A high prevalence of tylosin resistance among *Staphylococcus aureus* strains isolated from bovine mastitis

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**Article Info**

**Abstract**

The macrolides appear to have considerable effects for treatment of bovine mastitis because of excellent diffusion into the mammary gland, long half-life, low protein binding, high intracellular concentration and lipid solubility. Acquired resistance to macrolides in *Staphylococcus aureus* is primarily related to target-site modification through acquisition of an *erm* gene. In the present study the prevalence of both phenotypic and genotypic tylosin resistance in *S. aureus* isolates (n = 103) from subclinical mastitis in nine dairy farms belonging to three different provinces of Iran were investigated. Overall, *ermA*, *ermB* and *ermC* was found in 7.80%, 32.00%, and 20.40% of *S.aureus* isolates, respectively. A very high percent of isolates (56.90%) were resistant to tylosin. MIC90 and MIC50 values were 64 and 32 µg mL−1, respectively. Most of tylosin resistant isolates did not harbour any *erm* gene but *ermB* was dominant gene among 58 tylosin resistant isolates of *S. aureus*. In overall, tylosin resistance was prevalent in *S. aureus* isolates obtained from bovine mastitis in Iran.

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**Key words:** Bovine mastitis, *erm* genes, Macrolides, *Staphylococcus aureus*, Tylosin resistance

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چکیده

به نظر می‌رسد ماکرولیدها به دلیل نفوذ عالی به بافت پستان، نیمه عمر طولانی، اتصال کم به پروتئین‌ها، غلظت بالای داخل سلولی و حلالیت در چربی، اثرات قابل توجهی در درمان ورم پستان گاو دارند. مقاومت اکتسابی به ماکرولیدها در *استافیلوکوکوس اورئوس* بصورة اولیه مربوط به تغییر محل اتصال از طریق کسب یکی از ژن‌های *erm* می‌باشد. در مطالعه حاضر شیوع فنوتیپی و ژنوتیپی مقاومت به تایلوزین در سویه‌های *استافیلوکوکوس اورئوس* جدیداً جدیداً از ورم پستان تحت بالینی در 9 گله شیری متعلق به سه استان مختلف ایران مورد بررسی قرار گرفت. در مجموع *ermA*, *ermB* و *ermC* به ترتیب در 00/13, 00/30 و 00/78 درصد از جدایه‌های *S. aureus* و 00/13, 00/30 و 00/78 درصد از جدایه‌های *S. aureus* مورد بررسی قرار گرفتند. در مجموع مقاومت به تایلوزین وارد به 90.23%، 32.00% و 20.40% درصد از جدایه‌های *S. aureus* و 90.23%، 32.00% و 20.40% درصد از جدایه‌های *S. aureus* را نشان داد. با توجه به وجود *erm* به ترتیب 66 و 32 میلی گرم بر لیتر بود، اغلب جدایه‌های مقاوم به تایلوزین هیچ کدام از ژن‌های *erm* را نداشتند وی و وجود در *ermB* در میان 58 جدایه‌ای از *S. aureus* مقاوم به تایلوزین، غالب بود. در مجموع مقاومت به تایلوزین در جدایه‌های *S. aureus* به 90.23% درصد از ورم پستان گاو در ایران شیوع دارد.

واژه‌های کلیدی: استافیلوکوکوس اورئوس، ژن‌های *erm*، ماکرولیدها، مقاومت به تایلوزین، ورم پستان گاو

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Introduction

Bovine mastitis is the most prevalent production disease affecting dairy farms worldwide, accounting for 38.00% of direct costs incurred by the dairy industry.\(^1\) *Staphylococcus aureus* is considered one of the most important pathogens in bovine mastitis. Effective control of *S. aureus* mastitis relies on antibiotic treatment of intramammary infections at drying-off and treatment of clinical and sub clinical mastitis during lactation.\(^2\)

Beta-lactams, aminoglycosides, lincosamides, fluoroquinolones and macrolides are common antimicrobial compounds for treatment of *S. aureus* mastitis by the intramammary or systemic administration route. Given high bioavailability from the injection site, lipid solubility, long half-life, low protein binding and intracellular concentration in phagocytes, macrolides are of considerable interest for parenteral mastitis therapy.\(^1\) Tylosin and tilmicosin are routinely used for dry cow therapy in most of dairy farms in Iran.

Macrolide resistance in *Staphylococcus* species occurs by three primary mechanisms: Through target-site modification, through efflux of the antibiotics, and by drug inactivation. Target modification is predominant mechanism that is dependent on the *erm* genes.\(^3\) Because of extraordinary usage of macrolides for treatment of *S. aureus* mastitis in Iranian dairy farms for a long time, it is not surprising that resistance is prevalent among *Staphylococcus* species. In this study, we determined the prevalence of both phenotypic and genotypic tylosin resistance in *S. aureus* isolates from bovine subclinical mastitis.

Materials and methods

**Bacterial isolates.** The present study was performed in nine dairy farms belonging to three different province of Iran including Tehran (three farms, \(n = 33\)), Khorasan Razavi (three farms, \(n = 40\)) and Alborz (three farms, \(n = 30\)) from 2014 to 2015. The California mastitis test (CMT) was done and milk samples were taken from quarters with score 1 or more in CMT and cultured. A total number of 103 bovine *S. aureus* isolates from more than 500 individual quarter milk with subclinical mastitis were collected. Collection of milk samples and microbiological procedures were performed according to the National Mastitis Council procedures.\(^4\) Isolation and identification of these presumptive *S. aureus* colonies were based on conventional methods, including Gram staining, colony morphology, production of coagulase, catalase, DNase and fermentation of mannitol. To confirm the identity of the isolate as *S. aureus*, the *nucA* gene was amplified by a PCR-based method using primers listed in Table 1. The confirmed *S. aureus* isolates were stored at \(-70\) °C in brain heart broth plus 20% glycerol.

**DNA isolation and polymerase chain reaction (PCR) amplification.** For detecting macrolide resistance genes, the whole genomic DNA from cultured strains was prepared using DNA extraction kit (GeneAll, Seoul, South Korea). The PCR was carried out on the three related genes, including *ermA*, *ermB*, *ermC*, using specific oligonucleotide primers listed in Table 1. Amplification was performed in a final volume of 25 mL containing 10 μL of Taq DNA polymerase 2x master mix red containing; 2 mM MgCl\(_2\), Tris-HCl, (NH\(_4\))\(_2\)SO\(_4\), 0.20% Tween 20, 0.40 mM dNTPs, 0.20 units per μL ampliqon Taq DNA polymerase inert red dye and stabilizer (Ampliqon, Odense, Denmark), 0.50 mg mL\(^{-1}\) of each primer and 3μL of template DNA. The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min, followed by 30 cycles of 45 sec at 94 °C, 50 sec at 50 °C (for *ermA* and *ermC* genes) or 54 °C (for *ermB* gene) and 55 °C (for *nucA*) and 75 sec at 72 °C. A final extension step was performed at 72 °C for 5 min. After amplification, DNA bands were analyzed by staining with ethidium bromide and electrophoresis (Bio-Rad, California, USA) on 1% agarose gel.

**Antibiotic susceptibility testing.** The broth microdilution method was used to determine minimum inhibitory concentrations (MIC), in accordance with the Clinical and Laboratory Standards Institute (CLSI).\(^5\) Mueller-Hinton broth was used as the test medium. Plates were incubated at 35 °C for 18 hr. *Staphylococcus aureus* ATCC29213 was used as reference strain for MIC quality controls. MIC were tested in the concentration range of 0.125 to 128.00 μg mL\(^{-1}\). Tylosin breakpoint 20 μg mL\(^{-1}\) was also considered in our test.\(^6\)

**Statistical analysis.** SPSS software (version 16.0; SPSS Inc., Chicago, USA), was used for statistical analysis. Differences in the prevalence of macrolides resistant genes and distribution of MIC values in different provinces were calculated using the chi-square test. A p-value of 0.05 was considered as statistically significant.

| Gene | Primers (5’-3’) | Size (bp) | Reference |
|------|-----------------|-----------|-----------|
| *nucA* | F-CTGGCATATGTTAGCAATTGT | 613 | 7 |
|       | R-TATTGACCTGAACTCGGTTGTTCT | | |
| *ermA* | F-TATCTTATGGTGAAGAGATT | 139 | 8 |
|       | R-CTACCTTGGCTGATGAAA | | |
| *ermB* | F-CTACGATTGTGGAAAGGATT | 141 | 8 |
|       | R-GTTCATCTGTTTATGGATACAA | | |
| *ermC* | F-AATCGTAACTTCCGTGATGTT | 299 | 9 |
|       | R-TAATCGTGGAATAGGGTTTG | | |

**Results**

The prevalence of *S. aureus* in milk samples obtained from cows with subclinical mastitis were 22.00%, 23.50% and 25.00% in Tehran, Khorasan Razavi and Alborz, respectively. Among 103 *S. aureus* isolates 58 (56.86%)
and 31 (30.09%) isolates were resistant and inter-
mediately resistant to tylosin, respectively. The MIC
values ranges for the all strains were from 0.50 to
128.00 µg mL⁻¹. The MIC₉₀ values in our test were 64.00
µg mL⁻¹, while the MIC₅₀ values were 32.00 µg mL⁻¹. The
distribution of MIC values in different provinces
showed no significant differences (p > 0.05). The MIC
distribution data of tylosin for the 103 S. aureus isolates
and macrolide resistant genes are summarized in Table
2. The PCR analysis of macrolides resistant genes
revealed that 8(7.80%), 33(32.00%) and 21(20.40%)
isolates harboured the ermA, ermB and ermC genes,
respectively (Figs. 1 and 2).

Fifty-six isolates (54.36%) of 103 had at least one
erm gene. The most dominant resistant gene in Tehran
was ermC and in other provinces was ermB. Between
provinces, ermB and ermC have significant difference
(p < 0.05). Among 58 tylosin resistant S. aureus, ermB
gene was dominant and 27.60% of isolates harboured
this gene (Table 3).

**Table 2.** Comparison of macrolides resistant genes, tylosin resistant rates (%) and MIC (µg mL⁻¹) distributions of S. aureus isolates.

| Province | No. | ermA (%) | ermB (%) | ermC (%) | MIC₅₀ | MIC₉₀ | Range |
|----------|-----|----------|----------|----------|-------|-------|-------|
| Tehran   | 33  | 1(3.03)  | 1(3.03)  | 7(21.21) | 0     | 3     | 0.125 |
| Khorasan Razavi | 40 | 6(15.00) | 21(52.50) | 13(32.50) | 0     | 0     | 0.25  |
| Alborz   | 30  | 1(3.33)  | 11(36.67) | 1(3.33)  | 0     | 0     | 0.5   |
| Total    | 103 | 8(7.80)  | 33(32.00) | 21(20.40) | 0     | 7     | 16    |

The MIC breakpoint for tylosin (≥ 20 µg mL⁻¹) was based on the veterinary antimicrobial decision support (VADS); MICs indicating
susceptibility are exhibited on a white background, those indicating intermediate resistance on a light grey background, and those
indicating resistance on a dark grey background.

**ab** Values with different superscripts in the same column are significantly different from each other (p < 0.05).

**Discussion**

We have determined the prevalence of macrolides
resistant genes and the distribution of tylosin MICs in S.
aureus isolates from subclinical mastitis. The prevalence of
S. aureus isolated obtained from subclinical mastitis was
23.00%. This prevalence varies in different countries.

**Table 3.** Distribution of erm genes in isolates.

| Genotype | Tylosin resistant isolates (n = 58) | Tylosin intermediate isolates (n = 31) | Tylosin sensitive isolates (n = 14) |
|----------|-----------------------------------|---------------------------------------|----------------------------------|
| ermA⁺B⁻C⁻ | 1 (1.70%)                         | 0 (0.00%)                             | 0 (0.00%)                        |
| ermA⁺B⁺C⁻ | 9 (15.50%)                        | 9 (29.00%)                            | 5 (35.70%)                       |
| ermA⁺B⁺C⁺ | 5 (8.60%)                         | 3 (9.70%)                             | 0 (0.00%)                        |
| ermA⁻B⁺C⁻ | 1 (1.70%)                         | 0 (0.00%)                             | 0 (0.00%)                        |
| ermA⁻B⁻C⁻ | 4 (6.90%)                         | 0 (0.00%)                             | 0 (0.00%)                        |
| ermA⁻B⁻C⁺ | 6 (10.30%)                        | 1 (3.20%)                             | 0 (0.00%)                        |
| ermA⁻B⁺C⁺ | 1 (1.70%)                         | 1 (3.20%)                             | 0 (0.00%)                        |
| ermA⁻B⁻C⁻ | 31 (53.40%)                       | 17 (54.80%)                           | 9 (64.30%)                       |
Prevalence of S. aureus in China is reported to be as high as 25.20%, others reported a prevalence of 10.20% and 30.60% in Finland and Kenya, respectively. The same prevalence rate (25.00%) was found among nine farms in Alborz, Iran.

In the present study, frequency of acquired resistance to tylosin in S. aureus was high (56.86%). These results were completely in agreement with other investigation who reported 56% resistance in S. aureus isolated from cow milk samples with clinical and subclinical mastitis in Romania. Other studies from different geographical locations reported lower resistance to tylosin ranged from 4.40% to 40.30%.

Pourtaghi et al. determined antimicrobial resistance patterns of S. aureus isolated from bovine subclinical mastitis in Alborz province and showed that resistance to tylosin was 28.80% of isolates.

Furthermore, the MIC50 value of tylosin for S. aureus isolates in our study was higher than those measured by previous investigators. The overall resistance of S. aureus isolated from bovine mastitis to macrolides in different countries is low or scarce. Result of present study showed higher frequency of resistance to macrolides. High resistance of S. aureus isolates to tylosin might be associated with the increase in highly resistant strains and rapid transfer of cloned resistance which could result from introducing and extraordinary usage of macrolides in Iranian dairy farms. When a single resistance determinant was considered, ermB was the most common. These observations are contrary to most other findings especially in human research that reported ermA was more frequent genes and ermB was present in only a minority of strains. Others showed that ermC was the most frequently encountered gene responsible for macrolide resistance among S. aureus isolates.

Interestingly, in our investigation most of tylosin resistant isolates did not harbour any erm gene. It seems that other resistant genes are more important in tylosin resistance isolates obtained from bovine mastitis. Other resistant genes like ermF, ermY and lanA have also been detected in S. aureus isolates that were macrolide-lincosamides and streptogramin resistant. Also, it may suggest that one or several new resistance mechanisms for macrolides may be widespread among S. aureus isolates.

In conclusion, tylosin resistance was prevalent in S. aureus isolates obtained from bovine mastitis in Iran. The most frequent determinant of macrolides resistance was ermB and with ermC the next most common.

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