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Characteristics of Erythromycin Resistance in Methicillin-Resistant *Staphylococcus aureus* Isolated From Raw Milk

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are one of the most important multidrug resistant microorganisms that threaten human health.

Objective: The present study was conducted to evaluate genotypic and phenotypic characteristics of erythromycin resistance among MRSA isolates recovered from raw milk in Iran.

Materials and Methods: A total of 50 MRSA isolates were recovered from raw milk. Tests for erythromycin and clindamycin susceptibility and inducible clindamycin resistance were done. In addition, the presence of the methicillin resistance determinant (*mecA*), erythromycin resistance genes (*ermA*, *ermB*, *ermC* and *msrA*) and an important virulence gene (Panton–Valentine leukocidin) were investigated using polymerase chain reaction (PCR) method.

Results: Forty-eight percent (24/50) and 46% (23/50) of the isolates were resistant to erythromycin and clindamycin, respectively. Seven (14%) isolates showed inducible clindamycin resistance phenotype. The *mecA* gene was detected in 88% (44/50) of MRSA isolates. The incidence of the *ermA*, *ermB*, *ermC* and *msrA* genes was 14%, 64%, 12%, and 26%, respectively and the *PVL* gene was present in 18% (9/50) of MRSA isolates.

Conclusion: According to the results of the study, the incidence of erythromycin resistance genes and inducible clindamycin-resistant MRSA strains was high in raw milk samples in Iran.

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Background

Staphylococcus aureus is one of the most common causes of nosocomial, skin and purulent infections in humans and animals. This bacterium is responsible for septic arthritis, infections after burn, endocarditis, pneumonia, food poisoning, toxic shock syndrome, and folliculitis.[1] In recent years, the resistance of *S. aureus* to different types of antibiotics is steadily increasing around the world and nowadays there is a great risk of several resistant strains such as methicillin-resistant and vancomycin-resistant *S. aureus* (MRSA and VRSA). These strains spread through various origins, particularly livestock animals and their products.[1-3] On the other hand, the increase in the number of infections caused by oxacillin and methicillin resistant *S. aureus* implies superiority of macrolide and lincosamide antibiotics such as erythromycin and clindamycin as alternative agents.[4] *S. aureus* is present in a variety of foods including meat products, vegetables, salads, cooked and salty foods.[3] This microorganism is one of the most important pathogens in association with dairy products and raw milk can be a substantial source for resistant strains of *S. aureus*.[3,5,6] The resistance of *S. aureus* to macrolides is mediated by 2 mechanisms: the first mechanism is target alteration which is mediated by *erm* genes encoding 23S rRNA methylase that causes conformational change in the ribosome and can trigger resistance to macrolides, lincosamides and streptogramin B antimicrobials so this pattern called MLS_B.[7] The second resistance mechanism of the MLS antibiotics is mediated by the presence of macrolide efflux pumps which is encoded by *msrA* and *msrB* genes.[8] The presence of the erythromycin resistant-MRSA strains, particularly with virulence factor-related genes, in dairy products could be a serious risk to the public health. Since erythromycin can be a proper alternative for treatment of MRSA infections, it is essential to evaluate the resistance to this antibiotic among the isolates. In addition, Panton–Valentine leukocidin (PVL) is one of the important virulence factors of the organism.[9] This toxin affects macrophages and polymorphonuclear white blood cells. Its action increases

the permeability of the cell membrane and the result is lysis of the leukocytes and tissue necrosis.[9]

In order to simplify epidemiological studies essential to assess the MRSA threats, several important subjects must first be inspected, including the genetic structure of MRSA, the acquisition and evolution of methicillin resistance, the source of the infection, and the geographic spreading of this resistance. The present study was conducted to evaluate the phenotypic and genotypic characteristics of erythromycin resistance among MRSA isolates recovered from raw milk to evaluate the correlation between the presence of the resistance gene and resistance to the erythromycin and clindamycin. In addition, the presence of *PVL* gene was evaluated among the isolates.

Materials and Methods

Sample Collection

To evaluate the erythromycin resistance and related genes, the study was carried out on 50 methicillin resistant isolates recovered from raw milk samples. From December 2017 to February 2018, a total of 120 raw milk samples were collected from retail shops in Mazandaran province, northern Iran. The samples were transported in 2 mL sterile microtubes to the lab for subsequent microbiological procedures.

Isolation of *Staphylococcus aureus* and Identification of MRSA Isolates

One milliliter of each sample was added to the trypticase soy broth (TSB) containing 10% NaCl and incubated at 37°C for 18 hours. The culture was done on Baird-Parker agar (HiMedia, India) containing egg yolk tellurite emulsion (Liofilchem, Italy) and the plates were incubated aerobically at 37°C for 24 hours. Black colonies with transparent zone were subjected to gram staining, subculture on mannitol salt agar (HiMedia, India), and further biochemical examinations. *S. aureus* isolates were identified by conventional methods including gram staining, tests for catalase, coagulase, DNAse, and mannitol fermentation.[10] The identification of MRSA was done according to the Clinical and Laboratory Standards Institute (CLSI) protocol with 30 µg cefoxitin disk.[11] Isolates with ≤21 mm inhibition zones were identified as *mecA* positive or oxacillin resistant (methicillin resistant or MRSA).

Erythromycin and Clindamycin Susceptibility Test

The susceptibility of the isolates to erythromycin and clindamycin was examined according to the CLSI protocol using 15 µg erythromycin and 2 µg clindamycin disks and incubation at 37°C for 18 hours. For erythromycin, the inhibition zone diameter of ≥23, 14-22, and ≤13 were considered as susceptible (S), intermediate (I), and resistant (R), respectively. For clindamycin the inhibition zone diameter of ≥21, 15-20, and ≤14 were considered as susceptible (S), intermediate (I), and resistant (R), respectively.[11]

Inducible Clindamycin Resistance (D-test)

For distinguishing isolates with inducible clindamycin resistance, we used a D-test according to the CLSI. MRSA isolates were cultured on Muller Hinton agar until the bacterial culture reached 0.5 McFarland standards. Afterwards, 2 µg clindamycin and 15 µg erythromycin disks were placed on the agar with 15-26 mm distance. The flattening of the inhibition zone of the clindamycin disk on the side of the erythromycin disk (of the resistant isolates) was determined as inducible resistance.[11]

DNA Extraction

Total DNA was extracted from each pure isolate using a bacterial genomic DNA extraction kit (GTP Co, Iran) according to the manufacturer's recommendations. Briefly, 1 mL of overnight fresh bacterial culture was centrifuged at 10 000 g for 1 minute and the pellet was used for the procedure. Extraction was done using buffers and a reagent including RNase A, lysozyme, and proteinase K and finally, the DNA of the samples was eluted from spin columns using elution buffer and centrifugation at 10 000 g for 1 minute. The extracted DNA was stored at -20°C until use.

Detection of *mecA, pvl* and Erythromycin Resistance Genes

The presence of the methicillin resistance determinant (*mecA*), macrolide/lincosamide resistance genes including *ermA*, *ermB*, *ermC* and *msrA* and *PVL* gene was investigated by PCR using MJ mini thermal cycler (BioRad Co, USA). Each reaction mixture included 2.5 µL 10X PCR buffer, 1.5 µL $MgCl_2$, 1 µL dNTPs (50 µM), 1.5 U Taq DNA polymerase, and 1 µL (10 pmol) of both forward and reverse primers (Table 1)[12-16] and the volume was completed to 25 µL using distilled deionized water. All ingredients were obtained from Takapuzist Corporation, Iran. PCR programs were set according to the annealing temperature of the primers (Table 1), and 94°C and 72°C were used for denaturation and extension phase, respectively. After PCR, the products were resolved in 1.2% agarose gel, stained with ethidium bromide, and pictured using a UV transilluminator. The 100-bp DNA marker (Yektatajhiz Co, Iran) was used as the molecular size marker.

Statistical Analysis

Data were analyzed using SPSS version 16.1. Discrete variables were expressed as percentages and proportions were compared using the chi-square test. *P* values less than 0.05 were considered statistically significant.

Results

A total of 50 MRSA isolates were recovered from 120 samples isolated from raw milk. Among isolates, 48%

Table 1. Oligonucleotides Used as Primers in PCR

Name of Primer	Sequence (5′ to 3′)	Target gene	Annealing Temperature	Product Size (bp)	Reference
mecA-F mecA-R	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	*mecA*	47°C	162	(12)
ermA-F ermA-R	TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA	*ermA*	50°C	139	(13)
ermB-F ermB-R	CTATCTGATTGTTGAAGAAGGATT GTTTACTCTTGGTTTAGGATGAAA	*ermB*	50°C	142	(13)
ermC-F ermC-R	AATCGTCAATTCCTGCATGT TAATCGTGGAATACGGGTTTG	*ermC*	50°C	300	(14)
msrA-F msrA-R	GGCACAATAAGAGTGTTTAAAGG AAGTTATATCATGAATAGATTGTCCTGTT	*msrA*	51°C	940	(15)
Luk-PV-1 *Luk-PV*-2	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	*PVL*	55°C	433	(16)

(24/50) and 46% (23/50) were resistant to erythromycin and clindamycin, respectively and 9 phenotypes were determined according to the resistance, susceptibility or intermediate results of the isolates to erythromycin and clindamycin (Table 2). The most prevalent phenotype was R/R which was present in 14 (28%) isolates (Table 3). In total, seven (14%) isolates which were resistant to erythromycin and susceptible to clindamycin showed inducible clindamycin resistance. Moreover, one erythromycin susceptible isolate with *ermB* and *msrA* genes showed inducible resistance to clindamycin.

The incidence of the erythromycin resistance genes including *ermA*, *ermB*, *ermC* and *msrA* was 14%, 64%, 12%, and 26%, respectively. There were 10 distinct genotypes according to the presence of 4 resistance genes (Table 3). Based on the results, *ermB* was the most prevalent genotype which was present in 21 isolates (including 8 different phenotypes). Nine isolates did not have any *erm* genes, four of which were resistant to erythromycin and one resistant to clindamycin. The coexistence of two or more *erm* genes was detected in 20 (40%) isolates. The *mecA* gene was detected in 44 (88%) MRSA isolates. In addition, the *PVL* gene was present in 9 (18%) MRSA isolates.

There was not any significant correlation between the genotypic and phenotypic patterns ($P \geq 0.05$) of erythromycin resistance. In addition, there was not any significant correlation between the incidence of each one of the resistance genes and the phenotype of the erythromycin and clindamycin resistance ($P \geq 0.05$). There was a significant correlation between the RS phenotype and inducible clindamycin resistance ($P \leq 0.05$).

Discussion

MRSA is one of the most important pathogenic bacteria associated with food poisoning, hospital-acquired infections, infections after burn and other staphylococcal infections all around the world. The occurrence of MRSA isolates has significantly increased the possible hazards of livestock animals and humans as well.[17,18] It has been shown that animals can be the probable reservoirs of MRSA for human infections.[1,19] The results showed higher prevalence of the MRSA among samples recovered from raw milk in comparison with other studies.[17,20] Nowadays, MRSA isolates were identified using various methods including resistance to cefoxitin or the presence of *mecA* gene.[11] However, in contrast to other studies, none of the MRSA isolates recovered from raw milk possessed the *mecA* gene which is responsible for resistance to penicillinase-resistant beta-lactam antibiotics. The high level of this resistance can be due to the presence of additional genetic features[21] though the finding is in contrast with the results of other studies which have reported that all MRSA isolates possessed *mecA* gene.[20,22] This result can be due to the technical and personal errors during the examination although we double-checked *mecA* negative samples.

The results of the study showed a relatively high occurrence of erythromycin and clindamycin resistance among MRSA isolates obtained from raw milk. Different studies previously reported the emergence of high erythromycin resistance among *S. aureus* isolated from different origins which can be considered as a potential hazard for public health because the erythromycin is a proper alternative antibiotic for penicillin in human infections caused by *S. aureus*.[22,23] The mastitis of cattle is one of the main origins of MRSA in milk or dairy products.[24,25] Therefore, control and monitoring programs and avoiding utilization of such antibiotics in farms can be considered as essential tasks to combat these infections in human.

It seems that erythromycin-resistant MRSA isolates from various sources have different genotypic features around the world. For example, all clinical MRSA isolates in Japan were resistant to erythromycin and all of them had *ermA* while no isolates had *ermB* and *ermC*.[26] Akpaka et al, in a study on 309 *S. aureus* isolates, showed 40.1% were resistant to erythromycin and 36% were resistant to clindamycin. The MRSA isolates in the study were resistant to macrolides or macrolides/lincosamides and harbored

Table 2. The Incidence of Antibiotic Resistance and Different Genes Among MRSA Isolates

No.	Source	Resistance to Erythromycin	Resistance to Clindamycin	Inducible Resistance	Resistance Genes				*mecA* Gene	*PVL* Gene
					ermA	*ermB*	*ermC*	*msrA*		
1	Raw milk	R	S	-	+	+	-	-	+	-
2	Raw milk	R	R	-	-	+	-	-	+	-
3	Raw milk	I	S	-	-	+	-	-	+	-
4	Raw milk	R	R	-	-	+	-	-	-	-
5	Raw milk	I	S	-	-	-	-	-	+	-
6	Raw milk	I	S	-	+	+	-	+	+	+
7	Raw milk	I	R	-	-	-	-	+	-	-
8	Raw milk	S	R	-	-	+	-	-	+	-
9	Raw milk	R	R	-	-	+	-	+	+	+
10	Raw milk	R	S	+	+	+	-	-	+	+
11	Raw milk	R	S	-	+	+	+	+	+	-
12	Raw milk	R	S	-	-	+	-	+	+	-
13	Raw milk	R	R	-	-	-	+	-	+	-
14	Raw milk	R	S	+	+	+	-	+	+	-
15	Raw milk	R	R	-	-	-	-	-	+	-
16	Raw milk	R	S	+	-	-	-	-	+	-
17	Raw milk	R	S	+	-	+	-	+	+	-
18	Raw milk	R	R	-	-	+	-	-	+	-
19	Raw milk	S	S	-	-	+	-	-	+	-
20	Raw milk	R	R	-	-	+	+	-	-	-
21	Raw milk	S	S	-	-	-	-	-	+	-
22	Raw milk	S	S	-	-	+	-	-	+	-
23	Raw milk	R	S	+	-	-	-	-	+	-
24	Raw milk	I	S	-	-	-	-	-	+	+
25	Raw milk	I	S	-	+	-	-	+	-	-
26	Raw milk	I	S	-	+	-	-	+	-	-
27	Raw milk	S	I	-	-	+	-	+	+	-
28	Raw milk	I	R	-	-	-	-	-	+	-
29	Raw milk	R	R	-	-	-	+	-	+	+
30	Raw milk	S	I	-	-	+	-	-	+	+
31	Raw milk	I	R	-	-	+	-	-	+	+
32	Raw milk	I	R	-	-	+	-	-	+	-
33	Raw milk	I	I	-	-	-	-	-	+	-
34	Raw milk	R	R	-	-	+	-	-	+	-
35	Raw milk	I	I	-	-	+	-	-	+	-
36	Raw milk	R	R	-	-	-	-	-	+	-
37	Raw milk	I	S	-	-	-	-	+	+	-
38	Raw milk	R	R	-	-	+	-	-	+	-
39	Raw milk	R	R	-	-	-	+	-	-	+
40	Raw milk	S	R	-	-	+	-	-	+	-
41	Raw milk	I	R	-	-	+	-	-	+	+
42	Raw milk	R	R	-	-	+	-	-	+	-
43	Raw milk	S	S	-	-	+	-	-	+	-
44	Raw milk	S	R	-	-	+	-	-	+	-
45	Raw milk	S	R	-	-	-	+	-	+	-
46	Raw milk	R	R	-	-	+	-	-	+	-
47	Raw milk	I	S	-	-	+	-	-	+	-
48	Raw milk	R	S	+	-	+	-	-	+	-
49	Raw milk	S	I	-*	-	+	-	+	+	-
50	Raw milk	R	I	+	-	-	-	+	+	-
Total	Raw milk	**24**	**23**	**7**	**7**	**32**	**6**	**13**	**44**	**9**

* The isolate with inducible clindamycin resistance without resistance to erythromycin and with resistance genes.

Table 3. The Comparison of Different Phenotypes and Genotypes Among MRSA Isolates

Genotypic pattern	Phenotype of Resistance Pattern (Erythromycin/Clindamycin)									Total
	R/R	R/I	R/S	I/R	I/I	I/S	S/R	S/I	S/S	
ermB	7	-	1	3	1	2	3	1	3	**21**
ermC	3	-	-	-	-	-	1	-	-	**4**
msrA	-	1	-	1	-	1	-	-	-	**3**
ermA/ermB	-	-	2	-	-	-	-	-	-	**2**
ermA/ msrA	-	-	-	-	-	2	-	-	-	**2**
ermB/msrA	1	-	2	-	-	-	-	2	-	**5**
ermB/ermC	1	-	-	-	-	-	-	-	-	**1**
ermA/ermB/msrA	-	-	1	-	-	1	-	-	-	**2**
ermA/ermB/ermC/msrA	-	-	1	-	-		-	-	-	**1**
Without studied genes	2	-	2	1	1	2	-	-	1	**9**
Total	**14**	**1**	**9**	**5**	**2**	**8**	**4**	**3**	**4**	**50**

R: resistant; S: susceptible; I: intermediate.

ermA (n = 20), *ermC* (n = 2), and *msrA* (n = 17).[27] Schmitz et al reported that 93% of erythromycin resistant MRSA and 44% of erythromycin resistant MSSA, expression of macrolide-lincosamide-streptogramun B (MLS_B) resistance was constitutive, with *ermA* as predominant gene in European university hospitals. The *ermA* gene was more common in MRSA, while *ermC* was more common in MSSA isolates.[28] Some studies showed *ermC* as the predominant responsible gene for erythromycin resistance among isolates recovered from burn, environmental and clinical samples.[29,30] However, in the present study, the *ermB* gene was considered as predominant responsible gene for erythromycin resistance among *S. aureus* isolates recovered from raw milk. Nevertheless, there was not any significant correlation between the presence of any gene or/and any genotype with resistance phenotypes ($P \geq 0.05$). This can indicate that only the presence of resistance genes will not determine the resistances because a number of isolates (10/50) were still susceptible to erythromycin despite having one or more of these genes (8, 2, and 1 isolates with *ermB*, *ermB*/*msrA,* and *ermC* genotype, respectively). This finding was more prominent among clindamycin susceptible isolates (Table 2). In addition, 4 and 1 isolates without any studied genes showed resistance to erythromycin and clindamycin, respectively. These isolates may have another resistance mechanism such as the expression of efflux pumps for resistance to these antibiotics. The presence of MRSA isolates in the milk of food animals can be due to the extra use of similar antibiotics such as oxacillin or penicillin in breeding these animals. On the other hand, the utilization of macrolides, streptogramins B and lincosamides can increase the erythromycin and clindamycin resistance rate of strains in food animals and their products because it leads to cross-resistance.[7,29,30] These strains can be transmitted to humans through direct contact or the use of dairy products.

Some of the MRSA isolates had the ability to acquire the clindamycin resistance in relation to erythromycin resistant phenotype or/and the presence of the related genes. Although the inducible clindamycin resistance was significantly more observed in RS phenotypes than in other phenotypes, inducible resistance was observed in other phenotypes too (Table 2). In fact, it seems that the genetic content of the isolates is more likely to contribute to the phenomenon. In addition, there was no significant correlation between the inducible clindamycin resistance and the presence of any erythromycin resistance genes ($P \geq 0.05$).

Finally, the study results showed the presence of the *PVL* gene among the MRSA isolates recovered from raw milk samples. Other studies reported higher incidence of the gene in other sources including pediatric infections, hospital-acquired infections, skin infections and pneumonia.[16,31,32] However, the results indicated a remarkable occurrence of the gene in comparison with some studies.[33] There was not any significant correlation between the resistance genotypes/phenotypes and the presence of *PVL* gene in the present study. The investigation of the presence of the PVL producing MRSA isolates in dairy products in particular is of great importance because the consumption of raw dairy products and the numbers of local dairy retail stores in Iran have increased in recent years.[34]

Conclusion

Considering the fact that there are very few studies in the country in this regard, this study is the first report of the high incidence of erythromycin resistance genes and inducible clindamycin-resistant MRSA strains in raw milk samples in Iran. *PVL* appears to be a possible virulence factor associated with raw milk MRSA. Association of *PVL* genes with other resistance determinants is not identified. In conclusion, by increasing the awareness of

people about the use of pasteurized dairy products and the proper training of livestock breeders in observing hygienic precautions at the time of supplying dairy products and familiarizing people with diseases caused by dairy consumption, basic steps can be taken to reduce the incidence of infections caused by *S. aureus* with dairy origin.

Authors' Contributions

All Authors had equal contribution to the manuscript.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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