Values in the lectin patterns represent $OD_{492} \times 1000$; values < 200 are recorded as negative. Each strain is placed in the lectin pattern which approximated to the actual lectin value of that strain (see text for calculation of intervals between adjacent lectin-binding patterns).

pyranoside 1% w/v inhibited LCA but only by 50–70% and there was no inhibition with D-glucose or D-mannose. For all the lectins, inhibition was not found with carbohydrates not specific for that lectin.

Typing of isolates

As patients were not epidemiologically related, isolates were evaluated from each patient separately and defined as different if they originated from different patients even if test results showed identity. In the lectin-binding assay, all strains from one patient were examined in the same set of microtitration plates and, therefore, evaluation of identity and non-identity between isolates in this assay is based on intra-plate variations.

If all the applied typing methods, except the slime test, were taken into consideration, it was possible to group the 113 CNS isolates as 71 different strains of CNS, 64 of which were *S. epidermidis*. The slime test did not give any meaningful separation of strains, was difficult to read and was, therefore, omitted from further analysis. As subdivision of *S. epidermidis* isolates was the main purpose of the typing scheme, only these strains were analysed further.

Table IV. Typability of the 64 *S. epidermidis* isolates and number of different "strains" classified by the four typing methods alone and in different combinations

Typing method	Number (%) of strains typable	Number of strains classified
Resistance pattern (RP)	64 (100)	42
Phage typing (PT)	12 (18.8)	11
Plasmid profile (PP)	56 (87.5)	45
Lectin-binding assay (LBA)	62 (96.9)	49
RP + PT	64 (100)	45
RP + PP	64 (100)	54
RP + LBA	64 (100)	59
PT + PP	59 (93.3)	50
PT + LBA	62 (96.9)	54
PP + LBA	64 (100)	64
RP + PT + PP	64 (100)	55
RP + PT + LBA	64 (100)	61
RP + PP + LBA	64 (100)	64
PT + PP + LBA	64 (100)	64
RP + PT + PP + LBA	64 (100)	64

Typability and discriminatory power of the lectin-binding assay

Only two of the 64 *S. epidermidis* isolates did not react with any of the lectins, a typability of 96.9% with this method. The majority, (56, 87.5%), of these isolates reacted with WGA and ConA (50, 78.1%) but only seven (10.9%) and two (3.1%) reacted with SBA and LCA, respectively. The 64 *S. epidermidis* isolates could be grouped in 25 lectin-binding patterns (table III). These patterns were defined on the basis of day-to-day variations for the assay. The interval between adjacent values for the same lectin in table III is calculated as the maximal variation with 95% confidence limits [$\text{variation} \times (\sqrt{2} \times t_{95\%})$], i.e., ratios between values were 1.57, 1.92, 2.07 and 1.96 for WGA, SBA, LCA and ConA, respectively.

Comparison between the lectin-binding assay and other typing methods

To evaluate the clinical applicability of the lectin-binding assay compared to other typing methods, a comparison was performed on the 64 *S. epidermidis* isolates which had represented a clinical problem in the 21 different patients.

The highest number of "strains" classified by a single typing system was 49 in the lectin-binding assay. If assays were combined, the lectin-binding assay combined with plasmid profiles distinguished between the highest number of different "strains" (64), and addition of resistance patterns and phage typing did not enhance discrimination further (table IV).

When applied to the isolates from each of the 21 patients, there was total agreement between the lectin-binding assay and all other typing methods in the discrimination of the isolates from 12 patients. In five patients, one or more differences were found between the methods and discrimination of strains was enhanced. The multiple isolates from four patients

Table V. Typing results of 11 isolates of *S. epidermidis* from a patient with prosthetic valve endocarditis

Isolate no.	site	Resistance pattern	DNA-plasmid profile	Lectin reactions†
1	Blood	PTM	2/1/0*	1650/-/-/1103‡
2	Blood	PTM	3/1/1	1736/-/-/1128‡
3	Blood	PTM	3/1/1	1520/-/-/1154‡
4	Cardiac valve	PTM	3/1/1	1895/-/-/1068‡
5	Cardiac valve	PTM	3/1/1	1416/-/-/825‡
6	Blood	PGEM	0/0/2	370/-/-/-
7	Blood	PTM	3/1/1	346/-/-/837
8	Blood	PTGM	2/1/1	1995/-/-/1176‡
9	Blood	PTGM	0/1/0	1064/-/-/989
10	Blood	PTM	3/1/1	1735/-/-/1113‡
11	Blood	PGEM	3/1/1	1405/-/-/236

P, penicillin; T, tetracycline; M, methicillin; G, gentamicin; E, erythromycin.

* Small (≤ 3 MDA)/intermediate (> 3 MDA < 10 MDA)/large (> 10 MDA).

† Mean OD₄₉₂ $\times 10^3$; WGA/SBA/LCA/ConA; -, reaction < 200 .

‡ Isolates of same identity according to lectin reactions (based on intra-plate variation of the assay; see text).

All isolates were non-typable by phage typing.

offered particular problems and the details of one of these patients, a case of prosthetic valve endocarditis, are shown in table V. *S. epidermidis* isolates 2–5 and 10 from this patient were identical by all typing methods; isolates 1 and 8 had identical lectin-binding patterns but isolate 1 lacked one large and one small plasmid and isolate 8 the same small plasmid when compared to isolates 2–5 and 10. These seven isolates seemed closely similar and it was concluded that they comprised three closely similar strains, which possibly originated from the same clone. The remaining four isolates (6, 7, 9 and 11) probably represented a further four different strains. Although isolates 7 and 11 had plasmid patterns identical with isolates 2–5 and 10, they had different lectin reactions and isolate 11 was resistant to erythromycin. Isolates 6 and 9 were different from the rest and from each other in both plasmid profile and lectin reactions.

Discussion

All the isolates in this study originated from clinical specimens from patients with a clinical history which made *S. epidermidis* infection a distinct possibility. It is of crucial importance to test a typing system on relevant clinical isolates as only such tests will evaluate the applicability of the typing system for epidemiological purposes in clinical microbiology.

Previously described methods for typing of CNS are mainly derived from methods primarily designed for the typing of other bacterial species. Phage typing of CNS suffers from low typability;¹¹ in our study only 18.8% of *S. epidermidis* isolates were phage-typable. Plasmid-DNA profile analysis is not always conclusive¹⁰ and day-to-day reproducibility is a major prob-

lem; furthermore, the method is rather expensive and the number of isolates which can be examined daily is limited. Antibiotic resistance patterns are valuable but stability must be taken into consideration.⁵ Therefore, there is a need to develop other typing systems for CNS which can be used routinely in clinical microbiology laboratories. The lectin-binding assay fulfills these criteria; it is simple, inexpensive and reproducible, and results from different days are comparable by means of established lectin patterns. In this study, the typability of *S. epidermidis* isolates was high (96.9%) and the discriminatory power was sufficient to distinguish individual strains. The highest number of strains found in one lectin pattern was 13 (20.3%) and 25 different lectin patterns were observed. The lectin-binding assay clearly helped in both differentiating and enhancing the likelihood of identity between isolates from patients.

All isolates for comparison were examined in the same set of microtitration plates, so up to 20 isolates could be examined simultaneously. The lectin patterns, which were based on day-to-day assay variations, could be used in the daily routine to indicate isolates which should be re-examined in the same set of plates.

WGA and ConA were responsible for the high typability and discriminatory power of the assay, as 87.5% and 78.1% of the *S. epidermidis* strains reacted with WGA and ConA respectively. Only 10.9% of isolates reacted with SBA and only 3.1% (two strains) reacted with LCA; these two strains were cultured from the same blood culture and had reactions to all the lectins but to different degrees (lectin patterns 2995/200/414/772 and 2995/739/414/200; table III). WGA probably binds to N-acetyl-D-glucosamine-containing terminal oligosaccharides in the staphylococcal cell-wall and membrane.^{18,19} Some strains bound WGA only poorly; this may be due to the existence of a capsular substance which masked the oligosaccharide ligands. The binding of SBA to the bacteria may be via cell-wall teichoic acid, which can have N-acetyl-D-galactosamine as a component.¹⁸ Other cell-wall components, e.g., mannose polymers (high-mannose type), could contribute to ConA binding.

Interestingly, only two strains (see above) from one patient bound LCA indicating that biantennary complex type glycans are not normally present in the cell walls of *S. epidermidis*. The diversity of lectin-binding patterns suggests that the glycosylation of *S. epidermidis* surface components is as heterogeneous as glycosylation of mammalian cell-surfaces or glycoproteins. Two strains (3.1%) of *S. epidermidis* (table III) did not react with any of the lectins in our study. This was not due to insufficient bacteria bound to the microtitration plates and might well be a result of masking by capsular material.

Lectin patterns could not be correlated with the number or size of plasmids, antibiotic resistance patterns or slime production. Therefore, the lectin-binding assay is a valuable independent typing method

for *S. epidermidis* which can be combined with other methods. The most useful combination may be the lectin-binding assay and the plasmid-profile analysis. The criteria for identity between isolates should be evaluated carefully. Minor differences in plasmid profiles between two isolates combined with relevant differences in resistance patterns may not be a satisfactory criterion for non-identity, although such a criterion was used for the maximum strain differentiation in this study.

Other lectins with different carbohydrate-binding properties may be used to enhance the discriminatory power in the typing system and this method may also be applied to the typing of bacteria other than *S. epidermidis*.

This work was supported by the Danish Medical Research Council (grant no 12-9510). We thank Mrs Jette Mondrup and Miss Aase V. Meyer for technical assistance.

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