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Detection of Antibiotic Resistance Genes in *Staphylococcus aureus* Strains Isolated from Cow's Milk using Multiplex PCR Assay

Moslem Parvizi[1], Abbas Doosti[2]* and Payam Ghasemi Dehkordi[1,2]

[1]Islamic Azad University, Shahrekord Branch, Young Researchers Club, Shahrekord, Iran.
[2]Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran .

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Mastitis is an important disease in dairy herds, typically caused by bacterial infection such as *Staphylococcus aureus*. Antibiotic resistance in *S. aureus* is worldwide public health problem and considerable concern that continues to grow. The objective of the present study was to investigate the antibiotic susceptibility of *S. aureus* strains by disk diffusion test and determination of *mecA*, *ermA*, *ermB*, *ermC*, and *msrA* antibiotic resistance genes of this bacterium isolated from cow's milk using multiplex PCR method. A total of 100 cow's milk samples that suspected for mastitis were collected from traditional centers and cattle industries in the Chaharmahal Va Bakhtiari province (Southwest Iran). All specimens were cultivated in sheep blood agar (SBA) for isolation of *S. aureus* and then catalase, oxidase, and the coagulase tests were carried out. Bacterial DNA was extracted from colonies cultivated onto sheep blood agar using DNA extraction kit. Then multiplex PCR performed by specific primers for detection of antibiotic resistance genes of *S. aureus* and amplified products were separated on 1% agarose gel electrophoresis. The electrophoresis revealed 163, 174, 139, 224, and 190 bp(s) for *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, genes, respectively. Multiplex PCR assay showed that 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) of *S. aureus* samples, contained *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, genes, respectively. Furthermore, *msrA* gene is more frequent than other antibiotic resistance genes. These results indicated that *msrA* gene could be related to increasing of MLS antibiotics resistant in *S. aureus* and the findings of this study could be useful for foodstuff, public health, and dairy industry to decrease *S. aureus* antibiotic resistance strains. So, these data may be helpful for prescription of best drugs for control of the infection caused by this bacterium in cows for the reduction of mastitis.

Key words: *Staphylococcus aureus*, Antibiotic resistance, Cows Milk, Multiplex PCR.

Staphylococcus aureus is a Gram-positive facultative anaerobic bacterium that is both catalase and coagulase positive[1]. *S. aureus* is the most dangerous of staphylococcal bacterial infections that causes a variety of diseases in animals and humans.

* To whom all correspondence should be addressed.
Tel: +98-3813-361001; Fax: +98-3813-361001
E-mail: biologyshk@yahoo.com

The infections in animals include mastitis, suppurative disease, arthritis, and urinary tract infections[2]. This microorganism is a major cause of pneumonia, osteomyelitis, endocarditis, food poisoning, postoperative wound infections, and nosocomial bacteremia in humans[3]. This bacterium is resistant to temperatures as high as 50°C, to high salt concentrations, and to drying[4].

Foodborne diseases, commonly called food poisoning, are an important public health and hygiene in the world and *S. aureus* is the most reported cause of foodborne intoxications.

Furthermore, *S. aureus* is the most common etiological organism responsible for 30-40% of mastitis in dairy herds and its resistance against multiple antimicrobials[5].

Today rapid evolution of antibiotic resistance in *S. aureus* has become a major clinical and public health problem. Resistant bacteria, or genetic determinants of resistance, can be transmitted from animals to humans via foodstuffs[5]. Multidrug-resistant *S. aureus* strains are especially one of the greatest public concerns since the treatment of infections is more difficult when encountering resistance. Isolation of Methicillin-resistant *S. aureus* (MRSA) from animals was first reported in 1972 following its detection in milk from mastitis cows[6, 7]. MRSA is resistant to all penicillins, including semisynthetic penicillinase-resistant congeners, penems, carbapenems, and cephalosporins. MRSA known to be one of the most prevalent nosocomial pathogens throughout the world and is capable of causing a wide range of hospital-linked infections[8]. The β-lactam resistance of MRSA is determined by the function of the penicillin-binding protein 2a (PBP2a), which is encoded by the methicillin resistant gene, *mecA*[9]. Numerous studies have shown that the prevalence of methicillin resistance in Iran is rising however, regionally the rates differ dramatically[10, 11].

There are many genes associated to multidrug-resistant of *S. aureus* strains. Some of these genes include *mecA*, *ermA*, *ermB*, *ermC*, *msrA*/*msrB*, *ereA* and *ereB*. The *mecA* gene is part of a 21- to 60-kb staphylococcal chromosome cassette mec (SCCmec), a mobile genetic element that may also contain genetic structures such as Tn554, pUB110, and pT181 which encode resistance to non-β-lactam antibiotics[12]. Macrolide resistance can be caused by several mechanisms, the predominant form being target modification mediated by one or more *erm* genes encoding a 23S rRNA methylase[13, 14]. *S. aureus* contains three genes encoding MsrA-specific methionine sulfoxide reductase (Msr) activity (msrA1, msrA2 and msrA3) and an additional gene that encodes MsrB-specific Msr activity. Resistance to macrolides, lincosamides, and streptogramins (MLS) antibiotics caused by the presence of macrolide efflux pumps in staphylococci (encoded by *msrA* or *msrB*) has also been documented[15]. Furthermore, inactivation has been described in several organisms. For example, enzymes (EreA and EreB) that hydrolyse the lactone ring of the macrocyclic nucleus and phosphotransferases that inactivate macrolides have been reported in *S. aureus*[16, 17]. The purpose of present study was to determine the antibiotic resistance genes (*mecA*, *ermA*, *ermB*, *ermC*, and *msrA*) in *Staphylococcus aureus* strains isolated from cow's milk using multiplex PCR technique and disk diffusion test in southwest Iran.

MATERIALS AND METHODS

Collection of samples

In the present study, 100 cow's milk samples that suspected for clinical mastitis were collected from 8 cattle industries and 5 traditional centers in Chaharmahal Va Bakhtiari province located in Southwest Iran.

Staphylococcal isolates

The samples were cultivated in sheep blood agar (SBA) (Merck, Darmstadt, Germany) and incubated in 37°C for 24 hours. The black, grey or white colonies of *Staphylococcus* were cultivated onto blood agar plates containing 5% sheep blood and identified using the catalase, oxidase, and the coagulase tests. The isolates were stored in tryptose soy broth (TSB; Oxoid, Basingstoke, UK) supplemented with 20% glycerol at 80°C until studied.

Disk diffusion test

All *S. aureus* isolates were investigated for their antibiotic resistance or susceptibility by disk diffusion test (D-zone test) on SBA using the following antibiotics: clindamycin (Cc), *erythromycin*, methicillin (Met), and oxacillin (Ox). Antibiotic disks were applied by a dispenser within 15 min after inoculation. Inhibition zone diameters were measured after 16-18 h of incubation at 37°C, but 24 h for Met.

Minimal inhibitory concentrations (MICs)

The MICs were determined using a standardized microdilution test (Veterinary plate for staphylococci, Trios, Prague, Czech Republic). The MICs interpretative criteria were based on the recommendations given in document M100-S16 of the Clinical and Laboratory Standards Institute. *S. aureus* ATCC 25923 served as a reference strain for quality control purposes.

DNA isolation

Black, grey or white colonies of *S. aureus* that cultivated onto SBA were selected randomly for investigation of antibiotic resistance genes. Then, bacterial DNA was extracted from each colonies using DNP™ Kit (CinnaGen, Iran), according to the manufacturer's protocol. The isolated DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell[18]. The extracted DNA of each sample was kept frozen at -20°C until used.

Amplification of antibiotic resistance genes

In present study multiplex PCR technique were used for investigation of antibiotic resistance genes of *S. aureus* strains isolated from cow's milk. The oligonucleotide primers described by Martineau *et al.,* were used in this study[19]. The sequences of primers for amplification of *mecA*, *ermA*, *ermB*, *ermC*, and *msrA* genes of *S. aureus* strains are given in Table 1.

S. aureus ATCC 25923 DNA was used as a positive control. A negative-DNA control was performed by adding 1 µL of sterile ultrapure deionized water. For investigation of antibiotic resistance genes of *S. aureus* the specimens were amplified in a Gradient Palm Cycler (Corbett Research, Australia) and multiplex PCR reaction was performed in a total volume of 25 µL in 0.5 ml tubes containing 1 µg of genomic DNA, 1 µM of each primers, 2 mM $Mgcl_2$, 200 µM dNTP, 2.5 µL of 10X PCR buffer and 1 unit of *Taq* DNA polymerase (Roche applied science, Germany). PCR cycles consisted of an initial denaturation step (95°C for 5 min) followed by 30 amplification cycles (denaturation at 94°C for 1 min, annealing at 58°C, and elongation at 72°C for 1 min) with a final elongation at 72°C for 5 min.

Analysis of multiplex PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H_2O and stir to dissolve). Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder of Fermentas, Germany). After electrophoresis, the amplicons were visualized with ultraviolet light after ethidium bromide (5 µg.mL^{-1}) staining and photographed were obtained in UVIdoc gel documentation systems (UK).

Statistical analysis

Analysis of data and investigation of antibiotic resistance genes of *S. aureus* were performed using the SPSS version 17.0 computer software (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Cultivation of 100 cow's milk samples in sheep blood agar and investigation of catalase, oxidase, and the coagulase tests showed 85 specimens (85%) were *S. aureus* that related to mastitis. The results of present study showed multiplex PCR assay correlated very well with disk diffusion test and MIC determination. There was no discordance between conventional susceptibility testing and PCR for isolated *S. aureus* strains (Table 2). Electrophoresis of PCR products for detection of *S. aureus* antibiotic resistance genes revealed 163, 174, 139, 224, and 190 bp(s) for *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, genes, respectively (Fig. 1).

Multiplex PCR assay showed that from 85 positive samples for *S. aureus* 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) specimens, contained *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, genes, respectively (Table 3). The results showed that *msrA* gene (41.18%) is more frequent

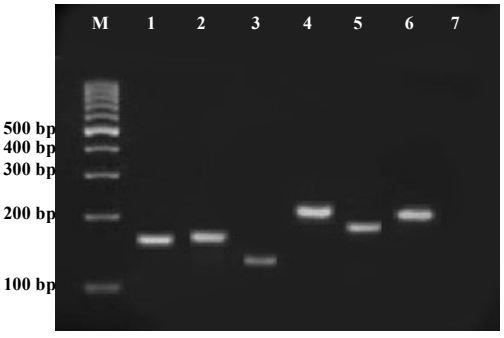

Fig. 1. Gel electrophoresis of multiplex PCR for detection of antibiotic resistance genes of *S. aureus* in cow's milk samples (Line M: 100 bp DNA ladder (Fermentas, Germany), lines 1-5: *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, genes, respectively, line 6: positive control (*S. aureus* ATCC 25923 strain), and line 7: negative control without DNA)

Table 1. Primers used for detection of antibiotic resistance genes in *S. aureus* strains isolated from cow's milk

Gene	Accession number (GenBank)	Oligonucleotide primers sequences	Amplicon size (bp)
msrA	X52085	msrA-F: 5′-TCCAATCATAGCACAAAATC-3′ msrA-R: 5′-AATTCCCTCTATTTGGTGGT-3′	163
mecA	X52594	mecA-F: 5′-AACAGGTGAATTATTAGCACTTGTAAG-3′ mecA-R: 5′-ATTGCTGTTAATATTTTTTGAGTTGAA-3′	174
ermA	K02987	ermA-F: 5′-TATCTTATCGTTGAGAAGGGATT-3′ ermA-R: 5′-CTACACTTGGCTTAGGATGAAA-3′	139
ermB	FN677479	ermB-F: 5′-CGTACCTTGGATATTCACCG-3′ ermB-R: 5′-GTAAACAGTTGACGATATTCTCG-3′	224
ermC	M17990	ermC-F: 5′-CTTGTTGATCACGATAATTTCC-3′ ermC-R: 5′-ATCTTTTAGCAAACCCGTATTC-3′	190

Table 2. Correlation between antibiotic susceptibility testing and PCR for investigation of *S. aureus* strains antibiotic resistance genes

Gene (Antibiotic)	Resistance genes (antibiotic used for susceptibility testing)									
	msrA methicillin		*mecA* oxacillin		*ermA*clindamycin		*ermB*erythromycin		*ermC* Erythr-omycin	
Susceptibility test results	PCR+	PCR–	PCR+	PCR–	PCR+	PCR–	PCR+	PCR–	PCR+	PCR–
Resistant	35	0	25	0	20	0	17	0	16	0
Susceptible	0	50	0	60	0	65	0	68	0	69

Table 3. Frequency of antibiotic resistance genes of *S. aureus* isolated from cow's milk samples using multiplex PCR assay

Gene	Frequency N (%)
msrA	35 (41.18)
mecA	25 (29.41)
ermA	20 (23.53)
ermB	17 (20)
ermC	16 (18.82)

than other antibiotic resistance genes of *S. aureus* isolated from cow's milk specimens.

S. aureus is non-motile, non-spore-forming, and catalase-positive bacteria. *S. aureus* expresses a variety of virulence factors. This pathogen is responsible for causing a wide array of diseases ranging from mild skin infections such as folliculitis and carbuncles to life-threatening conditions such as bacteremia, pneumonia, and endocarditis in human and animals[20]. The bacterium continues to demonstrate the ability to develop resistance to include a broad array of antimicrobial classes, and *S. aureus* is a prominent pathogen in both hospital and the community settings. *S. aureus* strains resistant to methicillin and many other antibiotics are major causes of mastitis and cow disease[19]. Resistance to methicillin is determined by the *mecA* gene, which encodes the low-affinity penicillin-binding protein PBP 2A. MRSA (methicillin resistant *S. aureus*) is present in the nose and on the skin and is shed into the environment by infected or colonized people and animals, indicating that airborne transmission is a possible route for infection[20].

There is no vaccine available, and the role of passive immunoprophylaxis is unclear. The population at risk increases with more elderly people and more patients receiving imunosuppression or having indwelling catheters and other foreign materials. The numbers of resistant bacteria, MRSA are rising. Within a year after the introduction of semi-synthetic penicillins such as methicillin, there were reports of resistant isolates in 1961[21, 22]. There are many genes role in

antibiotics resistance of *S. aureus*. In present study we investigated the antibiotic susceptibility by disk diffusion test and determined the *mecA*, *ermA*, *ermB*, *ermC*, and *msrA* antibiotic resistance genes of *S. aureus* strains isolated from cow's milk using multiplex PCR assay. Multiplex PCR technique showed that 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) samples, contained *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, antibiotics resistance genes of *S. aureus*, respectively. The disk diffusion test confirmed the results obtained from multiplex PCR method. The results of our study showed that *msrA* gene is more frequent than other antibiotic resistance genes of *S. aureus* isolated from cow's milk specimens and it could be related to increasing of macrolides, lincosamides, and streptogramins (MLS) antibiotics resistant.

There are many studies performed for detection of antibiotic resistance genes of *S. aureus* in human and animals. The frequency of antibiotic resistance genes of *S. aureus* is different in Iran and other parts of world. Zamani *et al.,* (2007) showed that out of a total of 70 *S. aureus* isolates obtained from the patients who consulted with the clinical centers of Hamedan Medical Science University and private laboratory in Iran 50% of the strains (35 cases) in PCR method and 31.4% (22 cases) in antibiotic resistance patterns with disc agar diffusion method were resistance to methicillin[23]. Heo *et al.,* (2008) in Korea showed that resistance to oxacillin and methicillin in *S. aureus* isolated from domestic and imported raw meat by the disk diffusion test and minimal inhibition concentration methods, but *mecA* gene not observed[24].

Evaluation of methicillin resistance *S. aureus* isolated from patients in Golestan province-north of Iran showed 67(36.2%) strains were MRSA, which demonstrated 100% resistance to penicillin, ampicillin and COAmoxyclav and 80, 96.2 and 75% resistance to cephotaxime, nalidixic acid and erythromycin, respectively[25].

The study of Mirzaei *et al.,* in 2011 on prevalence of methicillin-resistant *S. aureus* in raw milk and dairy products in Sarab by culture and PCR techniques showed that presence of coagulase positive *S. aureus* and MRSA have become remarkably widespread in dairy product samples[26]. They are no detected of *mecA* gene in raw milk and traditional butter isolates while in present results we detected *mecA* gene *S. aureus* isolated from cow's milk samples.

An antibiotic resistance strain of *S. aureus* is the major groups of bovine mastitis pathogens and worldwide public health problem. In current study *msrA* gene is more frequent than other antibiotic resistance genes and it could be related to increasing of MLS antibiotics resistant. In conclusion, investigation of antibiotic resistance genes of pathogen bacteria such as *S. aureus* is important for prevention and reduces of MRSA and other resistant strains. The results of present study generated a lot of useful information for public health, foodstuff, and dairy industry to control and decrease transmit of *S. aureus* antibiotic resistance strains to human. Furthermore, these data could be helpful for prescription of best drugs for control of *S. aureus* infection in cows.

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