

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

This field trial was conducted to evaluate two antibiotics at a close-up period in curing the existing IMI and to prevent new clinical and subclinical Intramammary infection (IMI). Two hundred and twelve Holstein cows were assigned to one of three treatment groups: TYLO, MARB and CONT. Cows in TYLO group received 10 mg/kg Tylosin for three days at the close-up period (21 days before calving), cows in MARB group received single SC injection of 8 mg/kg SC marbofloxacin at the close-up period and cows in CONT group remained untreated. Milk samples were collected for somatic cell count, microbial culture and Total oxidant/antioxidant capacity before drying-off, and 3 and 7days post calving. Antimicrobial susceptibility test and RAPT-PCR were performed on *S. aureus* isolates. No significant differences were detected in total cure rate within the groups, but *S. aureus* cure rates in TYLO and MARB were significantly higher than in CONT (74 and 73.5 % Vs 58.1 %). No significant differences in total new IMI were observed. Furthermore, the rate of new *S. aureus* IMI was higher in both treatment groups than in CONT group. Clinical mastitis rate in TYLO (3.8 %) and MARB (5.8 %) was significantly lower than 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 % dissimilarity. Administration of Tylosin or Marbofloxacin is not useful in all cases; however, they have the potential to reduce the incidence of post-calving clinical mastitis and improve *S.aureus* cure rate if used selectively.

Introduction

Streptococcus uberis and *Escherichia coli* are two common environmental bacteria that cause infections during the dry Period. IMI during the dry period may be related to untreated or chronic infections of previous lactations or those that had been developed between dry-off and calving. IMI during the dry period increases the risk of clinical mastitis after calving. On the other hand, clinical mastitis in cows with IMI during the dry period develops earlier compared to cows without an IMI during that time (Nitz et al., 2021).

Dry cow therapy (DCT) can reduce intramammary infections, both by eliminating the current infections and by preventing new infections during the dry period. New infection rates are highest in the early dry period and during colostrogenesis stage (Bradley & Green, 2004). Administration of antimicrobials in forms of intramammary infusion at

the beginning of dry off could cover the first half of dry period (Browning et al., 1990; Bradley & Green, 2001). However the level of administered compounds in the udder gland will decline to below the MIC (Oliver & Maki, 1987) resulting in a prophylaxis gap in the close up window (Smith et al., 1985; Bradley & Green, 2001). The practice of blanket DCT has been efficient in eliminating the existing IMI by 70 to 98 % and has provided short-term protection against new IMI during early dry period (reduction by 50 to 75 %) (Smith et al., 1985). Although DCT formulations are labeled effective against gram-positive bacteria, they are generally acknowledged as being less successful in eliminating IMI caused by *Staphylococcus aureus* (Østerås et al., 1999) and new IMI may still occur if the invasive pathogens resist the antimicrobial preparations. Furthermore, the active ingredients of antimicrobial agents do not remain at therapeutic threshold throughout the entire dry period (Browning et al., 1990; Bradley & Green, 2001). Some reports have been

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published regarding the efficiency of systemic dry cow therapy as a management practice (Soback et al., 1990b). Norfloxacin was reported effective for systemic treatment of *S. aureus* (Soback et al., 1990b). For the same pathogen, Bolourchi et al., discovered that systemic injection of enrofloxacin or tylosin are as efficient as intramammary infusion (Bolourchi et al., 1995). Zeconi reported that administration of tylosin, two weeks before calving, can significantly decrease IMI rate after calving, compared to those cases treated only at dry off (Zeconi et al., 1999). There is little evidence that blanket DCT or other antibiotic use in dairy cows are associated with an increased risk of antibiotic resistance (Makovec & Ruegg, 2003; McDougall, Penry, & Dymock, 2021). However, with increasing concern about the impact of antimicrobials on the emergence of antimicrobial-resistant, the use of blanket DCT is being questioned.

Some farmers use close-up antimicrobial therapies either alone or concurrently with teat sealants as preventive measure against IMI in herds with high mastitis rate after calving (Zeconi et al., 1999). Furthermore, some others use this strategy for strengthening the treatment of staphylococcal intramammary infection during dry period (Hovareshti, Bolourchi, & Tabatabayi, 2007).

Currently, due to the existing concerns on drug resistance, it is necessary to re-evaluate the treatments.

Based on pharmacokinetic characteristics of macrolides and fluoroquinolones, the passage from blood into the udder tissue is rapid and extensive (Ziv, 1980). Marbofloxacin as a fluoroquinolone is a broad-spectrum antimicrobial and there are controversial results of resistance to fluoroquinolones in *S. aureus* isolated from cases of bovine mastitis. While most articles have reported low resistance to this class of antibiotics, some articles have reported high resistance. O'Dea et al. (2020) reported that NorA gene that confers resistance to fluoroquinolones has been detected in most isolates; however, all *S. aureus* isolates were susceptible to enrofloxacin and marbofloxacin. In Europe during 2015–2016 (El Garch et al., 2020) showed that MIC value of Danofloxacin, Enrofloxacin and Marbofloxacin for *S. aureus* isolates from bovine mastitis were generally low. Kakooza et al. (2023) showed that 97 % (131 of 135) of staphylococcal isolates from bovine mastitis was susceptible to Ciprofloxacin. However, Neelam et al. (2022), reported high resistance against enrofloxacin, levofloxacin and moxifloxacin among *S. aureus* recovered from clinical mastitis in cattle.

Tylosin as a macrolide with basic pH and lipophilic characteristics can reach milk to plasma concentration ratio of 5:1 (Riviere, 2011). This feature would make this antibiotic an ideal parental treatment of IMI.

Many worldwide efforts have been made to reduce the use of antibiotics in dairy farms. However, some hard-to-treat microorganisms require special treatments or extended therapy to improve their cure rates. The objective of the present study was to assess the efficacy of two antibiotics in prepartum period in curing existing IMI and preventing new clinical and subclinical IMI in two farms with high prevalence of *S. aureus* IMI.

Materials and methods

Cow/ herd selection and sampling

This study was conducted using Two commercial Holstein dairy herds, based on convenience sampling and the availability and high bulk milk count of *S. aureus* (more than 60 CFU/mL). The sample size was calculated based on the cure rate of staphylococcal mastitis by Tylosin during dry period (Bolourchi et al., 1995), and a 95 % confidence level and 80 % test power were used for the calculation. The minimum sample size was determined to be 230 quarters per treatment. The cows were dried off gradually using BDCT at 60±3 days prior to the expected date of calving. The milk samples were collected individually from each quarter one week before and at the last milking of drying off, then all quarters were treated with a Dry cow ointment containing 100,000 IU Kanamycin acid sulfate, 500 mg Cloxacillin Benzathine and 300,000 IU

Penicillin G Procaine (Kanaclon DC®, Pars Dopharma, Iran). Individual quarter milk samples were taken at 3–7 days post-calving.

Study design

Following DCT, 841 quarters from 212 cows were randomly allocated to one of the three groups (TYLO, MARB and CONT). Cows in TYLO ($n = 291$) received SC injection of 10 mg/kg mg tylosin (Tyloject® 20 %, Razak Laboratories Co, Iran) daily for three days, and 21 days prior to calving; cows in MARB ($n = 275$) received single SC injection of 8 mg/kg SC marbofloxacin (Marbox®, Ceva Santé Animale BV, Netherlands) 21 days prior to calving, while cows assigned to CONT ($n = 275$) remained untreated (Fig. 1).

Somatic cell count and bacteriological culture

Somatic cells of composite milk samples from the previous month dry-off were counted by electric counter (Fossomatic Milk Analysis, Foss Electric, Hillerød, Denmark).

Identification of bacteria were performed according to instructions of National Mastitis Council. A sample was considered contaminated if more than 3 bacterial species were observed on the plate. In the case of *S. aureus*, an IMI was diagnosed when one colony was isolated (≥ 100 cfu/mL). To confirm the identification of isolates as *S. aureus*, the gene was amplified by *nucA* via conventional PCR method using a 613-bp primer (F; CTGGCATATGTATGGCAATTGTT, R; TATTGACCTGAATCAGCGTTGCT).

Clinical mastitis

In our study, a clinical case of mastitis was considered whenever there was heat, swelling or pain in the udder, or there were changes in the milk (wateriness or clots) that persist for more than three squirts of milk or the experience systemic signs including pain, reduced appetite, reduced rumen function, increased heart rate, fever and depression.

Antimicrobial Susceptibility Test. The Agar disk diffusion method (ADD) was used to determine the susceptibility of *S. aureus* isolates for the following antimicrobial agents: Penicillin (10 µg), Tylosin (30 µg), Kanamycin (1000 µg), Marbofloxacin (5 µg) and Cloxacillin (5 µg). Procedures were based on the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2020). *S. aureus* ATCC 29,213 was used as the reference strain for quality control.

RAPD-PCR. In quarters from which *S. aureus* was isolated from both dry-off and post-calving, the genomic variability of *S. aureus* isolations was analyzed by RAPD-PCR method based on primer AP4 (5' TCACGCTGCA 3'). The amplification conditions for AP4 primer was performed based on a study by Morandi et al. (2010).

PCR fingerprint images were analyzed 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) of skim milk samples was assessed by FRAP method (Chen et al., 2003). The reduction of Fe³⁺-TPTZ to Fe²⁺-TPTZ was followed by an absorbance increase at 595 nm via spectrophotometer (2150-UV, UNICO®, USA). Total Oxidant Capacity (TOC) of skim milk of Individual quarter samples was analyzed by measuring DTNB based on the method applied by Silanikove et al. (2014). The decrease in the absorbance at 412 nm was used to calculate the rate of oxidation of TNB to DTNB. The parameters have been 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 tested values. Between-series imprecision and bias have been evaluated as well.

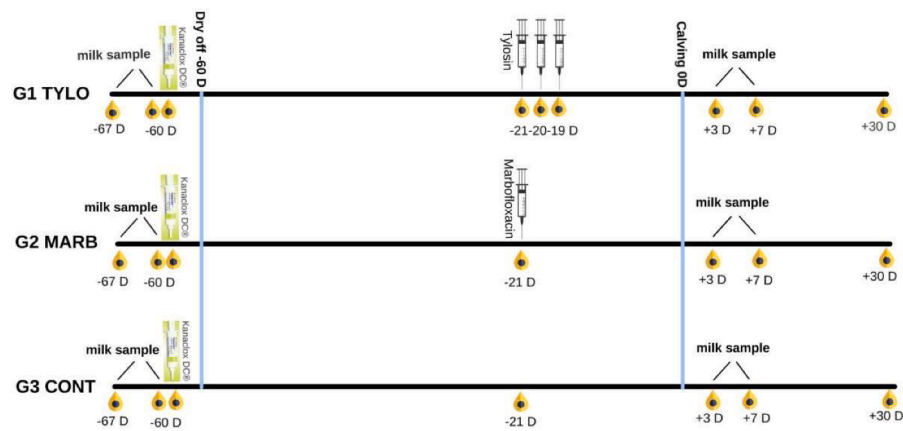


Fig. 1. Schematic design of study.

Statistical analysis

Univariate chi-square analysis was used to investigate the differences in cure, new infections and clinical mastitis rates among treatment groups. Mixed-effect logistic regression was carried out to determine the effect of treatments on the probability of cure, the new infection and clinical mastitis controlling for parity (1 or ≥ 2), milk yield (<30, 30–40 and >40), position of the quarter (Front and Rear) and oxidant/antioxidant balance during the dry period. Since cows were within two herds, the herds were considered to be random effects in the model. Kaplan-Meier survival curves were plotted to show survival distribution function of the quarters with clinical mastitis for each level of treatments. Mann-Whitney U test was used to compare DTNB and FRAP in cured, uncured and new infected and healthy quarters. All statistical differences were assessed at the two-sided 0.05 level of significance. The sample size was calculated based on confidence level of 95 % and power of 90 %. The statistical analysis was performed using SPSS software (version 21.0; SPSS Inc., Chicago, USA) and STATA Statistical Software, release 12.0 (Stata Corporation, College Station, Texas, USA).

Definition of terms for analysis

Cure: Cure was defined as the absence of pathogens that were present during the drying-off, on both days 3 and 7 of DIM. In cases where *S. aureus* was isolated on both drying-off and post-calving, RAPD-PCR pattern was used as a cure criterion.

New Infection: A new IMI was defined as the presence of pathogen at 3 or 7 DIM in a quarter that was not previously infected during the dry-off, or the presence of a different pathogen from those present at dry off (Huxley et al., 2002). 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 cases where RAPD-PCR revealed an *S. aureus* isolate during calving which belong to a different strain other than *S. aureus* isolate at dry off, they were considered as new IMI.

Developing Clinical Mastitis: A clinical mastitis 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 during dry-off and 30 DIM was classified as a positive clinical mastitis case.

Results

841 quarters of 212 cows were included in this study. The Mean length of dry period of cows in TYLO, MARB and CONT groups were 57, 56 and 57 days, respectively. No significant differences were observed in IMI of the two farms before the treatment ($P > 0.05$). The total prevalence of IMI at quarter level before drying-off was 67.8 %. In TYLO,

MARB and CONT quarters, the prevalence was 67.0, 60.7 and 72.4 %, respectively ($P > 0.05$). Total prevalence of IMIs at post-calving was 61.8 % and it was TYLO 62.2 %, MARB 60.4 %, CONT 62.9 % within each treatment group ($P > 0.05$).

Cure rate

The total cure rate was 72.3, 76.2 and 59.6 % for TYLO, MARB and CONT, respectively ($P > 0.05$). The *S. aureus* cure rate obtained with tylosin and marbofloxacin injections was 74 and 73.5 %, respectively; both of which were significantly higher than control group (58.1 %) (Table 1).

The odds ratio (OR) of the 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 by CNS that received tylosin had greater odds of cure (OD=4.1, $P = 0.04$). Quarters of cows with more than 30 kg of daily milk yield were less likely to be cured compared to those with less 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.

New infection rate

There were no statistically significant differences in new infection rates among the three groups (TYLO =40.9 %, MARB=38.5 % and CONT=42.5 %, $P > 0.05$). Surprisingly the quarters from TYLO and MARB groups had a significantly higher new *S. aureus* IMI compared to quarters from CONT group ($P < 0.05$). The percentage of new IMIs acquired by each treatment group regarding every specific pathogen are outlined in Table 2. Results of multivariate regression analysis of odds for acquiring new *S. aureus* IMI between dry-off and 7 \pm 3 DIM showed that 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 *S. aureus* infection.

Clinical mastitis rate

The proportion of quarters with the occurrence of clinical mastitis between dry-off and 30 DIM was significantly lower for both treatment groups (TYLO=3.8 %, MARB=5.8 %) vs. CONT (11.3 %) quarters (Fig. 2).

Disc agar susceptibility test

Disc agar susceptibility test was performed on 180 *S. aureus* isolated from dry-off and 7 & 3 DIM periods. Generally higher percentages of resistance were found in post-calving isolates compared to dry-off

Table 1
Total and pathogen specific cure rate for different groups.

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 (103/145)	75.9 ^a (41/54)
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 (107/142)	79.1 ^a (34/43)
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 (113/189)	59.0 ^b (26/44)

^{a-b} Different letters (a, b) above the numbers in each column indicates significant difference ($P < 0.05$).

Table 2
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)	24.5 ^a (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)	25.5 ^a (60/235)
MARB	38.5 (106/275)	24.1 ^a (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)	25.9 ^a (61/235)
CONT	42.5 (117/275)	13.8 ^b (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)	14.9 ^b (35/234)

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

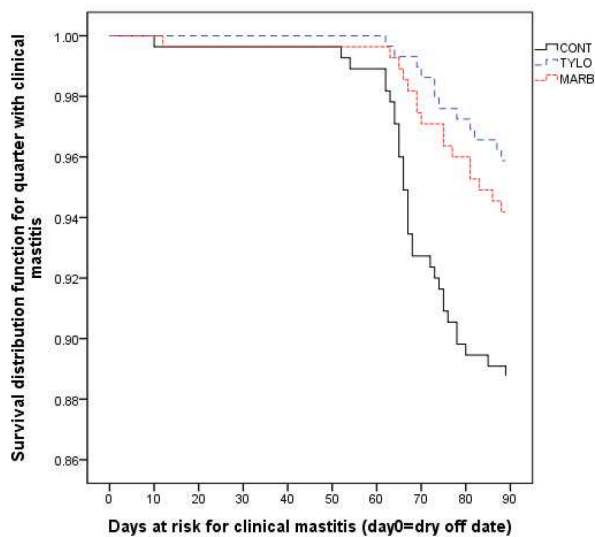


Fig 2. Kaplan-Meier survival curves providing survival distribution function of quarter with clinical mastitis for each of the treatment groups from drying off (day 0) until DIM 30 (day 90).

isolates. The differences were significant for both tylosin and cloxacillin ($P < 0.05$) (Fig. 3).

Total oxidant/antioxidant capacity in quarters with new infection

Post-calving DTNB concentrations in quarters with new infection were higher than that of 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 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. Data have been summarized in Table 3.

RAPD-PCR

Amplification of AP4 primer resulted in a multiple amplicon ranging from 100 bp to more than 2000 bp obtained from all 86 *S. aureus* isolates. The 43 *S. aureus* paired samples were selected from quarters which

were infected both before the dry-off and after parturition. Isolates were characterized into 9 different RAPD types (A-I), the majority of which were categorized as A, B, E and G type. Eight paired of the strains collected at dry off stage were identical to those collected at post-calving; moreover, 35 strains of the dry-off had more than 60 % dissimilarity to post-calving strains. The isolates' RAPD patterns in this study have been provided as a dendrogram in Fig. 4. Different patterns of antimicrobial resistance among four predominant RAPD types (A,B,E, G) in each herd are shown in Table 4.

Discussion

The cure rates for infected quarters in different groups of this study are similar to those reported in previous studies with calculated average of 78 % (71 to 85 %) (Halasa et al., 2009). The *S. aureus* cure rate for quarters received intra-mammary infusion solely is similar to those reported in initial drug efficacy studies (Halasa et al., 2009). We observed that the total cure rate 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 (1994), intramuscular oxytetracycline and intra-mammary cephapirin were administered to cattle and the cure rates of quarters did not improve compared to the quarters treated with cephapirin alone (Erskine et al., 1994). Bolourchi et al. (1995) discovered that the injection of systemic enrofloxacin or tylosin during dry-off period did not increase the efficacy of intra-mammary infusion. The aforementioned studies were also performed in herds with high prevalence of *S. aureus*. We have observed that both tylosin and marbofloxacin improved *S. aureus* cure rate, which is consistent with the results from another study in which systemic norfloxacin was administered at the start of the dry period which resulted in a better *S. aureus* cure rate compared to intramammary cephapirin (Soback et al., 1990a). Zeconi (1999) reported that administration of systemic tylosin two weeks before calving, alongside with traditional dry-cow therapy is an effective supplementary treatment for intra mammary therapy of *S. aureus* (Zeconi et al., 1999).

Antibiotic effectiveness often changes when two or more such drugs are administered simultaneously. Antagonism has an inhibitory mechanism where one antibiotic blocks or reduces the effect of another one. On the other hand, administration of DC ointment at the beginning of dry-off would cover the first half of the dry period. Systemic administration of antibiotic at the beginning of the close-up period reduces the probability of antibiotic interaction.

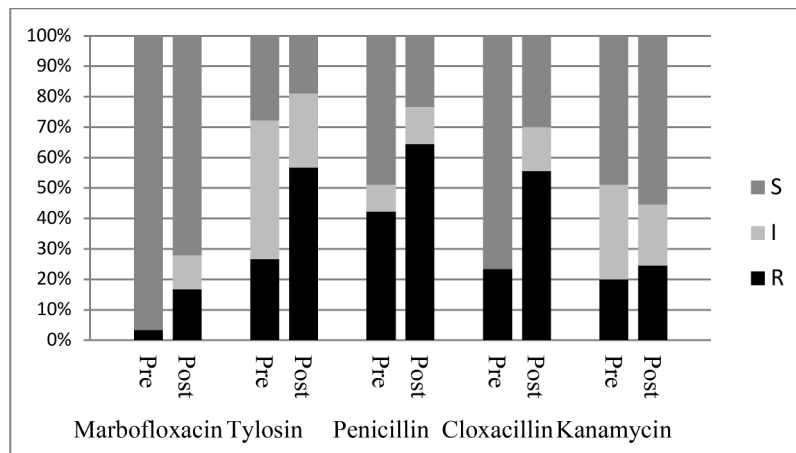


Fig 3. Antimicrobial susceptibility test results of 5 antimicrobial agents used as intra-mammary infusion or systemic injection. (S: susceptible, I: intermediate and R: resistant response. Pre: drying-off samples, Post: post calving samples).

Table 3

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

Strain-specific characteristics can be expected to affect the probability of cure of *S. 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 the strains, responded well to tilmicosin compared to the rest of the groups (Dingwell et al., 2004). More studies are warranted to examine the variability of treatment responses in different strains of *S. aureus*. Moreover, high proportion of the isolated strains in the present study were resistant to penicillin. It has been discovered that the probability of cure is lower for penicillin-resistant *S. aureus* than for penicillin-susceptible strains (Barkema et al., 2006).

The Odds of cure was lower in quarters with more 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 the infection (Barkema et al., 2006). The probability of cure for total and specific pathogens tended to reduce with LS enhancement. Similarly, others have observed that the probability of cure in quarters infected by *S. aureus* decreased as SCC increased (Sol et al., 1994; Dingwell et al., 2006). Higher-levels of SCC may also indicate that multiple quarters of the udder are infected, and if more quarters are infected, they are less likely to be cured (Østerås et al., 1999).

Result of the recent studies showed that 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. In most of previous studies, the systemic antimicrobial agents had been administered at the beginning of drying off (not during dry period), which made the comparison difficult. Contreras et al., used 12 g of systemic tylosin combined with intra-mammary infusion and teat

seal to prevent new infection, and it was discovered that the addition of systemic tylosin was not effective (Contreras et al., 2013). In that study, lower rate of new IMI might be due to lower prevalence of *S. aureus* (4.5 %) compared to our study (15.1 %) and teat seal was not used in the present study. In both herds, *S. aureus* was prevalent and it has been noted that the contagious bacteria, especially *S. aureus*, are likely to establish more new infections in those herds where they are prevalent (Berry & Hillerton, 2002).

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 group. Several authors stated that CNS bacteria can protect quarters against IMI caused by major pathogens, either when causing IMI (Matthews et al., 1992; Lam et al., 1997) or when colonizing bovine teats (De Vliegher et al., 2004). Data from Pankey and Nickerson suggest that quarters containing *C. bovis* are more resistant to *S. aureus* infections (Pankey et al., 1985). General activation of the immune system, competition for binding sites and alterations in teat canal keratin are suggested as possible explanations (Nickerson & Boddie, 1994).

Results of this study showed an increased new IMI in cows with more than two parities. A similar association between the risk of acquiring IMI in dry period and parity has been previously documented (Dingwell et al., 2004). Increased IMI associated with higher parity might be related to a decrease in the integrity of the streak canal (Cousins et al., 1980).

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 has been no study on the effect of close up systemic antimicrobial agents on the incidence of clinical

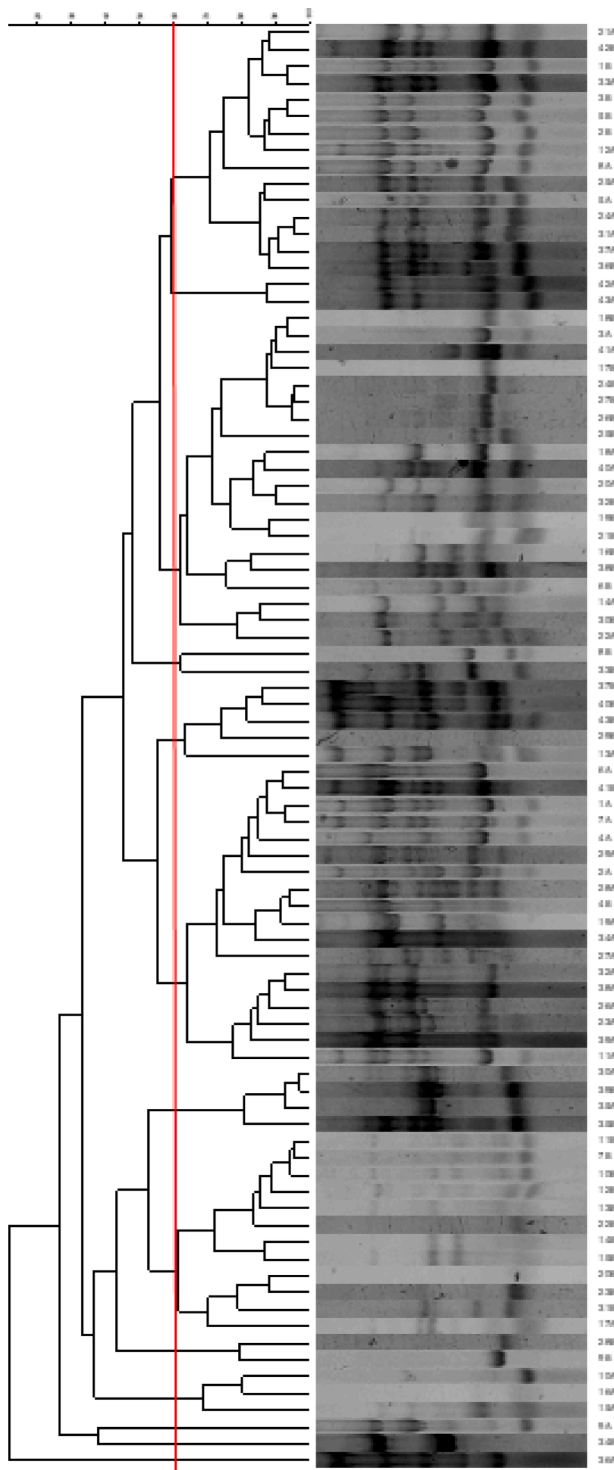


Fig 4. Dendrogram of RAPD-PCR types of *S. aureus* isolates from dry off and post calving. A: strains from dry off, B: strains from post-calving. 60 % similarity was considered for classification of RAPD types.

Genotyping methods have proven a useful tool to determine the diversity of *S. aureus* strains. different pathogenicity and antimicrobial susceptibility have been demonstrated using various typing methods (Fitzgerald et al., 2000; Haveri et al., 2005). Strain-specific characteristics significantly impact the cure rate of *S. aureus* IMI during dry period (Dingwell et al., 2006). Sommerhäuser et al. demonstrated that the real persistent and newly infected quarters could be determined using a genotyping method on *S. aureus* isolated before and after dry period (Sommerhäuser et al., 2003).

mastitis, some studies had previously compared the 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 100 DIM significantly reduced in cows that received tylosin (Ismail et al., 2018). Contradictory results have been reported from Australia, which showed more clinical mastitis rate after DCT (Browning et al., 1990). Pathogen isolation was not performed for clinical cases in this study but based on previous studies, it is presumed that most clinical mastitis cases are caused by environmental pathogens including streptococci and coliform bacteria (Bradley, 2002). Tylosin, as a member of Macrolides, has high MIC for gram-negative bacteria (Salmon et al., 1998) and is generally effective against gram positive bacteria. In our study, high doses of the drug (20 mg/kg) were administered for three consecutive days. It is worth mentioning here that marbofloxacin has a wide spectrum of activity and it has been proven that *E. coli* strains are very susceptible to marbofloxacin with a very low MIC90 (Schneider et al., 2004). Bradley and Green (2001) 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. The findings of the present study and other related studies confirm that dry cow therapy can play an important role in the clinical mastitis epidemiology during early lactation.

There is limited evidence showing that antimicrobial usage as DCT is associated with increased risk of antimicrobial resistance (McDougall et al., 2021). Furthermore, Oliver et al., showed that dry cow treatment was not entirely effective in reducing the new infection. There is a growing interest in new alternative methods for dry period management. For cows or quarters likely to be uninfected, infusion of only one teat sealant offers a nonantibiotic approach in reducing the rate of new IMI over dry period (Oliver, 1988).

The findings of the current investigation demonstrated elevated rates of resistance in isolates obtained after calving in contrast to isolates obtained during the dry-off. Bacteria can develop resistance towards antibiotics via genetic mutations which can subsequently modify the cellular targets of antibiotics, or by acquiring specialized resistance genes from fellow bacteria. In the presence of long act antibiotics, these resistant bacteria exhibit the ability to proliferate and reproduce, even when neighboring bacteria susceptible to the antibiotics are effectively eradicated. Under antibiotic treatment, resistant strains have the potential to quickly dominate pathogen populations through the process of Darwinian natural selection.

Furthermore, rather than penicillin and cloxacillin, a considerable percentage of isolates were resistant to tylosin, especially in post-calving samples. Both phenotypic (Pourtaghi et al., 2016) and genotypic (Bahraminia et al., 2017) 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 one of the underlying causes. Because of elimination of susceptible strains by dry-off treatment and introduction of some new strains, some discrepancies were detected in resistance characteristic of the strains from dry off and post-calving.

Based on results obtained by RAPD-PCR, in quarters in which *S. aureus* was isolated in both dry-off and post-calving periods, dry-off strains were not persisted in the udder and reinfection has been made by different *S. aureus* strains. Contradictory result reported by Myllys et al. (1997), showed that *S. aureus* strains have been persisted in udder without reinfection by other strains. They used ribotyping which was not used in our study. Moreover, in their study, the poor cure rates of *S. aureus* were associated to the persistence of the original strain. Four predominant RAPD types (A,B,E,G) were detected in the present study. Based on genotyping survey by Sommerhäuser et al. (2003), one type of strains are dominant in some herds, while in some others there are several dominant types.

In recent years, free radical damage has become increasingly significant as a complementary tool in the evaluation of inflammatory status. It

Table 4
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	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 %		

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 the healthy quarters, which is consistent with the results of Atakisi et al., study who reported that TOC levels were significantly higher in milk in glands with sub-clinical mastitis compared to normal glands (Atakisi et al., 2010). In another study performed on goat, the authors sustained similar results (Silanikove et al., 2014). Infection of mammary gland have caused expansion in the number of neutrophils and epithelial cells and cytokines in mammary tissue which resulted in the enhancement of free radicals in milk (Sadek et al., 2017).

According to data obtained 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 the probability of cure and acquiring new infection.

Conclusions

According to the global rise in antimicrobial resistance, the optimization of antibiotics use is important for controlling the antibiotic consumption and the release of antibiotic residues.

Administration of tylosin or marbofloxacin during dry period resulted in a reduction in post-calving clinical mastitis, but it could not improve cure rate or prevent new subclinical mastitis. By considering the probability of eliminating minor pathogens without affecting the major pathogens, this protocol appeared to be unsatisfactory for the prevention of new IMIs in herds with high prevalence of *S. aureus*, but it could improve the cure rate of *S. aureus* IMI, so that it can be useful if applied selectively. Conducting antimicrobial susceptibility test, and molecular typing for the isolated pathogens and analyzing the oxidant/antioxidant capacity of milk are valuable in selecting appropriate therapeutic compounds as a part of dry cow management.

Statement of animal rights

The present study included cows. Protocol of study was confirmed by animal welfare committee of Ferdowsi University of Mashhad (38,059) in accordance with institutional and national and/or international guidelines.

Ethical statement

Hereby, I Babak Khoramian, consciously assure that for the manuscript titled "Evaluation of close up antimicrobial therapies for treatment prevention of subclinical mastitis in the herds with high prevalence of *Staphylococcus aureus*" declare that this was approved by Ferdosi University Animal Experiments Local Ethics Committee (48,157).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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