Persistence of gentamicin residues in cow milk after intramammary treatment

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SUMMARY

The indiscriminate use of the drugs for the treatment of mammary gland infections can leave residues in cow milk destined for consumption. The objective of this study was to evaluate the persistence of antibiotic residues in milk of lactating cows with and without mastitis after intramammary treatment with gentamicin. Twenty Black and White Holstein cows, 10 with mammary quarter inflammation and 10 without inflammation diagnosed by the California Mastitis Test, were selected. Gentamicin sulfate was administered by intramammary infusion into each quarter once daily. The treatment was performed after afternoon milking and repeated for 3 days. After application of the last antibiotic dose, milk samples were collected from the mammary quarters and collecting balloons over a period of 6 days for the detection of antibiotic residues by the Delvotest® SP. The microorganisms most frequently isolated from milk samples of cows without and with inflammation were Corynebacterium spp. (48.27% and 38.46%, respectively), Streptococcus spp. (13.79% and 46.15%), and coagulase-negative Staphylococcus (24.14% and 15.38%). The Staphylococcus aureus isolates were resistant to neomycin and coagulase-negative Staphylococci were resistant to oxacillin and penicillin G. Antibiotic residues were detected after the withdrawal period established by the manufacturer of the drug in 44.4% and 55.5% of samples collected from cows without and with inflammation, respectively, and in 20.0% and 40.0% of collecting balloons. Milk producers in Brazil should be advised to perform tests for the detection of antibiotic residues before releasing milk for consumption and the veterinary pharmaceutical industry should reevaluate the withdrawal period of intramammary administered antibiotics.

Keywords: Milk, Mastitis, Milk quality, Delvotest®SP, residues

INTRODUCTION

Mastitis is an inflammatory process of the mammary gland that has a marked economic impact on milk production by dairy cows, causing losses to milk producers and the dairy industry. The costs of this disease vary according to its severity, with the indiscriminate use of drugs and the disposal of milk containing antibiotic residues being the main causes of economic losses [33, 12, 6]. Mastitis usually occurs in response to a bacterial infection, but can also be caused by other microorganisms or by physical and chemical trauma [38].

The indiscriminate use of antimicrobial agents for the treatment of mastitis can lead to residues of these drugs in milk and to the selection of bacterial strains that are resistant to antibiotic therapy [32, 37]. The main causes of the persistence of antibiotic residues in milk are the administration of not recommended drugs whose pharmacokinetics is not completely established, excess dose administration, lack of compliance with the milk discard period in the case of treated animals, identification errors of treated animals, and confusion of milk after milking treated animals [18, 17]. The withdrawal period of antimicrobial agents is reported on the information leaflet and should be followed to prevent the contamination of milk. However, the occurrence of antimicrobial residues in commercialized milk and milk derivatives has been reported [2, 1]. Intramammary treatment of dry cows and pre-calving heifers has been identified as another important factor for the occurrence of antibiotic residues in milk [23, 24].

Milk containing antimicrobial residues is inappropriate for consumption and presents a public health risk [31]. Antibiotic residues in milk can cause allergic reactions in susceptible individuals and problems to the processing industry, interfering mainly with the fermentation of milk derivatives [32]. In contrast to other countries [34], no...
restrictions exist in Brazil regarding the use of gentamicin for the treatment of mastitis. Therefore, the objective of the present study was to evaluate the persistence of antibiotic residues in milk of lactating cows after intramammary treatment with gentamicin sulfate.

**Material and methods**

**CHARACTERIZATION AND SELECTION OF THE ANIMALS**

Milk samples were collected from mammary quarters of 20 Black and White Holstein cows for the diagnosis of subclinical mastitis by the California Mastitis Test (CMT) [7]. The CMT reaction was classified as negative (-), weak (+), moderate (++), or strong (+++). Ten of the animals submitted to the test exhibited strong reactions and were selected as animals with subclinical mastitis. Animals presenting negative reactions in all mammary quarters were classified as having no inflammation.

**TREATMENT AND COLLECTION OF MILK SAMPLES**

The animals had not been treated with any antibiotic at least 30 days prior gentamicin administration. Before the treatment, each infected quarter was thoroughly milked out by hand and the teat end was cleaned using a cotton swab soaked with 70% alcohol.

The treatment consisted of intramammary infusion syringe with 10ml commercial product (150 mg gentamicin sulfate, 50 mg bromhexine hydrochloride, and 10 ml excipient, Mastifin®️, Ourofino Agronegócio Ltda, Brasil). The infusion was performed once daily after the afternoon milking and repeated for 3 days. The withdrawal period reported on the information leaflet is 96 hours after application of the last dose in agreement with the withdrawal time of gentamicin that is commercialized in Brazil and France.

The milk samples were collected from the mammary quarters before treatment for determination of the microbiological profile, antimicrobial susceptibility profile, and detection of antimicrobial residues. For this purpose, the mammary quarters were washed and dried with paper towel and antisepsis was performed with 70% alcohol. Twenty-four hours after application of the last drug dose, milk samples were collected daily during afternoon milking for 6 consecutive days. In addition, milk samples were collected from the collecting balloons for the determination of antimicrobial residues.

**ANALYSIS OF MILK SAMPLES**

The milk samples were seeded onto 5% defibrinated sheep blood agar and incubated at 37°C. The bacterial isolates were identified as described by Murray et al. [21]. The antimicrobial susceptibility profile of coagulase-negative staphylococci (CoNS) and Staphylococcus aureus isolates was determined by the Mueller-Hinton agar diffusion test using disks impregnated with the following antibiotics: oxacillin (1µg), gentamicin (10µg), kanamycin (30µg), cefaclor (30 µg), neomycin (10µg), and penicillin G (10µg). Antibiotic susceptibility was evaluated according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [22].

The presence of antibiotic residues in milk was evaluated using the Delvotest SP microbiological test system (DSM Foods Specialties,) according to manufacturer instructions. Before the test, the milk samples were treated for the inactivation of possible nonspecific inhibitors present in milk and prevention of false-positive results according to Kosikowski and O'Leary [15]. The occurrence of antibiotic residues in milk was evaluated in the selected animals before and after treatment with gentamicin. Antibiotic-free raw milk (obtained from an animal that had not been treated for a long period of time) served as negative control. The positive control consisted of negative milk samples enriched with a solution of gentamicin sulfate Sigma®️ (St. Louis, MO, USA) at concentrations of 10, 50, 100, 200, 300 and 500 µg/L.

**STATISTICAL ANALYSIS**

The chi-square test was used to determine differences between groups, adopting a confidence level of 95%. Statistical analysis was performed using the Minitab software, version 15.1.3. [19].

**Results**

Table I shows the CMT scores and results of the microbiological tests obtained for 80 mammary quarter milk samples collected from lactating cows selected for...
intramammary administration of gentamicin. Among the 54 samples with a negative CMT result, microbiological growth was detected in 26 (48.15%) and cultures were negative in 28 (51.85%). Among the 26 mammary quarter milk samples with a positive CMT, cultures were negative in seven (26.92%) and positive in 19 (73.08%).

Table II shows the relative and absolute frequencies of microorganisms isolated from milk samples of cows with mastitis (strong CMT) and without mastitis (negative CMT). Staphylococcus (38.89%), Corynebacterium spp. (38.89%), and CoNS (14.82%) were the most frequent bacteria isolated from animals with subclinical mastitis. The most frequent pathogens isolated from cows without inflammation were Corynebacterium spp. (50.0%), CoNS (28.57%), and Gram-negative bacilli (14.29%). The frequency of isolation of Staphylococcus aureus was low in the present study. The CoNS strains isolated from cows with and without inflammation were resistant to oxacillin and penicillin G and Staphylococcus aureus was resistant to neomycin (Table III).

Persistence of antimicrobial residues was observed 2 days after the withdrawal period recommended by the manufacturer of the drug (4 days) in 55.5% and 44.4% of mammary quarter milk samples from treated cows without and with inflammation, respectively (Table IV). In addition, antimicrobial residues were detected in 40% and 20% of milk samples collected from the collecting balloons of treated cows without and with inflammation, respectively (Figure 1). No significant differences in the presence of antimicrobial residues after the withdrawal period were observed between animals with a strong CMT and a negative CMT reaction (P>0.05).

**Figure 1: Percentage of gentamicin detected in milk from ballons collectors after treatment**

**Discussion**

With respect to the various infectious agents responsible for the development of mastitis, Leslie et al. [16] suggested the existence of minor and major pathogens depending on the cellular immune response to the pathogen triggered by the mammary gland. Minor pathogens cause low somatic cell counts and include Corynebacterium spp. and some CoNS. Major pathogens are responsible for high somatic cell counts and include Staphylococcus aureus, coliform bacteria, and Streptococcus spp.

Staphylococci and streptococci are the microorganisms most frequently isolated from animals with subclinical mastitis. Species of the genus Staphylococcus are the main etiological agents of contagious mastitis due to their ability to penetrate and establish deep infection in mammary gland tissues [3]. In the present study, Staphylococcus aureus was isolated from only 3.84% of milk samples of cows with inflammation. The most prevalent species were Corynebacterium spp., Streptococcus spp. and CoNS. The role of Corynebacterium bovis as a causative agent of mastitis is controversial. Some investigators consider this microorganism to be of minor importance. However, studies have drawn attention to the high percentage of isolation of C. bovis from mammary quarters with mastitis, with this microorganism often being the most prevalent agent in dairy herds [13, 35]. The high frequency of isolation of bacterial species of this genus from milk samples is often related to failure of teat disinfection and milking hygiene [5, 28]. In the present study, the highest frequency of isolation of Corynebacterium spp. was observed for mammary quarters with negative CMT reactions.

The percentage of isolation of secondary mastitis pathogens such as Corynebacterium spp. and CoNS was higher for mammary quarters with negative CMT reactions, a finding highlighting the importance of isolation of the causative agents of mastitis for the diagnosis of the disease [5]. The group of CoNS plays an important role in contagious mastitis and different species have been isolated from infected mammary quarter milk samples [27].

Aminoglycoside antibiotics are widely used in Brazil for the treatment of mastitis and no restrictions exist regarding their veterinary use. The Program for the Control of Biological Residues elaborated by the Ministry of Agricultures, the organ responsible for the control of milk quality in Brazil, does not provide recommendations regarding the upper limits for gentamicin in milk [4]. Despite the demonstration of the efficacy of aminoglycosides such as gentamicin in the treatment of mastitis [29], evaluation of the withdrawal period of these drugs is necessary since determinant factors are often not taken into account in preliminary pharmacokinetic tests. In the present study, antibiotic residues were detected in mammary quarters and collecting balloons of animals for an additional 2 days after the withdrawal period recommended by the manufacturer. Raiaand Costa [30] observed the persistence of gentamicin residues in milk beyond the withdrawal period in animals with clinical mastitis after intramammary treatment. In the study of Pedersoli et al.[25] evaluating the persistence of gentamicin after systemic and intramammary administration, this antibiotic was detectable for a period of up to 228 hours. The persistence of aminoglycoside residues seems to vary depending on factors such as the formulation used, dose...
administered, drug interval, health status of the animal, and physiological factors [9, 34].

The CoNS strains isolated from treated mammary quarters were resistant to oxacillin and penicillin G and Staphylococcus aureus was resistant to neomycin. The indiscriminate use of antibiotics for different therapeutic purposes in veterinary medicine, especially for the treatment of mastitis, has led to the selection of resistant strains [20, 10]. Among the different mastitis-causing species, Staphylococcus aureus is the most important and most common microorganism in dairy herds worldwide. This microorganism is difficult to control because of the production of beta-lactamases, enzymes that cleave beta-lactam antibiotics and thus confer resistance to these antibiotics [14, 11, 26]. The animals selected in the present study come from a farm which has a history of the indiscriminate use of beta-lactam antibiotics and the recent use of gentamicin. The high level of resistance of Staphylococcus aureus and CoNS to beta-lactam and aminoglycoside antibiotics reported in studies conducted in different countries clearly shows that the prolonged use of antimicrobial agents, in the absence of isolation and identification of the microorganisms involved and concomitant susceptibility testing, will lead to the selection of resistant strains [28, 8, 36].

In conclusion, antibiotic residues were detected in milk beyond the withdrawal period recommended by the

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<thead>
<tr>
<th>Pathogens</th>
<th>CMT +++</th>
<th>CMT negative</th>
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<tr>
<td></td>
<td>n</td>
<td>%</td>
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<tr>
<td>Corynebacterium spp.</td>
<td>10</td>
<td>38.46</td>
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<td>Streptococcus spp.</td>
<td>12</td>
<td>46.15</td>
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<tr>
<td>Coagulase-negative Staphylococci</td>
<td>4</td>
<td>15.38</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1</td>
<td>3.84</td>
</tr>
<tr>
<td>Bacil gram negative</td>
<td>0</td>
<td>0.00</td>
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<tr>
<td>Total</td>
<td>26</td>
<td>100</td>
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Table II: Number and percentage of pathogens isolates from mammary quarters with and without mastitis

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<th>Microorganism (%)</th>
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<tr>
<td></td>
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<tr>
<td>Coagulase-negative Staphylococci (n=11)</td>
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<tr>
<td>Antimicrobial</td>
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<tr>
<td>Gentamicin (10µg)</td>
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<tr>
<td>Kanamycin (30µg)</td>
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<tr>
<td>Oxacillin (1µg)</td>
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<tr>
<td>Penicillin (10UI)</td>
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<tr>
<td>Cefaclor (30µg)</td>
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<td>Neomicin (10µg)</td>
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Table III: Antimicrobial resistance profile of Staphylococci isolates from cow with and without mastitis

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<thead>
<tr>
<th>Mammary quarters</th>
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<tbody>
<tr>
<td>Days after treatment</td>
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N= Number of mammary quarters

Table IV: Antibiotic residues in milk after treatment with gentamicin intramammary and score CMT.
manufacturer of the drug; a finding posing a health risk to consumers. Milk producers should perform rapid tests before the release of milk for consumption to prevent its contamination with gentamicin sulfate residue. Veterinary pharmaceutical industry should reevaluate the withdrawal period of intramammary administered antibiotics as gentamicin.

References


