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Research article

# Relationship between methicillin resistance and the presence of *femA* and *mecA* genes in coagulase positive and negative staphylococci isolated from milk and cheese

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**ABSTRACT**

Three hundred milk and cheese samples were collected from Baghdad markets. Two hundred staphylococcal isolates were isolated from milk and cheese samples. *Staphylococcus aureus* was predominant species (97 isolates (48%)) followed by *S. chromogenes* (82 isolates (41%)) and *S. epidermidis* (21 isolates (11%)). The antibiotic susceptibility of coagulase positive staphylococci (COPS) and coagulase negative staphylococci (CONS) isolates to 3 antibiotics (Methicillin, Tetracycline and Vancomycin) was determined using disc diffusion method. The results revealed that 80 isolates of *S. aureus* (82.47%) were resistant to methicillin (MRSA), while 8 isolates (8.24%) were resistant to vancomycin (VRSA) and 18 isolates of *S. aureus* (19%) were resistant to tetracycline. Sixty four CONS isolates (62.13%) were resistant to methicillin, 28 CONS isolates (27.18%) resistant to tetracycline, and 5 CONS isolates (4.85%) resistant to vancomycin. Deoxyribo nucleic acid (DNA) was extracted from staphylococcal isolates. The genetic determinants of methicillin resistance genes (*femA* and *mecA)* were amplified using monoplex PCR technique to identify methicillin resistant (*mecA*+) and susceptible (lacking *mecA*) staphylococci and to identify *S. aureus* (*femA*+) and coagulase negative staphylococci (lacking *femA*). Ninety six *S. aureus* isolates (98.96%) were attributed as harbour *femA* gene. The *mecA* gene was detected in 91 (93.81%) MRSA isolates, while it was detected in 70 (67%) CONS isolates.

**Keywords: Methicillin resistance, milk, cheese, *femA*, *mecA*.**

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# INTRODUCTION

*Staphylococcus aureus* is involved in a wide variety of diseases in humans and animals and its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance [1]. In 2009, the European Food Safety Authority underlined the increasing concern for Public Health represented by the presence of methicillin-resistant *S. aureus* (MRSA) in food producing animals, and recommended that further work should be performed on sampling, detection and quantification of MRSA carriage in both humans and animals, as well as on the contamination of food and the environment [2].

MRSA is a major human pathogen, having emerged first in hospitals during the 1970's and then expanding into a worldwide public health problem. Over the past decade, a growing number of MRSA cases have been reported in companion and food animals and in their human associates, including pet owners, farmers and veterinary personnel [3]. MRSA in animals was first detected in milk from cows with mastitis and since then has been found in dogs, cats, horses, pigs, sheep, rabbits, chickens, and several exotic species [4].

Controlling this pathogen is very difficult because it expresses certain virulence factors as it contains a thick peptidoglycan layer and teichoic acid, gives the bacterium structure, rigidity and promotes colonization of host tissue. It invasions like Leukocidin causes the destruction of Leukocytes and promote bacterial spread in tissue and cause pus formation; surface factors (Capsule, Protein A) inhibit phagocytic engulfment. Carotenoids; catalase enhances their survival in phagocytes; membrane damaging toxins (haemolysin, leukocidin and leukotoxin) lyse the eukaryotic cell membrane; exotoxins (Staphylococcal enterotoxins, Toxic Shock Syndrome toxin (TSST), and Exfoloative Toxin (ET)) damage the host tissue and cause symptoms of disease. Coagulase, an enzyme that clots plasma and coats the Staphylococcal cells, prevents the cells from being phagocytosed and destroyed by macrophages [5].

The predominance of human MRSA strains in household pets suggests that animals become colonized through contact with infected or colonized people, and that pets could in turn pass MRSA back to humans or other species [6]. Considering the facts above and that at present little is known about the occurrence of the antibiotic resistance genes among the staphylococci isolated from foods in our country. The study aims to Isolate and identify the most prevalent staphylococcal species from milk and cheese samples using bacteriological and immunological methods, and confirming this identification by PCR technique through the amplification of the fimA gene. Detection of the *mecA* gene in coagulase positive (COPS) and coagulase negative staphylococci (CONS) using PCR technique. Moreover, Comparison between the genotypic contents of the *femA* and *mecA* genes with the phenotypic expression of these genes represented by methicillin resistance by Staphylococcal isolates.

# MATERIALS AND METHODS

## Isolation, Identification and Antibiotic Susceptibility of *S. aureus*

Samples of the milk and cheese were collected aseptically in sterilized plastic bags from different market areas of Baghdad and directly transported to the laboratory under cold conditions and analyzed within 4 hs. The standard method of Al-khafaji *et al.*, was followed with little modification (Kirby-Bauer method) was used for Isolation, Identification and Antibiotic Susceptibility of *S. aureus* [7,8].

## DNA Extraction

DNA extraction method described by previous method [9] and modified to be suitable for the DNA extraction from staphylococcal isolates. Five average sized pure isolated colonies from a fresh overnight culture plate were picked up and placed in an eppendorf tube containing 200 µl of distilled water, the tube was vortexed, then incubated at 85$^{o}$C for 20 min, then immediately frozen (-20$^{o}$C) for 10 min. Thereafter, it was centrifuged at 10000 rpm for 5 min. Supernatant was placed in a new sterilized eppendorf tube, and examined for quantity, purity and quality via the estimation of DNA concentration and purity, and agarose gel electro-phoresis. The standard strain *S. aureus* ATCC 25923 DNA was extracted by this method to be subjected to polymerase chain reaction (PCR) for confirmation of the results obtained by the primers used for the staphylococcal identification.

## Polymerase Chain Reaction

The PCR assay was performed in a monoplex patterns in order to amplify *femA* and *mecA* genes. The primers were selected for this study according to previous study: the forward (*femA* -F: 5′-CATGATGGCGAGATTACA GGT-3′) and the backward primer (*femA*-R: ′5-GTCATCACGATCAGCGAAAGC-3′) which gave a PCR product of 314 base pair (bp) [10]; and the forward (*mecA* -F: 5-AAAATCGATGGTAAAGGTTGGC-3′) and the backward primer (*mecA* -R: 5-AGTTCTGCAGTA CCGGATTTGC-3′) which gave a PCR product of 533 bp [11]. These primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmol/ µl as recommended by provider and stored in deep freezer until used in PCR amplification. The extracted DNA, primers and PCR premix (Accupower, Bioneer), were thawed at 4$^{o}$C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes. PCR mixture was set up in a total volume of 25 µl included 5 µl of PCR premix, 2 µl of each primer and 4 µl of template DNA were used. The rest volume was completed with sterile de-ionized distilled water, then vortexed and finally 4 µl of template

DNA was added. Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermal cycler PCR instrument. Initial denaturation was at 95$^{o}$C for 5 min followed by 30 cycles of 95$^{o}$C for 1 min, 63$^{o}$C for 1 min and 72$^{o}$C for 1 min and a final extension at 72$^{o}$C for 5 min).

### Determination of PCR Specificity

The concentration and purity of extracted DNA of staphylococcal isolates (which were isolated from milk and cheese samples) were checked. Thereafter, they were analyzed by PCR and the results confirmed by using 1.5% agarose gel electrophoresis.

## RESULTS AND DISCUSSION

Results of antibiotic susceptibility obtained by this study confirmed that 80 *S. aureus* isolates (82.47%) were methicillin resistant (MRSA), while 8 isolates (8.24%) were vancomycin resistant (VRSA). Eighteen *S. aureus* isolates (19%) were resistant to tetracycline.

### *femA* and *mecA* genes amplification by monoplex PCR technique

The accurate and rapid diagnosis of antibiotic resistance is very important in the treatment of staphylococcal infections. PCR-based molecular methods are often preferred for determination of antibiotic resistance genes. PCR technique was used to determinant the genes of methicillin resistance *femA* and *mecA* and to identify susceptible (lacking *mecA*) and resistant (*mecA*+) staphylococci and to differentiate *S. aureus* (*femA*+) from coagulase- negative staphylococci (lacking *femA*). The results of the present study showed that *femA* gene band detected with 314 bp, all MRSA isolated strains were produced 314 bp band (**Fig 1**).

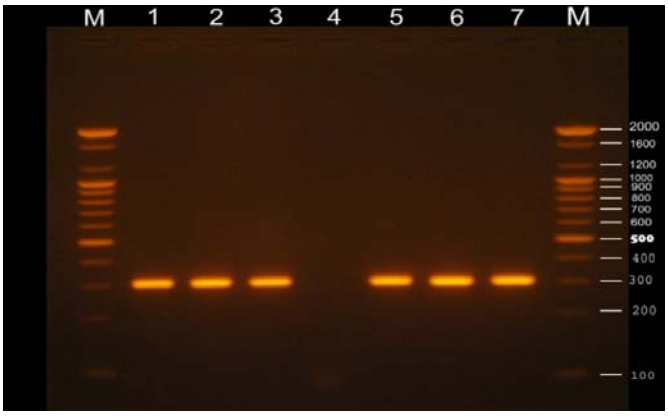


**Fig 1.** PCR products of *femA* gene (314bp) of *S. aureus* isolates in monoplex polymerase chain reaction (PCR). Ethidium bromide–stained 1.5% agarose gel electrophoresis. M, DNA molecular weight marker (100bp ladder "BIONEER"); Lane 1, Lane 2 and Lane 3, represent the 314 bp of *femA* gene of isolates S 21, S 30 and S 102, respectively; Lane 4, *femA* negative CONS isolate S213; Lane 5, Lane 6 and Lane 7, Positive amplification of 314 bp for *femA* gene of the isolates S 117, S 140 and S 205, respectively.

Ninety six *S. aureus* isolates (98.96%) were found to harbour *femA* gene, and all of them were MRSA (**Table 1**).

Regarding CONS, none of them carried *femA* gene, since it is species specific marker for *S. aureus*. *femA* is generally accepted as a species specific marker. This gene encodes a factor which is essential for methicillin resistance and is universally present in all MRSA isolates, its product, a 48-kDa protein, has been implicated in cell wall metabolism and is found in large amounts in actively growing cultures [12]. Analysis of the *femA* product indicated that this protein is associated with the expression of high levels of methicillin resistance without affecting Penicillin Binding Protein-2 (PBP-2`) production, the significance of the *femA* genes in the mechanism of methicillin resistance was supported by the demonstration that *S. aureus* strain with *femA* gene inactivated; lost the methicillin resistance trait, but with the transduction of *femA* genes restored the resistance [13]. Another biochemical analysis suggested that *femA* product may be involved in the metabolism of cell wall synthesis [14].

*mecA* gene, which encodes production of an altered PBP (PBP2a or PBP2) a modified transpeptidase that has a low affinity for all β-lactam antimicrobials (penicillins, cephalosporins and carbapenems) [15].

533 bp *mecA* was detected in 91 (93.81%) MRSA isolates, while it was detected in 70 (67%) CONS isolates (**Fig 2**).

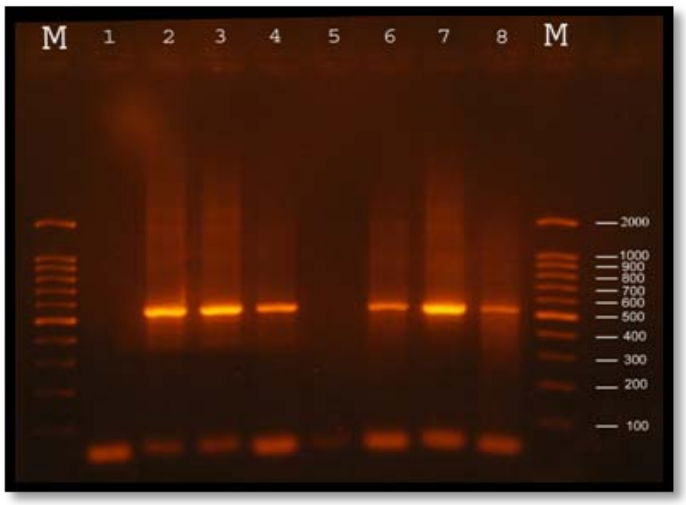


**Fig 2.** PCR products of *mecA* gene (533bp) of staphylococcal isolates in monoplex polymerase chain reaction (PCR). Ethidium bromide–stained 1.5% agarose gel electrophoresis. M, DNA molecular weight marker (100 bp ladder "BIONEER"); Lane 1, *mecA* negative S 112; Lane 2, Lane 3 and Lane 4, 533 bp *(mecA* gene) of the COPS isolates S 14, S 55 and S 117, respectively; Lane 5, Negative control; Lane 6, Lane 7 and Lane 8, 533 bp for *mecA* gene of the CONS isolates S 33, S 39 and S 174, respectively.

Results of *mecA* gene amplification by COPS and CONS isolates were demonstrated in the **tables 2** and **3,** respectively.

**Table 1**. *femA* gene amplification results of the COPS strains obtained by monoplex PCR.

| Id | Strain | *fem A* gene amplification | Id | Strain | *fem A* gene amplification | Id | Strain | *fem A* gene amplification |
|---|---|---|---|---|---|---|---|---|
| 1 | S 14 | + | 34 | S 97 | + | 67 | S 173 | + |
| 2 | S 15 | + | 35 | S 98 | + | 68 | S 174 | + |
| 3 | S 16 | + | 36 | S 100 | + | 69 | S 177 | + |
| 4 | S 17 | + | 37 | S 102 | + | 70 | S 180 | + |
| 5 | S 21 | + | 38 | S 103 | + | 71 | S 185 | + |
| 6 | S 27 | + | 39 | S 108 | + | 72 | S 191 | + |
| 7 | S 30 | + | 40 | S 109 | + | 73 | S 194 | + |
| 8 | S 31 | + | 41 | S 111 | + | 74 | S 195 | + |
| 9 | S 40 | + | 42 | S 112 | + | 75 | S 196 | + |
| 10 | S 41 | + | 43 | S 113 | + | 76 | S 198 | + |
| 11 | S 50 | + | 44 | S 117 | + | 77 | S 199 | + |
| 12 | S 51 | + | 45 | S 119 | + | 78 | S 200 | + |
| 13 | S 55 | + | 46 | S 120 | + | 79 | S 203 | + |
| 14 | S 56 | + | 47 | S 121 | + | 80 | S 204 | + |
| 15 | S 57 | + | 48 | S 125 | + | 81 | S 205 | + |
| 16 | S 58 | + | 49 | S 130 | + | 82 | S 206 | + |
| 17 | S 59 | + | 50 | S 132 | + | 83 | S 207 | + |
| 18 | S 60 | + | 51 | S 133 | + | 84 | S 208 | + |
| 19 | S 63 | + | 52 | S 136 | + | 85 | S 214 | + |
| 20 | S 64 | + | 53 | S 140 | + | 86 | S 215 | + |
| 21 | S 65 | + | 54 | S 144 | + | 87 | S 217 | + |
| 22 | S 68 | + | 55 | S 145 | + | 88 | S 219 | + |
| 23 | S 70 | + | 56 | S 147 | + | 89 | S 220 | + |
| 24 | S 73 | + | 57 | S 150 | + | 90 | S 223 | + |
| 25 | S 77 | + | 58 | S 155 | + | 91 | S 227 | + |
| 26 | S 80 | + | 59 | S 156 | + | 92 | S 228 | + |
| 27 | S 81 | + | 60 | S 157 | + | 93 | S 230 | + |
| 28 | S 83 | + | 61 | S 161 | + | 94 | S 232 | + |
| 29 | S 85 | + | 62 | S 163 | + | 95 | S 233 | + |
| 30 | S 91 | - | 63 | S 164 | + | 96 | S 234 | + |
| 31 | S 93 | + | 64 | S 165 | + | 97 | S 235 | + |
| 32 | S 94 | + | 65 | S 168 | + | | | |
| 33 | S 95 | + | 66 | S 171 | + | | | |

**Table 2**. *mecA* gene amplification results of the COPS strains obtained by monoplex PCR.

| Id | Strain | *mecA* gene amplification | Id | Strain | *mecA* gene amplification | Id | Strain | *mecA* gene amplification |
|---|---|---|---|---|---|---|---|---|
| 1 | S 14 | + | 34 | S 97 | + | 67 | S 173 | + |
| 2 | S 15 | - | 35 | S 98 | + | 68 | S 174 | + |
| 3 | S 16 | + | 36 | S 100 | + | 69 | S 177 | + |
| 4 | S 17 | + | 37 | S 102 | + | 70 | S 180 | + |
| 5 | S 21 | + | 38 | S 103 | + | 71 | S 185 | + |
| 6 | S 27 | + | 39 | S 108 | + | 72 | S 191 | + |
| 7 | S 30 | + | 40 | S 109 | + | 73 | S 194 | + |
| 8 | S 31 | + | 41 | S 111 | + | 74 | S 195 | + |
| 9 | S 40 | + | 42 | S 112 | - | 75 | S 196 | + |
| 10 | S 41 | + | 43 | S 113 | + | 76 | S 198 | + |
| 11 | S 50 | + | 44 | S 117 | + | 77 | S 199 | + |
| 12 | S 51 | + | 45 | S 119 | + | 78 | S 200 | + |
| 13 | S 55 | + | 46 | S 120 | + | 79 | S 203 | + |
| 14 | S 56 | + | 47 | S 121 | + | 80 | S 204 | + |
| 15 | S 57 | + | 48 | S 125 | + | 81 | S 205 | + |
| 16 | S 58 | + | 49 | S 130 | - | 82 | S 206 | + |
| 17 | S 59 | + | 50 | S 132 | + | 83 | S 207 | + |
| 18 | S 60 | + | 51 | S 133 | + | 84 | S 208 | + |
| 19 | S 63 | + | 52 | S 136 | + | 85 | S 214 | + |
| 20 | S 64 | - | 53 | S 140 | + | 86 | S 215 | + |
| 21 | S 65 | + | 54 | S 144 | + | 87 | S 217 | + |
| 22 | S 68 | + | 55 | S 145 | + | 88 | S 219 | + |
| 23 | S 70 | + | 56 | S 147 | + | 89 | S 220 | + |
| 24 | S 73 | + | 57 | S 150 | + | 90 | S 223 | + |
| 25 | S 77 | + | 58 | S 155 | + | 91 | S 227 | + |
| 26 | S 80 | + | 59 | S 156 | + | 92 | S 228 | - |
| 27 | S 81 | + | 60 | S 157 | + | 93 | S 230 | + |
| 28 | S 83 | + | 61 | S 161 | + | 94 | S 232 | + |
| 29 | S 85 | + | 62 | S 163 | + | 95 | S 233 | + |
| 30 | S 91 | + | 63 | S 164 | + | 96 | S 234 | + |
| 31 | S 93 | + | 64 | S 165 | + | 97 | S 235 | + |
| 32 | S 94 | - | 65 | S 168 | + | | | |
| 33 | S 95 | + | 66 | S 171 | + | | | |

**Table 3.** *mecA* gene amplification of the CONS strains

| Id | Strain | *mecA* gene amplification | Id | Strain | *mecA* gene amplification | Id | Strain | *mecA* gene amplification |
|---|---|---|---|---|---|---|---|---|
| 1 | S 1 | + | 36 | S 48 | + | 71 | S 124 | + |
| 2 | S 2 | + | 37 | S 49 | - | 72 | S 126 | - |
| 3 | S 3 | + | 38 | S 52 | + | 73 | S 127 | + |
| 4 | S 4 | - | 39 | S 53 | + | 74 | S 128 | + |
| 5 | S 5 | + | 40 | S 54 | + | 75 | S 129 | + |
| 6 | S 7 | + | 41 | S 61 | - | 76 | S 131 | - |
| 7 | S 8 | + | 42 | S 62 | + | 77 | S 134 | + |
| 8 | S 9 | + | 43 | S 66 | + | 78 | S 135 | + |
| 9 | S 10 | + | 44 | S 67 | - | 79 | S 160 | + |
| 10 | S 11 | - | 45 | S 69 | + | 80 | S 162 | + |
| 11 | S 12 | - | 46 | S 71 | - | 81 | S 175 | - |
| 12 | S 13 | + | 47 | S 72 | + | 82 | S 178 | + |
| 13 | S 18 | + | 48 | S 74 | + | 83 | S 179 | + |
| 14 | S 19 | + | 49 | S 75 | - | 84 | S 183 | + |
| 15 | S 20 | - | 50 | S 76 | - | 85 | S 190 | + |
| 16 | S 22 | + | 51 | S 78 | - | 86 | S 192 | - |
| 17 | S 23 | - | 52 | S 79 | + | 87 | S 193 | + |
| 18 | S 24 | + | 53 | S 82 | + | 88 | S 197 | + |
| 19 | S 25 | + | 54 | S 84 | - | 89 | S 201 | + |
| 20 | S 26 | - | 55 | S 86 | + | 90 | S 202 | + |
| 21 | S 28 | + | 56 | S 87 | - | 91 | S 210 | + |
| 22 | S 29 | + | 57 | S 88 | + | 92 | S 211 | + |
| 23 | S 33 | + | 58 | S 89 | + | 93 | S 212 | + |
| 24 | S 34 | + | 59 | S 90 | + | 94 | S 213 | - |
| 25 | S 35 | - | 60 | S 92 | - | 95 | S 216 | + |
| 26 | S 36 | - | 61 | S 96 | + | 96 | S 218 | + |
| 27 | S 37 | + | 62 | S 99 | + | 97 | S 221 | + |
| 28 | S 38 | - | 63 | S 101 | - | 98 | S 222 | - |
| 29 | S 39 | + | 64 | S 104 | + | 99 | S 224 | + |
| 30 | S 42 | - | 65 | S 105 | + | 100 | S 225 | + |
| 31 | S 43 | + | 66 | S 107 | + | 101 | S 226 | + |
| 32 | S 44 | - | 67 | S 114 | - | 102 | S 229 | + |
| 33 | S 45 | + | 68 | S 115 | - | 103 | S 231 | - |
| 34 | S 46 | - | 69 | S 122 | + | | | |
| 35 | S 47 | - | 70 | S 123 | + | | | |

The virulence genes *mecA* and *femA* were considered because they are believed to be the major contributors to methicillin resistance. It is known that methicillin resistance could manifest with or without *mecA*, auxillary genes like *femA* influence the extent of resistance implying that there could be other mechanisms that circumvent absence of gene to confer methicillin-resistance [16]. The inclusion of an internal positive control *femA* in the reaction provides assurance against false-negative results [17].

Food is a main source for the transfer of antibiotic resistance. The transfer of resistant food-borne pathogens, or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms [18,19]. The detection of *mecA* gene remains the gold standard for detecting methicillin-resistance, its detection alone does not confirm the presence of *S. aureus* and there is no consensus on the molecular target that could be used to confirm the *S. aureus* species, constitutively expressed genes such as *femA*, *femB* and nuc are being used as molecular targets for the identification of *S. aureus* polymorphisms within these constitutive genes have been reported worldwide,from another point of view, *mecA* alone does not solely confer the methicillin resistance, studies have shown that *fem* (factors essential for methicillin-resistance) or the auxiliary genes like *femA*/*B*/*X* in addition to *mecA* are also important in expression of methicillin resistance, the *femABX* operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of Staphylococci [2016].

In the present study, the results of antibiotic susceptibility by disk diffusion method were compared with gene analysis results in staphylococcal isolates (**Table 4**), because the phenotypic expression of antimicrobial resistance has been reported to be influenced by various factors.

The statistical analysis results showed that there was correlation between genotypic content of the *femA* and *mecA* genes and the phenotypic expression of them when tested by antibiotic disc diffusion method. There was no significance relationship between genotypic content and phenotypic expression of them represented by methicillin resistance. Gene analysis results confirmed that none of the CONS strains harboured the *femA* gene; all of them harboured the *mecA* gene, which means that CONS' methicillin resistance refers to the *mecA* gene only, while COPS' methicillin resistance refers to both of the *femA* and *mecA* genes.

**Table 4.** Relationship between Methicillin resistance and the presence of *femA* and *mecA* genes in both COPS and CONS. a, P > 0.05.

| Staphylococcal isolates | Positive No. of Methicillin resistant Staphylococci by phenotypic method | The distribution of methicillin resistance genes | | $X^2$-Test value |
|---|---|---|---|---|
| | | Positive No. of *femA* | Positive No. of *mecA* | |
| 97 COPS isolates (100%) | 80 (82.47%) | 96(98.96%) | 91(93.81%) | 1.506[a] |
| 103CONS isolates (100%) | 64 (62.13%) | 0 (0%) | 70 (67.96%) | 0.269[a] |

Nafisi *et al.* (2008) studied 52 isolates of coagulase positive *S. aureus* using agar screen and duplex PCR methods and reported that phenotypically 23 cases (44%) of the isolates, and genotypically *mecA* 27 cases of the isolates (52 %) are resistant to methicillin [21]. Duran *et al*. (2012) had reported that methicillin resistance was observed in 17.8 % isolates when tested by methicillin disk diffusion method, whereas 27.9 % isolates had *mecA* gene. Phenotypically methicillin susceptible 30 isolates also carried the *mecA* gene [22]. Chikkala *et al.* (2012) observed a rather high rate (58.3%) detection of *mecA* among MSSA isolates [16]. Standardized methods of susceptibility test have been used for the detection of MRSA strains. Kirby-Bauer antibiotic testing (disc diffusion antibiotic sensitivity test), oxacillin agar screen test, agar dilution and the Epsilometer test (E-test) are commonly used to determine the MRSA phenotype in the clinical microbiology laboratory, these tests have limitations and frequently show variations, however, phenotypic expression of methicillin-resistance can be heterogenous, in addition, methicillin resistance is influenced by culture conditions such as temperature, pH and sodium chloride (NaCl) content in the medium; these factors complicate the detection of methicillin resistance, especially for strains with low level resistance, the PCR methods have high sensitivity and specificity and are independent of the physical and chemical conditions of the culture [23,24].

The results of this study showed a high incidence of *S. aureus* resistant to methicillin in the milk and cheese, therefore, food handlers should take appropriate measures to prevent the spread of MRSA by contaminated raw foods and to prevent the occurrence, growth and survival of MRSA in prepared food.

**Conflict of interest**

The authors declare that they have no conflict of interests.

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