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Immunoreactivity of 80-kDa peptidoglycan and teichoic acid-like substance of slime producing *S. epidermidis* and specificity of their antibodies studied by an enzyme immunoassay

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Abstract

S. epidermidis is considered an important cause of nosocomial bacteraernia in immunocompromized hosts as well as the commonest agent of sepsis in patients with prosthetic devices. Pathogenesis is attributed to adherence and growth on bioniaterials facilitated by production of extracellular slime. The major macromolecules of slime are: a 20-kDa acidic polysaccharide (20-kDa PS) comprising the 60% of carbohydrate-containing slime macromolecules, a peptidoglycan with average molecular size of 80-kDa (30% of slime dry weight) and cell wall teichoic acid-like substance. In this study, antibodies to these macromolecules as well as crude slime were raised in rabbits and their immunological reactivity and specificity were studied by an enzyme immunoassay. All isolated macromolecules induced the production of specific antibodies. 20-kDa PS was less immunogenic than 80-kDa peptidoglycan and teichoic acid-like substance. However, 20-kDa PS was the most potent inhibitor of the reaction of slime with its homologous antibodies revealing that this polysaccharide is the major antigenic determinant of slime. All three antibodies specifically recognize ($p < 0.05$) and react with slime-producing *S. epidermidis* in comparison to other staphylococci species. Obtained results indicate that the 20-kDa PS may be distributed in the surface of the slime exposing most of its antigenic determinants to the immune system, whereas those of 80-kDa peptidoglycan and teichoic acid-like substance seem to be less accessible.

Keywords: Staphylococcus epidermidis; Slime; Antibodies; Enzyme immunoassay; Peptidoglycan; Polysaccharide

1. Introduction

Despite the presence of coagulase-negative staphylococcal species in the normal microflora of human, *Staphylococcus epidermidis* represents a

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frequent cause of nosocomial sepsis resulting in significant morbidity and mortality [1,2]. Particularly, *S. epidermidis is* by far the most predominant microorganism responsible for infections associated with implanted medical devices, such as intravascular and peritoneal catheters, prosthetic devices, cardiac valves, and haemodialysis and cerebrospinal shunts [1–3]. Additionally, it constitutes a major cause of infection in seriously ill and immunocompromised patients (i.e. preterm neonates, intensive care patients, cancer and transplant patients) [4]. Difficulties to differentiate between a true infection and contamination impede the accurate diagnosis of *S. epidermidis* infection from blood cultures. Treatment of infections is further complicated by the presence of antibiotic-resistant *S. epidermidis* strains [5].

The aetiology of biomaterial-related *S. epidermidis* infections has been a topic of intensive research in the last two decades. Colonisation of the foreign biomaterial involves an initial step of bacterial adhesion to the polymer surface and the subsequent production of an extracellular highly adhesive material known as slime [1,6]. One of the most important roles of slime is the formation of a biofilm, which may act as a penetration barrier to antibiotics and components of the host immune system [7]. Furthermore, the inhibitory effect of slime to opsonophagocytosis and T- and B-cell proliferation has been reported [1]. However, it has been suggested that a foreign body is not essential for the expression of virulence by slime-producing *S. epidermidis* strains, although there is still controversy over the issue [14,15]. The exact mechanism(s) by which slime contributes to *S. epidermidis* pathogenicity have not yet been elucidated and is a topic of extensive research.

Knowledge of the exact chemical structure of slime is necessary in order to understand the pathogenic mechanisms and suggest new diagnostic and therapeutic options. Studies on the extracellular slime produced by *S. epidermidis* in our laboratories showed that it is composed of distinct macromolecules, including glycoproteins, peptidoglycans and polysaccharides [8]. Particularly, the major polysaccharide component (approximately 60–65% of total carbohydrates) of slime is a sulphated 20-kDa acidic polysaccharide (20-kDa PS) [8,9]. This polysaccharide was found to be immunogenic in humans, since 20-kDa PS-specific antibodies have been detected in preparations of intravenous immunoglobulins (IVIG) and blood sera derived from IVIG-treated preterm neonates and patients with established slime-producing *S. epidermidis* bacteraemia [10,11]. Other macromolecules identified in slime are: (a) teichoic acid-like polysaccharides; (b) a polydisperse but homogeneously charged acidic 80-kDa peptidoglycan containing a covalently bound polysaccharide; (c) two non-anionic glycoproteins (degradable with papain) of molecular masses 250- and 125-kDa; (d) a papain-degradable 60-kDa macromolecule bearing acidic carbohydrates covalently bound to protein, and (e) an acidic polysaccharide with molecular mass of 12.5-kDa [8].

In search of specific markers that could differentiate between slime-producing *S. epidermidis* from other staphylococci, the immunogenicity in rabbits of the major slime macromolecules, i.e. teichoic acid-like polysaccharides, 80-kDa peptidoglycan and 20-kDa PS, and the specificity of their homologous antibodies have been studied.

2. Materials and methods

2.1. Bacterial strains

Slime-producing *S. epidermidis* reference strain 35983 obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and a slime-producing clinical isolate obtained from a patient with catheter-related bacteraernia were used for the isolation of crude slime. Other staphylococci spp (*S. aureus*, *S. saprophyticus* and *S. haemolyticus)* isolated from hospitalized patients were also used in the present study. Identification of clinical isolates was determined by Gram staining, catalase reaction, tube coagulation tests and, ultimately, by the API Staph-Trac system (Analytab Products, USA). The tube adherence test described by Christensen et al. [12] was used to determine the ability of all strains to adhere to polystyrene surface and, subsequently, to produce slime. Stock cultures of all strains were

maintained in Tryptic Soy Broth (TSB) (BBL Microbiology, Cockeysville, MD, USA) supplemented with 20% glycerol at −70°C without serial passage.

2.2. Isolation of crude slime

Extracellular slime was obtained as previously described [8,9]. In brief, bacteria were grown in TSB, which has been shown to promote slime production, for 24 h at 37°C and with 5% CO_2 (v/v) in humidified chambers. Extracellular product was removed from cells by gentle shaking with glass beads in 0.15 M NaCl. The extract was precipitated with a mixture of ethanol, sodium acetate, and acetic acid at final concentrations of 80% (v/v), 0.26 and 0.05 M, respectively. The precipitate was dissolved in double-distilled water, insoluble material was removed by centrifugation at 27 000 × *g* for 30 min, and the supernatant was extensively dialysed against 100 vols of double-distilled water. The dialysate was centrifuged at 105 000 × *g* for 3 h and the supernatants were freeze-dried and stored at −70°C. All steps were performed at 4°C in the presence of the following proteinase inhibitors: 6-aminohexanoic acid, Na_2EDTA, and phenylmethanesulfonyl fluoride at final concentrations of 50, 10, and 20 mM, respectively. The amount of crude slime obtained from 10 l of culture ranged from 15 to 20 mg.

2.3. Fractionation of crude slime macromolecules

Fractionation of slime macromolecules was carried out according to a previously described procedure [8]. Aqueous solutions of crude slime were chromatographed on DEAE–Sephacel (50 × 1.6 cm i.d.), which was eluted with one volume of each, distilled water and 0.2 M NaCl, followed by a NaCl gradient elution (10 vols) and, finally, with 2 vols of 2.0 M NaCl. Collected fractions were assayed for neutral Sugars (A_{625}) [13], protein (A_{280}) and conductivity to plot the NaCl gradient. Fractions containing carbohydrates and/or proteins were pooled, concentrated on a YM-10 membrane (Amicon), and then extensively dialysed against 10 vols of 0.5 M ammonium formate, pH 7.0, at 4°C for 24 h. In order to check the purity of each population, isolated populations were chromatographed on Sepharose CL-4B. The column was eluted by 0.5 M ammonium formate, pH 7.0, and fractions were assayed for neutral sugars and protein. The homogeneity of each population was checked by electrophoresis on precast linear gradient polyacrylamide gels (5–20%) in the presence of 0.1% (w/v) SDS and staining with Alcian blue and silver nitrate, electrophoresis on cellulose acetate and staining with Toluidine blue and finally electrophoresis on pure agarose 1% (w/v) and staining with both Toluidine and Coomassie blue, as earlier described [8].

2.4. Immunization and enrichment of antibodies

Antibodies to 80-kDa peptidoglycan, teichoic acid-like polysaccharides, 20-kDa PS and crude slime were raised in 4–5 months' old New Zealand White rabbits weighing 2–3 kg. Animals were kept one per cage under a 07:00 light on, 19:00 light off cycle having *ad libitum* access to food and water. Filter-sterilized antigens (0.5 ml in PBS) were emulsified with equal volumes of Freund's complete adjuvant for the first injection and incomplete Freund's adjuvant for the following challenges which were performed subcutaneously 1, 2 and 4 weeks later. At the first time, each animal was challenged with 0.4, 0.4, 0.6 and 5.0 mg dry wt of 80-kDa peptidoglycan, teichoic acid-like polysaccharide, 20-kDa PS and crude slime, respectively. The following injections were performed with half dose of the above mentioned amounts. Sera obtained in the fifth week were enriched for immunoglobulins by precipitation with a $(NH_4)_2SO_4$ saturated solution. Inimunoglobulin populations were then purified by gel chromatography on Sephadex G-25 (PD-10 prepacked column, Pharmacia, Uppsala, Sweden) where they were eluted with the void volume, as it was assessed by dot-immunobinding assay [10,11]. The obtained immunoglobulin populations were concentrated on a YM-2 membrane and ultimately used in PB S in the presence of 0.1% (v/v) Tween-20 at a volume equal to the initial antisera volumes.

2.5. Immunological methods

The specificity of produced antisera and their reactivity with other staphylococci species were determined by enzyme-linked immunosorbent assays (ELISA). In order to find the optimum working conditions, sterile 96-well round bottomed microplates were coated with antigens (100 μl/well) at concentrations of 5.0, 1.5, 0.5 and 0.15 μg/ml in phosphate buffer saline, pH 7.2, (PBS) at 4°C for 16 h. Microplates were washed three times with PBS containing 1% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin (BSA) and 0.4 M NaCl (PBS-Tween) (120 μl/well). Non-specific binding was blocked by incubation with a 3% (w/v) solution of BSA in PBS (200 μl/well) at 37°C for 1 h. After three washings (220 μl/well), microplates were incubated at 37°C for 1 h with homologous antibodies at dilutions of 1:3000, 1:1000, 1:300, 1:100, 1:30 and 1:10 in PBS. Plates were washed with PBS-Tween for three times. Peroxidase H-conjugated goat anti-rabbit IgG (Sigma Chemical Company, St Louis, MO, USA), diluted 1:2000 were used as detection antibodies. After incubation at 37°C for 1 h and thorough washings, colour was developed by adding (100 μl/well) 0.1 M citric acid–phosphate buffer, pH 5.0, containing 0.067% (w/v) *ortho*-phenylenediamine and 0.03% (v/v) H_20_2. The mixture was incubated for 15 min at room temperature in the absence of light. The reaction was terminated with 1 M H_2SO_4 and the optical density was measured at 490 nm in a Molecular Devices E-max photometer. Calibration and validation of results were performed using the SOFT max PRO software (version 1.2.0).

The specificity of antibodies against their homologous and heterologous antigens was studied with competitive ELISA. Each antigen was coated to the ELISA microplate and its binding to homologous antibodies was measured by inhibiting the reaction of antibodies with their antigens. In brief, each antisera was separately incubated with the various antigens in capped polypropylene tubes at different concentrations ranging from 0.5 to 100 μg/ml for 16 h at 4°C. The final concentration of antisera, in these mixtures as well as the concentrations of antigens for coating the microplates were determined previously as the optimum working conditions. After coating and thorough washing, microplates were incubated with BSA, as stated above. Following three washings to remove unbound BSA, microplates were incubated with the antigen–antibodies mixtures (100 μl/well). The amount of antibodies that finally bound to antigens on the microplate was detected with peroxidase H-conjugated goat anti-rabbit IgG. Results are expressed as the percent decrease of the absorbance produced by incubation of antigens on the microplates with antibodies in the absence of antigens in solution.

The reactivity of produced antisera with clinical isolates of *S. epidermidis*, *S. aureus*, *S. saprophyticus* and *S. haemolyticus* was determined by ELISA. Bacterial suspensions (100 μl/well) of initial $A_{600} = 1.0$ (ca. 2.5×10^5 cells) diluted 1:1, 1:10, 1:30, 1:100 and 1:300 in carbonate buffer, pH 9.6, were coated at microplates and incubated with the optimum dilutions of antisera.

3. Results

3.1. Isolation of distinct slime macromolecules and study of their immunogenicity

Fractionation and isolation of slime macromolecules was performed as earlier described [8]. In brief, anion-exchange chromatography of crude slime from reference strain ATCC 35983 differently charged populations (data not shown). Each population was chromatographed on Sepharose CL-4B and electrophoretic studies were performed for all populations. Combination of all analyses showed that the composition of slime was identical to that described by Karamanos et al. [8]. Particularly, the population, that eluted with 0.2 M NaCl from DEAE–Sephacel, constitutes 30% of slime dry weight and contains an acidic polysaccharide covalently bound to a small peptide. This macromolecule is homogeneously charged, but polydisperse with molecular size ranging from 35 to 120-kDa and an average 80-kDa. This macromolecule is referred as 80-kDa peptidoglycan. An acidic polysaccharide population containing small amounts of protein was

eluted from DEAE–Sephacel with 0.6 M NaCl. The presence of phosphate groups in this population and its mobility led us to refer to it as teichoic acid-like polysaccharide. Finally, a homogeneous acidic carbohydrate population was eluted with 0.7 M NaCl, has a molecular size of 20-kDa (determined by gel chromatography) and constitutes approximately 60–65% of total slime carbohydrates. Further chemical studies confirmed that this polysaccharide was the 20-kDa PS earlier described [8,9].

In order to study the immunogenicity of the isolated discrete macromolecules, rabbits were challenged once every week for four consequent weeks with these antigens and crude slime in the presence of Freund's adjuvant according to the protocol described. Samples of blood sera were obtained from the first to the fourth week after the first challenge and their reactivity with the homologous antigens was tested by ELISA (Fig. 1. All antigens induced the production of antibodies without any modification of their structure. Relatively high reactivity was observed on the third week, but sera were finally collected on the fourth week, since observed titres were 1.5–1.7 times higher than those on the third week. The immune response induced by teichoic acid-like

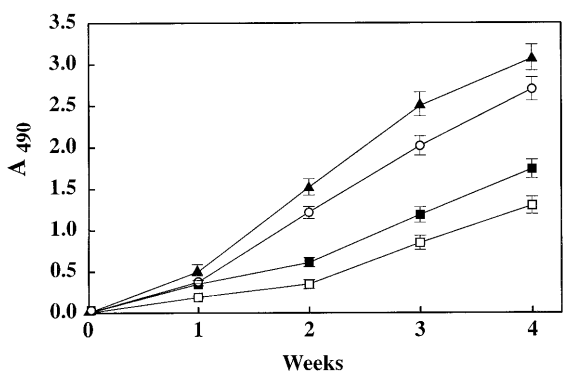

Fig. 1. Reactivity of antisera to crude slime (■), 20-kDa PS (□), 80-kDa peptidoglycan (▲) and teichoic acid-like substance (○) with the homologous antigens at 1, 2, 3 and 4 weeks after immunization. 20-kDa PS was coated to ELISA microplates at a concentration of 5.0 μg/ml and the other antigens at a concentration of 1.5 μg/ml. Antisera were used at a dilution of 1:300. Every point represents the average value ± SD of four experiments in triplicate.

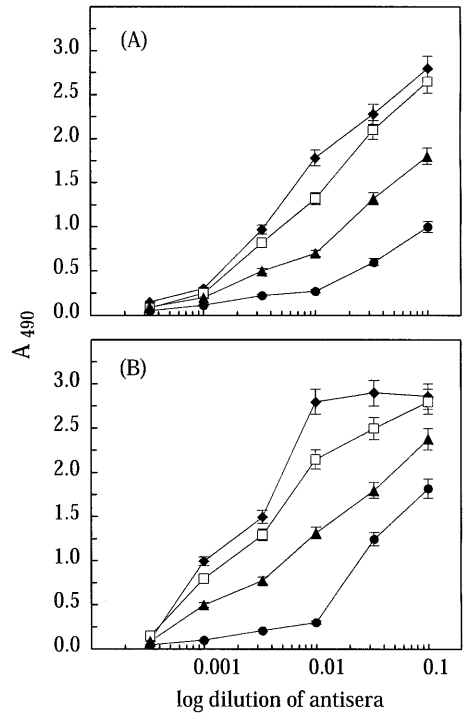

Fig. 2. Determination of optimum antigen coating concentrations and dilutions of antisera. 100 μl of teichoic acid-like polysaccharide (A) and 80-kDa peptidoglycan (B) were coated to ELISA microplates at concentrations of 5.0 (◆), 1.5 (□), 0.5 (▲) and 0.15 (●) μg/ml and incubated with their respective antisera diluted 1:3000, 1:1000, 1:300, 1:100, 1:30 and 1:10. Every point represents the average value ± SD of four experiments in triplicate.

polysaccharide and 80-kDa peptidoglycan was significantly higher ($P < 0.001$) than the antibody response to 20-kDa PS suggesting that their structures are more immunogenic than the latter macromolecule.

3.2. Determination of optimum working conditions and specificity of antibodies

Determination of optimum ELISA conditions was performed using incremental concentrations of antigens for microplate coating and incubating with serial dilutions of the homologous antibodies. The corresponding absorbance values were plotted on a semi-log paper against the concentration of the antigens for every antibody dilution

(Fig. 2). Conditions for all consecutive experiments were chosen from those curves that gave the highest inclination and best linearity. The coating concentrations of slime, 80-kDa peptidoglycan and teichoic acid-like polysaccharide were 1.5 μg/ml, whereas that of 20-kDa PS was 5 μ/ml. The optimum dilutions of antisera were 1:600 for antibodies to crude slime and 20-kDa PS, 1:700 for antibodies to teichoic acid-like polysaccharide and 1:900 for 80-kDa peptidoglycan-specific antibodies.

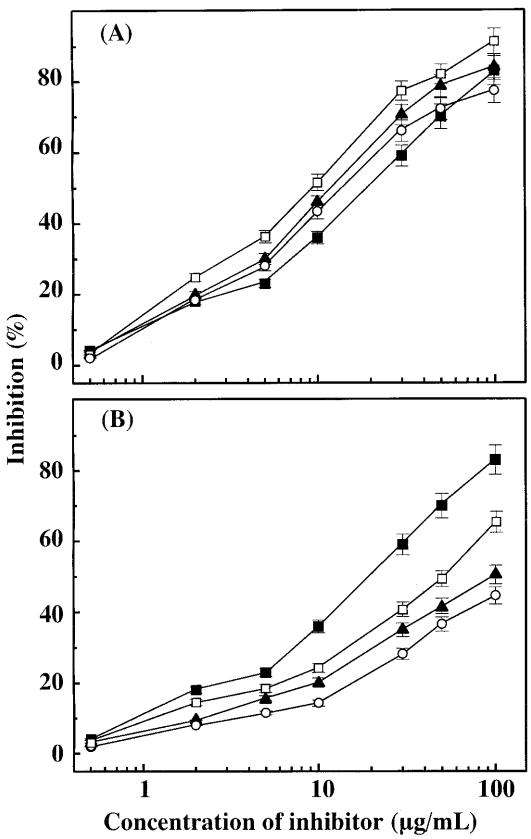

Fig. 3. Displacement curves from competitive ELISA experiments. Study of the inhibitory effect of various concentrations of crude slime (■), 20-kDa PS (□), teichoic acid-like substance (▲) and 80-kDa peptidoglycan (○) to binding of their homologous antisera to the same antigens on the ELISA microplate (A). The lower panel shows the inhibitory action of these antigens to the reaction of slime coated to the ELISA microplate with its homologous antisera (B). Every point represents the average value + SD of four experiments in triplicate.

The specificity of antibodies for the homologous antigen was studied by competitive ELISA. Separate incubation of antibodies with increasing concentrations of their homologous antigen (inhibitor) reduced the amount of antibodies in the mixture that could interact with the antigen on the microplate, and, therefore, the recorded absorbance (Fig. 3A). Resulting sigmoid curves on semi-log graph of inhibition of antibody binding versus the concentration of the inhibitor revealed that produced antibodies are specific to the corresponding slime macromolecule that induced their production.

The same experimental approach was applied to study the cross-reactivity of anti-slime antibodies with the three distinct slime macromolecules, i.e. 80-kDa peptidoglycan, teichoic acid-like polysaccharide and 20-kDa PS. All three antigens inhibited the binding of anti-slime antibodies to their homologous antigen, a fact that was expected since the three macromolecules are components of crude slime. However, the degree of inhibition was different for each macromolecule (Fig. 3B). The 20-kDa PS exhibited the highest inhibitory effect and gave a high value of cross-reactivity (1:2.5), despite the fact that it was not so immunogenic in rabbits as the other two macromolecules. The 80-kDa peptidoglycan was the weakest inhibitor since the degree of cross reactivity of anti-slime antibodies with this peptidoglycan was 1:10. The respective cross-reactivity value of anti-slime antibodies with teichoic acid-like polysaccharide was 1:5. These results demonstrate that the 20-kDa PS is the major antigenic determinant of slime, which is in accordance with previous findings [10].

3.3. Reactivity of antibodies to S. epidermidis slime macromolecules with other staphylococci species

The ability of specific antibodies against the three distinct slime macromolecules to recognize whole slime-producing *S. epidermidis* cells was studied by ELISA. Furthermore, their reactivity with other clinical staphylococci isolates (*S. aureus*, *S. haemolyticus* and *S. saprophyticus)* was also studied. For this purpose, four different dilu-

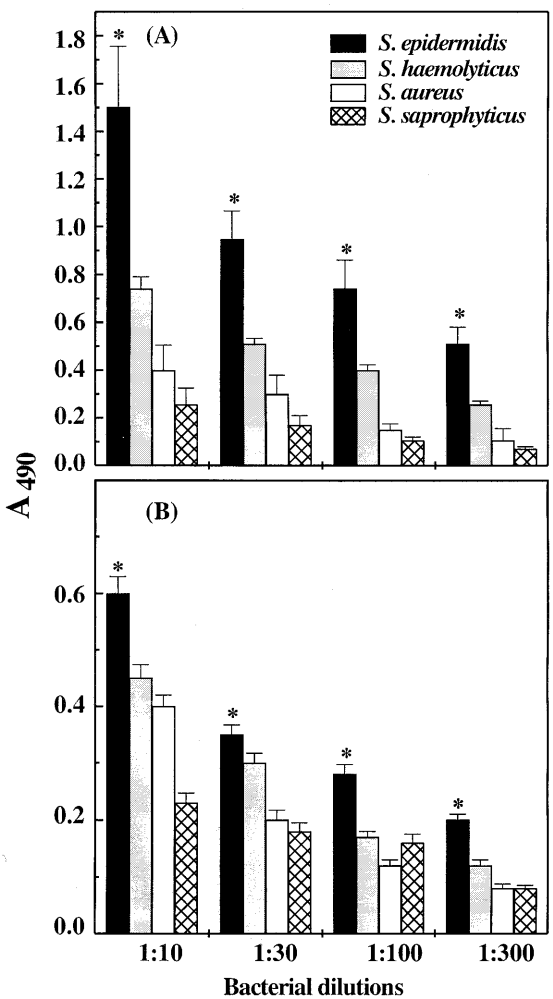

Fig. 4. Study of the reactivity of antibodies to teichoic acid-like substance (A) and 80-kDa peptidoglycan (B) with slime-producing *S. epidermidis* and other staphylococci species. Bacteria at 1:10, 1:30, 1:100 and 1:300 dilutions in carbonate buffer, pH 9.6 were coated to microplates and incubated with the respective antisera diluted 1:700 (A) and 1:900 (B), respectively. Results represent the average of nine experiments for each dilution of each bacterial species. Asterisks show the statistically significant ($P < 0.05$) higher reactivity.

tions (1:10, 1:30, 1:100 and 1:300) of bacterial suspensions with an absorbance value equal to 1.0 at 600 nm were used to coat the ELISA microplates. As shown in Fig. 4, the best coating bacterial dilution was 1:10, since a plateau was obtained after this value. All antibodies could recognize slime-producing *S. epidermidis* suggesting that 80-kDa peptidoglycan, teichoic acid-like polysaccharide and 20-kDa PS are organized in such a way in slime that are exposed to the surface of the cell. Moreover, the antibodies specifically recognize slime-producing *S. epidermidis* among other staphylococci species, since only *S. epidermidis* exhibited a statistically significant ($P < 0.05$) reactivity for the whole dilution range. Antibodies react with other staphylococci in the ascending order: *S. haemolyticus*, *S. aureus* and *S. saprophyticus* The observed cross-reactivity may be explained by the presence of surface carbohydrate(s) bearing common epitopes with the slime macromolecules.

4. Discussion

In this study, the crude slime and its major components, 80-kDa peptidoglycan, teichoic acid-like polysaccharides and 20-kDa PS, were isolated from one reference strain (ATCC 35983) and one clinical isolate. Polyclonal antibodies were raised in rabbits and their specificity was studied by an enzyme immunoassay.

Teichoic acid-like polysaccharides and 80-kDa peptidoglycan elicited higher immunogenicity than the 20-kDa PS and crude slime. In a previous study of Kojima et al. [16], a strong immune response to teichoic acids, but not to the capsular polysaccharide/adhesin (PS/A) was observed within the first 14 days after catheter implantation and development of bacteraemia in unimmunized rabbits [16]. Specificity studies showed that 20-kDa PS was the strongest inhibitor of binding of anti-slime antibodies to slime on an ELISA microplate despite its poor immunogenic capability. These results may be explained by a specific structural organisation of slime that allows the presence of 20-kDa PS molecules in the outer surface but hinders the exposure of the epitopes of 80-kDa peptidoglycan and teichoic acid-like polysaccharides. This provides an explanation for a previous study in which antibodies to teichoic acids were not protective [16]. Possibly, antibodies could not react with teichoic acids because they

are embedded in the thick slime layer. On the other hand, the protective efficacy of antibodies to 20-kDa PS has been clearly shown both in vitro and in humans [11]. Rabbit antibodies to 20-kDa PS in vitro exhibit higher opsonophagocytic activity than antibodies to slime. Furthermore, prophylactic administration of intravenous immunoglobulin with high titres of 20-kDa PS-specific antibodies to preterm neonates significantly protect them from slime-producing *S. epidermidis* infection [11], Therefore, it is obvious that the 20-kDa PS is not only the major polysaccharide constituent of *S. epidermidis* slime but also the dominant antigenic determinant. This result explains previous reports in which great differences (4:1) were observed among reactivity of 20-kDa PS specific antibodies with slime-positive and slime-negative strains [10].

The fact that all antibodies specifically recognize *S. epidermidis* may be exploited in order to design new diagnostic methods. It should be noted that the proposed enzyme immunoassay can be used for qualitative purposes (positive/negative test results) i.e. differentiating slime-producing *S. epidermidis* among other species and detecting whether a positive blood culture represents a true infection or contamination.

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