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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 803–810

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Potential use of solid phase immunoassays in the diagnosis of coagulase-negative staphylococcal infections

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Accepted 1 August 2003

Abstract

Staphylococcus epidermidis is a major nosocomial pathogen, even though it is a member of the normal bacterial flora of skin and the mucous membranes. A major complication is the development of biofilms on implanted medical devices. Diagnosis of coagulase-negative staphylococcal infections relies on the presense of clinical manifestation of infections and on microbiologic evidence, usually obtained after the removal of the biomaterial. Solid-phase immunoassays have not yet been used for routine diagnosis of coagulase-negative staphylococcal infections and distinction between pathogenic and normal cocci. The enzyme immunoassays developed in the last decade are presented in this review article. Serodiagnosis has been attempted by determining antibodies against bacterial cells, mixtures of *S. epidermidis* slime antigens and discrete slime antigens. Detection or typing of staphylococcal cells has been performed by specific antibodies and lectins. There is still a long way until the application of such assays in the routine clinical laboratory and large clinical studies are necessary.
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Keywords: Enzyme immunoassay; *Staphylococcus epidermidis*; Coagulase-negative staphylococci; Diagnosis; Typing

1. Introduction

The incidence of infections due to coagulase negative staphylococci (CNS) has risen dramatically in the last decades [1,2]. According to the 1998 National Nosocomial Surveillance System Report (Center for Disease Control and Prevention, Public Health Service, US Department of Health and Human Services, Atlanta, 1998), *Staphylococcus aureus*, CNS and enterococci accounted for 34% of nosocomial infections in the time period of 1990–1996 [3]. *Staphylococcus epidermidis*, the most frequently isolated member of CNS, has emerged as a major nosocomial pathogen even though it is a member of the normal bacterial flora of skin and mucous membranes. *S. epidermidis* is responsible for nosocomial bloodstream infections, cardiovascular infections and infections of the eye, ear, nose, and throat especially in compromised patients (drug abusers, AIDS patients, premature newborns and patients under immunosuppressive therapy) [1,4].

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doi:10.1016/S0731-7085(03)00564-8

Moreover, *S. epidermidis* is a major cause of infections associated with implanted medical devices, such as intravascular and peritoneal catheters, prosthetic devices, cardiac valves, and haemodialysis and cerebrospinal shunts [4,5]. The mortality associated with true CNS bacteraemia varies between 4.9 and 28% [2]. Infections caused by *S. epidermidis* are often subacute, persistent and relapsing. Development of antibiotic resistance in staphylococci complicates therapy [6,7].

In contrast to *S. aureus*, *S. epidermidis* does not produce many toxins and tissue-damaging exoenzymes, giving rise to questions about how and when it becomes virulent. Biomaterial related *S. epidermidis* infections are associated with the formation of staphylococcal biofilms on the implanted devices and dead tissues [8]. Biofilms favor bacterial survival because they act as penetration barriers to antibiotics and to components of the host immune system [8–10]. Colonization of the foreign biomaterial involves an initial step of bacterial adhesion to the polymer surface and the subsequent formation of multi-layered cell clusters through the production of an extracellular highly adhesive material, known as slime [11,12]. Although it has been suggested that a foreign body is not essential for the expression of virulence by slime producing *S. epidermidis* strains, there is still controversy over the issue [13,14]. Staphylococcal interference with the host's immune system is also intriguing and an inhibitory effect of slime components to opsonophagocytosis and T- and B-cell proliferation has been reported [2,11].

Diagnosis of CNS infections relies on the presence of clinical manifestations of infection and on microbiologic evidence. The latter is usually accomplished by multiple blood cultures or by the withdrawal of the biomaterial (e.g. catheter) for culturing. Biomaterial withdrawal causes inconvenience and is costly. Antibiotic treatment prior to blood sampling can give false negative results. Sampling for cultures is difficult in cases of endocarditis or complicated bacteremia with formation of intra-organ abscesses. New diagnostic methods to assess biomaterial colonization without withdrawal are under investigation [15]. CNS are the most frequent contaminants (58–83%) of positive blood cultures [5]. Distinguishing pathogenic *S. epidermidis* strains from contaminant strains is one of the major challenges in the clinical microbiology laboratories.

Inclusion of enzyme-linked immunosorbent assays (ELISAs) for diagnostic purposes is desirable because of the simplicity, repeatability, speed of performance and the fact that withdrawal of biomaterial is not necessary. Furthermore, immunoenzymic assays of antibodies to *S. aureus* antigens identify cases of *S. aureus* bacteraemia with false negative blood cultures due to prior antibiotic treatment [16–18]. Ryding et al. [18] conducted a comprehensive study of ELISAs measuring IgG antibodies against seven *S. aureus* antigens (peptidoglycan, teichoic acid, *S. aureus* ultrasonicate, whole *S. aureus* cells, α-toxin, lipase and capsular polysaccharide) in 129 patients with *S. aureus* bacteraemia, 78 patients with non-*S. aureus* bacteraemia and 100 febrile non-bacteraemic controls. According to their results, whole-cell ELISA was the most sensitive assay, but the specificity of all assays was low. Two different combinations of ELISAs for whole cells, teichoic acid, α-toxin, lipase and capsular polysaccharide did distinguish between *S. aureus* and non-*S. aureus* endocarditis, but not between uncomplicated and complicated *S. aureus* bacteraemia [18]. In this review article, the ELISAs developed in the last decade for diagnosis of CNS and in particular *S. epidermidis* infections are presented.

2. Detection of antibodies against whole bacteria

Determination of antibodies against whole bacterial cells is considered a sensitive way of serodiagnosis [18]. This technique is an easy way to screen large numbers of samples without extracting antigens. An ELISA for determining antibodies to surface antigens of whole bacterial cells in intravenous immunoglobulin (IVIG) preparations and blood sera was developed by our group [19]. Microplates were coated with suspensions of various bacterial species (ca. 8000 cells/ml) (100 μl per well) at 4 °C for 16 h. The bacteria-coated microplates were incubated with various dilutions of IVIG or blood sera. Detection of bound antibodies was performed by peroxidase H-conjugated anti-human IgG. The titre of specific antibodies to certain bacterial surface antigens was defined as the lowest sample concentration giving an absorbance of 0.2 units at 490 nm [19]. The ELISA method showed low intra- (C.V.: 5% for six wells) and inter- (C.V.: 7.5% for three repetitions)

assay variation. It was applied to screening 20 lots from two IVIG preparations for antibodies to frequent clinically isolated strains of *Escherichia coli*, *S. aureus*, *S. epidermidis*, *Klebsiella pneumoniae* and *Enterococci* spp. [20]. The two tested preparations showed significant differences in their content of specific antibodies that ultimately affected the levels of these antibodies in treated immunodeficient patients [20].

An easy and rapid ELISA system, filtration ELISA, to detect antibodies against bacterial cell surface antigens was developed using a 96-well filtration plate fitted with a 0.22 μm membrane (MultiScreen®-GV, Millipore) by Itoh et al. [21]. Alive bacterial cells were used as antigen in the presence of NaN_3; the cell suspensions were applied over the filter of the filtration plate wells. Buffers could be removed by vacuum filtration and the cells resuspended in appropriate buffer. After blocking and inactivating of endogenous cellular peroxidases, cells were incubated with serum or fecal samples. Assay reactions were performed in the wells without losing the solution. Detection of bound antibodies was accomplished by peroxidase-labeled anti-human antibodies and transfer of the solution to transparent 96-well microplates after enzymic action on chromogenic substrate [21].

3. Antibodies against mixtures of *S. epidermidis* slime antigens

Instead of using intact cells, mixtures of slime antigens have been used as probes for the detection of *S. epidermidis*-specific antibodies. The structure of these antigens is not necessarily determined. Ultrasonic extracts of *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, and non-haemolytic streptococci were used by Kjerulf et al. [22] for the detection of antibody responses in sera from normal persons ($n = 275$), patients with various types of bacteraemia ($n = 137$), and patients with endocarditis ($n = 28$). The ELISA was 72% successful in identifying cases of bacterial endocarditis caused by *S. aureus* (diagnostic sensitivity 84%; diagnostic specificity 89%), while in other cases of bacterial endocarditis it was less successful. Moreover, antibody levels against *S. aureus* increased with age, while antibodies against other bacteria showed no age variation. Furthermore, the different ELISA systems showed many serological cross-reactions [22].

Exocellular carbohydrate antigens of *S. epidermidis* were prepared by gel filtration chromatography of concentrated brain heart infusion culture supernatants [23]. The antigenic material appeared as diffuse bands between 24 and 32 kDa on immunoblots. Thirteen of 16 patients with *S. epidermidis* prosthetic joint infection showed an elevated serum IgG level by ELISA as compared to controls with not infected joints. However, the antigen was not specific for *S. epidermidis* bone infection; high levels of IgG were also detected in patients with other serious staphylococcal and streptococcal infections. The authors suggest that the ELISA test might be valuable in distinguishing between staphylococcal infection of joints and aseptic loosening by excluding cases of infection [23].

Selan et al. [24] developed an ELISA to detect serum antibodies against staphylococcal slime polysaccharide antigens (SSPA). The SSPA preparation could detect antibodies in late-onset synthetic vascular graft infection caused by different staphylococcal species. Polystyrene microtitre wells sensitized with 0.5 μg of SSPA/well were blocked with PBS containing 10% calcium-free soymilk. Serum dilutions (1:160 for IgM and 1:320 for IgG) were left to react for 1 h at 37 °C. Antigen-bound immunoglobulins were detected with peroxidase-conjugated antibodies against human IgG or IgM. Antibody titres are expressed as units of optical density [24].

Titers of IgM antibodies against SSPA were higher in patients with an ongoing staphylococcal late-onset synthetic vascular graft infection (LO-SVGI) (*S. aureus*, *S. epidermidis*, CNS other than *S. epidermidis* and mixed infection by one more staphylococcal species plus enterococci, *P. aeruginosa* or *E. coli*) than in controls [24]. An almost complete absence of overlap between the values of the former patients and those of other controls was observed. IgM antibody titres against SSPA of 0.4 ELISA units or more indicated an ongoing staphylococcal late-onset synthetic vascular graft infection with detection rates of 97% and without any false-positive result. This detection rate was increased to 100% and the false-positive result rate to 2% when antibody titres were 0.35 ELISA units or more. The false-positives detected by a threshold titre set at 0.35 ELISA units arose in patients with a late-onset synthetic vascular graft

infection caused by bacteria other than *S. epidermidis*, and were possibly related to a polymicrobic nature of infection, including some staphylococcal component eventually replaced by other bacteria or missed by microbiologic testing. Although the highest IgG antibody titres to SSPA were noted in individuals with an ongoing staphylococcal LO-SVGI, high titres were also seen in controls, which precluded the use of IgG titres for diagnostic purposes.

4. Antibodies against discrete slime antigens

4.1. Antibodies to lipid S

A glycerophosphoglycolipid (termed lipid S) produced by *S. epidermidis* has found application in serodiagnosis of infections due to CNS. Lipid S is an exocellular short chain length form of the lipoteichoic acid which resides in cell wall and membrane [25]. It is composed of six glycerophosphate units linked to a glycolipid, in contrast to 40–42 units in lipoteichoic acid (Fig. 1). Determination of antibodies against lipid S seems to be of value in diagnosis of central-venous and intravascular catheter-associated sepsis, staphylococcal infection around prostheses and staphylococcal endocarditis [26–29]. Microplates are easily coated with lipid S through physical absorption (4 °C for 18 h) and the ELISA detection of antibodies in serum samples proceeded as usual. Sixty-seven patients diagnosed as having central venous catheter-associated sepsis, based on strict clinical criteria including positive blood cultures, were compared to 67 patients with a central venous catheter in situ who exhibited no evidence of sepsis [26]. Patients' sera were titrated and compared with a standardized positive serum sample (IgG titre: 100,000 and IgM titre 25,000). Significant difference ($P < 0.001$) was observed among both the mean IgG and IgM titres of the septic patients and control subjects [26]. When a cut-off of 20,000 was used for the IgG titre, the test had a sensitivity of 75% and a specificity of 90% as compared to diagnosis based on clinical and microbiologic criteria. However, when a cut-off of 5000 was used for the IgM titre, the sensitivity and specificity of the IgM test was 52 and 85%, respectively. Similar results were obtained from the measurement of serum IgG titres in 40 patients with a clinical and microbiologic diagnosis of intravascular catheter-related sepsis by CNS and 40 control patients [27]. The serum IgG titres of septic patients were raised and were significantly higher than the control group ($P < 0.001$). Optimization of assay conditions and titration of sera through comparison with a reference negative control serum increased the assay specificity to 100% [27]. The mean antibody titre in patients with sepsis caused by CNS was 10,429, whereas serum IgG was not detected in the control group of patients.

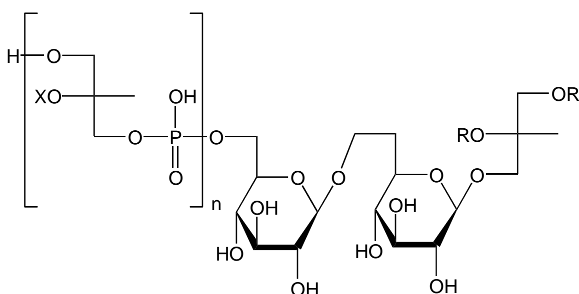

Fig. 1. Proposed structure of lipid S from NCIMB 40896. R: ester-linked fatty acids, X: ester-linked D-alanine or *N*-acetylglucosamine, $n = 6$. Reproduced from Lambert et al. [25].

The value of lipid S as a serological marker of bone infection was also investigated by this research group in 15 patients with culture-proven infection of prostheses caused by gram-positive bacteria [28]. There were significant differences ($P < 0.0001$) between the serum IgG and IgM levels in patients with infection due to staphylococci and those of a control group of 32 patients with no infection. The ELISA test may be valuable in distinguishing between staphylococcal infection around prostheses and aseptic loosening. Furthermore, serum IgG levels to lipid S were significantly elevated in 34 patients with gram-positive bacterial endocarditis confirmed as 'definite' by the Duke criteria as compared to 50 control patients [29]. The test had a sensitivity of 88% and a specificity of 88%. The authors further suggest an algorithm, which incorporates lipid S serology into the positive diagnostic strategy (Fig. 2). The emergence of lipid S as a posible serodiagnostic marker of staphylococcal infections is in contrast to previous studies which have shown limited clinical usefulness of assays of lipoteichoic acid and wall teichoic acid in diagnosis of *S. aureus*, owing to the considerable overlap in the range of antibody concentrations between patient and blood donor sera [30].

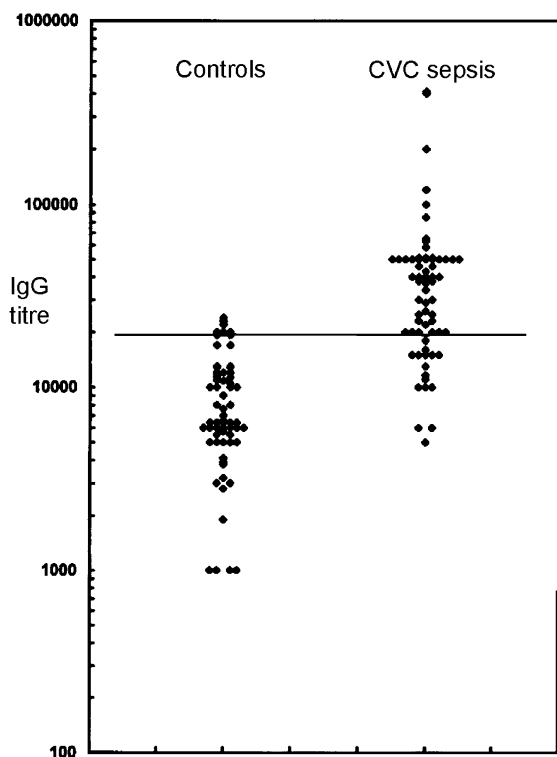

Fig. 2. Scatter plots of the levels of IgG against lipid S from 67 patients with central venous catheter-associated sepsis and control patients. The 20,000 cut-off point for positivity is indicated. Reprinted with permission from reference [24].

4.2. Antibodies to a slime 20 kDa acidic polysaccharide

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 [31]. Particularly, the major polysaccharide component (approximately 60–65% of total carbohydrates) of slime is a sulfated 20 kDa acidic polysaccharide (20 kDa PS) [31,32]. 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 (30% of slime dry weight); (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 [31].

In search of specific markers that could differentiate between slime producing *S. epidermidis* from other staphylococci, antibodies to the major macromolecules as well as crude slime were raised in rabbits and their immunological reactivity and specificity were studied by ELISA [33]. All isolated macromolecules induced the production of specific antibodies. The immune response induced by teichoic acid like 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. However, competitive ELISA experiments showed that 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 [33]. All three antibodies specifically recognise ($P < 0.05$) and react with slime producing *S. epidermidis* in comparison to other staphylococci species. Antibodies react with other staphylococci in the ascending order: *S. haemolyticus*, *S. aureus*, and *S. saprophyticus*. 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 [33].

In our preliminary experiments, the presence of anti-20 kDa PS antibodies in patients' sera was investigated by ELISA [34]. Microplates were easily coated with 20 kDa PS (5 μg/ml) through physical absorption (overnight incubation at 4 °C). These were incubated with serial dilutions of sera from 25 patients obtained 10–15 days after confirmation of slime-producing *S. epidermidis* bacteremia or sera collected from 10 healthy individuals. Bound antibodies were detected by peroxidase-conjugated anti-human antibodies. Obtained results showed the presence of anti-20 kDa PS in healthy individuals and statistically significant increase in patients' sera. Specifically, at an absorbance $A_{492} = 1.0$, the serum dilution in healthy individuals was approximately 1:800, while the respective value in patients was 1:13,000 [34].

The ELISA was optimized in order to determine quantitatively the levels of antibodies against the 20 kDa PS of *S. epidermidis* slime [35]. The content

of antibodies to 20 kDa PS in samples was expressed in relation to rabbit anti-20 kDa PS sera (reference serum). For quantification of the amount of specific antibodies towards the 20 kDa PS, it was assumed that the reference serum has 1000 units/ml. A reference curve was constructed by plotting the optical density at 490 nm versus serial dilutions of reference antiserum in a semi-log graph. Antibody titres in IVIG lots and blood sera were determined by selecting dilutions that generated absorbance values within the linear part of the reference curve. The X-intercept of the absorbance values in units/ml was multiplied by the appropriate dilution factor and the titers were expressed in units/ml of the preparation. As an internal control, a reference curve was created in every microplate using dilutions 1:32,000, 1:16,000 and 1:8000 of the reference anti-20 kDa PS serum, which corresponded to the linear part of the curve [35].

Calculated specific antibody titers in 27 lots of an IVIG preparation ranged from 113.8 to 465.24 units/ml varying from lot-to-lot [35]. The amount of 20 kDa PS specific antibodies correlated well with their in vitro opsonic activity against slime-producing *S. epidermidis*. The majority of lots (75%) having titers higher than 200 units/ml showed significant opsonic activity (50–75%) towards slime-producing *S. epidermidis*. Sandoglobulin® lots with titers higher than 200 units/ml of 20 kDa PS specific IgG were administered as a prophylactic agent to low-birth weight (lower than 1700 g) preterm neonates immediately after birth. The levels of 20 kDa PS specific IgG in neonates' blood sera were significantly higher than those found in the control group, even 10 days after the last infusion. The rate of slime-producing *S. epidermidis* bactereamia in neonates who received IVIG was also considerably lower than those in the control group [35].

Additionally, the protective and therapeutic effects of both active immunization, using 20 kDa PS as antigen, and passive administration of specific antibodies towards the 20 kDa PS was studied in a rabbit keratitis model [36]. Clinical observations were made weekly for 1 month and levels of 20 kDa PS antibodies in serum and aqueous humor in both immunization groups were determined by ELISA. The levels of specific anti-20 kDa PS IgG in serum and aqueous humor following either active or passive immunization were significantly higher as compared to control groups ($P < 0.001$). Although actively immunized rabbits showed significantly less corneal damage than control animals, passively immunized ones were significantly better protected as compared to both control and actively immunized animals [36].

5. Detection of *Staphylococcus cells*

In search for a method to detect slime-positive *S. epidermidis* strains, a polyclonal rabbit anti-serum against the slime-producing *S. epidermidis* strain RP62A was absorbed with the slime-negative phase variant of this strain, PV1, in order to remove non slime-specific antibodies [37]. The ELISA showed high absorbance when extracts from slime-positive strains (confirmed in the tissue culture tube test) were used as antigens. The high absorbance of slime-positive strains was greatly reduced by periodate oxidation of the extracts and was resistant to proteinase digestion suggesting that the detected antigen is composed of polysaccharides. These antibodies were used for the development of an ELISA for detection of *S. epidermidis* slime in the presence of human serum [37].

The use of anti-20 kDa PS serum to differentiate between slime-positive and slime-negative strains of *S. epidermidis* has been studied [34]. Whole cells of 25 slime-positive and 10 slime-negative *S. epidermidis* clinical isolates, by the method of Christensen et al. [38], were coated on microtitre plates at a dilution 1:10 of an initial suspension of 250,000 cells/ml. Further incubations with rabbit anti-20 kDa PS serum and goat anti-rabbit IgG showed that the absorbance at 490 nm of all slime-producing strains was within the absorbance range of 0.75–1.10, while the respective value for the slime-negative strains ranged from 0.22 to 0.30 (Fig. 3) [34]. Among the different staphylococci species tested, anti-20 kDa PS-antibodies bound to *S. epidermidis* at a statistically significant ($P < 0.05$) degree as compared to *S. scleiferi*, *S. saprophyticus*, *S. aureus*, and *S. haemolyticus*. These results demonstrated that antibodies to 20 kDa PS can be used for specific recognition of slime-producing *S. epidermidis* strains among staphylococci species [34].

The potential of ELISA for detection of staphylococci in milk was studied by Yazdankhah et al. [39]. *Staphylococcus* spp. are the single most important

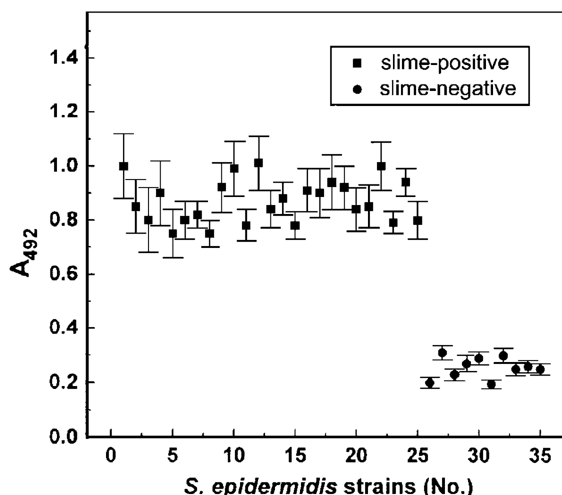

Fig. 3. Reactivity of clinical strains of *S. epidermidis* with anti-20 kDa PS serum. Bacterial cells were coated at a dilution 1:10 in carbonate buffer, pH 9.6, and incubated with anti-20 kDa PS (1:200). 25 slime-producing *S. epidermidis* strains (■) and 10 slime-negative strains (●) were studied. Every point of the plot represents the average value ± S.D. of three experiments in triplicate. Reprinted with permission from reference [34].

cause of mastitis in dairy cows. Monoclonal antibodies (5G3) were raised against whole cells of *S. aureus* that could bind not only to *S. aureus* (100%) but also to 14/16 *Staphylococcus* spp. tested (*S. epidermidis*, *S. hyicus*, *S. simulans*, *S. cohni*, *S. capitis*, *S. caprae*, *S. hominis*, *S. intermediu* and *S. haemolyticus*), while they did not react with other bacterial strains. Monoclonal antibodies recognized both intact staphylococcal cells and soluble antigens. Pretreatment of samples with lysostaphin increased the sensitivity of the assay since it increased the number of target antigens. Samples (1 ml) were incubated for 30 min in the presence of monoclonal antibody-coated tosylactivated magnetic beads (5 μg antibody/mg beads). Detection of captured antigens was performed by monoclonal antibodies conjugated with alkaline phosphatase. The effective test time for magnetic bead-based ELISA was about 3 h and the detection limit of the assay was shown to be 10^4–10^5 cells/ml [39]. The use of this assay for routine screening of milk samples was suggested because, apart from the short analysis time, staphylococcal infection can be diagnosed even in the absence of cells by recognition of released staphylococcal soluble antigens.

The binding ability of lectins to carbohydrates has been also used for detection and typing of staphylococci. Four biotinylated lectins (wheat germ agglutinin, soy bean agglutinin, lentil agglutinin and concanavalin A) were used to detect immobilised whole cells of CNS in microtitration plates [40]. The amount of bound lectin was measured by peroxidase-conjugated avidin. The method was compared to other typing methods, such as antibiotic-resistance analysis, phage typing, plasmid DNA profile and slime production. Seventy-one strains of CNS, including 64 strains of *S. epidermidis*, out of a total of 113 isolates were detected if all typing methods were taken into consideration. If only one typing method was used, the highest discriminatory power among *S. epidermidis* isolates was obtained by the lectin-binding assay since it 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 [40]. Phosphatase-labeled wheat germ agglutinin has been also used for quantification of in situ biofilm produced by *S. epidermidis* in polystyrene 96-well tissue culture plates with potential applications in screening strains for their capacity to adhere to plastic, produce slime, and form biofilm [41].

6. Future perspectives

Application of ELISAs in the routine diagnosis of CNS infections has not yet been realised although it is desirable due to the rapid analysis time and ease of performance. Studies conducted in the last years reveal that ELISA diagnosis of staphylococcal infections is feasible but larger and well-organised clinical studies are necessary. Two research directions have been utilised; detection of antibodies against whole cells, mixtures of antigens or distinct antigens, and detection of *S. epidermidis* cells. Applications in *S. epidermidis* typing have been also described. Specific diagnosis of *S. epidermidis* infections is even more difficult due to the fact that many virulence factors or antigenic determinants are transferred from one staphylococcal species to the other.

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