

Herd-specific autovaccine and antibiotic treatment in elimination of *Staphylococcus aureus* mastitis in dairy cattle

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Abstract: The aim of the present study was to compare the effectiveness of cefuroxime, herd-specific autovaccine, and cefuroxime/herd-specific autovaccine treatments in eliminating *S. aureus* from the milk of 45 cows with subclinical mastitis. Prior to this study, cows with *S. aureus* mastitis were divided into 3 groups of 15 cows each. Group 1 received cefuroxime, group 2 received the autovaccine, and group 3 received cefuroxime and the autovaccine. The antibiotic was infused into the teat canal, whereas the autovaccine was administered in the upper udder lymph node region. It was revealed that after day 35 of the treatment *S. aureus* was not detected in the milk of 40% of the cows treated with antibiotic, 60% of the cows treated with the autovaccine, and 100% of the cows that underwent combined therapy. Further observation of cows selected for the current study showed that in cows treated with both cefuroxime and a herd-specific autovaccine *S. aureus* mastitis was not recorded for at least 2 years. In conclusion, this investigation revealed that the administration of an anti-*S. aureus* herd-specific autovaccine into the upper udder lymph node region combined with infusion of an appropriate antibiotic into the teat canal eliminates *S. aureus* and leads to prolonged udder protection against invasion by these microorganisms.

Key words: *S. aureus*, autovaccine, antibiotic, mastitis, cattle

1. Introduction

Staphylococcus aureus is considered the major etiological factor in mammary gland inflammation (1,2). To date, several different vaccines have been used for prophylaxis of mastitis (3–5). Nevertheless, it was shown that commercial anti-*S. aureus* vaccines administered subcutaneously were incapable of providing complete protection against mastitis (6). Moreover, anatoxins based on staphylococcal leukocidin or hemolysin triggered a large number of side effects (7). However it should also be noted that Leitner et al. (8) observed a statistically significant increase in the concentration of anti-*S. aureus* IgG in serum and milk as a result of Mastivac I vaccine with Freund adjuvant administration into the upper udder lymph node region.

A range of antibiotics are used in mastitis treatment, but due to increasing antibiotic resistance in mastitis-causing bacteria the therapy is not always effective. Therefore, attempts have been made to use autovaccines as an alternative to antibiotics (9). Autovaccines are known to be useful in treating many different infections (10), including those caused by intracellular pathogens (11,12). *S. aureus* can be considered an intracellular pathogen because it is able to survive inside neutrophils

after phagocytosis (13). As phagocytosed bacteria are inaccessible to antibiotics, antibiotic therapy can therefore be insufficient. The administration of an autovaccine enhances phagocytic effectiveness and leads to the destruction of staphylococcal cells.

Preparing autovaccines for individual cows is expensive and labor-intensive. However, as was proved in the study by Nawrotek et al. (9), *S. aureus* mastitis in a herd of cows is often caused by only one clonal type of bacteria. Thus, in this study it was assumed that the administration of an autovaccine containing bacteria representing one clonal type of mastitis causing *S. aureus* to all of the mastitic cows from a herd will reduce the effort and cost of production while retaining its effectiveness. We called this preparation a herd-specific autovaccine.

The present study was based on the comparison of the effectiveness of cefuroxime, a herd-specific autovaccine, and a mixed cefuroxime/herd-specific autovaccine treatment in the elimination of *S. aureus* from the milk of cows with subclinical mastitis. The purpose of this work was to examine which therapy leads to the most effective elimination of *S. aureus* from mastitic bovine milk.

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2. Materials and methods

2.1. Animals

Forty-five Polish Holstein-Friesian cows from one herd in Western Pomerania (Poland) were selected. The cows were 2–3 years old and had subclinical mastitis. The criteria to qualify cows for the experiment were: the same stage of lactation, inflammation limited to 2 quarters, and isolation of *S. aureus* alone from milk. The diagnoses of subclinical mastitis were provided by the local veterinarian. A California mastitis test (Vet-Agro, Lublin, Poland) was used as the indicator of the subclinical inflammation. Selected animals were separated from their herd.

2.2 Isolation and identification of bacteria

The milk samples were collected from 2 infected quarters of each cow into 1 container before treatment (day 0) and the 7, 21, and 35 days after treatment. Samples were collected aseptically according to standards and stored at 4 °C until proceeding. Firstly, decimal dilution with 0.85% NaCl (from 10^{-1} to 10^{-9}) was performed. Then 0.1 mL of milk from each dilution was plated onto Baird–Parker agar (Oxoid, Cambridge, UK) and cultivated for 48 h at 37 °C. The isolates were identified as *S. aureus* on the basis of the presence of colonies on Baird–Parker agar, their biochemical properties (api Staph, bioMérieux, Lyon, France), and their ability to coagulate rabbit plasma and produce a clumping factor. Additionally, all the isolates were examined by PCR using primers for the *S. aureus* specific *coa* gene, as described by Aarestrup et al. (14).

2.3. Analysis of the clonal relationships between strains of *S. aureus*

The Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) assays were carried out as originally described by Reinoso et al. (15) and modified later by Nawrotek et al. (16). 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. Each reaction mixture (25 µL) consisted of 1 U GoTaq DNA Polymerase with 1X Green GoTaq Flexi Buffer (Promega, Madison, WI, USA), 100 nM of dNTP mix (Promega), 0.4 µM of each primer, and 50 ng of template DNA. RAPD-PCR was carried out with the following thermal cycling conditions: an initial denaturation of DNA at 95 °C for 5 min was followed by 40 cycles of amplification (95 °C for 1 min, 37 °C for 1.30 min, and 72 °C for 1 min), ending with a final extension at 72 °C for 10 min. The PCR products were run on 1.5% agarose gel electrophoresis in 1X Tris-borate-EDTA buffer, and ethidium bromide was used as the DNA stain. Bands were visualized under UV light and analyzed by GeneTools software (Syngene, Cambridge, UK) using the unweighted pair group method with arithmetic mean (UPGMA) and Jaccard's similarity coefficient.

2.4. Antimicrobial resistance

The susceptibility of *S. aureus* to antibiotics commonly used to treat mastitis was determined with the disk diffusion method according to the Clinical and Laboratory Standards Institute (17). The following antibiotics were used: amoxicillin (AMX 10 µg, Oxoid), amoxicillin with clavulanic acid (AMC 30 µg, Oxoid), penicillin G (P 10U, Oxoid), oxytetracycline (OT 30 µg, Oxoid), cephalexin (CL 30 µg, Oxoid), cefuroxime (CXM 30 µg, bioMérieux), ceftiofur (CEFTIO 30 µg, Rosco, Taastrup, Denmark), gentamicin (CN 10 µg, Oxoid), amikacin (AK 30 µg, Oxoid), streptomycin (S 10 µg, Oxoid), ampicillin with cloxacillin (AX 25 µg, Oxoid), neomycin with bacitracin and tetracycline (NBT 70 µg, Mast Diagnostics, Liverpool, UK), penicillin with novobiocin (PEN/N 40 µg, Rosco), and lincomycin with neomycin (LIN/N 75 µg, Rosco).

2.5. Preparation of herd-specific autovaccine

The herd-specific autovaccine was prepared from the clonal strain of *S. aureus* isolated from the milk of cows selected for the experiment. *S. aureus* isolate was inoculated onto brain heart infusion agar (Oxoid) and incubated at 37 °C for 24 h. Following incubation, bacterial colonies were washed from the agar surface with a sterile 0.85% saline solution. The bacterial suspension in a volume of 10 mL was transferred to a new sterile tube, standardized to 1 McFarland standard (3×10^8 CFU/mL) and inactivated by the addition of 0.05 mL of formalin. In order to confirm sterility, 0.5 mL of the autovaccine was spread onto a Columbia agar base with 5% sheep blood (Graso, Starogard Gdański, Poland) and incubated at 37 °C for 72 h. The absence of a colony was interpreted as lack of viable bacteria in the prepared autovaccine. The *S. aureus* autovaccine was also tested for toxicity on randomly selected white laboratory mice and revealed negative results. The effectiveness of treatment was estimated on the basis of the CFU of *S. aureus* per 1 mL of milk of the investigated cows, according to bacteriological procedures previously described.

Additionally, clinical observation and microbiological examination of milk of cows from the experimental groups were continued for 24 months.

2.6. Treatment of animals

The animals were divided into 3 groups of 15 cows each. Each group of animals received a different kind of therapy. Group 1 received cefuroxime (Spectrazol, Intervet, Warsaw, Poland), group 2 received the herd-specific autovaccine, and group 3 received both cefuroxime (Spectrazol, Intervet) and the herd-specific autovaccine. The antibiotic was infused into the teat canal 5 times after milking (every 12 h). The autovaccine was administered once (at the time of administration of the first dose of antibiotic) in the upper udder lymph node region, in the amount of 3 mL per node.

2.7. Statistical analysis

Data are presented as the number of *S. aureus*-infected cows before and after treatment in particular groups. The statistical significance of the differences between the number of *S. aureus*-infected cows (including the number of CFU of *S. aureus* in their milk) in different groups of treatment in the following days of the experiment was analyzed by a chi-squared test. All statistical analyses were conducted with Statistica 7.1 software.

3. Results

All *S. aureus* isolates collected from milk of investigated cows and used to produce herd-specific autovaccine showed a typical phenotypic pattern and possessed the *coa* gene. The RAPD-PCR proved also that all of these isolates belonged to one genotype. A pattern of 6 amplification products of RAPD-PCR, defined as a characteristic for strains of that genotype, was published earlier by Nawrotek et al. (16). On the basis of the antibiotic resistance analysis it was found that all the investigated *S. aureus* isolates showed high in vitro susceptibility to cefuroxime (second generation cephalosporin). For that reason Spectrazol (Intervet), containing 250 mg cefuroxime sodium, was chosen for further research.

The results showing the effect of the treatment on the elimination of *S. aureus* from the milk of investigated cows in particular groups are presented in the Table. The statistical analysis showed statistically significant differences ($P < 0.01$) between the number of *S. aureus*-infected cows in different groups of treatment on days 7, 21, and 35 of the experiment. It was found that, in the group receiving cefuroxime, 7 days after the last antibiotic administration *S. aureus* was not found in the milk of 9 cows (60%), whereas after 21 and 35 days the number of cows free of *S. aureus* decreased to 6 (40%). Furthermore, the present study showed that, after 7 days of treatment, *S. aureus* was not isolated from the milk of 6 cows (40%) treated with herd-specific autovaccine. It is also interesting to note that on day 35 of the experiment the

number of cows that received the autovaccine and from which *S. aureus* was not isolated increased to 9 (60%). The most effective therapeutic effect was found in the group receiving cefuroxime and the herd-specific autovaccine, in which between days 7 and 35 all animals were free of *S. aureus*. In addition, this system of therapy resulted in long-term protection against staphylococcal mastitis, as further observation of cows selected for the study showed that in cows treated with the cefuroxime/herd-specific autovaccine combination there were no signs of mastitis observed for at least 2 years.

4. Discussion

In the present study we evaluated the effectiveness of cefuroxime, herd-specific autovaccine, and cefuroxime/herd-specific autovaccine treatment efficacy in eliminating *S. aureus* mastitis in dairy cattle. We found that, in the group receiving cefuroxime, 7 days after the last antibiotic administration *S. aureus* was not isolated from milk of 9 cows (60%). Antibiotics take effect in the place where bacterial cells are accessible and do not destroy bacteria inside phagocytic cells. Cefuroxime inhibits peptidoglycan biosynthesis (18), leading to the death of unphagocytized bacteria. Nevertheless, the staphylococci located inside phagocytic cells could survive due to lack of antibiotic accessibility. Following the destruction of neutrophils *S. aureus* multiplies, which explains why after days 21 and 35 of antibiotic treatment the number of *S. aureus* infection-free cows decreased in the antibiotic group from 9 to 6 animals (40%).

The present study showed that, after 7 days of treatment, *S. aureus* was not isolated from milk of 6 cows (40%) treated with herd-specific autovaccine and after 35 days this number increased to 9 (60%) animals. The efficacy of the autovaccines is associated with the presence of antigens, which are the same as the antigens on the surface of *S. aureus* strains that cause the disease. Moreover, in our study the autovaccines were administered during chronic infection and the resulting immune reaction triggered

Table. Effect of the treatment on the elimination of *S. aureus* from the milk of investigated cows in particular groups.

Group	CFU of <i>S. aureus</i> per 1 mL of milk											
	before treatment			7 days after treatment			21 days after treatment			35 days after treatment		
	0	1-10 ⁴	> 10 ⁴	0	1-10 ⁴	> 10 ⁴	0	1-10 ⁴	> 10 ⁴	0	1-10 ⁴	> 10 ⁴
Number of cows (%)												
Antibiotic	0	9 (60)	6 (40)	9 (60)	6 (40)	0 (0)	6 (40)	8 (53)	1 (7)	6 (40)	8 (53)	1 (7)
Autovaccine	0	8 (53)	7 (47)	6 (40)	8 (53)	1 (7)	8 (53)	7 (47)	0 (0)	9 (60)	6 (40)	0 (0)
Antibiotic/Autovaccine	0	5 (33)	10 (67)	15 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
χ^2 test	$\chi^2 = 2.31; P = 0.31$			$\chi^2 = 13.6; P < 0.01$			$\chi^2 = 14.2; P < 0.01$			$\chi^2 = 13.6; P < 0.01$		

a secondary response, which resulted in a short-time synthesis of large amounts of highly specific antibodies IgG₂. The effectiveness of autovaccines also depends on the place and method of their administration. The efficacy of Mastivac I vaccine in *S. aureus* elimination reaches 70% (8), whereas the effectiveness of the autovaccines used in the current study after 35 days of administration was 60%. The autovaccines used in our study and the Mastivac I vaccine used by Leitner et al. (8) were administered in the same way: into the upper udder lymph node region. As is well known, the upper udder lymph nodes play a strategic role in protecting the mammary gland. It was earlier proved that during mastitis IgG₂ antibodies mainly pass in to the mammary gland from the supramammary lymph nodes (19). In the case of intracellular pathogens antigen presentation is not possible and the production of IgG₂ is insufficient. Therefore, autovaccine administration enables antigen presentation through the antigen presenting cells to Th1 lymphocytes, which in turn stimulates B cells to produce specific IgG₂ through production of IFN- γ . The antibodies appear more quickly after re-vaccination, remain longer, and consist mainly of IgG₂ (20–22). As we recorded, during our experiment the supramammary lymph nodes of cows treated with autovaccine were enlarged for 8 days. This could be a result of a strong reaction to immunization. Vaccination that induces an acute Th1 response results in increased inflow of IgG₂ to the infected mammary gland and an influx of neutrophils into the mammary gland (23). IFN- γ secreted by Th1 lymphocytes is also an important factor in an increase of Fc receptors for IgG₂ on the surface of neutrophils and macrophages. Polymorphonuclear neutrophils play the most important role in mammary gland protection against microorganisms flowing into the gland and settling in the gland tissue (24–26). The increase in phagocytic activity observed after autovaccine administration kills the staphylococci located in and outside neutrophils (27–29). However, lack of full effectiveness in elimination of *S. aureus* after autovaccination can be a result of defensive strategies of *S. aureus*. For example, surfactant protein A

binds to the Fc fragment of immunoglobulin G making *S. aureus* “invisible” to phagocytic cells. *S. aureus* also produces toxins that inhibit neutrophil phagocytic activity (30).

In the present study the most encouraging results were found in the group receiving both cefuroxime and the herd-specific autovaccine, in which, between days 7 and 35, all animals were free of *S. aureus*. In addition, this system of therapy resulted in long-term protection against staphylococcal mastitis, as further observation of cows selected for the current study showed that in cows treated with cefuroxime/herd-specific autovaccine no signs of mastitis were observed for at least 2 years. The influence of a combination of subcutaneous vaccination and extended intramammary antimicrobial treatment in elimination of chronic intramammary *S. aureus* infections in lactating dairy cows were evaluated by Smith et al. (31). These authors demonstrated a significant reduction in mastitis cases in vaccinated and pirlimycin-treated cows, providing evidence that an autovaccine and an antibiotic can have synergistic action. As suggested by these authors, the antibiotic eliminates extracellular bacteria, whereas the vaccine stimulates synthesis of anti-*S. aureus* immunoglobulin G₂ and enhances phagocytic activity, leading to digestion of engulfed (intracellular) bacteria.

To summarize, the present study revealed that the administration of anti-*S. aureus* herd-specific autovaccine into the upper udder lymph node region, combined with infusion of the appropriate antibiotic into the teat canal, eliminates *S. aureus* and leads to prolonged udder protection against invasion by these microorganisms. Therefore it can be concluded that a herd-specific autovaccine administered with an antibiotic is the most effective method in the treatment of mastitis caused by *S. aureus*. Furthermore, it was also shown that a herd-specific autovaccine could be as effective as an antibiotic, which is of particular importance when considering rapidly developing pathogen resistance to most of the known antibiotics.

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