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Short communication

The effect of auto-vaccination therapy on the phenotypic variation of one clonal type of *Staphylococcus aureus* isolated from cows with mastitis

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ABSTRACT

The aim of this study was to demonstrate the effect of auto-vaccine therapy on selected properties of *Staphylococcus aureus* strains, isolated from milk of cows with subclinical mastitis. The experiment was based on auto-vaccines which were prepared from *S. aureus* strains isolated from 16 cows. *S. aureus* strains isolated from cows on the 7th, 21st and 35th day following auto-vaccination, were analyzed phenotypically and genotypically. The isolated strains represented 17 biotypes all belonging to one clonal type. Increases of new biotypes of *S. aureus* were detected on the 35th day of therapy. Among 48 re-isolated strains, 18.75% (9/48) revealed single and 12.50% (6/48) multiple phenotypical changes. The present study demonstrated that during auto-vaccine therapy, *S. aureus* strains can change phenotypically, pointing out the necessity for using precise diagnostic methods, that would make possible a better assessment of the used therapy.

1. Introduction

Inflammation of the bovine mammary gland is known as a functional disorder caused by a wide range of etiological factors. Mastitis causes considerable economical loss which is the result of decreased productivity, diminished milk quality, increased costs of diagnostics and therapy and problems with utilization of antibiotic contaminated milk (Gruet et al., 2001; Rainard and Riollet, 2006).

The epidemiology of bovine mastitis has been characterized worldwide by an increase in the prevalence of staphylococci (Sommerhäuser et al., 2003; Moret-Stalder et al., 2009). Additionally, coagulase-negative staphylococci (CNS), one of the etiological factor of mastitis, are considered in many countries as the main cause of bovine mastitis (Taponen et al., 2006). *Staphylococcus aureus* is the most frequently isolated, coagulase-positive microorganism among the forty-four species belonging to the *Staphylococcaceae* family, and is considered the main cause of chronic, subclinic bovine mastitis (Kauf et al., 2007; Saei et al., 2009; Wang et al., 2009). Its particular properties, especially the presence of a wide range of virulence factors, makes this organism one of the most dangerous animal pathogen (Iwatsuki et al., 2006; Rall et al., 2010; Seo et al., 2010).

Trials of alternative therapy of staphylococcal mastitis have been conducted, especially in cases when *S. aureus* was the main cause of this disorder (Castagliuolo et al., 2006). In these cases mastitic cows were vaccinated with inactivated *S. aureus* strains. These vaccines caused increased immunological responses, but did not guarantee a persistence of total immunity to new staphylococcal infections. Results from these studies suggest that the isolated staphylococcal strains produced a slightly different array of immunogenic antigens. Thus, standardization of an anti-staphylococcal vaccine could be very difficult and its commercial production economically unprofitable (Talbot and Lacasse, 2005; Kerro-Dego et al., 2006).

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Another method which can be used as an alternative to classic therapy, could be auto-vaccination. In this case, an auto-vaccine would be prepared from the bacterial strain isolated from an individual cow and administered to the same cow, which the strain was isolated from. The aim of this study was to demonstrate the influence of auto-vaccine therapy on selected properties of *S. aureus* strains, isolated from milk of cows with subclinical mastitis.

2. Materials and methods

2.1. Bacterial strains and procedure of auto-vaccination

Sixteen *S. aureus* strains were isolated from milk samples taken from 16 cows with subclinical mastitis. Cows were selected for use in the experiment on the basis of a positive California Mastitis Test score. Initially, isolated bacterial strains were used to produce auto-vaccines (the procedure described below). Cows were auto-vaccinated and milk samples were collected and microbiologically tested from all cows on the 7th, 21st and 35th day following auto-vaccination. Moreover, on the 21st day, each cow was re-vaccinated with the same auto-vaccine. All cows were vaccinated with 3 mL of the appropriate auto-vaccine by injection in the area of each supramammary lymph node. The vaccination was performed by a veterinarian. Sixteen foremilk samples were collected aseptically and immediately shipped to the laboratory on ice. A hundred microliters of each sample was spread onto the surface of a Baird Parker agar plate (Oxoid, UK). Following incubation for 24 h at 37 °C, shiny black colonies with pale shaded areas surrounding the colony were examined using standard microbiological procedures for identification and characterization of staphylococci (phenotypic properties and genetic typing procedures are described below).

2.2. Auto-vaccine preparation

S. aureus auto-vaccines were prepared individually for each cow. The strains were inoculated onto bacteriological agar plate (Oxoid) and incubated at 37 °C for 24 h. Following incubation, bacterial colonies were washed from the agar surface with sterile 0.85% saline solution. The bacterial suspension in a volume of 10 mL was transferred to a new sterile tube, standardized to 1° of McFarland scale (3×10^8 cfu/mL) and inactivated by addition 50 μL of 40% formalin solution. In order to confirm sterility, 0.5 mL of each auto-vaccine was spread onto a brain heart infusion agar plate (Oxoid), and incubated at 37 °C for 72 h. Absence of a colony was interpreted as lack of viable bacteria. The *S. aureus* auto-vaccines were tested for toxicity on randomly selected white laboratory mice and revealed negative results.

2.3. Phenotypic properties

The ability for carbohydrate utilization, nitrate to nitrite reduction, acethyl-methyl-carbinol production, secretion of phosphatase, arginine dihydrolase and urease synthesis were investigated using API Staph (bioMérieux, France). The production of yellow pigment was checked after incubation of all bacterial strains on Mueller–Hinton Agar (Oxoid) for 48 h at room temperature. The ability to coagulase production was tested according to manufacturer's instruction (Oxoid). Readings were performed after 1, 3, 6 and 24 h of incubation. All the test tubes were incubated for another 96 h in order to confirm fibrinolytic activity of the bacteria strains. The presence of clumping factor was analyzed using Staphytect Plus™ (Oxoid). Haemolytic properties of investigated strains were detected on Columbia Agar with 5% sheep blood (Graso, Poland) after incubation of bacterial strains for 24 h at 37 °C.

2.4. Genetic analysis

Genomic DNA was isolated using Genomic Mini Kit (A&A Biotechnology, Poland) according to manufacture instructions. The presence of the *nuc* gene (encoding staphylococcal thermo-resistant nuclease), characteristic for *S. aureus*, was determined with primers and reaction conditions described previously by Wilson et al. (1991). The RAPD assays were carried out as described by Reinoso et al. (2004). Briefly, the oligonucleotides OLP11 (5′-ACGATGAGCC-3′) and OLP13 (5′-ACCGCCTGCT-3′) were used for the amplification of DNA simultaneously in the same mixture for the purpose of acquiring a wide range of amplicons and higher assay repeatability. The amplification was performed under the following conditions: predenaturation at 94 °C for 5 min, then 40 cycles of 1 min at 93 °C, 1.30 min at 37 °C and 1 min at 72 °C. A final elongation step at 72 °C for 8 min was included. All amplified products were separated by agarose gel electrophoresis and the product analyzed using GenTools software (Syngene, UK). Chemicals used in the PCR reactions were purchased from Fermentas (Lithuania). PCR primers were provided by IBB PAN (Poland).

3. Results

Sixteen *S. aureus* strains, isolated from 16 cows, were used for preparation of 16 monovalent auto-vaccines (one for each cow). Auto-vaccines were administered to animals (day 0 of experiment), according to the procedure described in Section 2. On the 7th day after auto-vaccination, only nine cows were infected with *S. aureus*. On the 21st day after auto-vaccination the number of infected cows was reduced to eight. On the 35th day (14 days after re-auto-vaccination, on day 21), the number of mastitic cows infected by *S. aureus* increased to 15 (Table 1). Each strain revealed a typical phenotypic pattern and possessed the *nuc* gene. RAPD-PCR analysis confirmed that all isolated *S. aureus* strains represent only one clonal type. Based on phenotypic examination, 17 biotypes of *S. aureus* were established during the experiment. Strains isolated before the auto-vaccine therapy (day 0 of experiment) were classified into six biotypes (A, B, C, D, K and M). Seven days after auto-vaccination, the number of biotypes decreased from six to four and three new biotypes (E, G and H) were detected, however, biotype A remained the dominant biotype. Twenty-one days after auto-vaccina-