Original Article

Biotyping and enterotoxigenicity of coagulase-positive and coagulase-negative staphylococci isolated from different raw meat

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Abstract

Background: Staphylococcal enterotoxins (SEs) are produced in foods under favorable conditions and considered a potential biological threat. **Aims:** The study was performed to detect enterotoxigenic genes of A and B in coagulase-positive (CPS) and coagulase-negative staphylococci (CNS) to evaluate biotypes and antibiotic resistance of isolated *Staphylococcus aureus* from different meat. **Methods:** A total of 160 meat swab samples were collected from lamb, water buffalo, cattle, and chicken carcasses. Presumptive colonies on Baird Parker agar were subjected to biochemical identification, including Gram staining, catalase, oxidase, and coagulase activity. Relevant colonies separately were subjected to the polymerase chain reaction (PCR) assay for identification of *Staphylococcus* genus, enterotoxigenic genes (*sea* and *seb*) and the thermonuclease gene (*nuc*) specific for the *S. aureus*. The antibiotic susceptibility test was also carried out using five antibiotics. **Results:** Totally, 150 *Staphylococcus* spp. were isolated from the samples among which 135 (90%) isolates harbored *sea* gene, meanwhile, none of the isolates contained *seb* gene. Twenty-five *S. aureus* confirmed by PCR from which 15 isolates (60%) belonged to host specific (HS), 7 isolates (28%) belonged to non-host specific (NHS) biotypes, while 3 isolates (12%) were non-typable. Overall, 68%, 56%, 16%, 12%, and 8% of isolates were resistant to penicillin, trimethoprim, gentamicin, oxacillin, and erythromycin, respectively. **Conclusion:** The meats provided from this area were contaminated with enterotoxigenic and antibiotic-resistance staphylococci, which can threaten the health of the consumers. The study showed that not only CPS contain enterotoxin A gene, but also CNS isolates possess this gene, especially in buffalo meat, and thus they are recognized as potential hazards in different meats.

Key words: Coagulase, Enterotoxin, Meat, PCR, *Staphylococcus*

Introduction

Staphylococci are Gram-positive bacteria, generally present on the skin and the mucus membranes of humans and animals. More than 40 species and 30 subspecies of *Staphylococcus* genus have been identified so far. Based on their ability to coagulate human and rabbit plasma, staphylococci are divided into two main groups including coagulase-positive (CPS) and coagulase-negative (CNS) strains (Podkowik *et al*., 2013). Among the pathogenic strains of *Staphylococci*, *Staphylococcus aureus* is one of the most important pathogens that cause food poisoning in thousands of people each year (Hennekinne *et al*., 2012).

Staphylococcal enterotoxins (SEs) are produced in foods by enterotoxigenic strains under favorable temperature and time. In addition to five classical enterotoxins (SEA to SEE) discovered in the 60th century, more than 18 SEs have been identified.

It is reported that the staphylococcal classical enterotoxins are responsible for 95% of staphylococcal food poisoning cases and the other known enterotoxins make the rest of cases (Jay *et al*., 2005). SEA is recognized as the most common cause of *Staphylococcus* food-borne disease worldwide (Kadariya *et al*., 2014).

Coagulase-negative staphylococci have been considered as nonpathogenic bacteria in humans and animals for many decades. However, many studies have shown that a relatively high number of CNS isolates could be associated with human and animal infections, as well as in patients with immunodeficiency (Piette and Verschraegen, 2009; Vasconcelos *et al*., 2011). It has been proven today that not only CPS strains can produce toxins, but also CNS isolates can potentially create enterotoxins (Cunha *et al*., 2006) due to possessing similar genes.

The importance of CNS has been recognized in human infections and the most related researches have been performed in human cases. It is noticeable that *Staphylococcus* is host-adapted, and about one-half of

the discovered strains are indigenous to humans and animals (Kloos and Bannerman, 2005).

Based on four biochemical characteristics including staphylokinase presence, bovine plasma coagulation, growth type on crystal violet agar, and β-haemolysin production, *S. aureus* strains are divided into bovine, ovine, poultry, human, and five other non-host specific (NHS) biovars (Devriese *et al*., 1984). The transmission of antibiotic-resistant bacteria through the food has previously been reported (Angulo *et al*., 2004). Therefore, food-related bacteria can be a source of antibiotic-resistant genes (Atanassova *et al*., 2001). It is noticeable that limited strains of *S. aureus* are responsible for food poisonings (Fitzgerald *et al*., 2003). Thus, performing phenotypic and genotypic studies may aid in finding the source of contamination. Although it is not so difficult to isolate and identify these bacteria in the laboratory, the genotypic and phenotypic studies are critical due to the inter-species high heterogeneity of the bacteria (Dastmalchi Saei *et al*., 2009). According to the World Health Organization (WHO), meat and its by-products are classified as the high-risk foods group, and their contamination with pathogenic bacteria such as *S. aureus* can make health problems.

Most researchers have focused on the molecular characterization, virulence factors, and antimicrobial susceptibility of staphylococci isolated from dairies in the world (Al-Ashmawy *et al*., 2016; Baniardalan *et al*., 2017; Kukhtyn *et al*., 2017; Bellio *et al*., 2018). However, few studies have been performed on biotyping and enterotoxigenicity of staphylococci isolated from raw meat. In a Japanese study (Kitai *et al*., 2005) on 444 samples of raw chicken meat, *S. aureus* was isolated from 292 (65.8%) of the samples and most of the enterotoxigenic isolates belonged to the human and poultry biotypes. In another study, of a total of 286 fresh and processed meat samples marketed in Jordan, 33.6% had CPS. Biotyping of *S. aureus* subsp. *aureus* isolates revealed that 12%, 24%, 8%, and 27% were human, bovine, ovine, and NHS biovars, respectively (Al-Tarazi *et al*., 2009). In Mexico, sixty-two *S. aureus* isolates were examined in which 79% biotype C (human), 11.3% biotype A (bovine), 6.5% biotype E (canine) and, 3.2% NHS were recognized (Manjarrez López, 2012).

This study was designed to identify the enterotoxigenic genes of A and B from CPS and CNS, to perform biotyping studies, and to examine the antibiotic resistance of *S. aureus* isolated from different meat samples in Ahvaz, Iran.

Materials and Methods

Sample collection

In this cross-sectional survey, a total number of 160 meat swab samples were collected from lamb, water buffalo, cattle, and chicken carcasses (40 samples each) in Ahvaz abattoirs, South-West Iran during four months. Samples were collected using three sterile cotton-tipped swabs for each carcass. Swabs were first soaked in phosphate buffer solution (PBS) and then rubbed several times on three different parts of each carcass. The swabs were then placed in the test tubes containing 10 ml trypticase soy broth (TSB) medium (Merck, Darmstadt, Germany) supplemented with 7.5% sodium chloride (Kim *et al*., 2011). The tubes were transferred to the laboratory under a cool condition in less than 2 h and then incubated for 24 h at 37°C for enrichment.

Isolation of *Staphylococcus*

An aliquot (100 µL) of the enriched culture was spread onto Baird Parker agar (Hi-media, India) and incubated at 37°C for 24 h. Presumptive colonies (black or brown, with or without halo around) were subjected to biochemical identification experiments including Gram staining, catalase, oxidase, and coagulase activities.

Tube coagulase test

In a test tube, 0.5 ml of 1:4 diluted bovine plasma was mixed with 0.5 ml of the bacterial suspension. After incubation at 37°C, the tubes were examined periodically after 1, 2, 3, and 6 h for coagulation. Negative tubes were placed at room temperature (25°C) overnight and then were reviewed for clot formation (Devrise *et al*., 1984; Murray *et al*., 2003).

Polymerase chain reaction (PCR) procedures

DNA extraction

DNA extraction was performed using the method described by Adwan (2014). Briefly, presumptive staphylococci colonies were separately grown overnight in 5 ml of TSB at 37°C. One ml of each culture was centrifuged (800 g, 5 min), the pellet was resuspended in 1 ml of distilled water and samples were heated at 100°C for 10 min. After heating, the suspension was again centrifuged and the supernatant was used as the PCR template.

PCR and electrophoresis procedure

Each extracted DNA was subjected to the PCR assay for amplification of four genes including specific *16S rRNA* for *Staphylococcus* genus identification, enterotoxigenic genes (*sea* and *seb*) and the thermonuclease gene (*nuc*) specific for *S. aureus*, using specific primers. The primers, product size, and PCR conditions are shown in Table 1.

Each 25 µL PCR reaction consisted 12.5 µL of master mix (2x, Ampliqon, Denmark), 1 µL of a mixture of the two forward and revised primers, 6.5 µL of ddH_2O, and 5 µL of template extracted DNA. The mixture was processed in a thermocycler (Bioer Technology Co., China). The amplified PCR products were detected by electrophoresis (Paya Pajoohesh Pars, Iran), stained, and visualized under ultraviolet light illumination (Kiagen, Iran). The following standard strains of *S. aureus* were used as positive controls: ATCC 25923 for *nuc* and *sea* genes; strain 85065 provided by Ataee *et al*. (2011) for *seb* gene; and deionized water was used as a negative control.

Table 1: Target genes, product size (bp), sequence of primers, and PCR conditions

Primer	Size (bp)	Sequence	PCR condition	Reference
16S rRNA	228	F: GTAGGTGGCAAGCGTTATCC R: CGCACATCAGCGTCAG	Denaturation 94°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 45 min, 72°C for 1 min Final extraction 72°C for 5 min	Brakstad *et al*. (1992)
sea	120	F: TTGGAAACGGTTAAAACGAA R: GAACCTTCCCATCAAAAACA	Denaturation 95°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 45 min, 72°C for 1 min Final extraction 72°C for 5 min	Johnson *et al*. (1991)
seb	478	F: TCGCATCAAACTGACAAACG R: GCAGGTACTCTATAAGTGCC		
nuc	359	F: TCGCTTGCTATGATTGTGG R: GCCAATGTTCTACCATAGC	Denaturation 95°C for 6 min, 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C 30 s Final extraction 72°C for 5 min	Takashi *et al*. (2010)

PCR: Polymerase chain reaction

Biotyping of *S. aureus* isolates

Biotyping of *S. aureus* isolates were performed according to Devriese's system (1984). This method is based on four different biochemical tests including staphylokinase detection, β-haemolysin, type of growth on crystal violet agar, and coagulase activity on bovine plasma. The tested strains using this system were divided into five host specific (HS) ecovars (human β^+ and β^-, poultry, sheep, and bovine), and five different NHS ecovars.

Antibiotic susceptibility testing

According to the CLSI method (2006), antimicrobial susceptibility tests were carried out using the Kirby-Bauer disk diffusion method. The antimicrobial agents were penicillin (10 µg), oxacillin (1 µg), trimethoprim (23.75 µg), erythromycin (15 µg), and gentamicin (10 µg). A swab was taken from each bacterial suspension (1×10^7 CFU/ml) and stroked trolley on Mueller-Hinton agar (Merck, Germany). Then, the antibiotic discs (Padtan Teb, Iran) were placed on the agar. After incubation at 35°C for 24 h, the diameter of the inhibition zone was measured for each antibiotic for classification of the isolates to resistant, intermediate (reduced susceptibility), or sensitive.

Results

Isolation and molecular characterization of *Staphylococcus* spp. and *S. aureus*

According to the biochemical profile (catalase, oxidase, Gram staining and colony morphology), 176 suspected isolates were purified and identified by the PCR technique. Amplification of the *16S rRNA* gene showed that 150 isolates belonged to the genus *Staphylococcus* (Fig. 1).

Staphylococcal isolates were subjected to the second stage of PCR for detection of toxigenic genes. Data showed that 135 (90%) isolates harboring *sea* gene (Fig. 2) whereas none of them contained *seb* gene (Table 2).

Among 150 isolated staphylococci, only 25 isolates (16.6%) possessed thermonuclease gene (*nuc*) considered as *S. aureus* (Fig. 3).

Biotyping test

All 25 isolated *S. aureus* were typed by Devriese biotyping system; the number and the prevalence of each biovar are presented in Table 3. This table indicates that 28%, 12%, 16%, and 4% of the isolates were explicitly allocated to sheep, bovine, human (one β^+), and poultry biotypes, respectively, whereas seven strain (28%) were of NHS biovars (K-β^+CV: A). The remaining three non-typable isolates (2 from buffalo and one from cattle carcasses) were classified according to different results of their reactions in the biotyping system.

Antibiotic-resistant phenotypic characteristics

Antibiotic susceptibility tests were carried out on all *S. aureus* isolates. The susceptibility, intermediate, and resistance profiles of the isolates to six tested antibiotics are shown in (Table 4). Overall, 68% of the isolates were resistant to penicillin, 56% to trimethoprim, 16% to gentamicin, and 12% to oxacillin. While, only two isolates (8%) were resistant to erythromycin.

Discussion

Coagulase-negative staphylococci have been well documented as infectious agents (Podkowik *et al*., 2013). However, in many cases, the CNS isolates of clinical environments have little clinical significance. Studies

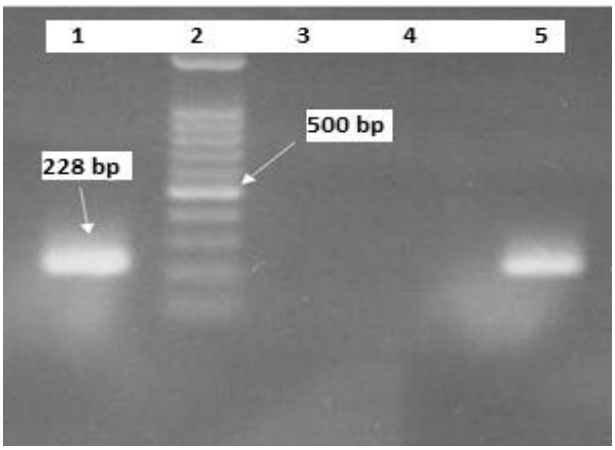

Fig. 1: PCR product electrophoresis; detecting the specific gene of *Staphylococcus* in several suspected isolates. Line 1: Positive control of *Staphylococcus* (ATCC 25923), Line 2: Gene ladder (100 bp), Line 3: Negative control, Line 4: Negative sample, and Line 5: *Staphylococcus* spp. positive sample (228 bp)

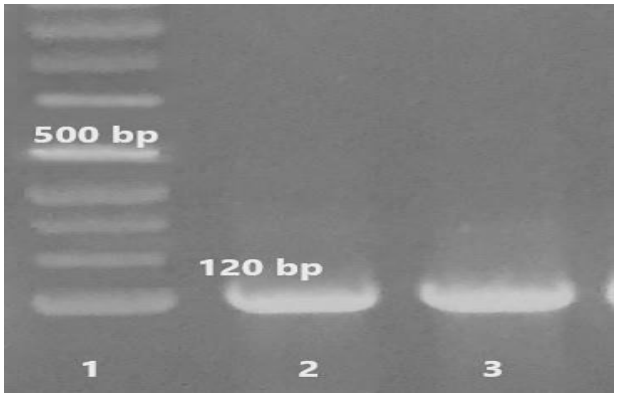

Fig. 2: The *sea* gene identification. Line 1: Gene ladder (100 bp), Line 2: Positive sample (120 bp), and Line 3: Positive control (ATCC 25923)

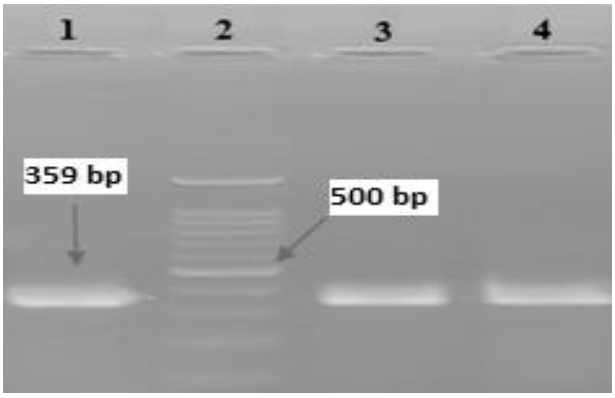

Fig. 3: The PCR product electrophoresis to detect *S. aureus*. Line 1: Positive control (ATCC 25923), Line 2: Gene ladder (100 bp), and Lines 3 and 4: Positive samples (359 bp)

Table 2: Number and molecular characteristics of isolated *Staphylococcus* spp.

Meat samples source	Number of *Staphylococcus* spp. isolates (%)	Number of isolates containing *sea* gene (%)	Number of isolates without *sea/seb* gene (%)	Number of CPS (%)	Number of CNS (%)	Number of CPS isolates containing *sea* gene (%)	Number of CNS isolates containing *sea* gene (%)
Lamb carcasses	51 (34)	49 (32.6)	2 (1.3)	39 (26)	12 (8)	39 (26)	10 (6.6)
Buffalo carcasses	32 (21.3)	30 (20)	2 (1.3)	15 (10)	17 (11.3)	14 (9.3)	16 (10.6)
Cattle carcasses	38 (25.3)	35 (23.3)	3 (2)	31 (20.6)	7 (4.6)	29 (19.3)	6 (4)
Poultry carcasses	29 (19.3)	21 (14)	8 (5.3)	18 (12)	11 (7.3)	12 (8)	9 (6)
Total	150 (100)	135 (90)	15 (10)	103 (68.6)	47 (31.3)	94 (62.6)	41 (27.3)

CPS: Coagulase-positive staphylococci, and CNS: Coagulase-negative staphylococci

Table 3: The results of *S. aureus* biotyping isolated from slaughterhouse samples

Meat samples source	Number of isolates	HS biotypes					NHS biotypes					Cannot be biotyped
		Poultry ecovar	Sheep ecovar	Bovine ecovar	Human (He)	Human (β+)	K-β-CV:C	K+β+CV:A	K+β-CV:A	K-β+CV:A	K-β-CV:C	
Lamb carcasses	7	0	2	0	3	0	0	0	0	2	0	0
Buffalo carcasses	5	1	1	0	0	0	0	0	0	1	0	2
Cattle carcasses	9	0	3	3	0	0	0	0	0	2	0	1
Poultry carcasses	4	0	1	0	0	1	0	0	0	2	0	0
Total	25	1	7	3	3	1	0	0	0	7	0	3

HS: Host specific, NHS: Non-host specific

Table 4: The resistance profile of *S. aureus* isolated from various meat samples

Type of antibiotic	*S. aureus* isolated from lamb carcass			*S. aureus* isolated from buffalo carcass			*S. aureus* isolated from cattle carcass			*S. aureus* isolated from poultry carcass			Total number of resistant isolates (%)
	S	I	R	S	I	R	S	I	R	S	I	R	R
Penicillin	2	-	5	3	-	8	1	-	3	2	-	1	17 (68)
Oxacillin	3	3	1	8	1	2	2	2	-	2	1	-	3(12)
Erythromycin	2	4	1	3	7	1	2	2	-	2	1	-	2 (8)
Gentamicin	6	-	1	9	-	2	3	-	1	3	-	-	4 (16)
Trimethoprim	4	-	3	3	1	7	2	-	2	1	-	2	14 (56)

S: Susceptible, I: Intermediate resistant, and R: Resistant

concerning the CNS strains have shown that they produce a variety of metabolites including toxins and enzymes (Cunha *et al*., 2006).

In the current study, 94 CPS and 41 CNS from 150 staphylococci isolates harbored *sea* genes (Table 2). These findings indicate a high number of CNS in the raw meat samples of which a high percentage contain enterotoxigenic gene A in Ahvaz abattoirs. These results are consistent with the findings of Huber *et al.* (2011) who detected a large number methicillin resistant CNS (48.3%) in samples of livestock, chicken carcasses, bulk tank milk, minced meat, and humans obtained in Switzerland. In another study, Turchi *et al.* (2020) isolated 37 CNS from 120 samples of ovine bulk-tank milk in Italy. They highlighted that CNS from ovine milk represented a reservoir of environmental persistence factors. Our findings are consistent with the study of Veras *et al.* (2008) who reported the highest rate of

enterotoxigenic genes, *sea* and *seb*, from confirmed CPS and CNS by examining 16 outbreaks of food poisoning. In a study by Rall *et al*. (2010), 22.2% of tested dairy products were contaminated with CNS strains among which 12 strains (26.2%) contained enterotoxigenic genes. Surprisingly; the highest frequency (22%) was related to the genes producing enterotoxin A.

Several reports addressing the isolation of *S. aureus* encoded enterotoxin A in Iran. For example, in Tehran, the bacterium isolated from 29 (15.6%) meat samples including beef (14.8%), mutton (15%), chicken (15.7%) and turkey meat (16.6%) among which five samples encoded enterotoxin A (Sarrafzadeh Zargar *et al*., 2014). In another study (Havaei *et al*., 2015), 450 milk samples were obtained in Esfahan city (central Iran). Totally, fifty-four (12%) *S. aureus* were isolated and the *sea* and *seb* genes were detected in 19 and 2 isolates, respectively. Meanwhile, one isolate possessed both *sea* and *seb* genes.

All of the mentioned data suggest that animal origin products could be important reservoirs of enterotoxigenic CNS and more attention should be paid to the toxigenic capacity of these bacteria. The presence of toxins producer *S. aureus* localized on the skin, mucosal surface, and foodstuffs is a potential risk for food poisonings. Hence, the typing of *S. aureus* isolates seems to be necessary to determine the source of contamination and to identify its dominant types for better control and treatment of the resultant disease (Normanno *et al*., 2007). Biotyping is considered as a pattern of expressing the metabolic properties of an isolate. The biotyping approach proposed by Devriese (1984) is known as a simple and conventional procedure accepted by various researchers. This method has the potential to differentiate the source of staphylococci based on the relevant host (Kitai *et al*., 2005). Researchers believe that humans play a very important role in food contamination and poisoning caused by *S. aureus* (Aycicek *et al*., 2005).

In this study, the contamination of samples to HS and NHS species was 60% and 28%, respectively, and 3 isolates were not classified in the biotyping procedure. The biotyping revealed that 16% of the isolates were of the human biotype.

The findings can be compared with other similar studies in the world and Iran. For example, Normanno *et al*. (2007) found that, out of 125 analyzed strains among the isolates from meat and dairy products in Italy, the prevalence of the human biotype was 50.4% followed by the ovine (23.2%), bovine (7.2%), poultry-like ecovar (1.6%), and NHS strains (17.6%). In another study, CPS from pig carcasses from Swedish slaughterhouses were studied. The biotyping classified 56 isolates as NHS, 29 as human, five as poultry, one as ovine, one as bovine biotype and eight isolates were unclassified (Neskovic, 2008). In Iran, a study conducted by Soltan Dallal *et al*. (2010) showed that out of 100 isolates obtained from food (dairy and meat products), 29 isolates belonged to the human ecovar, 11 to the poultry ecovar, 9 to the bovine ecovar, 47 to NHS ecovars, and 4 isolates were not allocated. All the above data show that the prevalence of human ecovar in foodstuffs is high. Although some studies found no differences between the human strains and the NHS isolates regarding the ability to cause illness (Isigidi *et al*., 1992), the frequency of some of the severity factors is higher among the human ecovar than NHS ecovars (Soltan Dallal *et al*., 2010).

The high occurrence of human biotypes in the meats could be due to the manipulation of the carcass during the slaughtering process. The visceral evacuation process is a stage causing the contamination transmission via workers' hands. Regarding the high rate of slaughterhouses contamination to human ecovar, due to more manipulation of these products, the human contamination sources are considered as sources for further contribution to human food poisoning. Particularly, some studies have highlighted the frequency of a number of factors involved in staphylococcal poisoning among the human ecovar (Soltan Dallal *et al*., 2010).

In Ahvaz abattoir, sheep and cattle are slaughtered in separate but adjacent saloons. Our data showed that the ovine biotypes are rarely found in cattle carcasses, which can be explained by human contamination during the slaughtering process. This type of contamination is often limited by the prevention of contamination by the hands. Also, all isolated human ecovar contained the enterotoxin A gene, which suggest a possible relationship between the ecovar and the enterotoxin gene. However, none of the ecovars contained the enterotoxin B gene. In addition, the poultry ecovar contained none of the enterotoxin genes.

The antibiotic susceptibility test on *S. aureus* isolates showed a high prevalence of resistance among the isolates to trimethoprim (56%) and penicillin (68%) (Table 4). Resistance to trimethoprim may be a health hazard found in more than half of the isolates. In medicine, trimethoprim is used in combination with sulfamethoxazole in a formulation called co-trimoxazole. The major clinical usage of co-trimoxazole against *S. aureus* is for treating cystic fibrosis patients, treatment of endocarditis and bacteremia (Foster, 2017). Although resistance to trimethoprim in Europe is low (den Heijer *et al*., 2013), studies report that it is widespread in methicillin-resistant *Staphylococcus* (MARSA) in Africa (Nurjadi *et al*., 2014) and in Asia (Nurjadi *et al*., 2015). In a study, resistance against co-trimoxazole (85.3%) was observed in CNS recovered from broiler chicken populations in Egypt (Younis *et al*., 2017). Similar results (83.78%) were reported where methicillin-resistant *S. aureus* isolated from hospital food in Iran (Dehkordi *et al*., 2017). These data are consistent with the current study.

Resistance to penicillin at high levels has been reported from different countries such as Iran (Dehkordi *et al*., 2017), China (Wang *et al*., 2018), and Malaysia (Mahyudin, 2019). Also, Pereira *et al*. (2009) in Portugal performed an antibiotic resistance test on 148 CPS strains isolated from different food origins and showed that 73% of strains were resistant to penicillin. They reported that the virulence pattern was origin and strain

dependent. In our study the HS, NHS, and bovine biotype showed high prevalence in penicillin resistance. All of the above data emphasize the need to prevent the contamination of foods to *S. aureus*.

The present study revealed a high rate of staphylococcal contamination in meat supplied in the area of the study. The biotyping revealed that 16% of the isolates were of the human biotype. Human contamination sources are considered as the primary sources that further contribute to human food poisoning. The meats are also contaminated with enterotoxigenic and antibiotic-resistance staphylococci, which can threaten the health of the consumers. The study showed that not only CPS contain enterotoxin A gene, CNS isolates also possess this gene in high proportions and may be potential hazards in various meats, particularly buffalo meat. Preventing meat contamination during slaughter operations and the meat supply chain by human agents as well as avoiding the consumption of undercooked meat is recommended to prevent food poisoning.

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Conflict of interest

The authors declare that they have no conflict of interest.

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