Characterization of soluble antigens of *Staphylococcus spp* isolated from dairy farms in Venezuela

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Abstract

The genus *Staphylococcus* contains many pathogenic strains that are difficult to differentiate. Given the absence of a specific immunological test to identify autochthonous species, we have characterized soluble antigens (SAGs) using hyperimmune sera from BABL/c mice. Ten samples were taken from the farmers' hands and cattle udders on three different farms. The isolated species were identified using the API kit (Staph) and their ability to form biofilms was determined. The species most commonly found in the isolates (90%) corresponded to the coagulase-negative bacteria and *Staphylococcus sciuri* (*S. sciuri*), which presented the ability of biofilm formation, representing the majority (60%) in this group. We produced SAGs from those *Staphylococcus* species present in a higher frequency, such as *S. sciuri*, *S. aureus*, and the reference strain, *S. aureus* ATCC 6835. Polyclonal antibodies (PAb) from mice allowed SAGs characterization by enzyme-linked immunoassay (ELISA) and immunoblotting. The humoral response obtained with the PAb by indirect ELISA tests indicated that our hyperimmune sera have a high recognition for all SAGs produced. We also evaluated the hyperimmune sera cross-reactivity between different SAGs by indirect ELISA and immunoblotting assays. The ELISA experiments showed a significant statistical difference in the recognition of *S. sciuri* when compared to SAGs from *S. aureus*. These results showed a high antigenicity and specificity from *S. sciuri* SAGs in immune tests. We identified a specific immunodominant polypeptide of ~31 kDa (p31) from *S. sciuri* SAG, which do not presented cross-reactivity between different SAGs. We concluded that the p31 polypeptide from *S. sciuri* SAG could be used as antigen in a differential diagnosis test for different staphylococcal species

Introduction

Staphylococci are widely distributed in nature and can be found in the mucus from different animals, foodstuffs, such as meat, milk and cheese, and in the environment, soil, air and water. Some species of staphylococci may present some risks for humans being responsible for pathologies such as peritonitis, endocarditis and infections of the urinary tract.¹⁻³ In addition, pathogenic species belonging to the staphylococci genus can cause clinical and subclinical mastitis in dairy cattle.¹ Based on their ability to produce the enzyme coagulase, these microorganisms can be classified into coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci. Among the CPS staphylococci, the most pathogenic species is *Staphylococcus aureus* (*S. aureus*), which is currently considered an emerging pathogen in cases of subclinical mastitis in dairy cows.² Given the absence of a specific immunological test for autochthonous species from Venezuela, we characterized a *S. sciuri* soluble antigen (SAG) as a potential antigen for staphylococcus differential identification. A total of 10 isolates were taken from farm workers' hands and cattle udders on three different farms in Guárico State. The isolate species were identified using the API kit (Staph). We produced SAGs from *Staphylococcus* species present in higher frequency, where *S. sciuri* was the most common. In order to characterize these antigens, polyclonal antibodies (PAb) were produced in BALB/c mice. SAGs were evaluated by enzyme-linked immunoassay (ELISA) and immunoblotting. Since biofilm formation is a characteristic virulence factor, the ability of *S. sciuri* isolates to form biofilms was also evaluated.^{3,4} Our results showed a high antigenicity and good specificity for *S. sciuri* SAGs in ELISA tests, suggesting the use of this antigen in a differential diagnosis for different CNS species.

Materials and Methods

Staphylococcus isolates

The study consisted of 10 staphylococcal isolates from the hands and noses of farm workers and cattle udders on three different farms in the north-central state of Guárico, Venezuela. Samples were collected from the palm of the hands and anterior region of the nose of the operators and from cattle udders using a moist swab (Nitrogen) and transported to the laboratory under sterile conditions. The swabs were planted on mannitol salt agar plates (Difco). Typical colonies of *Staphylococcus spp* were selected and transferred to the

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Universidad Simón Bolívar using sterile swabs (Nitrogen). The swabs were streaked on Trypticase soy agar plates (Difco) immediately after collection. These plates were incubated at 37°C for 48 h and each isolate was grown to check for purity and identified by a Gram staining and catalase test using 30% hydrogen peroxide.⁵ *Staphylococcus* identification to species level was performed by the commercial API Staph® method (BioMérieux, France) according to the manufacturer's instructions, and included the reference strain *S. aureus* ATCC 6538. Also, oxidase activity was determined by a commercial oxidase diagnostic kit (BBL DrySlide) and Staphylocoagulase (free coagulase) activity was determined by using rabbit plasma and the tube method.⁶

Soluble antigen preparation

Each isolate was grown in *Luria Bertani* broth in sterile tubes. The tubes were incubated at 37°C in continuous shaking for 48 h. Bacterial cultures were harvested by centrifugation at 3000 g for 10 min at 4°C in a centrifuge (Beckmann, model J221M) and then the pellet was washed three times with phosphate buffered saline (PBS) (8% NaCl, 0.2% KCl, 0.93% Na₂HPO₄, 0.2% KH₂PO₄), pH 7.0.

The supernatant from each isolate was discarded and the pellets were resuspended in 10 mL of PBS. To each pellet of bacteria previously isolated, we added a cocktail of protease inhibitors (1 mM iodoacetamine, 1 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetraacetic acid, 1 mM benzamide) and 3 M urea in 5 mL of PBS. Bacterial cell suspensions were vigorously pipetted to break up aggregates and samples were placed on ice and subjected to sonication for 3 min using an ultrasonic probe (Ultrasonics Ltd., USA) emitting 20 W. This procedure was performed five times and between each pulse, the samples were subjected to freezing and thawing using liquid nitrogen. Finally, the suspensions were centrifuged at 3000 g for 30 min at 4°C, the supernatants were collected as Sags, and their protein concentrations were determined using the Bradford method.⁷ Sags were stored at -20°C until use.

Hyperimmune sera and ascites fluid

Ten week-old female BALB/c mice bred under specific-pathogen-free conditions were used in groups of 6. The first inoculations of SAgS were at 100 µg/µL with 100 µL of Freund's Complete Adjuvant (FCA) per mouse; subsequently, four inoculations were prepared in the same way but with Freund's Incomplete Adjuvant (FIA). All these inoculations were administered by intraperitoneal injection. Another group of 10 healthy mice were used as control. Water and food were allowed *ad libitum*. After the last inoculation, animals were sacrificed under deep anesthesia with ether and exsanguinated by cardiac puncture to collect the serum. In order to obtain antibodies against relevant immunogenic proteins, the most frequently reactive protein, recognized by SAgS serum in immunoblots (see below), was selected to obtain ascitic fluid. This protein was isolated from the nitrocellulose paper, which was pulverized in a mortar and used as antigen after adding 5% dimethylsulfoxide to the solution. A group of mice were then inoculated intraperitoneally with this antigen in FCA, four times. The ascitic fluid (AF) was collected and polyclonal antibodies (PA) were purified by ammonium sulfate as indicated in the protocol by Harlow and Lane.⁸

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SAGs were analyzed by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) on 12.5% (w/v) polyacrylamide gels.⁹ Protein samples were solubilized in SDS sample buffer (0.15M Tris-Cl, pH 6.8, 4.6% SDS, 23% glycerol, and 0.2 M dithiothreitol (DTT) and 0.1% w/v bromophenol blue) and heated at 100°C for 5 min. Protein samples, 10 µg per track, were then loaded onto a SDS-PAGE gel; the same concentration of protein was loaded for each isolate. The SDS-PAGE gel consisted of a separating gel (12.5% (w/v) acrylamide/bis-acrylamide, 0.37 M Tris-Cl, pH 8.8, 0.1% (w/v) SDS, set by the addition of 0.3% w/v TEMED, 0.03% (w/v) ammonium persulfate) with a 3% stacking gel (3% w/v acrylamide/bis-acrylamide, 0.1 M Tris-Cl pH 6.8, 0.1% (w/v) SDS, plus TEMED and 0.05% w/v ammonium persulfate). Electrophoresis was carried out at 120 V for 1 h using a Mighty Small II vertical slab gel unit (Bio Rad Instruments). Wide molecular mass markers from Invitrogen (18.3 kDa to 109.5 kDa) were used. After electrophoresis, proteins were revealed with silver staining.⁸

Immunoblotting

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membrane (NCP, Schleicher and Schuell, 0.45 µm pore size) to allow immunodetection of the proteins by the antibodies in the sera. The proteins were transferred at 120 mA in a transblotting chamber (Bio-Rad, Instruments), for 1 h at 4°C, using 25 mM Tris-HCl, 150 mM glycine, and 20% (v/v) methanol.¹⁰ After transfer, blots were blocked by incubation with a solution of

3% (w/v) non-fat milk in PBS for 30 min at room temperature, and washed three times (3x) in PBS/T, containing 0.05% (v/v) Tween-20. Blots were then incubated for 1 h at room temperature with a solution of primary antibody (hyperimmune serum) diluted 1:200 in PBS, washed three times and incubated in a solution of affinity purified goat antimouse immunoglobulin conjugated to horseradish peroxidase, at a 1:2000 dilution (Sigma), for 1 h more. The blots were washed again three times and antibody binding was detected by incubation in a solution of 3 mg/mL 4-chloro-1-naphthol in methanol, mixed with 50 mL of 50 mM Tris-HCl pH 7.5, and 30 L of 30 % H₂O₂. The color reaction was stopped by washing with H₂O.

Enzyme-linked immunosorbent assay

The indirect ELISA test was used to detect specific antibodies present in mouse sera against SAGs. Ninety-six well plates (Immulon 4 from Dynatech) were coated with 100 µL per well of SAGs solution, using 1, 5, 10 and 20 µg/mL of the soluble antigen solution diluted in coating buffer (0.1 M sodium carbonate/bicarbonate pH 9.6). Plates were incubated overnight at 4°C and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS/T). Plates were then blocked by the addition of PBS containing 1% (v/v) bovine serum albumin for 30 min at room temperature, followed by further washing with PBS (3x). One hundred µL of serum diluted 1:200 were added to each well and the plate was incubated at 37°C for 1 h. Then, plates were washed three times with PBS and 100 µL/well of horseradish

Figure 1. Biofilm formation phenotype. A) This figure shows the formation of biofilm at the air-medium interface, monitored over a 24 h period for *S. sciuri*. B) Absorbances from crystal violet-stained, surface-attached cells. Dye was solubilized in ethanol and absorbances were read at 595 nm, after 24 h. These values represent the median from six individual experiments. *Absorbance exceeds the threshold limit values. Control used was the *Pseudomonas aeruginosa* strain, which forms biofilm and the other strains correspond to seven different isolates from *S. sciuri* (S.s) and *Staphylococcus aureus* ATCC 6538 (S. a).

peroxidase-conjugated goat anti-mouse polyvalent immunoglobulin (IgG; H+L) (Sigma) were added at 1:1000 and 1:2000 dilutions. After incubation for 1 h at 37°C the plates were washed again with PBS. The bound conjugated antibody was detected by the addition of 100 µL of azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, Sigma) as substrate, pH 5.0 containing 0.05% (w/v) H₂O₂. The reaction was stopped by the addition of 50 µL 2M H₂SO₄ per well. The plates were read in a microplate reader (Bio-RAD i-Mark), using Titer-software (Flow) to measure the absorbance at 405 nm.⁸ Ten normal mice sera were used as a negative control. The conditions of the indirect ELISA test were determined using different concentrations of antigens and conjugates, and conditions were selected that showed significant differences between NS and hyperimmune sera using different SAGs.

Biofilm formation assay

Screening for biofilm formation in *S. sciuri* isolates was also evaluated. This assay is based on the ability of the bacteria to form extracellular polysaccharides on polyvinylchloride microplate wells. The biofilm was detected by staining with crystal violet, solubilizing the dye in ethanol and determining the absorbance at 595 nm.¹¹ *S. sciuri* was assessed for biofilm formation as a potential virulence factor and the bacterium *Pseudomonas aeruginosa* (*P. a*) was used as positive control.^{3,12} All tests were carried out three times.

Statistical analysis

Each experimental value is presented as the mean of six replicates ± standard deviation. Once normality and homogeneity criteria were satisfied, statistical analyses were carried out by one-way analysis of variance; α=5% (P<0.05) was considered significant.

Results

In this study, 10 isolates from three farms from Zaraza, Guarico State were identified using commercially available API *Staph* tests and other complementary tests, such as, Gram staining, coagulase, catalase, hemolysis, and oxidase tests (*data not shown*). These results are summarized in Table 1. The most frequently identified isolate was *S. sciuri*, (6 out of the 10 isolates) and the remaining isolates were: *S. hominis* (2 out of 10 isolates), *S. epidermidis* and *S. aureus* (1 out of 10). Figure 1 shows the biofilm formation of the most frequently isolated species which was *S. sciuri*. Panel A shows the biofilm formation as a potential of this sample monitored over a 24 h period. Panel B shows the

absorbance at 595 nm from the crystal violet-stained isolates and *S. aureus* ATCC 6538, which was used as a reference. The control test was *P. aeruginosa*, which produced an absorbance over 3 units, similar to S.s 4 isolate. Isolates S.s 5, S.s 6, and S.s. 7, showed absorbances between 1.5 to 2.5, and the isolates S.s 1, S.s. 2 and S.s 3 isolates were under 1 unit of absorbance. These isolates (S.s 1,2 and 3) did not produce a biofilm (*data not shown*). Considering these results, the isolate S.s. 4 was disrupted by sonication to prepare soluble antigens. The SAGs polypeptide profiles from the *S. sciuri* isolates were analyzed by SDS-PAGE. After silver staining, several polypeptides were observed and the SAG from *S. sciuri* and *S. aureus* species gave characteristic and distinct polypeptide profiles (Figure 2).

The hyperimmune sera from inoculated

Figure 2. Soluble antigens polypeptide profiles from *S. sciuri* (S.s) and *S. aureus* (S.a) isolates prepared with protease inhibitors after SDS-PAGE (12.5%) and silver staining. Molecular mass standards (KDa) are indicated at the right. The arrow shows the ~31 KDa polypeptide.

Figure 3. Hyperimmune sera immunoblots showing the IgG responses produced after six weeks of inoculation with soluble antigens isolates. Hyperimmune serum from *S. sciuri* recognizing the *S. sciuri* soluble antigen (Line 1). Hyperimmune serum from *S. sciuri* recognized the soluble antigens *S. aureus* ATCC 6538 (Line 2). Hyperimmune serum from *S. aureus* recognizing the *S. sciuri* soluble antigen (Line 3), and hyperimmune serum from *S. aureus* ATCC 6538 recognizing the *S. aureus* ATCC 6538 soluble antigen (Line 4). Molecular mass standards (KDa) are indicated at the right. The p31 is indicated with an arrow.

Table 1. Biochemical tests applied to *Staphylococcus* strains isolated from three cattle farms located in Zaraza city, Guarico State, Venezuela.

Species	Sample origin	No. of isolates	Coagulase test	Catalase test	Hemolysis test	Oxidase test
<i>S. sciuri</i>	Udder and tail of cattle Nose of worker Milk	6	-	+	γ -hemolysis	+
<i>S. epidermidis</i>	Hands of worker	1	-	+	γ -hemolysis	-
<i>S. hominis</i>	Hands of worker	2	-	+	γ -hemolysis	-
<i>S. aureus</i>	Udder	1	+	+	β -hemolysis	-
<i>Staphylococcus aureus</i> ATCC 6538	Reference		+	+	β -hemolysis	

Species were identified using the commercial Kit API *Staph* (BioMerieux, Marcy-l'Etoile, France). In addition, we used other tests such as coagulase, oxidase and catalase to characterize the isolates. The most frequent species isolated in animals and workers was *S. sciuri*.

mice produced after six weeks of immunization with different SAGs were evaluated by immunoblot to demonstrate the IgG responses. In addition, the cross-reactivity between the different antigens was evaluated by developing the blots with heterologous hyperimmune sera (Figure 3).

Hyperimmune serum from *S. sciuri* recognized three polypeptides of 50, 40 and 31 kDa in *S. sciuri* SAG, with the highest recognition for the 31 kDa polypeptide (line 1). In the same way, the serum from SAG S.a ATCC 6538 immunized mice recognized five polypeptides in the homologous antigen (line 4). The hyperimmune serum from S.s recognized two bands in *S. aureus* ATCC 6538 SAG (line 2) and hyperimmune serum from *S. aureus* ATCC 6538 recognized four bands in *S. sciuri* SAG (line 3). The intensity of the cross-reaction in immunoblotting was very faint. The most immune dominant polypeptide from SAG *S. sciuri* detected by immunoblot was p31.

The optimal conditions obtained from ELISA standardization were: 10 g/mL for all SAGs and a 1:2000 dilution of conjugated antibodies; these conditions were used in all ELISA tests. Figure 4 shows the ELISA test results from *S. sciuri* SAGs and antibodies from the different sources. The titers of AF, PA and hyperimmune sera were determined down to a 1:12800 antibody serial dilution to establish the antigenicity. All the IgGs recognized the *S. sciuri* SAG and showed high titers in all cases, especially the IgG from S.s hyperimmune serum with an absorbance approximately 4-6 times higher than that of NS. Indirect ELISA was used to determine the specificity of antibodies from different sources to each SAG and the cross-reaction between the sera (Figure 5). In this experiment, there was a significant recognition of the S.s SAG by the antibody against p31 from AF when compared to antigens from S.a and S.a ATCC 3568. Purified antibodies showed lower absorbance values (Figure 5) that could be due to a low yield from the purification technique. The recognition of S.s SAG by its own antibodies is specific (Figure 5). The results with S.a SAG showed a cross-reaction with all the sera (Figure 5), but recognition was always lower when the hyperimmune sera from S.a were tested against other antigens.

Discussion

We have isolated 10 *Staphylococcus* strains from farmers' hands and cattle udders, and identified them by biochemical tests as shown in Table 1. These results showed a high prevalence of CNS bacteria such as *S. sciuri*, followed by *S. hominis* and *S. aureus*. Thorberg and co-workers studied the ability of different

CNS species in dairy herds to induce a persistent infection of subclinical mastitis.¹³ They showed that *S. epidermidis* was mainly found in multiparous cows and *S. chromogenes* in primiparous cows. Also, they reported that *S. sciuri* is one of the most common findings in udder, which agrees with our results. In addition, CNS bacteria have been considered as a group of emerging pathogens that are responsible for subclinical mastitis in some countries.^{2,13,14} Many pathogenic strains have the capacity to form biofilms so this characteristic is considered a potential virulence factor. Despite the fact that *S. sciuri* has never been reported as an etiological infectious agent, our results showed the ability of this species to form biofilm (Figure 1). This property could

allow *S. sciuri* to adhere to and colonize the mammary gland.^{4,15,16} *S. sciuri* has been isolated from central venous catheters in humans and this species produced biofilms, suggesting that *S. sciuri* could be involved in human infections.¹⁷ These results, together with the low milk production of the cattle reported by farmers, could indicate in the present study a correlation with subclinical mastitis. Nevertheless, Oliver and colleagues investigated the invasive potential of biofilm-formation *Staphylococci* from bovine subclinical mastitis isolates and compared them with primary cell cultures with fluorescent rRNA probes.¹² They found that biofilm formation does not influence the invasion capacity of *S. aureus* in mastitis. Although biofilm formation is not fully implicated as a

Figure 4. Titers for hyperimmune sera bound to *S. sciuri* soluble antigens (10 ug/mL) in enzyme-linked immunoassay test. Bars represent the absorbance read at 405 nm and the discontinuous line the cut off value for negative sera (0,117 D.O). NS, negative serum; AF, ascitic fluid; PA, antibody against p31 purified by ammonium sulfate precipitation; S. Ss, serum from *S. sciuri* soluble antigen.

Figure 5. Evaluation of soluble antigens from *Staphylococcus* strains from isolates and reference strain *S. aureus* ATCC 6538 by indirect enzyme-linked immunoassay using different sources of polyclonal antibodies. Bars represent the absorbance read at 405 nm and the discontinuous line the cut off value for negative sera. AF, ascitic fluid from p31; PA, purified antibodies by ammonium sulfate precipitation; S. Sa ATCC 6538, serum from *S. aureus* ATCC 6538; S. Sa, serum from *S. aureus*; S.Ss, serum from *S. sciuri*; NS, negative serum; SAG, soluble antigen.

pathogenic characteristic, it could be an important factor in allowing bacteria to adhere and colonize the mammary gland. This study showed that SAg preparations from different isolates induced high antibody responses in ELISA and immunoblotting tests. We isolated several *S. sciuri* and the S.s 4 isolate was chosen to evaluate the humoral response in experimental BALB/c mice. Our results demonstrated that SAgS from isolates were good immunogens because they were able to activate the mice's humoral response and produced hyperimmune sera that could be used to characterize the different isolates. As shown in Figures 2 and 3, the SAg preparations demonstrated different protein patterns for *S. aureus* and *S. sciuri*, a *S. sciuri* polypeptide of about 31 kDa that could be observed with silver staining (Figure 2). Immunoblot analyses using SAg hyperimmune sera against *S. sciuri* SAg strongly recognized a polypeptide of ~31 kDa (p31) which did not show a cross reaction with *S. aureus* ATCC 6538 SAgS (Figure 3). In the same way, hyperimmune sera from *S. aureus* also recognized a polypeptide of ~28 kDa that was not recognized in *S. sciuri* SAg. This polypeptide could be observed in the silver stained gel (Figure 2). We obtained polyclonal antibodies against p31 and demonstrated that this polypeptide was a good antigen for immunodiagnosis and could be used as a differential diagnostic tool. Previous studies reported the use of whole-cell SDS-PAGE band patterns in the identification of Staphylococcal species.^{18,19} However, Sacilik and colleagues analyzed different CNS species by SDS-PAGE and concluded that the polypeptides patterns obtained from whole-cell preparations were difficult to interpret.²⁰

Conclusions

This is the first study to report using soluble antigens to assess their potential in a *Staphylococcus* differential diagnosis. Our results suggest that, in Venezuela, an ELISA test based on the p31 molecule could be used as a differential diagnostic test between staphylococcal species. Future research should

be orientated toward purifying the p31 polypeptide in order to use it as a specific antigen for ELISA tests or to producing a monoclonal antibody for capture ELISA. Each geographical region should study its own isolates of staphylococcal species in order to obtain the best antigen to identify their native species.

References

- Pitkälä A, Haveri M, Pyörälä S, et al. Bovine mastitis in Finland 2001 - prevalence, distribution of bacteria, and antimicrobial resistance. *J Dairy Sci* 2004;87: 2433-41.
- Taponen S, Pyörälä S. Coagulase-negative staphylococci as cause of bovine mastitis. Not so different from *Staphylococcus aureus*? *Vet Microbiol* 2009;134:29-36.
- Hensen SM, Pavičić MJ, Lohuis JA, et al. Location of *Staphylococcus aureus* within the experimentally infected bovine udder and the expression of capsular polysaccharide type 5 in situ. *J Dairy Sci* 2000;83: 1966-75.
- Almeida RA, Oliver SP. Interaction of coagulase-negative *Staphylococcus* species with bovine mammary epithelial cells. *Microb Pathog* 2001;31:205-12.
- Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates. A quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 1985;22:996-1006.
- Mulder JG, Degener JE. Slime-producing properties of coagulase-negative staphylococci isolated from blood cultures. *Clin Microbiol Infect* 1988;4:689-94.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976;72:248-354.
- Harlow E, Lane D. *Antibodies: a laboratory manual*. New York: Cold Spring Harbor Laboratory; 1988.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350-4.
- O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 1998;23:449-61.
- Oliveira M, Bexiga R, Nunes SF, et al. Invasive potential of biofilm-forming *Staphylococci* bovine subclinical mastitis isolates. *J Vet Sci* 2011;12:95-7.
- Thoberg BM, Danielsson-Tham ML, Emanuelson U, et al. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J Dairy Sci* 2009;92:4962-70.
- Pyörälä S, Tapponen S. Coagulase-negative staphylococci. Emerging mastitis pathogens. *Vet Microbiol* 2009;134:3-8.
- Davison TJ, Dohoo IR, Donald AW, et al. A cohort study of coagulase negative staphylococcal mastitis in selected dairy herds in Prince Edward Island. *Can J Vet Res* 1992; 56:275-80.
- Hensen SM, Pavičić MJ, Lohuis JA, et al. Use of bovine primary mammary epithelial cells for the comparison of adherence and invasion ability of *Staphylococcus aureus* strains. *J Dairy Sci* 2000; 83:418-29.
- Crump JA, Collignon PJ. Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* 2000;19:1-8.
- Clink J, Pennington TH. Staphylococcal whole-cell polypeptide analysis: evaluation as a taxonomic and typing tool. *J Med Microbiol* 1987;23:41-4.
- Pennington TH, Harker C, Thomson-Carter F. Identification of coagulase-negative staphylococci by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and rRNA restriction patterns. *J Clin Microbiol* 1991;29:390-2.
- Sacilik SC, Osmanaoglu Ö, Sayar AH, et al. Availability of use of total extracellular proteins in SDS-PAGE for typing *Staphylococcus aureus* and coagulase-negative *Staphylococci*. *Turk J Biol* 2001;25: 145-51.