Virulence genes and antimicrobial resistance profiles of *Staphylococcus aureus* isolated from chicken meat in Isfahan province, Iran

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Primary Audience: Researchers, Veterinarians

SUMMARY

The objectives of the current study were to detect virulence factors and determine antimicrobial susceptibility of *Staphylococcus aureus* by using 360 fresh raw chicken meats, collected from 133 chicken shops in Isfahan, Iran, from January 2011 to March 2012. The *Staph. aureus* isolates were identified using culture and phenotypical methods. The PCR assays were developed with specific primers for the detection of different virulence and antibiotic resistance genes of *Staph. aureus*. The agar disk diffusion method was used for evaluation of antibiotic susceptibility of *Staph. aureus* isolated from chicken meat samples. In this survey, 101 out of 360 samples were positive for *Staphylococcus* (28.05%). In our results indicated, out of 360 samples, 82 (22.77%) were positive for *Staph. aureus* and, out of 82 positive samples, 96.34% had *X-region*, 76.92% had fibrinogen clumping factor A, 63.41% had staphylococcal coagulase virulence genes, 26.82% had IgG binding region, and the toxic shock syndrome toxin-1 gene was not isolated in any sample. The methicillin was the highest (82.92%), whereas macrolides was the lowest (34.14%) antibiotic-resistant genes in *Staph. aureus*-positive samples. Tetracycline had the highest resistant profile (97.56%) in *Staph. aureus* isolates, followed by methicillin (75.6), sulfamethoxazol (31.7%), trimethoprim (31.7%), streptomycin (31.7%), gentamicin (29.26%), enrofloxacin (28.04%), ampicillin (26.82%), chloramphenicol (20.73%), and cephalothin (17.07%). Statistical analysis showed significant differences between presences of various virulence and antibiotic resistance genes in *Staph. aureus* isolated from chicken meat samples. It seems that inspection of chicken meat using multiplex PCR is a useful technique for detection of *Staph. aureus* virulence and antibiotic resistance genes.

Key words: *Staphylococcus aureus*, multiplex PCR, virulence gene, antibiotic-resistance property, chicken meat, Isfahan province

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DESCRIPTION OF PROBLEM

Staphylococcus aureus is a facultative anaerobic gram-positive coccal bacterium that can easily contaminate meat, food, and the environment [1, 2]. *Staphylococcus aureus* causes severe animal diseases such as mastitis, suppurative disease, arthritis, and urinary tract infections [3, 4]. In humans, this bacterium is a major cause of food poisoning, pneumonia, postoperative wound infections, and nosocomial bacteremia [1]. The onset of symptoms in staphylococcal food poisoning is usually rapid and in many cases acute, depending on individual susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food ingested, and the general health of the victim. The most common symptoms are nausea, vomiting, retching, abdominal cramping, and prostration. Some individuals may not always demonstrate all the symptoms associated with the illness. In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse rate may occur. Recovery generally takes 2 d; however, it is not unusual for complete recovery to take 3 d and sometimes longer in severe cases. Food spoilage caused by *Staph. aureus* is more important in children and the elderly. Death from staphylococcal food poisoning is very rare, although such cases have occurred among the elderly, infants, and severely debilitated persons [5].

In most cases of disease due to *Staph. aureus*, virulence genes have a major role. This bacterium can cause a disease that is associated with the presence of various virulence genes. In many cases of disease due to *Staph. aureus*, proteins with affinity to fibronectin (*fnbA*), fibrinogen (i.e., clumping factors A and B, encoded by the *clfA* and *clfB* genes, respectively), sialoprotein (*bbp*), collagen (*cna*), adhesins with unknown function (*sdrC and sdrE*), and elastin (*ebpS*) have been isolated [6–12]. Bacterial proteins with superantigen activity including toxic shock syndrome toxin-1 (*TSST-1*, encoded by *tst*), enterotoxins A to E, G to R, and U (encoded by the genes *sea–see*, *seg–ser,* and *seu*), exfoliative toxins A and B (*eta* and *etb*), and other toxins, such as a-, b-, c-, and d-toxin and the Panton–Valentine leukocidin (*pvl*), have a role in causing disease [13–15]. The *X-region* gene of *Staph. aureus* has a high degree of importance in causing diseases, and it may have a variation rate (or clock speed) that provides suitable discrimination for outbreak investigation [16]. The IgG binding region is responsible for causing host specificity and various immunological responses against *Staph. aureus* [17, 18]. The *X-region* and IgG binding region of virulence genes have been isolated from various types of *Staph. aureus* infections [19–24].

The rapid evolution of antibiotic resistance in *Staph. aureus* is a global concern. Results from a study demonstrated that the *Staph. aureus* has multiple resistances to different antimicrobial agents [25]. Antibiotic resistance depends on the presence of genes, which encode the antibiotic resistance. Another study reported the rapid appearance of penicillin and methicillin resistance in *Staphylococcus* spp. [26]. Other investigations showed that *Staph. aureus* has multidrug resistance, such as resistance to aminoglycosides, macrolides, lincosamides, streptogramins, and tetracyclines [27, 28]. The possible transfer of *Staph. aureus* genes encoding antibiotic resistance has been reported from humans and other animals [29].

This bacterium prevents phagocytosis, indirect cell immunity [30], and produces enzymes that limit the effectiveness of penicillin treatment [31]. The antibiotic-resistant genes, including *mecA* (methicillin), *aacA-D* (aminoglycosides), *tetK*, *tetM* (tetracyclines), *ermA*, *ermB*, *ermC* (macrolide– lincosamide–streptogramin B), *msrA* (macrolides), and *linA* (lincosamides), have been reported in last decade among the isolates of *Staph. aureus* [27, 32]. Among these antimicrobial resistance genes, *mecA* encodes *PBP2a*, *aacA-D* encodes a bifunctional enzyme [28], *msr* causes resistance to macrolides and streptogramin B, which effects efflux pump activity [28, 33], as well as *tetK*, *tetM*, which modify tetracycline efflux activity and ribosome function, respectively [28].

Staphylococcus aureus has been tested in meat and poultry products to assess the microbiological and storage quality of products. It is necessary to know which endemic strains of *Staph. aureus* in chicken meat samples are highly pathogenic and antibiotic resistant, but no previous data exists for the Isfahan province of Iran. Therefore, the present study was carried

out for detection of virulence factors and antimicrobial resistance properties of *Staph. aureus* isolated from chicken meat in the Isfahan province of Iran.

MATERIALS AND METHODS

Samples and Identification of Staph. aureus

In this study, which took place from January 2011 to March 2012, a total of 360 chicken shops were studied randomly from 522 total chicken shops in Isfahan province. One chicken meat sample was taken from each shop; therefore, 360 chicken meat samples were collected. Each chicken shop was dedicated solely to the sale of chicken. All of the chicken meats sampled in the Isfahan province were apparently healthy and were immediately transferred to the laboratory in cool packs.

Twenty-five grams of chest muscle were weighed into sterile stomacher bags and diluted with 225 mL of sterile Butterfield's phosphate buffered dilution water. All samples were homogenized in a stomacher for about 1 min. Ten milliliters of homogenized Butterfield's phosphate buffered dilution water was inoculated into tryptic soy broth [34] with 10% NaCl and incubated at 37°C for 18 h. One loopful of the tryptic soy broth was streaked on Baired-Parker agar [35], supplemented with egg yolk-tellurite emulsion [36], and incubated at 37°C for 24 h. Typical colonies of *Staph. aureus* were isolated from each plate and cultured separately on brain-heart infusion agar [37].

The identification was carried out using Gram staining, production of coagulase, and fermentation of mannitol. The strains were further identified as *Staph. aureus* by PCR amplification of the 23S rDNA according Straub et al. [38].

DNA Extraction

Isolates were grown on blood agar [39] for 24 h, then a single colony was picked, resuspended in 100 mL of sterile deionized water, and heated at 99°C for 15 min with mild shaking in a Thermomixer comfort [40]. The tubes were then centrifuged at 1,000 × *g* for 5 min at 14°C to remove the sediment and supernatant containing crude extract of bacterial DNA was transferred into a new tube and frozen until used for PCR amplification.

PCR for Detection of Virulence Genes in Staph. aureus

For PCR amplification, the reaction mixture (30 µL) contained 1 µL of primer F (10 pmol/µL), 1 µL of primer R (10 pmol/µL), 0.6 µL of deoxynucleoside triphosphate (10 mmol/L) [41], 3 µL of 10× PCR buffer [42], 1.8 µL of $MgCl_2$ (25 mmol/ L) [43], 0.1 µL of *Taq* DNA polymerase (5 U/µL) [44], and 20 µL of distilled water. Finally, 2.5 µL of DNA preparation was added to each 0.2-mL reaction tube. The tubes were subjected to thermal cycling [45] with the program shown in Table 1.

The PCR amplification was performed for the genes encoding staphylococcal coagulase (*coa*), clumping factor (*clfA*), IgG binding region, TSST-1 (*tst*), ETA (*eta*), and ETB (*etb*). The presence of PCR products was determined by electrophoresis of 12 µL of the reaction product in a 2% agarose gel with trisacetate electrophoresis buffer (0.04 mol of Tris/L, 1 mmol of EDTA/L; pH 8) and a 100-bp DNA ladder [51] as a molecular marker [52].

PCR for Detection of Antibiotic Resistance Genes of Staph. aureus

All oligonucleotide primers used in this study were selected from earlier reports, as mentioned in Table 2. The presence of the *mecA*, *aacA-D*, *tet K*, *tet M*, *msrA*, and *msrB* genes encoding methicillin, aminoglycosides, tetracyclines, and macrolides resistance was examined using multiplex PCR assay introduced by Kumar et al. [28].

Antibiotic Susceptibility Test

Antimicrobial susceptibility tests were performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar [53] according to the Clinical and Laboratory Standards Institute guidelines. All *Staph. aureus* isolates were investigated for their antimicrobial resistance by the agar disk diffusion test using the antibiotics ampicillin, chloramphenicol, enrofloxacin, gentamicin, sulfamethoxazole, tetracycline, streptomycin, trimethoprim, cephalothin, and methicilin. After incubating the inoculated plate aerobically at 35°C for 18 to 24 h in an aerobic atmosphere, the susceptibility of the *Staph.*

Table 1. The oligonucleotide primers and amplification conditions for detection of virulence genes of *Staphylococcus aureus* isolated from chicken meat

Gene	Oligonucleotide sequence (5′-3′)	PCR program	Product size (bp)	Reference
coa	Forward: CGA GAC CAA GAT TCA ACA AG Reverse: AAA GAA AAC CAC TCA CAT CA	30 times (94°C, 1 min; 58°C, 1 min; 72°C, 1 min)	970,730	[46]
clfA	Forward: GGC TTC AGT GCT TGT AGG Reverse: TTT TCA GGG TCA ATA TAA GC	35 times (94°C, 1 min; 57°C, 1 min; 72°C, 1 min)	980	[47]
X-region	Forward: CAA GCA CCA AAA GAG GAA Reverse: CAC CAG GTT TAA CGA CAT	30 times (94°C, 1 min; 60°C, 1 min; 72°C, 1 min)	320	[48]
IgG binding region	Forward: CAC CTG CTG CAA ATG CTG CG Reverse: GGC TTG TTG TTG TCT TCC TC	30 times (94°C, 1 min; 58°C, 1 min; 72°C, 1 min)	920	[49]
tst	Forward: ATG GCA GCA TCA GCT TGA TA Reverse: TTT CCA ATA ACC ACC CGT TT	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	350	[50]
etA	Forward: CTA GTG CAT TTG TTA TTC AA Reverse: TGC ATT GAC ACC ATA GTA CT	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	119	[50]
etB	Forward: ACG GCT ATA TAC ATT CAA TT Reverse: TCC ATC GAT AAT ATA CCT AA	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	200	[50]

aureus isolates to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by NCCLS [54]. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as control strains of this test.

Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet [55] for analysis. Using the SPSS 18.0 statistical software [56], Chi-squared test analyses were performed, and differences were considered significant at values of $P < 0.05$.

RESULTS AND DISCUSSION

From a total of 360 chicken shops studied, the chickens of 101 shops were found to be contaminated with staphylococcal bacteria (28.05%). Among the 101 staphylococcal isolates from chicken meat samples, 82 were identified as *Staph. aureus* (81.18%) according to the results of phenotypical assays. These strains were confirmed by PCR amplification of the 23s rDNA specific to *Staph. aureus*. Therefore, out of 360 specimens collected, 82 *Staph. aureus* (22.77%) isolates were identified.

After PCR, it was recognized that from a total 82 *Staph. aureus* isolates, 79 samples (96.34%) had the *X-region* and 63 samples (76.82%) had the *clfA* virulence genes. The presence of the IgG binding region (26.82%), *etA* (30.48%), and *etB* (30.48%) had lower frequency (Table 3). The *tst* gene was not found among the 82 positive samples. This study is the first preva-

Table 2. Oligonucleotide primers and amplification conditions for detection of antibiotic resistance genes of *Staphylococcus aureus* isolated from chicken [28]

Gene	Oligonucleotide sequence (5′-3′)	Product size (bp)
mecA	Forward: AAAATCGATGGTAAAGGTTGGC Reverse: AGTTCTGCAGTACCGGATTTGC	532
msrA	Forward: GGCACAATAAGAGTGTTTAAAGG Reverse: AAGTTATATCATGAATAGATTGTCCTGTT	940
msrB	Forward: TATGATATCCATAATAATTATCCAATC Reverse: AAGTTATATCATGAATAGATTGTCCTGTT	595
aacA-D	Forward: TAATCCAAGAGCAATAAGGGC Reverse: GCCACACTATCATAACCACTA	227
tetK	Forward: GTAGCGACAATAGGTAATAGT Reverse: GTAGTGACAATAAACCTCCTA	360
tetM	Forward: AGTGGAGCGATTACAGAA Reverse: CATATGTCCTGGCGTGTCTA	158

Table 3. Distribution of virulence genes in strains of *Staphylococcus aureus* isolated from chicken meat

Gene	Presence (%)
coa	52 (63.41)
clfa	63 (76.82)
X-region	79 (96.34)
IgG binding region	22 (26.82)
tst	0
eta	25 (30.48)
etb	25 (30.48)

lence report of *X-region* and IgG binding region in chicken meat samples.

The presences of antibiotic resistance genes were detected by single-step PCR (Table 4). A total of 68 of the 82 positive samples (82.92%) had the *mecA* resistance gene, whereas 28 samples (34.14%) had the *msrA* gene (Table 4). The *mecA* gene had the highest frequency of antibiotic resistance genes. The *msrA* had the lowest frequency of antibiotic resistance genes in *Staph. aureus* isolated from chicken meat in the Isfahan province of Iran.

Antimicrobial resistance profiles in *Staph. aureus* isolated from chicken meats in the Isfahan province of Iran showed that *Staph. aureus* strains had the highest antibiotic resistance to tetracycline (97.56%), followed by methicilin (75.60%), sulfamethoxazol (31.70%), trimethoprim (31.70%), and streptomycin (31.70%). The *Staph. aureus* isolates had the lowest antibiotic resistance to cephalothin (17.07%) and chloramphenicol (20.73%; Table 5). In the current study, multiple resistances were found in *Staph. aureus* isolated from chicken meats (Table 6). All isolates were resistant to an antibiotic, but only 10.96% of isolates were resistant to more than 3 antibiotics.

Seasonal patterns were observed for the prevalence of this bacterium from January 2011 to March 2012 (Table 7). The chicken meat samples collected in summer had the highest and samples collected in winter had the lowest prevalence of *Staph. aureus*, respectively (Table 7). The high prevalence of *Staph. aureus* in summer season showed that this bacterium needs a proper temperature (warm weather) to survive and contaminate chicken meats. The relative temperature during summer in this area of Iran was 40°C on average, whereas average temperatures were 15, 6, and 19°C in autumn, winter, and spring, respectively. Significant differences ($P < 0.05$) were observed between the summer and winter levels of chicken meat contamination with *Staph. aureus* and also ($P < 0.05$) between the relative temperature of the summer and winter seasons.

Prescription of tetracycline, methicilin, streptomycin, trimethoprim, and sulfamethoxazol is not currently effective for treatment of cases of *Staph. aureus* in the Isfahan province of Iran. Cephalothin and chloramphenicol are the best antibiotics for treatment of *Staph. aureus*.

The high presence of *Staph. aureus* in chicken meat samples (22.77%) in our study was in

Table 4. Distribution of antibiotic resistance genes in strains of *Staphylococcus aureus* isolated from chicken meat

Gene	Presence (%)
mecA	68 (82.92)
msrA	28 (34.14)
msrB	39 (47.56)
aacA-D	32 (39.02)
tetK	43 (52.43)
tetM	38 (46.34)

Table 5. Application of the disk diffusion method for study of the antimicrobial resistance profiles in *Staphylococcus aureus* isolated from chicken meat

Antimicrobial agent	*Staph. aureus* (82)
Streptomycin	26 (31.70%)
Tetracycline	80 (97.56%)
Trimethoprim	26 (31.70%)
Enrofloxacin	23 (28.04%)
Gentamicin	24 (29.26%)
Sulfamethoxazol	26 (31.70%)
Cephalothin	14 (17.07%)
Ampicillin	22 (26.82%)
Chloramphenicol	17 (20.73%)
Methicilin	62 (75.60%)

Table 6. Multiple resistances of *Staphylococcus aureus* to 1, 2, 3, and more than 3 antibiotics

Item	*Staph. aureus* (82)
Resistance to 1 antibiotic	82 (100%)
Resistance to 2 antibiotics	30 (36.58%)
Resistance to 3 antibiotics	17 (20.73%)
Resistance to more than 3 antibiotics	9 (10.96%)

Table 7. Seasonal distribution of *Staphylococcus aureus* isolated from chicken meat samples in Iran

Staphylococcus aureus-positive chicken meat	Seasonal distribution of *Staph. aureus*			
	Spring (%)	Summer (%)	Autumn (%)	Winter (%)
82	14 (17.07)	61 (74.39)	5 (9.75)	2 (2.43)

agreement with previous investigations [57, 58]. Only 2 studies have been done on detection of *Staph. aureus* in poultry meat samples in Iran [59, 60] that are in agreement with our results. Javadi and Safarmashaei [60] reported that 65% of poultry meat samples were positive for presence of *Staph. aureus*, which was lower than our results (81.18%).

Based on the results of the present study, consumption of these infected chicken meat samples can cause gastrointestinal illness. Bennett et al. [61] estimated that 1,200 deaths occur yearly due to staphylococcal food poisoning. Therefore, it is important to investigate the presence of this bacterium in food samples. Chicken meat is a common food among the Iranian people, and this study will help to raise the health awareness of people about the consumption of chicken meat.

Staphyloccocal infections are usually associated with the presence of virulence genes. A study in Abidjan, Côte d'Ivoire, showed that, from a total of 34 strains of *Staph. aureus* isolates, 70.6% had the *mecA* gene, 67.7% had the *lukS* gene, and the *eta*, *etb* and *tst* genes coding for exfoliatine and TSST-1 were less frequent [62], which was in contrast with our results. The genes *mecA* (82.92%) and *tetk* (52.43%) had the highest prevalence of antibiotic resistance genes in our study. The *Staph. aureus* isolates of our study had the highest antibiotic resistance to methicillin (75.6%) and tetracycline (97.56%). The methicillin-resistant *Staph. aureus* (**MRSA**) was previously isolated from 9.6% of pork, 5.6% of beef, and 1.2% of chicken samples [63], which was in agreement with our results.

In one study of 143 *Staph. aureus* isolates from pork and chicken meat, *seg*, *sei*, *sem*, and *sen* were the most frequently virulence genes (53 isolates, 37%) [64], whereas our results indicated that *X-region*, *clfA*, and *coa* were the most frequently detected virulence genes (96.34, 76.82, and 63.41%, respectively). Fessler et al. [65] reported that 25% of fresh chicken meat, 21.1% of chicken meat products, 50% of fresh turkey meat, and 52.4% of turkey meat products displayed signs of MRSA. The presences of *mecA*, *msrA*, *msrB*, *aacA-D*, *tetK*, and *tetM* antibiotic resistance genes and *coa*, *clfA*, *X-region*, IgG binding region, *tst*, *etA*, and *etB* virulence genes have been frequently reported from mastitic milk samples [66–68]. The present study is the first report of direct detection of *coa*, *clfA*, *X-region*, IgG binding region, *tst*, *etA*, and *etB* virulence genes in chicken meat samples. In our study, resistance to tetracycline and methicilin was 97.56 and 75.6%, respectively, which is in agreement with previous studies [60, 63, 65]. All isolates of our study that had the IgG binding protein and all isolates with *X-region* factors were resistant to more than 3 antibiotics. Therefore, these virulence factors may play an important role in resistance of *Staph. aureus* isolates.

In a previous study, 185 (92.5%) out of the 200 isolates had resistance to antibacterial agents, including penicillin G (82.0%), tetracycline (19.0%), erythromycin (2.5%), clindamycin (2.0%), trimethoprim (7.5%), kanamycin (2.5%), streptomycin (1.5%), ciprofloxacin (1.5%), fusidic acid (1.0%), and cadmium acetate (68.0%) [69]. In a study in Portugal, 38% of the *Staph. aureus* isolates were resistant to oxacillin (≥6 mg/mL; MRSA positives), but only 0.68% showed the presence of *mecA* gene, whereas 70 and 73% of the *Staph. aureus* strains were resistant to β-lactams, ampicillin, and penicillin, respectively [70]. It seems that our study is the first prevalence report of direct detection of virulence factors and antibiotic resistance properties of *Staph. aureus* isolated from chicken meat in the Isfahan province of Iran.

In our study, chloramphenicol is a forbidden antibiotic drug. Our results showed that chloramphenicol had a low resistance percentage, because of its infrequent use. Unfortunately, veterinarians in various fields, such as large animal internal medicine, poultry, and even aquaculture, use this antibiotic as a main drug. We

suggest the use of florfenicol instead of chloramphenicol, as florfenicol is not banned for use in farm animals and has no adverse effects such as those for chloramphenicol.

CONCLUSIONS AND APPLICATIONS

1. *Staphylococcus aureus* can easily contaminate chicken meat and this contamination is usually associated with a high presence of virulence and antibiotic resistance genes.
2. All strains of *Staph. aureus* isolated from chicken meat samples had resistance to more than one antibiotic drug. It seems that using raw chicken meat, processing with unsanitary methods, and a lack of proper hygiene practices by the poultry slaughterhouse staff are the main factors for contamination.
3. The best methods for *Staph. aureus* prevention are poultry vaccination against pathogen, improving methods of processing, monthly monitoring of poultry slaughterhouses to detect *Staph. aureus* (especially in the chicken meat and on surfaces), fumigating poultry slaughterhouses frequently, observing hygiene during processing, complete cooking of chicken meat before consumption, keeping chicken meat in a cool and dry place away from sunlight, and prevent contamination of chicken meats with extrinsic factors such as insects and dust.
4. The multiplex PCR assay can be used as an accurate, safe, and fast technique for the detection of *Staph. aureus* and its virulence and antibiotic resistance genes in chicken meat samples.
5. It is important to determine the important virulence and antibiotic genes and also the antibiotic resistance properties of *Staph. aureus* isolated from chicken meat. In our study, the *X-region* virulence genes, *mecA* antibiotic resistance genes, and resistance to tetracycline had the highest frequencies.
6. Due to antibiotic resistance, especially in *Staph. aureus*, veterinarians should pay closer attention when prescribing antibiotics.

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