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Identification of Methicillin-Resistant Staphylococcus aureus in poultry meat portions using Multiplex PCR

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A B S T R A C T

Staphylococcus aureus is a prevalent bacterial colonizer with zoonotic potential, affecting humans and various animal species, including livestock, poultry, and pets. This study investigates methicillin-resistant *S. aureus* (MRSA) isolates in poultry meat portions using Multiplex PCR methods. A total of 210 samples, including 70 wings, 70 thighs, and 70 necks, were collected from Mashhad, Iran markets. *S. aureus* identification employed culture and phenotypical methods, while the disk diffusion method assessed antibiotic susceptibility using 14 different disks. The Multiplex PCR assay was developed to confirm *S. aureus* isolates and detect antibiotic resistance genes. Among the 210 samples, 52 (24.76%) tested positive for *S.* aureus. Antibiotic susceptibility testing revealed that 17 (32.69%) of the *S. aureus* isolates were resistant to methicillin. Tetracycline exhibited the highest resistance, followed by ampicillin (61.5%) and penicillin (57%). Conversely, chloramphenicol demonstrated the lowest resistance at 3.8%. All isolates were susceptible to gentamicin, vancomycin, imipenem, and ciprofloxacin. PCR analysis confirmed the presence of *16S rRNA* and *femA* genes in all isolates, while 14 (26.92%) harbored the methicillin-resistant gene (*mecA*). The study suggests multiplex PCR is a valuable and sensitive technique for detecting antibiotic resistance genes in *S. aureus* within chicken meat, emphasizing its utility in surveillance and control efforts.

Keywords: *Staphylococcus aureus, methicillin-resistant, antibiotic resistance, Poultry meat*

1 Introduction

Staphylococcus aureus is one of the most prominent bacterial colonizers and zoonotic bacterial infections among the human population and various animal species, including livestock, poultry, and pets. (1-4) *S. aureus* is a gram-positive bacterium that can produce various virulence factors, including staphylococcal enterotoxins (SEs) (5, 6).

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Staphylococcal food poisoning and several infections that are getting harder to treat may result from *S. aureus* (7, 8). Antibiotics are potent drugs against infectious diseases and have tremendously improved effects on health care around the world and saved millions of lives. (9). The presence of antimicrobial drug resistance (AMR) in food constitutes a widespread concern with a direct threat to public health and global relevance to overall animal health, especially livestock (10-12). Leading to a range of diseases, underscores the importance of implementing effective prevention and control measures for public health (13, 14). S. *aureus* has rapidly emerged due to a wide range of mechanisms for adaptation and resistance to a variety of classes of antibiotics (11, 15, 16). Nowadays, methicillin-resistant *S. aureus* (MRSA) is one of the most important threats to human health (17). Antimicrobial susceptibility testing (AST) is an essential function for optimizing patient care and preventing resistance development (18, 19).

The primary purpose of this study is to investigate methicillin-resistant *Staphylococcus aureus* isolates from poultry meat portions by Multiplex PCR methods.

2 Materials and Methods

2.1 Sampling, isolation, and identification of S. aureus

A total of 210 chicken meat portions, including 70 wings, 70 necks, and 70 thighs, were collected from October 2014 to March 2015 from different food stores in Mashhad, Iran. The samples were referred in sterile plastic bags and transferred to the laboratory at the Department of Food Hygiene of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran, at 4 °C for *S. aureus* and MRSA microbiological analyses.

Firstly, 25 g of samples were individually added to 225 mL of 0.1 % peptone water and homogenized for 1 min by using a stomacher. Then, 10 ml was added to the tryptic soybean broth (TSB) containing 10% NaCl and incubated at 37 °C for 18 to 24 h. (5, 20-23)

Samples were plated onto Baird Parker Agar (BPA) and Blood Agar, followed by incubation at 37 °C for 30-48 h. Colonies were seen by light microscope after Gram staining to detect *Staphylococcus* spp. Biochemical tests were performed, including Gram staining, catalase, Mannitol salt agar, DNase, and coagulase (24)

2.2 Antibiotic susceptibility testing

In order to detect methicillin-resistant *S. aureus* (MRSA) strains, the antibiotic resistance of *S. aureus* isolates against 14 common antibiotics (Padtan Teb co., Tehran, Iran) was determined by disk diffusion method. Breakpoints were measured and classified according to the guidelines of the Institute of Clinical and Laboratory Standards (CLSI, 2021). Antibiotic discs used and their medicinal content include methicillin (ME, 5µg), vancomycin (VAN, 30µg), ampicillin (Am, 10µg), tetracycline (TE, 30µg), erythromycin (E 15µg), gentamicin (GEN, 10µg), ciprofloxacin (CP, 5µg), chloramphenicol (C, 30µg), cephalothin (CF, 30µg), penicillin (P, 10µg), lincomycin (L, 2µg), kanamycin (K,30µg), sulfamethoxazole (SXT, 25µg), imipenem (IMP, 10µg).

2.3 Molecular Detection of MRSA

Staphylococcus aureus ATCC 25923, was used as the reference strain. The boiling method was modified for the DNA extraction and PCR detection as follows: at least five fresh colonies of *S. aureus* were suspended in 200 µL sterile distilled water and then boiled for 15 min at 100 °C. After centrifugation, the supernatant was collected as the DNA for PCR. The isolates that were biochemically identified as *S. aureus* were subjected to species-specific PCR using *16S rRNA* primers: Forward (5' - AGA GTT TGA TCC TGG CTC AG - 3') and Reverse (5' - CCC ACT GCT GCC TCC CGT AG - 3') (25). A set of primers including *FemA* F: 5 - GCA AAC TGT TGG CCA CTA TG -3 and *FemA* R 5 - TCA TCA CGA TCA GCA AAA GT -3 were used for the detection of the *femA* gene (26). PCR detection of the *mecA* gene was amplified by appropriate primers: *mecA* F 5-AAAATCGATGGTAAAGGTTGGC-3 and *mecA* R 5-AGTTCTGCAGTACCGGATTTGC-3 (27).

Multiplex Polymerase Chain Reaction (Multiplex-PCR) was conducted using a 36 µl reaction volume, which included 12 µL (100 pmol) forward and reverse primers, 20 µL of 2x master mix (Ampliqon, Denmark), two µL each of $MgCl_2$, and 2 µL of sterile distilled water. Approximately five µl of genomic DNA, the template DNA, was added to the mixture.

The amplification program in the thermocycler starts with an initial denaturation at 94 °C for 5-min, 38 cycles of denaturation at 94 °C, 30 s; 45 s annealing step follows within the cycle at a temperature set about 51.5 °C, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. The PCR products were then analyzed by 1% agarose gel

electrophoresis and studied with a (GDAS -1200 System) UV lamp. The ATCC reference strains *S. aureus* ATCC 43300, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 35984 were utilized as positive quality control, and distilled water was used as negative quality control.

3 Results

3.1 Isolation and identification of S. aureus

A total of 52 (24.76%) S. aureus isolates were identified out of the 210 samples. The prevalence of S. aureus in wings, thighs, and necks was 19 (36.53%), 19 (36.53%), and 14 (26.92%), respectively.

3.2 Antibiotic resistance

The findings from the antimicrobial susceptibility test revealed that out of the 52 S. aureus isolates obtained from chicken meat portions, 17 (33%) exhibited methicillin resistance. The highest resistance level among MRSA isolates was observed in tetracycline (84.6%). In contrast, the lowest resistance was observed in the case of chloramphenicol with 3.8%. No resistance was observed against vancomycin, gentamicin, ciprofloxacin, and imipenem. Resistance to penicillin, ampicillin, cephalothin, erythromycin, sulfamethoxazole, lincomycin, and kanamycin was 57%, 61.5%, 34.6%, 34.6%, 5.7%, 30.8%, and 7.7%, respectively (Table 1). All isolates showed resistance to 2 or more than six antimicrobial agents (Table 2).

Table 1. Antimicrobial resistance of Staphylococcus aureus isolates (N=52).

Samples	Number of *S. aureus* isolates (%)	Antimicrobial agent No (%)												
		ME	P	AM	CF	TE	E	CP	K	C	SXT	V	IPM	GM	L
Wings (%)	19(36.5)	3(5.7)	8(15)	8(15)	5(9.6)	16(30.7)	9(17.3)	0(0)	1(1.9)	1(1.9)	3(5.7)	0(0)	0(0)	0(0)	9(17.3)
Tights (%)	19(36.5)	7(13.4)	11(21)	12(23)	7(13)	15(28.8)	6(11.5)	0(0)	2(3.8)	1(1.9)	0(0)	0(0)	0(0)	0(0)	6(11.5)
Neck (%)	14(27)	7(13.4)	11(21)	12(23)	6(11.5)	13(25)	3(5.7)	0(0)	2(3.8)	0(0)	0(0)	0(0)	0(0)	0(0)	1(1.9)
Total (%)	52(100)	17(32)	30(57.7)	32(61.5)	18(34.6)	44(84.6)	18(34.6)	0(0)	5(9.6)	2(3.8)	3(5.7)	0(0)	0(0)	0(0)	16(30.7)

Table 2. Antibiotic resistance pattern of Staphylococcus aureus strains isolated from chicken meat portions (N=52).

Samples	Number of S. aureus isolates	Resistant to antimicrobial agents					
		1	2	3	4	5	≥6
Wings (%)	19(36.5)	4(7.6)	3(5.7)	6(11.5)	1(1.9)	2(3.8)	3(5.7)
Tights (%)	19(36.5)	2(3.8)	3(5.7)	7(13.4)	3(5.7)	3(5.7)	2(3.8)
Neck (%)	14(27)	2(3.8)	0(0)	3(5.7)	3(5.7)	4(7.6)	2(3.8)
Total (%)	52(100)	8(15%)	5(9.6%)	16(31%)	7(13.4%)	9(17%)	7(13.4%)

3.3 Molecular Detection:

Using PCR and specific primers targeting the 16S rRNA (361 bp) and femA (594 bp) genes, successful amplification was achieved in all the isolates. Of the 52 S. aureus isolates, 14 (26.92%) exhibited mecA genes (533 bp), indicating methicillin-resistant genes (Table 3). This observation confirmed the presence of S. aureus (Figure 1). Each isolate underwent a minimum of four repetitions for the PCR reaction.

Table 3. Results of multiplex PCR of Staphylococcus aureus strains isolated from chicken meat portions (N=52).

Samples	Number of S. *aureus* isolates	Target gene		
		16S rRNA	*femA*	*mecA*
Resistant N (%)	17(32.6)	17(32.6)	17(32.6)	14(26.9)
IntermediateN (%)	2(3.8)	2(3.8)	2(3.8)	0
Susceptible N (%)	33(63.4)	33(63.4)	33(63.4)	0
Total (%)	52(100)	52(100)	52(100)	14(26.9)

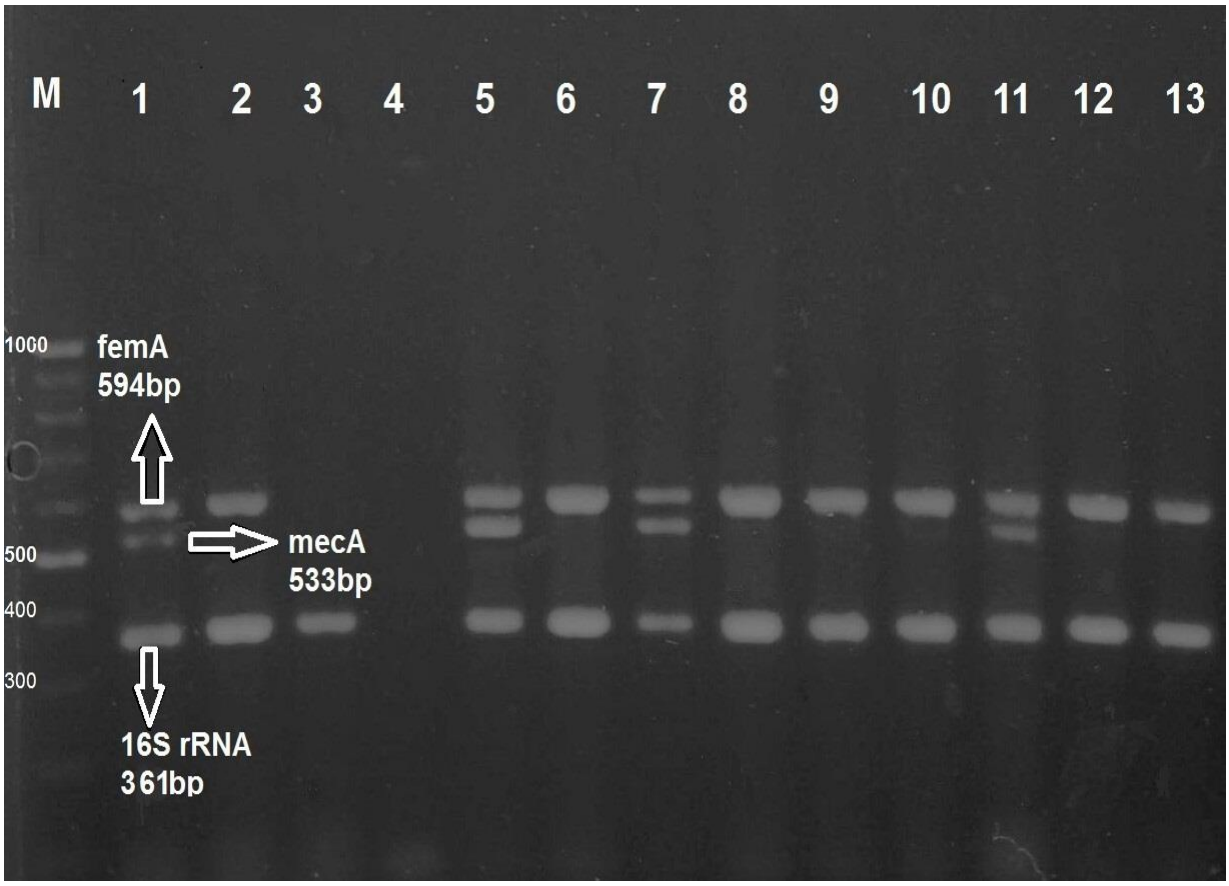

Figure 1. *S. aureus* genes (16S *rRNA*, *mecA*, *femA*) detected by gel electrophoresis.

Lanes: M, Marker, 100 bp ladder;1, 2, 3,4, negative control (Distilled water); 1: Methicillin-Resistant *S. aureus* (ATCC:43300); 2: Methicillin-Sensitive *S. aureus* (ATCC:25923); 3: *S. epidermis* (ATCC: 35984); 5,7 and 11: Methicillin resistant isolates; 6,8,9,10,12,13: Isolates sensitive to methicillin.

4 Discussion and Conclusion

This study was conducted to identify methicillin-resistant *S. aureus* isolated from poultry meat portions employing the Multiplex PCR technique. The findings of our research showed that multiplex PCR is a valuable and sensitive tool for detecting antibiotic resistance genes in *S. aureus* isolated from chicken meat portions. This method underscores the significance of employing advanced molecular methods for robust antimicrobial resistance surveillance in food sources, particularly in the context of potential public health implications.

Kansaen et al. (2023) noted that their findings highlight potential public health concerns related to the environmental contamination of staphylococci in the food chain. These bacteria in food, particularly meat, may contribute to antimicrobial resistance and the spread of enterotoxin genes, fostering cross-contamination between humans and livestock. Therefore, it is crucial to carefully control and implement preventive measures to mitigate the associated risks (1, 31). Consuming contaminated meat can result in food poisoning and the acquisition of genes associated with antibiotic resistance. In chicken meat, *S. aureus* is the predominant bacteria, contributing to severe cases of foodborne diseases (2, 32). Considering the habits of Iranian people regarding the consumption of this foodstuff, it is necessary to investigate the contamination of chicken meat with this bacterium.

In another study, Momtaz et al. (2013) reported that the prevalence of *S. aureus* in chicken meat was 22.8%, which is approximately similar to our findings (5). In the present study, the prevalence of *S. aureus* in chicken meat is 24.76%. The results of the study by Hamad et al. (2022) showed that the prevalence of *S. aureus* in chicken breast and thigh samples reached 92% and 84%, respectively (7). In our study, the prevalence of *S. aureus* in wings, thigh, and neck was 19 (36.53%),19 (36.53%), and 14 (26.92%), respectively.

S. aureus in bovine and poultry isolates exhibiting diverse genotypes could give rise to a distinctive form of infection (9, 33). There was a notable correlation between the expression of methicillin resistance at the phenotypic level and the detection of the *mecA* gene at the genotypic level (10, 34). This study conducted genotypic detection of the *mecA* and *femA* genes, which is consistent with previous studies. Various studies have shown that *Staphylococcus* spp. particularly *S. aureus*, are resistant to methicillin in clinical settings (16, 35). The antimicrobial susceptibility findings of the present study revealed that out of the 52 *S. aureus* isolates obtained from chicken meat portions, 17 (33%) exhibited methicillin resistance. Igbinosa et al. (2023) reported that out of the samples tested, 110 (29.9%) were positive for MRSA (17). Of the samples examined in the study of Parvin et al. (2021), 54.9% were positive for *S. aureus*, and 37.1% of isolates were identified as MRSA (18). In the present work, MRSA isolates exhibited the highest resistance to tetracycline (84.6%), while chloramphenicol showed the lowest resistance with 3.8%. It should be noted that no resistance to vancomycin, gentamicin, ciprofloxacin and imipenem was observed, which is contrary to the findings of Gaddafi et al. () in Iran who reported high resistance of isolates to penicillin, gentamicin and oxytetracycline. (10). The isolates were resistant to penicillin (57%), ampicillin (61.5%), erythromycin (34.6%), and sulfamethoxazole (5.7%). Our findings from this study further agree with Amoako (2020) for results of tetracycline (61.67%), penicillin G (55.83%), and also the result for erythromycin 54.17%, ampicillin 34.17%, trimethoprim-sulfamethoxazole 30.00% (21). This finding, however, differed from the reports of Ogundipe (2020), where resistance to β-lactams (100%), ciprofloxacin (33.9%), and gentamicin (32.1%) (22). In this study, we utilized the Multiplex PCR method to evaluate the presence of the *16S rRNA* and *mecA*, genes. Among the isolates, 14 (82.3%) tested positive for *mecA* genes. In 2023, Khoramian and Razmyar conducted isolation and identification of *S. aureus* using conventional methods, which were later confirmed through PCR (9). Out of the 220 isolates identified using later agglutination in Rao et al. (2022), 217 were verified as *S. aureus* through PCR targeting the nuc gene, with 21.4% testing positive for the *mecA* gene (23).

In conclusion, the study demonstrated that S. aureus isolates from poultry meat portions exhibit diverse genotypes, with a notable correlation between methicillin resistance at the phenotypic level and the detection of the *mecA* gene at the genotypic level. The antimicrobial susceptibility findings of the present study revealed that out of the 52 *S. aureus* isolates obtained from chicken meat portions, 17 (33%) exhibited methicillin resistance. Therefore, there is widespread concern about methicillin-resistant *S. aureus* isolated from poultry meat. Inspection of chicken meat using multiplex PCR is a valuable and sensitive technique for detecting *S. aureus* antibiotic resistance genes.

Conflict of Interest

The authors declared no conflicts of interest.

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Author Contributions

MJ: Visualization, Investigation. Data curation, Writing-Original draft preparation. NM: Data curation, Formal analysis, Validation, Writing- Original draft preparation. JM: Methodology, Data curation, Software, Validation, Writing- Reviewing and Editing. MM: Supervision, Resources, Conceptualization, Methodology, Data curation, Software, Validation, Writing- Reviewing and Editing. All authors contributed as the main contributors of this work.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

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