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Short communication:

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Serological proteome analysis of *Staphylococcus aureus* isolated

4

from subclinical mastitis

5

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16 Short Title: *SERPA of S.aureus*

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21

22 **Abstract**

23 *Staphylococcus aureus* is the most common aetiologic agent of contagious bovine
24 mastitis. Studies of the molecular epidemiology of *S. aureus* strongly suggest that
25 some genetic subsets of strains are particularly well adapted for causing infections
26 in cattle.

27 This communication reports the set up of experimental protocols to identify the
28 immunogenic proteins expressed by one of the most common field isolated strain
29 of *S. aureus* responsible for sub-clinical mastitis cases. The serological proteome
30 analysis (SERPA) approach applied consists of three main steps: two dimensional
31 electrophoresis-based separation of the proteins contained in field-isolated *S.*
32 *aureus* extracts enriched for surface proteins, detection of immunogenic spots
33 using anti-serum collected from sub-clinical mastitis cases and identification of
34 antigens by mass spectrometric-based methodologies.

35 The study allowed to identify three immunogenic proteins: DNAase translocase
36 FtsK, ribosomal proteins S1 and a Tell-like protein.

37

38 **Keywords:** *Staphylococcus aureus*, *mastitis*, *proteomics*, *serological proteome*
39 *analysis*, *MALDI-TOF*

40

41 **1. Introduction**

42 *Staphylococcus aureus* is associated with human, animal and foodborne
43 diseases (Balaban and Rasooly, 2000; Honeyman et al., 2001). Moreover, it is the
44 most common aetiologic agent of contagious bovine mastitis, a disease that causes

45 relevant worldwide losses in the dairy industry (Zecconi et al., 2006). *S. aureus*
46 expresses a wide array of surface-associated virulence factors (Foster, 2005), and
47 secretes various exotoxins (Rainard et al., 2003; Schuberth et al., 2001; von Eiff et
48 al., 2004). Therefore, it has been suggested that effective vaccines should be
49 comprised of antigens present in most of the strains and inducing high immune
50 response (Vytvytska et al., 2002).

51 Proteomics includes the study of proteins expressed by an organism,
52 specifically the amount of particular proteins expressed and the potential
53 interactions can be evaluated (Nilsson, 2002).. When combined with an
54 immunological read-out (immunoproteomics), it is a valuable tool for developing
55 molecularly defined vaccines (Klade, 2002; Kudva et al., 2006; Pedersen et al.,
56 2005). We recently set up experimental protocols to obtain reproducible two
57 dimensional electrophoresis maps of cell wall associated proteins and to identify
58 the most abundant proteins by mass spectrometric analysis (Taverna et al., 2007).
59 The aim of this study was to apply the experimental conditions and information
60 obtained from our previous research to the detection of immunogenic proteins
61 from a *S. aureus* strain responsible for subclinical mastitis in cows by
62 SERological Proteome Analysis (SERPA).

63

64 2. Materials and Methods

65 2.1 Bacterial strains and growth condition

66 *Staphylococcus aureus* strain 1673 was isolated from a case of bovine subclinical
67 mastitis. The strain is genetically classified within the cluster (V) which has been

68 recovered in most of the herds, as observed in a previous molecular
69 epidemiological study (Zecconi et al., 2005). Identification as *S.aureus* was
70 performed according to the following scheme: catalase positive, Gram-positive
71 cocci, haemolytic on blood agar, coagulase positive in 4-18 h. The presumptive
72 identification was confirmed by API ID32 Staph (99.9%.T=0.63) (BioMerieux,
73 Marcy L'Etoile, France). The genetic characteristics of the isolate are: coagulase
74 positive, collagen binding protein negative, fibrinogen binding protein positive,
75 protein A positive (9 repetitions) and enterotoxin G and I positive.

76 The isolates were stored at -20°C in BHI broth with 15% (v/v) glycerol. Before
77 proteomic analysis, the isolate was thawed and cultured on blood agar plate with
78 5% bovine blood for 18h, washed, suspended in physiological saline and
79 harvested by centrifuging at 12000 x g for 30 min at 4 °C.

80

81 2.2 2-Dimensional Electrophoresis

82 Surface associated proteins were prepared by lysostaphin treatment under isotonic
83 conditions and reduced and alkylated as described in (Taverna et al., 2007).
84 Following precipitation in an anhydrous solution of acetone and methanol (8:1
85 v/v) at -20 °C, the precipitate was resuspended in the 2-D PAGE sample buffer (7
86 M urea, 2 M thiourea, 4% CHAPS, 0.5% Resolyte 4-8, bromophenol blue) and
87 sonicated for 1 min. For the first dimension, 1000 µg (preparative 2-DE) or 250
88 µg (analytical 2-DE) of protein were applied to rehydrated IPG strips (Immobiline
89 Dry strip pH 4-7, 180 mm; GE Healthcare, Cologno Monzese, Italy) by sample
90 cups. IEF was carried out at 14 °C, 74650 V total voltage, for 23 h. with the

91 Multiphor II system (GE Healthcare, Cologno Monzese, Italy). The second
92 dimension was performed on home-made 9-16% polyacrylamide linear gradient
93 gels (170 x 190 x 1.5 mm), at 40 mA/gel constant current (Protean II, BioRad,
94 Segrate, Italy).

95 Following 2-DE gels were either stained with ammoniacal silver (analytical 2-
96 DE), or with colloidal blue CBB G (Sigma, Milano, Italy) (preparative 2-DE to be
97 used for mass spectrometry based protein identification) or blotted. For blotting,
98 gels were splitted in two parts (A and B of Fig. 1) due to their large dimensions
99 and each part blotted separately onto a nitrocellulose membrane (Hybond, GE
100 Healthcare, Cologno Monzese, Italy) at 20 mA over night at 4 °C, using a trans-
101 blot apparatus (BioRad, Segrate, Italy). Blotted membranes were immunodetected
102 as follows. Prior to the addition of foetal or infected animal anti-serum, the
103 membrane was saturated in 5% non-fat dry milk in Phosphate Buffered Saline pH
104 7.5 with 0.1% Tween 20 (PBS-T), at room temperature, for 1 hour. The
105 membrane was washed 2 x 15 min and 1 x 5 min in PBS-T 0.1%. Primary
106 antibody incubation was performed for 2 hours, at room temperature, in a solution
107 of 5% non-fat dry milk in PBS-T 0.1% with 1:20000 serum collected from
108 animals infected with *S. aureus* or foetal serum. After washing with TBS-T 0.1%
109 the membrane was incubated with horseradish peroxidase-linked anti-bovine IgG
110 (whole molecule) (Sigma, Milano, Italy) diluted 1:30000, for 1 hour in 5% non-fat
111 dry milk in PBS-T 0.2%. After washing the reaction was developed using
112 enhanced chemiluminescence system (ECL, GE Healthcare, Cologno Monzese,
113 Italy) and proteins were visualized on an autoradiography film (Biomax Kodak).

114 Additional control experiments were run by omitting incubation with anti-sera to
115 evaluate staining due to the secondary antibody.

116 Stained gel and film images were digitized using an EPSON 1660 densitometer
117 (Bruker Daltonics, Milano, Italy). Computer aided 2-DE image analysis to match
118 signals from immunoblots and protein spots was carried out using Melanie 5
119 software (Bruker Daltonics, Milano, Italy).

120

121 *2.3 In situ digestion and protein identification by MALDI-TOF analysis*

122 Matrix Assisted Laser Desorption Ionization – Time Of Flight (MALDI-TOF)
123 analysis was carried out on the spot to be identified following 2-DE, in-gel
124 digestion and peptide extraction as described in Taverna et al., (2007), using a
125 Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Milano, Italy).
126 Measured peptide masses were used to search the Swiss-Prot, MSDB, TrEMBL,
127 and NCBI sequence databases for protein identifications by the Mascot program
128 (<http://www.matrixscience.com>). The predicted transmembrane regions in
129 proteins were identified by the program TMPred from the ExPASy server
130 (<http://www.expasy.org>).

131

132 **3. Results and Discussion**

133 *3.1 Identification of immunogenic proteins by SERPA*

134 The analysis was focused on the surface sub-proteome for two main reasons: first,
135 surface proteins are obviously better suited for vaccine development; second,

136 samples are less complex than total lysates, thus ensuring better separation and
137 more reliable identification of the immunogenic proteins.

138 Preliminary 1-DE experiments using the same material were performed to set up
139 the proper conditions for Western blot and immunostaining (data not shown). As
140 expected, and later confirmed by 2-DE experiments, broad immunostained spots
141 were detected also using foetal serum or secondary antibody alone. In order to
142 limit such unspecific signals, conditions using relatively high dilution of primary
143 anti-serum were chosen. Immunoblots using either cows-infected or foetal serum
144 are shown in Fig. 2. The strict conditions used allowed detecting five
145 immunogenic spots recognized only by infected serum (Fig. 1 and 2 and Table 1).
146 No immunostaining signal was detected in the region corresponding to portion B
147 of Fig. 1. Mass spectrometric analysis of the material corresponding to the broad
148 signal present in the upper right of the Western blot probed with infected serum
149 (Fig. 2) indicated that no proteins are present in such region. This observation,
150 confirmed by the absence of spots in the same portion of the silver stained gel
151 shown in Fig. 1, suggests that this immunoreaction might be due to residual
152 lipoteichoic acid or peptidoglycan present in the preparation, both known to be
153 highly immunogenic.

154

155 *3.2 Characterization of immunogenic proteins of S. aureus*

156 In order to evaluate the reliability of mass spectrometric data an example of mass
157 peptide fingerprint is shown in Fig. 3 and statistical data allowing protein
158 identification are reported in Table 1. Three highly immunogenic proteins were

159 identified searching the genome of the bovine mastitis-associated SA strain ET3-
160 1: DNA translocase FtsK, ribosomal protein S1 and a hypothetical protein which
161 shows high similarity to Tell-like protein family that are associated with Tellurite
162 resistance (Te^R).

163 DNA translocase FtsK is a 88 kDa polypeptide consisting of an N-terminal
164 domain with 5 predicted transmembrane helices. DNA translocase are required for
165 DNA replication, recombination and transfer within and between cells (Errington
166 et al., 2001). In addition, since the sequence of FtsK has extensive similarity to
167 that of the SpoIIIE protein of *B. subtilis* (Wu and Errington, 1994; Wu et al.,
168 1995), FtsK might have a similar role in peptidoglycan synthesis. (Dryla et al.,
169 2005) described DNA translocase as an immunogenic protein expressed during
170 early *S. aureus* infection in patients.

171 Ribosomal protein S1 is a 43 kDa polypeptide present in the small subunit of
172 bacterial ribosome. As a ribosomal protein, S1 is strikingly atypical. Association
173 of S1 to the ribosome is weak and reversible, whereas most other ribosomal
174 proteins are strongly bound. Also in this case a hypothetical transmembrane
175 domain is predicted. Ribosomal protein, such as ribosomal protein S1, are
176 considered molecules presenting good antigenic properties (Dryla et al., 2005). In
177 addition, using an approach very similar to the one that we applied, (Shin et al.,
178 2004)), showed that the ribosomal protein S2 in *Neospora caninum* is a good
179 antigen for bovine IgG and IgM.

180 The database search identifies spot 5 as a hypothetical protein showing high
181 similarity to Tell-like protein family that is associated with tellurite resistance

182 (Te^R). Again, the presence of this protein in a fraction enriched in surface proteins
183 is supported by the presence of a predicted transmembrane domain.

184 The presence of a protein of the Tell-like family was already detected in the
185 proteome of different *S. aureus* strains and classified as a virulence and drug-
186 resistance factor (Cordwell et al., 2002). Genes that mediate Te^R are found both
187 on plasmid and in the genomes of many unrelated bacteria, including pathogens.
188 The maintenance of several unrelated sets of genes in many different micro-
189 organisms, all of which confer Te^R, implies that they are important for survival
190 (Taylor, 1999). It has been proposed that their primary role might be to protect
191 against mammalian host defences, possibly by counteracting toxic substance
192 produced by host cellular defences.

193 In summary, application of the SERPA approach to field isolates responsible for
194 sub-clinical mastitis cases revealed three newly identified highly immunogenic
195 proteins for which data available from literature confirm possible association to
196 cell wall and antigenic or virulence features. The highly stringent conditions
197 applied allowed to detect only the most immunogenic proteins which, in our case,
198 do not include proteins commonly associated to adherence and resistance, in
199 keeping with the observation of Vytvytska et al. (2002) who applied the same
200 approach to identify highly immunogenic proteins in *S. aureus* COL isolated from
201 human patients.

202

203

204

205 **4. Conclusions**

206 The availability of reproducible proteomic procedures to evaluate the pattern of
207 antibody expressed by infected subjects is considered a valuable tool for the
208 development of vaccines and diagnostic tools (Etz et al., 2002; Sellman et al.,
209 2005; Vytvytska et al., 2002; Weichhart et al., 2003). In particular, the SERPA
210 approach offers the additional advantage of allowing direct identification of
211 antigenic proteins in a single experiment coupling immunostaining and mass
212 spectrometry based analysis of two aliquots of a sample analysed in the same
213 experiment. To our knowledge this communication reports for the first time
214 results obtained by applying the SERPA approach to a field isolated *S. aureus*
215 strain responsible for sub-clinical bovine mastitis cases.

216

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219

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314 **Figure captions**

315

316 **Figure 1 :** Silver stained 2-DE of cell wall associated proteins of *S. aureus* strain

317 1673. The identity of the infected cows specific immunoreactive proteins is

318 reported in Table 1. The dashed line marks the position at which a corresponding

319 gel run in parallel was split before Western blot analysis.

320 **Figure 2:** Serological proteome analysis of the A region of the same sample

321 shown in Fig. 1 run in parallel and immunostained using serum from infected

322 cows or foetal serum. Numbers correspond to the proteins of Fig. 1 and Table 1.

323 **Figure 3.** MALDI-TOF mass spectrum of peptides obtained after tryptic digestion

324 of spot 4. The mass spectrum corresponds to ribosomal protein S1. Molecular

325 masses are reported as monoisotopic values.

326

327

1

2

3 **Table I. Highly immunogenic antigens identified from *S. aureus* strain 1673**
4 **isolated from a subclinical mastitis case. Spot numbers refer to Figure 1**

5

Spot	Name	No. peptides/Seq coverage (%)	Mass (kDa)	SA no ¹ .	Accession no.
1, 2, 3	Ftsk/SpoIIIE	8/32	88.0	1138	P64165
4	30S ribosomal protein S1	14/61	43.3	1338	Q5HFU7
5	Hypothetical protein, Tell-like protein	18/54	43.4	1262	Q6G9F5

6

7

¹ SA no: cattle mastitis associated *S. aureus* RF122 genome

8

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11

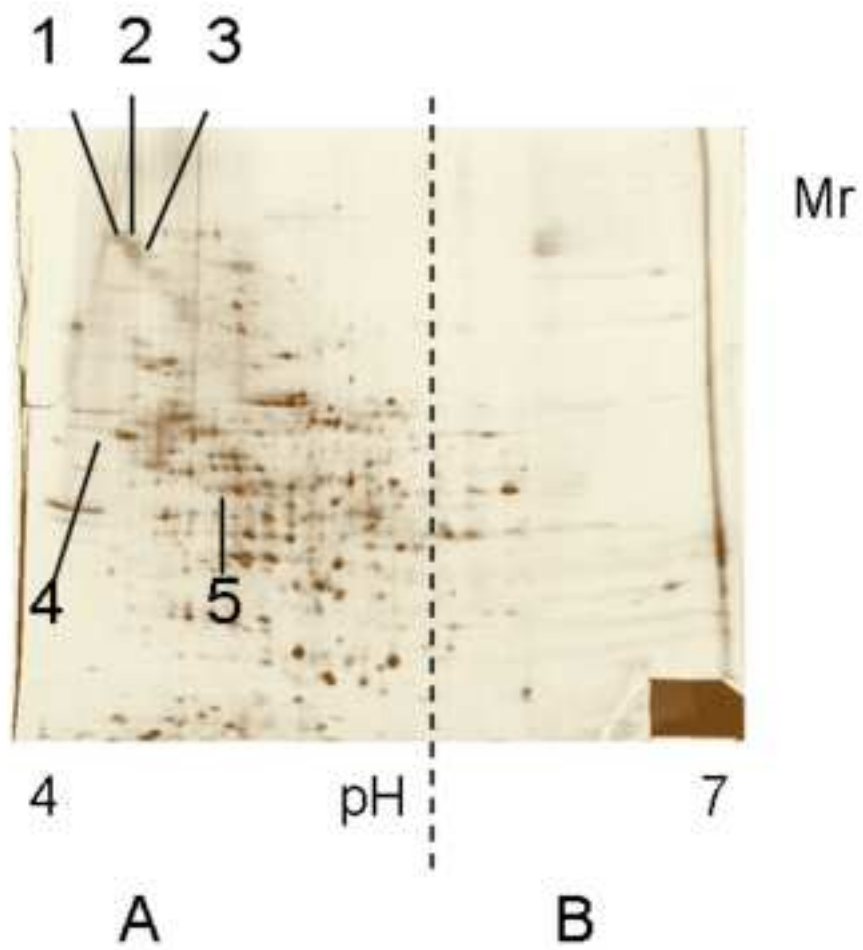
12

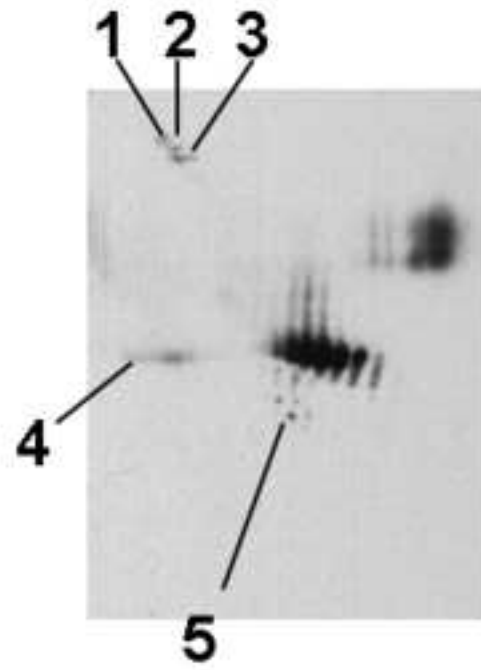
13

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16





Infected



Foetal

