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Proteome-wide antigen discovery of novel protective vaccine candidates against *Staphylococcus aureus* infection

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a rapidly growing problem, especially in hospitals where MRSA cause increased morbidity and mortality and a significant rise in health expenditures. As many strains of MRSA are resistant to other antimicrobials in addition to methicillin, there is an urgent need to institute non-antimicrobial measures, such as vaccination, against the spread of MRSA. With the aim of finding new protective antigens for vaccine development, this study used a proteome-wide *in silico* antigen prediction platform to screen the proteome of *S. aureus* strain MRSA252. Thirty-five different *S. aureus* proteins were identified, recombinantly expressed, and tested for protection in a lethal sepsis mouse model using *S. aureus* strain MRSA252 as the challenge organism. We found that 13 of the 35 recombinant peptides yielded significant protection and that 12 of these antigens were highly conserved across 70 completely sequenced *S. aureus* strains.

Thus, this *in silico* platform was capable of identifying novel candidates for inclusion in future vaccines against MRSA.

1. Introduction

Staphylococcus aureus is a ubiquitously occurring microorganism that can be isolated from approximately 20% of the population [1]. Although it is often harmless, *S. aureus* can cause serious illnesses, such as pyogenic infections, osteomyelitis and infective endocarditis.

S. aureus infections are a rapidly growing problem, especially in hospitals where methicillin-resistant (MRSA) and multi-drug-resistant *S. aureus* infections constitute an increasing challenge [2]. In recent years, there has been focus on the transmission of livestock-associated MRSA from pigs to humans. Although most of these cases were mild infections [3,4], serious and even lethal infections have now been reported [5].

Multi-drug-resistant MRSA infections are particularly difficult to treat, and the problem has been further complicated by the emergence of vancomycin-resistant *S. aureus* strains [6,7]. There is an urgent need for non-antimicrobial actions, such as vaccination, to control the spread of resistant staphylococcal infections [8,9]. Designing such a vaccine is particularly challenging because of the very nature of *S. aureus* with its multiple and sometimes redundant virulence factors that make it such a crafty pathogen. It has been predicted that if an anti-staphylococcal vaccine is to prove effective in humans, it will necessarily be a multicomponent vaccine that incorporates a number of surface proteins, toxoids, and perhaps also surface polysaccharides [10,11].

In this study we have used the software program VacFinder®, a proteome-wide *in silico* antigen prediction platform, to screen the proteome of *S. aureus* strain MRSA252, with the aim of finding useful vaccine candidates [12]. A panel of 35 proteins predicted by VacFinder to be potential vaccine candidates were recombinantly expressed, purified and used to immunize groups of 8–20 NMRI mice. The immunized mice were challenged with MRSA252. Thirteen of the 35 potential vaccines candidates were able to induce protective immunity against a lethal dose of bacteria.

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2. Materials and methods

2.1. VacFinder® technology

To predict the protective potential of each protein in the proteome, each of the proteins were analyzed using the proprietary VacFinder® *in silico* technology platform (NovVac Aps). VacFinder® is a data-driven machine-learning method aiming at identifying novel protective B-cell protein antigens with a neutralizing and/or opsonizing profile. The system was trained by protein property-pattern recognition on known protective B-cell protein antigens (excluding exotoxins). The machine-learned prediction is based on specific protein-property features of protein sequences rather than on sequence similarity, allowing antigen classification based solely on protein properties [12,13]. The input to VacFinder® was the proteome of *S. aureus* strain MRSA252 (http://www.ncbi.nlm.nih.gov/nuccore/NC_002952.2) [14]. The output was a list of proteins from the proteome that were ranked by their expected ability to elicit a highly protective antibody response. We excluded proteins that had been found ineffective in human trials by other investigators.

2.2. Protein expression

DNA sequences corresponding to the 35 selected proteins and 2 sham controls were synthesized by the company GenScript (New Jersey, USA) and inserted into the pQE1 expression vector (Qiagen). The synthesized sequences were without the coding sequence for the transmembrane domain and signal peptide.

The plasmids were transformed into *E. coli* (M15) cells, according to the protocol described by the manufacturer (Qiagen). One liter of *S. aureus* protein-producing *E. coli* cells was cultivated in a 2-liter flask with constant shaking until OD at 600 nm reached 0.5–1. Cells were induced with isopropyl β-D-1-thiogalactopyranoside (1 mM) and harvested by centrifugation (9000*g*, 4 °C, 20 min) after 4 h. Cell pellets were suspended and lysed in a denaturing solution (binding buffer) containing 8 M urea, 100 mM $NaHPO_4$, 100 mM Tris, 300 mM NaCl, 5 mM imidazole and 1 mM 2-mercaptoethanol (pH 8.0). The lysate was clarified by centrifugation (15,000*g*, 4 °C, 30 min). His-tagged *S. aureus* peptides were purified from the clarified lysate by chromatography in a column packed with ProBond™ Nickel-Chelating Resin (Invitrogen, Denmark). The column was washed with the binding buffer and the peptides were refolded with a linear gradient from lysis buffer to refolding buffer containing 100 mM $NaHPO_4$, 100 mM Tris, 300 mM NaCl, 5 mM imidazole and 1 mM 2-mercaptoethanol (pH 8.0). The peptides were then eluted in elution buffer containing 100 mM $NaHPO_4$, 100 mM Tris, 300 mM NaCl, 250 mM imidazole (pH 8.0), dialyzed against PBS and filtered through a 0.45 μm pore-size filter. The resulting purity of the peptides was shown to be >85% by means of SDS–PAGE and Coomassie Brilliant Blue staining and the concentrations were estimated by measuring OD_{280nm} and converting by means of a specific coefficient calculated from individual amino-acid composition.

2.3. Bacteria

S. aureus strain MRSA252 was obtained from Statens Serum Institut (SSI), Denmark and *S. aureus* strain *Newman*/*Staphylococcus aureus* subsp. *aureus Rosenbach* (ATCC® 25904™) were used for challenge trials.

Staphylococci were cultured on tryptic soy agar or in broth at 37 °C. To ensure that a consistent dose of bacteria was given in each experiment, bacteria were frozen in aliquots and stored at -80 °C. The dosage corresponding to 90% lethality (LD90) of an infection was determined in sham-immunized mice before the actual challenge.

2.4. Animal immunization and bacterial challenge

Groups of 8–20 NMRI mice (6–8 week-old outbred females) were immunized subcutaneously with 25–50 μg of purified peptide (potential protective antigens or sham proteins) adsorbed on to aluminum hydroxide gel together with Freund's incomplete adjuvant and boosted twice with only aluminum hydroxide gel as adjuvant on days 14 and 28 after the first immunization. Negative control mice were immunized with PBS mixed with aluminum hydroxide gel and Freund's incomplete adjuvant in the same way as for the antigens. To determine that survival was due to protective vaccination and not to biological variation or other factors, a group of 8–20 negative control mice (PBS) was included in every challenge trial.

Immunized, sham-immunized, and negative control (PBS) mice were challenged with $1.5–2 \times 10^9$ bacteria per animal, corresponding to LD90. The injection was given intraperitoneally on day 45 after the first vaccination, and the mice were afterwards monitored for 7 days.

Moribund mice were sacrificed according to the criteria described in the approved animal study protocol, and counted as dead along with any animal deaths that occurred between monitoring periods.

2.5. Study approval

Animal maintenance and experimental protocols were approved by the Danish Veterinary and Food Administration of the Ministry of Food, Agriculture and Fisheries (2012-15-2934-00632).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were drawn on day 10 after the last vaccination, and ELISA was used to test the level of specific antibodies in plasma from mice immunized with the *S. aureus* peptide of interest. Briefly, recombinant peptide was coated onto the wells of 96-well microtiter plates (Nunc) (1 μg/ml) overnight at 4 °C. Unbound peptide was removed by washing, and the wells were blocked with Tween (PBS-0.05% Tween 20) for 1 h. EDTA plasma from immunized mice diluted 1:200 was added to the wells of the ELISA plate, further diluted in by serial doubling 2-fold dilutions and incubated for 2 h. Plates were then washed with washing buffer (PBS- 0.05% Tween 20) three times, and rabbit anti-mouse IgG HRP conjugate (DAKO) was added to the wells and incubated for 1 h. After washing as described above, the plates were developed with OPD (*o*-phenylenediamine dihydrochloride from Sigma, Denmark) and H_2O_2 was added, the color intensity was read with an ELISA reader at OD 490 nm. The antibody titer was determined as the reciprocal of the dilution at which the absorbance decreased to 50% of the maximum measureable OD-value.

2.7. Statistical analysis

For analyzing the challenge data, a statistical analysis was performed by log-rank test and illustrated as Kaplan–Meier survival curves. $P \leqslant 0.05$ was regarded as significant. Spearman correlation was used to assess whether there was any correlation between the antibody titer and survival at an individual level.