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Comparative immunosecretome analysis of prevalent *Streptococcus suis* serotypes

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ABSTRACT

Streptococcus suis is a major Gram-positive swine pathogen associated with a wide variety of diseases in pigs. The efforts made to develop vaccines against this pathogen have failed because of lack of common cross-reactive antigens against different serotypes. Nowadays the interest has moved to surface and secreted proteins, as they have the highest chances to raise an effective immune response because they are in direct contact with host cells and are really exposed and accessible to antibodies. In this work, we have performed a comparative immunosecretomic approach to identify a set of immunoreactive secreted proteins common to the most prevalent serotypes of *S. suis*. Among the 67 proteins identified, three (SSU0020, SSU0934, and SSU0215) were those predicted extracellular proteins most widely found within the studied serotypes. These immunoreactive proteins may be interesting targets for future vaccine development as they could provide possible cross-reactivity among different serotypes of this pathogen.

1. Introduction

Streptococcus suis is a Gram-positive bacterium responsible for major infections in pigs, which causes different diseases in these animals that include meningitis, arthritis, bronchopneumonia, endocarditis, serositis and septicaemia [1]. Besides the important economic losses in the swine industry, it is also an important zoonotic agent for persons that are in direct contact with diseased pigs or their by-products [2]. Actually, two important outbreaks affecting humans occurred in China in 1998 and 2005, causing several tens of deaths [3,4].

S. suis serotyping is based on the capsular polysaccharide. According to this, 35 serotypes of this pathogen have been described [5]. Serotypes 2 (SS2) and 9 (SS9) are considered the most prevalent and virulent types, being frequently isolated from diseased animals [6]. Nevertheless, other serotypes have been associated to different clinical manifestations in pigs. The attempts to control *S. suis* infections are still hampered by the lack of sufficient knowledge about the pathogenesis of the disease and the lack of sensitive diagnostic methods and effective cross-protective vaccines. Various approaches to obtaining an effective vaccine, especially against the highly prevalent and virulent SS2, have been developed, including the use of bacterins or live-attenuated strains [1]. However, the identification of surfaced-attached proteins (i.e. those having any anchoring or retention motif to the bacterial surface) and secreted factors have received attention during the last years as vaccine candidate agents, because they are expressed in large quantities and are more exposed and thus more accessible to antibodies. Therefore, they have the best chances to raise a high and effective immune response which can be also cross-protective among different serotypes [7]. Extracellularly secreted proteins, which can be surface-associated, also contribute to adapt to and survive in their environment, are important during the course of infection and are in direct contact with host cells and really exposed and accessible to antibodies, therefore having also a good potential for vaccine development [7–9].

The detection of immunoreactive proteins is very helpful to identify potential immunogenic proteins as they are usually the best candidates for the discovery and development of vaccines, as well as for diagnostics purposes. Proteomics provides excellent means to identify in a fast and reliable way large numbers of proteins. Immunoproteomics, an approach involving two-dimensional electrophoresis followed by immunoblotting and further MALDI-TOF MS analysis, has been widely used to identify immunogenic proteins and pathogenicity factors in different bacterial species [10–14]. In this study, a comparative

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Table 1
Streptococcus suis clinical isolates analyzed in this work and recovered from infected pigs.

Reference	Year	Clinical sign	Organ	Serotype
638/03	2003	Arthritis	Joint	1
P1/7	–	Meningitis	Brain	2
235/02	2002	Septicaemia	Kidney	2
365/03	2003	Meningitis	Brain	2
123/11	2011	Arthritis	Joint	2
225/00	2000	Arthritis	Joint	3
14/03	2003	Septicaemia	Kidney	4
636/03	2003	Bronchopneumonia	Lung	4
204/03	2003	Meningitis	Brain	7
40/03	2003	Artritis	Joint	8
160/03	2003	Bronchopneumonia	Lung	8
233/01	2001	Arthritis	Joint	9
228/06	2006	Meningitis	Brain	9
34/11	2011	Meningitis	Brain	9
546/05	2005	Meningitis	Brain	14
232/06	2006	Arthritis	Joint	14
609/02	2002	Bronchopneumonia	Lung	15
668/02	2002	Meningitis	Brain	15
226/03	2003	Nervous	Brain	16

immunoproteomics approach was used to identify a set of secreted immunoreactive proteins (i.e. the immunosecretome) common to the most prevalent serotypes of *S. suis*, aiming at revealing proteins which can be used in further studies as vaccine candidates and also for diagnostics.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Nineteen strains belonging to 10 different serotypes (1–4, 7–9, 14–16) of *S. suis* isolated from diseased pigs were used in this study (Table 1). All strains, kept at −80 °C, were plated on Columbia agar blood base containing 6% (v/v) sheep blood and then grown in Todd-Hewitt broth at 37 °C and 5% CO_2 until an $OD_{600} = 0.25$ (mid-exponential phase) was reached.

2.2. Preparation of convalescent sera

One pig was challenged intravenously through the ear vein with a dose of 2×10^6 CFU of log-phase 235/02 SS2 of *S. suis* in 1 ml PBS. Blood sample was collected after 15 days of infection in 5 ml vacutainers from the anterior vena cava and centrifuged at 10,000 × *g* for 10 min to obtain the serum. The infection was conducted according to the European Union guidelines for the handling of laboratory animals and was approved by the Ethics Committee of the University of Córdoba.

2.3. Precipitation of secretomes

Secretomes, i.e. secreted protein fractions were prepared as already described by our group [15]. Briefly, the culture supernatants were collected by centrifugation at 3500 × *g* for 15 min at 4 °C, and filtered through a 0.22-μm membrane (Millipore) to remove residual bacteria. Then, the supernatants containing the soluble extracellular proteins were precipitated using trichloroacetic acid (TCA) solution (100% TCA and 0.4% sodium deoxycholate). The filtrates were mixed 9:1 (v/v) with prechilled 100% TCA solution and incubated in ice overnight at 4 °C. After centrifugation at 13,000 × *g* for 10 min at 4 °C, the pellets were resuspended in 450 μl 50 mM Tris pH 8 and washed twice; after that they were washed twice again in 450 μl acetone. The final pellets were air-dried. Finally, the precipitated proteins were stored at −20 °C until further analysis.

2.4. Two-dimensional gel electrophoresis

Secretomes were treated with the 2D Clean-up kit (GE Healthcare) following the manufacturer's instructions, and the precipitated proteins were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 20 mM DTT, 1.6% ampholytes, and 0.0001% bromophenol blue). The protein contents were determined using the Bradford assay [16]. For the first dimension, 7 cm Immobiline DryStrips (IPG, Immobilized pH Gradient, pH range 4–7; GE Healthcare) were rehydrated at 50 V for 12 h, in rehydration/sample buffer containing 150 μg of the protein samples in a total volume of 125 μl. Isoelectric focusing was performed using a Bio-Rad PROTEAN IEF cell (Bio-Rad) and focusing was conducted at 20 °C by stepwise increase of the voltage as follows: 250 V for 0.5 h, 4000 V for 2 h, and 4000 V until 17,000 V h were finally reached. Then, each IPG strip was washed in 1 ml of equilibration buffer 1 (50 mM Tris−HCl pH 8.8; 6 M urea, 2% SDS, 20% v/v glycerol, 2% DTT) for 15 min and then in 1 ml of equilibration buffer 2 (50 mM Tris−HCl pH 8.8; 6 M urea, 2% SDS, 20% v/v glycerol, 135 mM iodoacetamide) for other 15 min. For the second dimension, IPG strips were then placed over a 12% polyacrylamide gel and 100 V were applied until the tracking dye reached the bottom of the gels. Gels were stained with Coomassie brilliant blue G-250 solution (Sigma-Aldrich) according to manufacturer's instructions and scanned with a GS-800 densitometer (Bio-Rad). For each 2-D gel, a replicate was made for Western blotting, in order to identify immunoreactive proteins. Gel evaluation and data analysis were carried out using PDQuest v 7.3 (Bio-Rad) and ImageJ1 software.

2.5. Two-dimensional Western blotting

After SDS-PAGE, proteins in the gels were electrophoretically transferred onto nitrocellulose membranes in blotting buffer (50 mM Tris−HCl, 40 mM glycine, 20% methanol and 0.04% SDS). Electrotransfer time was 15 min at 15 V. After transfer, membranes were blocked with 5% skimmed milk in TBS (Tris-buffered saline, 50 mM Tris-Cl, pH 7.6; 150 mM NaCl) for 1 h at room temperature. The membranes were incubated with the serum from the convalescent pig (1:1000 dilution in T-TBS) for 1 h at room temperature and subsequently washed three times with 0.05% w/v Tween-20 in TBS (T-TBS) for 5 min. Total IgG binding was detected by incubation with horseradish peroxidase-conjugated anti-pig IgG (Sigma-Aldrich) as secondary antibody (1:5000 dilution in TBS, 1 h at room temperature). After washes with TBS the membranes were developed using the ECL Plus Western blotting System (GE Healthcare) and then exposed to a Molecular Imager® ChemiDoc™ XRS$^+$ (Bio-Rad).

2.6. MALDI-TOF/TOF analysis and protein identification

Protein spots of interest were manually excised from the 2-D gels and digested automatically with an Investigator ProPic and ProGest robotic Workstations (Genomic Solutions). Briefly, gel pieces were destained by two washes at 37 °C for 30 min with 200 mM ammonium bicarbonate in 40% (v/v) acetonitrile (ACN). Then, the pieces were washed twice, first with 25 mM ammonium bicarbonate for 5 min and later with 25 mM ammonium bicarbonate in 50% (v/v) ACN for 15 min, dehydrated with 100% ACN and finally dried at room temperature for 10 min. Then, 12.5 ng/μl sequence-grade trypsin (Promega) in 25 mM ammonium bicarbonate was added to the gel pieces and left at 37 °C overnight. Digestion was stopped by adding 10 μl of 0.5% trifluoroacetic acid (TFA); peptides were desalted using μC-18 ZipTip columns (Millipore) and then eluted directly with the matrix solution (α-cyano-4-hydroxycinnamic acid at 5 mg/ml in 70% ACN/ 0.1% TFA) onto a MALDI plate using the dry droplet method. The mass spectra were acquired in a 4800 Proteomics Analyzer MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems), in the *m/z* range from 800 to 4000, with an accelerating voltage of 20 kV in reflectron mode. Spectra were

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