Evaluation of close up antimicrobial therapies for treatment and prevention of subclinical mastitis in the herds with high prevalence of *Staphylococcus aureus*

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Abstract

Background: This field trial was conducted to describe and compare the efficacy of two treatment regimens comprising of pre-calving systemic administrations of marbofloxacin and tylosin in combination with dry cow therapy. For more precise detection of *Staphylococcus aureus* IMI, PCR (nucA gene) and RAPD-PCR genotyping have been applied. A total of 841 quarters from 212 dry cows within two herds were assigned to three groups TYLO, MARB and CONT. At 21±3 days prior to parturition, tylosin was injected for three days (10 mg/kg, SC) to cows from TYLO and at the same time, single injection of marbofloxacin (8 mg/kg, SC) was administered to cows from MARB. Cows assigned to CONT served as an untreated control. All quarters allocated to the groups received intra-mammary infusion of Kanaclox DC® at drying off. Milk samples from all quarters at one week, before last milking of dry off, 3 and 7 DIM were obtained for bacteriological and oxidative analyses.

Results: Despite the fact that no significant differences in total cure rate within the groups was demonstrated, the *S. aureus* cure rate achieved in TYLO and MARB were 74 and 73.5% respectively both of which being significantly higher than in CONT (58.1%). No significant differences in total new IMI were observed in TYLO (40.9%) and MARB (38.5%) groups compared with CONT group (42.5%). Furthermore the rate of new *S. aureus* IMI was higher in TYLO (24.5%) and MARB (24.1%) than in CONT (13.8%) group. Clinical mastitis rate in cows that received systemic injections (TYLO=3.8%, MARB=5.8%) was significantly lower than those detected in control cows (CONT=11.3%). Paired *S. aureus* isolates from dry off and post-calving have been clustered into 9 different RAPD types (A-I). 8 paired strains collected at dry off were identical to those at post-calving and 35 strains had more than 60% of dissimilarity.

Conclusion: The study demonstrates that dealing with herds in which *S. aureus* IMIs are prevalent, using close up broad-spectrum systemic antimicrobial agents can be unrewarding for preventing new *S. aureus* IMIs. However close up administration of systemic antimicrobials have the potential to reduce the incidence of clinical mastitis during lactation.

Background

The majority cases of mastitis in early lactation are the result of new dry period infections (1). Dry cow therapy (DCT) can reduce the proportion of intra-mammary infections, both by eliminating existing infections and preventing new infections during the dry period (2).

New infection rates are highest in the early dry period, and are lowest when involution is complete, and increase again as parturition approaches (3). During the colostrogenesis stage the protective factors of udder become diluted, the keratin plug breaks down and leukocyte function becomes impaired (4). Administration of antimicrobials in forms of intra-mammary infusion or systemic injection at the beginning of dry off would cover the first half of dry period (5, 6). However the level of administered compounds in udder gland will decline to below the MIC (7) resulting in a prophylaxis gap in the close up window (3, 5).

The practice of blanket DCT has been efficient in eliminating existing IMI by 70 to 98% and provided short-term protection against new IMI during early dry period (reduction by 50 to 75%) (3). Even though the DCT formulations are labeled effective against gram-positive bacteria they are generally acknowledged as being less successful in eliminating IMI caused by *Staphylococcus aureus* (8) and new IMI may still occur if offensive pathogens are not susceptible to the antimicrobial preparations. Furthermore the active ingredients of antimicrobial agents do not maintain at therapeutic levels throughout the entire dry period (5, 6).

Some reports have been published regarding efficiency of systemic dry cow therapy as management practice (9). Norfloxacin was reported as an effective drug for systemic treatment of *S. aureus* (9). For the same pathogen, Bolourchi et al found that systemic injection of enrofloxacin or tylosin are as efficient as intra-mammary infusion (10). Zecconi reported that administration of tylosin, two weeks before calving, can significantly decrease the IMI rate after calving, compared to those cases treated only at dry off (11).

Based on pharmacokinetic characteristics of macrolides and fluoroquinolones, passage from blood into the udder tissue is rapid and extensive (12). Marbofloxacin as a fluoroquinolone is a broad spectrum antimicrobial and no development of resistance has been found over time to which all the mastitis *S. aureus* isolates were susceptible (13).

Tylosin as a macrolide with basic pH and lipophilic character can reach milk to plasma concentration ratio of 5:1 (14). This feature would make this antibiotic an ideal parental treatment of IMI.

Genotyping methods have proven a useful tool to determine the diversity and spread of *S. aureus* strains which are not feasible by common bacteriological examination solely. Furthermore using various typing methods, differences regarding pathogenicity and antimicrobial susceptibility have been demonstrated (15, 16). Strain-specific characteristics significantly impact the success rate of cure of *S. aureus* IMI after implementing DCT (17).

Sommerhäuser et al, isolated *S. aureus* strains before and after DCT and using genotyping method reported that the actual persistent and newly infected quarters were able to be realized (18). Using RAPD-PCR on isolates of *S. aureus* from quarters during acute phase of mastitis and after treatment, it has been revealed that paired isolates were identical and original infective strains persisted (19, 20).

The main objective of this study was to describe and compare the effectiveness of two close up protocols of systemic antimicrobial agents in conjunction with routine DCT to cure existing IMI and the prevention of new clinical and subclinical IMI during the dry and early lactation period. Identification of the strains of *S. aureus* through DNA technologies was not commonly incorporated into previous DCT trials, so current study tried to reach a more precise assessment of DCT by taking advantage of a genotyping method.

Results

At the beginning 900 quarters from 227 cows enrolled at dry off and we excluded cows because of death, abortion, culling and quarters due to contaminated sample. Finally 212 cows representing 841 quarters remained in the study for analysis. TYLO: 291, MARB: 275, CONT: 275 quarters. Mean dry period length of cows included in TYLO, MARB and CONT groups were 57 ± 3 , 56 ± 3 and 57 ± 3 days respectively.

IMIs at Dry off and Post-calving

At dry off, total prevalence of IMIs was 67.8%. In TYLO, MARB and CONT quarters the prevalence was 67.0, 60.7 and 72.4% respectively which neither of mentioned values was statistically different among all groups ($P > 0.05$). Total prevalence of IMIs of post-calving was 61.8% and within each treatment group was as follows: TYLO 62.2%, MARB 60.4%, CONT 62.9%, which no differences were found within the three groups ($P > 0.05$).

Clinical Mastitis rate

The proportion of quarters with occurrence of clinical mastitis between dry off and DIM 30 was significantly lower for both treatment groups (TYLO=3.8%, MARB=5.8%) vs. CONT (11.3%) quarters (figure2).

New Infection rate

The percent of new IMIs acquired by each treatment group regarding every specific pathogen are outlined in Table1. Considering the total pathogens no significant differences were identified for new IMI rates in TYLO (40.9%), MARB (38.5%) and CONT (42.5%) groups ($P > 0.05$). Quarters from TYLO and MARB groups acquired significantly higher new *S. aureus* IMI compared to quarters from CONT group ($P=0.003$, $P=0.004$).

Factors Affecting New *S. aureus* Infection rate. Among the factors that were analyzed with regression model, the odds of developing a new *S. aureus* IMI were 1.8 and 1.5 times higher for quarters from TYLO ($P<0.05$) and MARB ($P<0.05$) respectively. Furthermore, cows with <30 kg milk yield (OR=5.4, $P<0.05$) and parity of >2 (OR=2.3, $P<0.05$) had greater odds of developing a new infection. Data summarized in table2.

Cure rate

The total cure rate was 72.3, 76.2 and 59.6% for TYLO, MARB and CONT respectively which the differences were not significant among them. The *S. aureus* cure rate achieved with tylosin and marbofloxacin injections was 74 and 73.5% respectively which both of them were significantly higher than control group (58.1%). Data of cure rate has been summarized in table3.

Factors Affecting Cure rate. The logistic regression model for cure of quarters is shown in Table4. The odds ratio (OR) of experiencing total cure in quarters that received tylosin and marbofloxacin was 1.3 and 1.4 respectively, which had a tendency to be significant. Quarters infected with CNS that received tylosin

had greater odds of cure (OD=4.1, $P=0.04$). Quarters of cows with higher than 30 kg of daily milk yield less likely to cure compared with those lower than 30 kg ($P=0.003$). There was a negative relationship between LS at dry off and likelihood of cure regarding major (OD=-1.34, $P=0.07$) and minor (OD=-1.29, $P=0.04$) pathogens.

Disc Agar Susceptibility Test

Disc agar susceptibility test was performed on 180 *S. aureus* isolated from dry off and 7 ± 3 DIM. Based on antimicrobial agents used in-vivo, results of 5 antimicrobial discs have been provided in figure3. Generally higher percentages of resistance were found in post-calving isolates compared to dry off isolates. The differences were significant for both tylosin and cloxacillin ($P<0.05$).

Total Oxidant/Antioxidant Capacity in Quarters with New Infection

Considering total pathogens, post-calving DTNB[1] concentrations in quarters with new infection were higher than that of in healthy quarters in TYLO ($P<0.01$), MARB ($P<0.01$) and CONT ($P<0.05$) groups. Same results were found for *S. aureus* in TYLO ($P<0.01$), MARB ($P<0.01$) and CONT ($P<0.01$) groups. FRAP[2] concentrations were higher in cured quarters compared to uncured quarters in TYLO ($P<0.01$), MARB ($P<0.05$) and CONT ($P<0.01$) groups. Furthermore FRAP concentrations had a tendency to impact 1.3 times on odds of experiencing cure ($P=0.09$). Data have been summarized in table5.

RAPD[3]-PCR

The RAPD-PCR technique was shown to be efficient in typing the studied strains. Out of the two primers used, Ap4 showed a better discriminatory power than T3. Amplification of the AP4 primer resulted in a multiple amplicon with size range from 100 bp to more than 2000 bp from all of 86 *S. aureus* isolates. The 43 *S. aureus* paired samples selected from quarters that were infected before (dry off) and after parturition (7 ± 3 DIM). Isolates were characterized into 9 different RAPD types (A-I) with instead of which the majority of isolates being categorized as A, B, E and G type. 8 paired strains collected at dry off stage were identical to those collected at post-calving and 35 strains from dry off had more than 60% of dissimilarity to post-calving strains. RAPD patterns for isolates in this study have been provided as a dendrogram in figure4. Different patterns of antimicrobial resistance among four predominant RAPD types (A,B,E,G) in each herd have been given in table6. Penicillin resistance in type E was lower compared to other types in both herds and tylosin resistance in type G was lower than other types in both herds. Penicillin and tylosin resistance in type E from herd1 were considerably higher than same type from herd2.

[1] (5,5'-Dithiobis(2-nitrobenzoic acid)

[2] Ferric Reducing Ability of Plasma

Discussion

The object of this study was to evaluate the efficacy of systemic antimicrobial agents at close-up period for treatment of existing infections that had not been cured by dry off intra-mammary preparations and the control of new IMI with emphasize on *S.aureus* strains.

Clinical Mastitis rate

In the present study pre-calving systemic injection of tylosin and marbofloxacin effectively reduced the occurrence of clinical mastitis within 30 days of calving. Although there is no study on the effect of close up systemic antimicrobial agents on incidence of clinical mastitis, some studies had previously compared effects of blanket or selective DCT. Ismail et al. evaluated the effect of systemic 12 g of tylosin in conjunction with intra-mammary infusion of beta lactam antibiotics and framycetin. These authors stated that the rate of clinical mastitis within the 100 DIM was significantly reduced in cows received tylosin (21). Contradictory results have been reported from Australia indicating more clinical mastitis rate after DCT (6). Pathogen isolation was not performed for clinical cases in this study but based on previous studies it is presumed that the most of clinical mastitis cases are caused by environmental pathogens including streptococci and coliform bacteria (22). Tylosin as member of Macrolides have high MIC for gram-negative bacteria (23) and generally is effective against gram positive bacteria. In case of our study the high doses of the drug (20mg/kg) was administered for three consecutive days. On the other hand marbofloxacin has a wide spectrum of activity and it has been proven that *Escherichia coli* strains are very susceptible to marbofloxacin with a very low MIC90 (24). Bradley et al. demonstrated that using an antimicrobial agent with a gram negative spectrum as DCT can influence the incidence of clinical coliform mastitis in the subsequent lactation (5). The findings of present study and other related studies confirm that dry cow therapy can play a role in the clinical mastitis epidemiology in early lactation. In respect to lack of DC intra-mammary products specific to gram negative bacteria in the market, it might be beneficial to take advantage of potent systemic antimicrobial agents with proper effectiveness on gram negative bacteria.

New Infection rate

Based on our data regarding total pathogens, systemic therapy with tylosin or marbofloxacin prior to parturition did not significantly decrease the total rate of new infection. Furthermore the rate of new infection by *S. aureus* was higher in quarters which received tylosin or marbofloxacin. Unlike to the present study which the adjunct systemic antimicrobials were administered three weeks before calving, in the most of previous studies the systemic antimicrobial agents had been administered at the beginning of drying off which made the comparison difficult. Contreras et al, used 12g of systemic tylosin combined with intra-mammary infusion and teat seal to prevent new infection and it was established that addition of systemic tylosin was not effective (25). Lower rate of new IMI in that study might be because of lower prevalence of *S. aureus* (4.5%) compared to our study (15.1%) and taking the advantage of teat seal

which was not used in present study. In both herds of under present investigation *S. aureus* was prevalent and it has been noted that contagious bacteria, especially *S. aureus*, are likely to establish more new infections in those herds where they are prevalent (26).

The findings from Erskine et al. suggested that intra-mammary infusion of cephapirin benzathine combined with four consecutive days of oxytetracycline injections were no more effective than intra-mammary infusion alone in preventing infection (27). In contrast to our study findings, Soback et al. reported significantly fewer *S. aureus* new IMIs occurrence in both systemic norfloxacin and oxytetracycline groups than in the group receiving intramammary infusion of cephapirin and the control group. It should be noted that in that study the systemic treatments were not used concurrently with intra-mammary infusion instead, they were administered at the onset of dry period. Another study revealed that combination of a high persistency product and pre-partum treatment with a lactating cow product did not significantly reduce the number of *S. aureus* infections postpartum when compared with intra-mammary infusion alone (28).

One explanation for the relatively poor preventive success of pre-calving systemic antimicrobial agents against *S. aureus* IMI in the present study might be related to the higher cure rate of CNS and *C. bovis* infections in treatment groups compared to control. Several authors stated that CNS bacteria can protect quarters against IMI caused by major pathogens, either when causing IMI (29, 30) or when colonizing bovine teats (31). Data from Pankey and Nickerson suggest that quarters containing *C. bovis* are more resistant to *S. aureus* infections (32). It is possible that elimination of minor pathogens by systemic antimicrobial agents has increased the susceptibility of treated quarters and higher percentages of CNS and *C. bovis* in the control quarters had protective effects against *S. aureus*. General activation of the immune system, competition for binding sites and alterations in teat canal keratin are suggested as possible explanations (33).

Another explanation could be the development of resistance to antimicrobial agents. *S. aureus* isolates typical of present IMI cannot be directly compared with those identified of 20 to 30 years ago. Additionally according to in-vitro susceptibility data, post-calving and dry off *S. aureus* isolates showed notable resistance behaviors against the antimicrobial agents used.

RAPD-PCR fingerprint types from post-calving isolates were mostly different from those isolated at dry off. It is noteworthy that all of 4 main RAPD types were resistant against penicillin. A and B types were more resistant to tylosin and marbofloxacin than E and G types (table 9). Dingwell et al. indicated that certain genotypes of *S. aureus* show different responses to systemic antimicrobial agents during dry period (17).

Factors Affecting New Infection rate. We saw increased proportion of new IMI in cows with higher than two parities. A similar association between the risk of acquiring IMI in dry period and parity has been previously documented (34). Increased IMI associated with higher parity might be related to a decrease in the integrity of the streak canal (35).

Cure rate

Generally the cure rates for infected quarters in different groups are similar to those reported in previous studies with the calculated average of 78% (71 to 85%) (36). The *S. aureus* cure rate for quarters received intra-mammary infusion solely is similar to those reported in initial drug efficacy studies (36). We observed that the total cure rate after pre-calving injections of tylosin or marbofloxacin alongside with intra-mammary infusion compound were not significantly different compared to intra-mammary infusion alone. Similarly in a study by Erskine et al. cows administered intramuscular oxytetracycline and intra-mammary cephapirin and the cure rates of quarters did not improved compared to quarters treated with cephapirin alone (27). Bolourchi et al., found that systemic enrofloxacin or tylosin at drying off approached but did not increase the efficacy of intra-mammary infusion (10). The aforementioned studies were performed in herds with high prevalence of *S. aureus*. We observed that both tylosin and marbofloxacin improved *S. aureus* cure rate which is consistent with results from another study in which systemic norfloxacin was administered at the start of the dry period that achieved a better *S. aureus* cure rate compared to intramammary cephapirin (37). Zecconi et al., reported that administration of systemic tylosin 2 weeks before calving, alongside with traditional dry-cow therapy is an effective supplementary treatment for intra mammary therapy of *S. aureus* (11).

Strain-specific characteristics can be expected to affect the probability of cure of *Staph. aureus* IMI, but we did not detect strain-specific response in RAPD types. Dingwell et al. observed a strain-specific response to tilmicosin or cloxacillin as dry cow treatment and reported that the three predominant groups of strains, responded well to tilmicosin compared to the rest of groups (34). More studies are warranted to examine the variability of treatment responses in different strains of *S. aureus*. Moreover, high proportion of strains isolated in present study were resistant to penicillin. It has been found that the probability of cure is lower for penicillin-resistant *S. aureus* than for penicillin-susceptible strains (38).

Factors Affecting Cure rate. The Odds of cure was lower in quarters with higher than 30 kg of daily milk yield. Higher milk production is usually associated with larger mammary gland size while the antimicrobial agent must diffuse through a larger tissue and a larger amount of tissue needs be cleared of infection (38). Probability of cure for total and specific pathogens tended to reduce with LS enhancement. Similarly, others observed that the probability of cure in quarters infected with *S. aureus* decreased as SCC increased (17, 39). High cow-level SCC may also indicate that multiple quarters within the udder are infected which more infected quarters means less chance of cure (8).

Antimicrobial Susceptibility Test

In the present study rather than penicillin and cloxacillin resistance considerable percentage of isolates were resistant to tylosin particularly in isolates from post-calving samples. Both phenotypic (40) and genotypic (41) tylosin resistance in *S. aureus* isolates have been reported from subclinical mastitis cases in Iran. Extraordinary usage of macrolides for treatment of mastitis in Iranian dairy farms in recent years might be as a one of underlying causes. Because of elimination of susceptible strains by dry off

treatment and introducing some new strains, there were some discrepancies in resistance character of strains from dry off and post-calving.

RAPD-PCR

Based on results of RAPD-PCR, in quarters in which *S. aureus* was isolated in both dry off and post-calving, dry-off strains were not persisted in the udder and reinfection has been made by different *S. aureus* strains. Contradictory result has been reported by Myllys et al. who stated that *S. aureus* strains have been persisted in udder without reinfection by other strains (19). These authors implemented ribotyping as well which was not used in our study. Moreover in that study the poor cure rates of *S. aureus* were associated to persistence of the original strain. Four predominant RAPD types (A,B,E,G) were detected in present study. Based on genotyping survey by Sommerhäuser et al., in some herds one type of strains are dominant while in some herds multiple dominant types exist (18). The later scenario was found in both herds of present study.

Milk Oxidant and Antioxidant Capacity

In recent years, free radical damage has become increasingly important as a complementary tool in the evaluation of inflammatory status. It was not an objective of this study to describe the mechanism but we have investigated possible connection of oxidant/anti-oxidant markers to the new infected, cured quarters or even as a related risk factor.

Generally post-calving total oxidant capacity (TOC) (DTNB) in quarters with new infection was higher than that of in healthy quarters which is consistent with results of study by Atakisi et al., who reported that TOC levels were significantly higher in milk of glands with subclinical mastitis compared to normal glands (42). In another study performed on goat the authors sustained the similar results (43). Infection of mammary gland causing expansion in the number of neutrophils, epithelial cells and cytokines in mammary tissue which resulted in enhancement of free radicals in milk (44).

According to data from regression analysis, TAC tended to impact 1.3 times on odds of cure. Probably the quarters with higher antioxidant capacity have more potency to overcome infections. Future studies are needed to determine the effects of various blood and milk oxidant/antioxidants markers on probability of cure and acquiring new infection.

Conclusion

The results generated in present study suggest that close up systemic administration of tylosin or marbofloxacin resulted in reduction of post-calving clinical mastitis rate but were not able to prevent new IMIs. Considering the probability of eliminating minor pathogens without affecting the major pathogens, this protocol appeared to be unsatisfactory for prevention of new IMIs in herds with high prevalence of *S. aureus*. Conducting antimicrobial susceptibility test, molecular typing for isolated pathogens and

assessment of oxidant/antioxidant capacity of milk could be a valuable tool for selecting appropriate therapeutic compounds as a part of dry cow management.

Methods

Cow/ herd Selection and Sampling

Two commercial free-stall Holstein dairy herds with high bulk milk count of *S. aureus* (ranged from 50 to 60 CFU/mL), milking 800 (herd 1) and 300 (herd 2) cows were selected for this study. Daily milk yield were 38 and 40 L respectively. Cows eligible for study had to be in fine health with functional quarters free of clinical mastitis and notable teat lesions without receiving antimicrobial or anti-inflammatory agents within the last two weeks. The cows were dried off by gradual cessation of milking at 60 ± 3 days prior to expected date of calving. Three foremilk samples from each quarter aseptically collected one week before and at the last milking of drying off and immediately transferred to the laboratory in a styrofoam box containing ice. Following the last milking, all quarters were disinfected and infused with Kanaclox DC[®] (Pars Dopharma, Iran); Kanamycin acid sulfate 100000 IU, Cloxacillin Benzathine 500 mg, Penicillin G Procaine 300000 IU and Oilbase: 10 gr). Post-calving milk samples were taken at 3 and 7 DIM.

Study Design

Following DCT cows were randomly allocated to one of the three groups (TYLO, MARB and CONT) via block randomization. Cows in TYLO received once daily SC injection of 10 mg/kg mg tylosin (Tyloject[®] 20%, Razak Laboratories Co, Iran) for three days, at 21 ± 3 days prior to calving and cows in MARB received single SC injection of 8 mg/kg SC marbofloxacin (Marbox[®], Ceva Santé Animale BV, Netherlands) at 21 ± 3 days prior to calving while cows assigned to CONT remained untreated (Figure1).

Milk Sample Analysis

Somatic Cell Counts. Composite milk samples from last month before dry off were counted by electric counter (Fossomatic Milk Analysis, Foss Electric, Hillerød, Denmark) and linear score (LS) was calculated subsequently.

Laboratory Bacteriological Culture. Culture techniques and identification of bacteria were performed according to instructions from National Mastitis Council (45). Diagnosis of various pathogens was made based on morphology, growth on selective and differential media, Gram staining, hemolysis, catalase, MSA, hippurate and CAMP. A sample was considered contaminated if more than 3 bacterial species were present on a plate.

Identification of *S.aureus* Isolates. The isolates were submitted to VP (Voges Proskauer) and coagulase tests. The quarters considered to be infected if >100 CFU/ml of *S. aureus* isolated from each cultures of

any one milk sample taken at dry off and post-calving. To confirm the identification of isolates as *S. aureus*, the gene was amplified by *nucA* via conventional PCR method.

Antimicrobial Susceptibility Test. The Agar disk diffusion method (ADD) was used to determine the susceptibility of *S. aureus* isolates. Procedures were based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (46). *S. aureus* ATCC 29213 was used as the reference strain for quality control.

***nucA* PCR.** DNA extraction was performed by rapid boiling according to method by Abdelhai et al.(47). Primers were selected on the basis of the published nucleotide sequence of the 613-bp *nuc* gene (F- CTG GCA TAT GTA TGG CAA TTG TT, R- TAT TGA CCT GAA TCA GCG TTG TCT).

RAPD-PCR. In quarters from which *S. aureus* was isolated from both dry off and post calving, the genomic variability of the *S. aureus* isolations was analyzed by RAPD-PCR method based on two primer AP4(5' TCACGCTGCA 3') and T3 (5'ATTAACCCTCACTAAAGG3'). The amplification conditions for AP4 primer performed based on study by Morandi et al. (48). Amplification cycle of the T3 primer has been described by Pinto et al. (49). Thermal cycler (BIO RAD®, USA).

Amplification products were electrophoresed on a 1.8% agarose gel (Bio-Rad, California, USA) in TAE buffer. Gels were stained with ethidium bromide and photographed under UV light. PCR fingerprint images were analyzed estimated band sizes using Gel Compar®II (Applied Maths, Inc, Austin, USA) software. Strains were clustered and displayed in dendrogram form with consideration of 60% genetic similarity.

Biochemical Analysis

Total Antioxidant Capacity (TAC). Antioxidant capacity of skim milk samples was assessed by the method of FRAP(50). The reduction of Fe³⁺-TPTZ to Fe²⁺-TPTZ was followed by the absorbance increase at 595 nm via spectrophotometer (2150-UV, UNICO®, USA).

Total Oxidant Capacity (TOC). Oxidant capacity of skim milk samples was analyzed by measuring DTNB based on the method applied by (43). The decrease in absorbance at 412 nm was used to calculate the rate of oxidation of TNB to DTNB. The parameters have measured in each sample three times and coefficient of variation (CV) was calculated by the formula (SD/mean×100). Within-run CV were <1.0% at all values tested. Between-series imprecision and bias have been evaluated as well.

The investigator of the tests was blinded to group allocation.

Statistical Analysis

In general, the statistical analysis was performed using SPSS software (version 21.0; SPSS Inc., Chicago, USA). The sample size calculated based on confidence level of 95% and power of 90%. Chi-square analysis was used to investigate the differences in cure, new infection and clinical mastitis rates among

treatment groups. Logistic regression was carried out to determine the effect of treatments on the probability of cure, new infection and clinical mastitis controlling for parity, milk yield, position of quarter and oxidant/antioxidant balance at dry period. Kaplan-Meier survival curves were plotted to show survival distribution function of quarters with clinical mastitis for each level of treatments. Mann-Whitney U test was used for comparison of DTNB and FRAP between cured and uncured and new infected and healthy quarters. All statistical differences were assessed at the two-sided 0.05 level of significance.

Definition of Dependent Variables

Developing New Infection Between Dry off and 7±3 DIM. A new IMI was defined as the presence of pathogen at 3 or 7 DIM in a quarter that was not previously infected at dry off, or the presence of a different pathogen from those present at dry off (51). In cases of mixed infections, a quarter was counted only once as having a new IMI, even if 2 new bacterial pathogens were cultured. In the cases which RAPD-PCR revealed an *S. aureus* isolate at calving belonged to different strain than *S. aureus* isolate at dry off, they were considered to new IMI.

Developing Clinical Mastitis. A clinical mastitis occurrence was defined as the appearance of obvious abnormal milk (with/without abnormal mammary gland or systemic signs of illness). A quarter developing one or more episodes of clinical mastitis between dry off and 30±3 DIM was classified as a positive clinical mastitis case.

Experiencing Cure. Cure was defined as the elimination of one or more pathogens at both 3 and 7 DIM that previously had been existed at dry off. The RAPD-PCR results were considered for the calculation of cure rate regarding the quarters which *S. aureus* isolated from both dry off and post-calving.

Abbreviations

DIM: days in milk

IMI: Intra-mammary infection

MIC: Minimum inhibitory concentration

Declarations

Ethics approval and consent to participate

The present study included cows. Protocol of study was confirmed by animal welfare committee of Ferdowsi University of Mashhad (38059) in accordance with institutional and national and/or international guidelines.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Research concept and design: BK, AHFR; Statistical analysis: MA; Samples collection: PA;

LAB Tests: MH, BK; Experiment performance: PA; Manuscript writing: PA, BK; All the

authors read and approved the final manuscript.

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Tables

Table1: Results of total and pathogen specific new IMI rate considering uninfected quarters and quarters infected by different pathogen between dry off and 7±3 DIM.

group	Total %(n)	<i>S.aureus</i>	CNS	<i>C. bovis</i>	<i>S.agalactiae</i>	Yeast	Other	Minor	Major
TYLO	40.9 (119/291)	^a 24.5 (59/241)	24.8 (49/197)	6.5 (17/261)	0.0 (3/287)	0.0 (0/290)	0.3 (1/289)	29.3 (44/150)	^a 25.5 (60/235)
MARB	38.5 (106/275)	^a 24.1 (58/241)	21.9 (43/196)	2.0 (5/251)	1.5 (4/266)	0.4 (1/270)	0.7 (2/275)	31.1 (47/151)	^a 25.9 (61/235)
CONT	42.5 (117/275)	^b 13.8 (32/232)	29.2 (38/130)	3.7 (10/267)	1.1 (3/274)	0.4 (1/270)	1.1 (3/274)	34.2 (41/120)	^b 14.9 (35/234)

(^{a-b}Means with different superscripts in each column indicates significant difference ($P < 0.05$))

Table2: Results of multivariate regression analysis of odds for acquiring new *S.aureus* IMI in uninfected and infected with different pathogen between dry period and 7±3 DIM

Variable		B	S.E	Wald	df	Sig.	Exp(B)	95% CI Exp(B)	
								lower	upper
treatment	control								
	Tylosin	0.618	0.270	5.228	1	0.022	1.855	1.092	3.150
	Marbofloxacin	0.423	0.280	2.290	1	0.030	1.527	0.883	2.642
Quarter position	Front								
	Rear	0.040	0.214	0.035	1	0.852	1.041	0.685	1.581
Milk yield	>40 kg								
	30-40 kg	1.043	0.370	7.967	1	0.005	2.838	1.375	5.856
	<30 kg	1.689	0.364	21.539	1	0.000	5.416	2.654	11.055
Parity	1								
	>2	0.833	0.227	13.431	1	0.000	2.300	1.473	3.591
Oxidant/antioxidant marker	DTNB ¹	0.068	0.100	0.456	1	0.500	1.070	0.879	1.302
	(pre-calving)								
	FRAP ²	-0.33	0.243	1.890	1	0.169	0.716	0.444	1.153
	(pre-calving)								
Constant		-3.15	0.508	38.489	1	0.000	0.043		

¹DTNB: 5,5-dithiobis-2-nitrobenzoate

²FRAP: ferric-reducing ability of plasma.

Table3: Results of cure rate for the different groups (Different letters (a, b) above the numbers in each column indicates significant difference)

Group	Total %(n)	<i>S.aureus</i>	CNS	<i>C.bovis</i>	<i>S.agalactea</i>	Yeast	Other	Minor	Major
TYLO	72.3 (199/144)	74.0 ^a (50/37)	62.5 ^a (112/70)	100.0 ^a (30/30)	100.0 (4/4)	100.0 (1/1)	100.0 (2/2)	71.0 ^a 103/14 (5	75.9 ^a 41/5 (4
MARB	76.2 (185/141)	73.5 ^a (34/25)	65.1 ^a (106/73)	90.3 ^a (31/29)	100.0 (9/9)	100.0 (2/2)	100.0 (3/3)	75.3 ^a 107/14 (2	79.1 ^a 34/4 (3
CONT	59.6 (233/139)	58.1 ^b (43/25)	52.8 ^b (140/74)	76.7 ^b (43/33)	100.0 (1/1)	100.0 (5/5)	100.0 (1/1)	59.7 ^b 113/18 (9	59.0 ^b 26/4 (4

(^{a-b}Means with different superscripts in each column indicates significant difference ($P < 0.05$))

Table4: Results of final logistic regression model for the probability of cure of infected quarters during the dry period.

Variables		Total pathogens		<i>S.aureus</i>		CNS		Minor		Major	
		P	OR	P	OD	P	OD	P	OD	P	OD
Treatment	Tylosin	0.095	1.353	0.261	1.811	0.043	1.794	0.309	1.307	0.270	1.889
	Marbox	0.096	1.427	0.190	1.905	0.045	1.811	0.47	1.722	0.380	1.298
Quarter position	Rear	0.103	-0.713	0.256	1.721	0.618	0.889	0.249	0.777	0.633	1.244
Parity	>2	0.127	-0.721	0.249	-1.865	0.644	-1.121	0.377	-1.219	0.189	-1.990
Milk yield	<30 kg	0.003	-0.426	0.011	0.207	0.887	0.951	0.673	-1.144	0.000	-0.126
	30-40 kg	0.174	-0.695	0.728	-0.793	0.944	-0.976	0.658	1.146	0.242	0.474
LS ¹ at Drying off	≥5	0.07	-1.12	0.076	-1.31	0.041	-1.22	0.042	-1.299	0.079	-1.344
DTNB ²		0.230	1.126	0.414	1.216	0.209	1.158	0.085	1.203	0.378	1.231
FRAP ³		0.094	1.321	0.095	1.336	0.339	0.980	0.261	0.897	0.096	1.289
Constant		0.147	0.574	0.419	0.440	0.653	0.821	0.367	0.691	0.936	0.924

¹LS: linear score

²DTNB: 5,5-dithiobis-2-nitrobenzoate

³FRAP: ferric-reducing ability of plasma.

Table5: median (max-min) concentrations of DTNB and FRAP as oxidant and antioxidant markers in quarters within the groups of study considering total pathogen and *S. aureus* IMI. (DTNB1 and FRAP1: dry off, DTNB2 and FRAP2: post-calving).

Markers	Quarter	TYLO		MARB		CONT	
	Condition	Total pathogen	<i>S. aureus</i>	Total pathogen	<i>S. aureus</i>	Total pathogen	<i>S. aureus</i>
DTNB2 μmol/s	New	3.11(0.22-	3.97(1.01-	2.31(0.56-	3.11(0.63-	4.73(3.11-	4.65(2.88-
	infected	7.22) ^a	7.22) ^a	7.80) ^a	7.8) ^a	5.12) ^a	5.1) ^a
	Healthy	1.21(0.34-	1.2(0.34-	1.55(0.37-	1.7(0.37-	2.40(1.23-	2.8(1.23-
		4.2) ^b	5.78) ^b	4.5) ^b	7.22) ^b	5.83) ^b	5.83) ^b
FRAP2 μmol/L	New	1.0(0.45-	1.3(0.38-	0.67(0.33-	0.77(0.33-	0.62(0.23-	0.83(0.44-
	infected	2.71)	2.71)	2.65)	2.6)	2.33)	1.37)
	Healthy	0.89(0.39-	1.12(0.34-	0.63(0.24-	0.63(0.24-	0.67(0.23-	0.67(0.23-
		2.05)	4.25)	2.11)	2.1)	4.03)	4.03)
DTNB1 μmol/s	Uncured	1.78(0.41-	1.94(0.89-	1.79(0.3-	1.79(0.47-	1.71(0.54-	3.12(1.04-
		5.34)	3.87)	5.7)	5.7)	5.26	5.01) ^a
	Cured	1.44(0.4-	2.09(0.4-	1.61(0.35-	1.5(0.58-	1.45(0.4-	1.5(0.69-
		5.05)	5.95)	4.89)	3.98)	5.02)	2.45) ^b
FRAP1 μmol/L	Uncured	0.55(0.23-	0.46(0.25-	0.67(0.22-	0.41(0.25-	0.49(0.31-	0.44(0.30-
		1.38) ^a	2.30) ^a	3.11) ^a	2.5) ^a	1.34) ^a	0.74) ^a
	Cured	0.87 (0.33-	0.9(0.34-	1.12(0.45-	1.05(0.4-	0.87(0.23-	1.03 (0.55-
		1.32) ^b	3.07) ^b	1.8) ^b	2.01) ^b	1.66) ^b	2.06) ^b

(^{a-b}Medians with different superscripts in each column indicates significant difference ($P < 0.05$))

FRAP: ferric-reducing ability of plasma, DTNB: 5,5-dithiobis-2-nitrobenzoate.

Table6: Antimicrobial resistance of the four predominant RAPD types (A,B,E and G) in herd 1 and herd 2.

Antimicrobials	No of isolates of indicated RAPD type							
	A		B		E		G	
	Herd 1	Herd 2	Herd 1	Herd 2	Herd 1	Herd 2	Herd 1	Herd 2
Marbofloxacin	4/12	1/5	4/15	1/5	2/11	1/7	2/8	0/4
	33%	20%	27%	20%	18%	14%	25%	0.0%
Tylosin	10/12	3/5	9/15	3/5	5/11	1/7	1/8	0/4
	83%	60%	60%	60%	45%	14%	12.5%	0.0%
Penicillin	7/12	4/5	7/15	3/5	5/11	2/7	2/8	1/4
	58%	80%	47%	60%	45%	29%	25%	25%
Cloxacillin	6/12	3/5	8/15	1/5	2/11	1/7	0/8	0/4
	50%	60%	53%	20%	18%	14%	0.0%	0.0%
Kanamycin	2/12	0/5	2/15	0/5	1/11	0/7	0/8	0/4
	17%	0.0%	13%	0.0%	9%	0.0%	0.0%	0.0%

Figures

Figure1: schematic design of study

Figure 1

Figure 2

Kaplan-Meier survival curves providing survival distribution function of quarter with clinical mastitis for each of the treatment groups from drying off (day 0) until DIM 30 (day 90).

Figure 3

Antimicrobial susceptibility test results of 5 antimicrobial agents used as intra-mammary infusion or systemic injection. (S: susceptible, I: intermediate and R: resistant response. Pre: dry off samples, Post: post calving samples).

Figure 4

dendrogram of RAPD-PCR types of *S. aureus* isolates from dry off and 7±3 DIM. A: strains from dry off, B: strains from post-calving. 60% similarity was considered for classification of RAPD types.

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