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Genotyping and antibiotic resistance of methicillin-resistant staphylococcus aureus strains isolated from raw and frozen meat samples and assessment of the antimicrobial effects of origanum vulgare against MRSA isolates

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Abstract--Background: Methicillin-resistant Staphylococcus aureus is a resistant bacterium responsible for food poisoning. The present survey was done to assess the genotyping and antibiotic resistance of MRSA strains isolated from raw and frozen meat samples and assessment of the antimicrobial effects of Origanum vulgare against MRSA isolates. Methods: Two-hundred raw and frozen meat samples were collected and presence of MRSA was assessed using the culture. Disk diffusion was used to assess the antibiotic resistance of isolates. Isolates were subjected to PCR to obtain the virulence characters. Gas chromatography was used to determine chemical components of O. vulgare. Minimum Inhibitory Concentration and Minimum Bacterial Concentration of O. vulgare essential oil was also assessed. Results: Thirty two out of 200 meat samples (16%) were positive for MRSA. MRSA isolates had the highest resistance toward penicillin (100%), ceftaroline (100%), tetracycline (87.50%), and erythromycin (68.75%). Distribution of seA and eta genes amongst the raw and frozen meat samples were 75% and 58.33% and 50% and 33.33%, respectively. B-Caryophyllene (31.10%), 1-Octen-3-ol (18.41%), 1,8-Cineole (15.19%), and Thujopsene (8.20%) were the most commonly identified chemical components.

The dimeter of the growth inhibition zone of MRSA isolates treated with O. vulgare (1%) essential oil was statistically higher than penicillin, gentamicin, ampicillin, and tetracycline (P< 0.05) and insignificantly lower than azithromycin (P> 0.05). Conclusion: Role of meat as a reservoir for transmission of MRSA strains was determined in this survey. O. vulgare can be used as an edible film to extend the shelf-life of different meat-based products.

Keywords---methicillin-resistant, staphylococcus aureus, raw meat, frozen meat, genotyping, antibiotic resistance.

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a significant cause of human nosocomial infections (1) and more recently foodborne diseases (2). The bacterium is responsible for severe and resistant cases of hospital infections, including urinary, respiratory, burn, wound, and soft tissue infections and septicemia (3). Foodborne diseases caused by MRSA are mainly known with vomiting, nausea, abdominal cramps, weakness and diarrhea and also toxic shock syndrome (4). MRSA strains isolated from human clinical specimens and also food samples harbored high resistance toward commonly used antibiotics, especially penicillins and cephalosporins (5). Studies showed that MRSA strains harbored 10-100% resistance toward different antimicrobials, including aminoglycosides, tetracyclines, macrolides, and fluroquinolones (6-9). Thus, attention to synthesis and production of novel antimicrobial agents can diminish the risk of occurrence of antibiotic resistance.

Origanum vulgare (O. vulgare), also called wild marjoram (family Lamiaceae), whose name comes from Greek origanon, is typical to North America, originating from Mediterranean, and have a long history of use as food and a medicinal plant. Oregano is a perennial herb (up to 80 cm in height), with dark oval fragrant leaves and white, pink, or purple flowers formed in spikes (10). O. vulgare (oregano) EO contains high amounts of monoterpenoidic phenols (about 90% thymol and carvacrol) reducing the microbial population (11). It can also use as a spice with high antimicrobial effects on food stuffs. According to the high importance of MRSA and role of food in its transmission, the present survey was done to assess the genotyping and antibiotic resistance of MRSA strains isolated from raw and frozen meat samples and assessment of the antimicrobial effects of O. vulgare against MRSA isolates.

Materials and Methods

Samples

During summer of 2021, two-hundred samples of raw (n= 80) and frozen (n= 120) meat samples were collected from slaughterhouses of Isfahan province. Tight muscle samples were selected for this purpose. A total of 100 g raw and frozen meat were collected from each sample. Samples were transferred to laboratory using ice-boxes.

S. aureus isolation

Each sample was aseptically weighed in an analytical balance and 25 g were transferred into a sterile plastic bag. Then, 225 mL of buffered peptone water (Merck, Germany) was added and homogenized in a Stomacher Bagmixer 400 W (Interscience, Saint-Nom, France) for 2 min. Five milliliter aliquot of the enriched homogenate was transferred into 50 mL Trypticase Soy Broth (TSB, Merck, Germany) supplemented with 10% NaCl and 1% sodium pyruvate. After incubation at 35 °C for 18 h, a loopful of the culture was plated onto Baird-Parker agar supplemented with egg yolk tellurite emulsion (Merck, Germany) and incubated overnight at 37 °C. Black shiny colonies surrounded by 2 to 5-mm clear zones were further identified on the basis of Gram staining, hemolytic activity on sheep blood agar (Merck, Germany), catalase activity, coagulated test (rabbit plasma), oxidase test, glucose O/F test, resistance to bacitracin (0.04 U), mannitol fermentation on Mannitol salt agar (Merck, Germany), urease activity, nitrate reduction, phosphatase, deoxyribonuclease (DNase, Merck, Germany) test, voges-proskaver (Merck, Germany) test and carbohydrate (xylose, sucrose, trehalose and maltose, fructose, lactose, mannose) fermentation tests (12).

MRSA identification

Cefoxitin (30 µg) and oxacillin (1 µg) susceptibility tests were used to distinguish the MRSA strains from *S. aureus* isolates. All tests were performed using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (12, 13).

Antibiotic resistance

Patterns of antimicrobial resistance of the MRSA strains were studied using the simple disk diffusion technique (14). The Mueller–Hinton agar (Merck, Germany) medium was used for this purpose. Susceptibility of MRSA isolates were tested against several types of antibiotics including penicillin (10 µg/disk), ceftaroline (30 µg/disk), gentamicin (10 µg/disk), azithromycin (15 µg/disk), erythromycin (15 µg/disk), tetracycline (30 µg/disk), and ciprofloxacin (5 µg/disk) antibiotic agents (Oxoid, UK) using the instruction of Clinical and Laboratory Standards Institute. The plates containing the discs were allowed to stand for at least 30 min before incubated at 37 °C for 24 h. The diameter of the zone of inhibition produced by each antibiotic disc was measured and interpreted using the CLSI zone diameter interpretative standards (15-18).

DNA extraction and analysis

MRSA isolates were sub-cultured on the TSB medium (Merck, Germany) and further incubated for 48 h at 37 °C. In addition, genomic DNA was extracted from bacterial colonies using the DNA extraction kit (Fermentas, Germany), according to the manufacturer's instructions. Furthermore, the purity (A260/A280) and concentration of the extracted DNA were checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The truth of the DNA was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany) (19-25).

PCR amplification

Gene detection of virulence factors of the MRSA strains was done to determine their genotyping. For this purpose, the method described previously was used (26, 27). Hemolysin genes (hla and hlb), exfoliative toxins (etA and etB), and toxic shock syndrome toxin (tsst) and enterotoxin A (seA) genes were identified using the PCR assay (26, 27).

Plant materials

Aerial parts of the O. vulgare were collected from Isfahan province. Plants were confirmed by an expert professor of the field of medicinal plants. The aerial parts of plant were dried in shade at room temperature. They were then ground. The essential oil was obtained by hydro-distilling of ground material with boiling water up to 4 h utilizing a Clevenger-type apparatus. The extracted oils were dried over anhydrous sodium sulfate followed by filtering and stored at 4 °C in sealed glass vials for further use.

GC-MS

GC-MS analysis of the plant essential oil was done using the method described by Gong et al. (2014) (28).

Antimicrobial activities of plants materials

The simple disk diffusion method was used to assess the antimicrobial effects of O. vulgare against MRSA isolates. For this purpose, isolated bacteria were cultured on Muller Hinton agar media. A total of 1000 µl of 1% O. vulgare essential oil were poured into the blank disk and located at the surface of each media. For comparison, tetracycline (30 µg/disk), penicillin (10 µg/disk), gentamicin (10 µg/disk), azithromycin (15 µg/disk), and ampicillin (10 µg/disk) (Oxoid, UK) antibiotic disks were accompanies. The Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) of O. vulgare essential oil were also assessed. For this purpose, 0.5, 1, 2, and 4 mg/ml concentrations of O. vulgare essential oil were prepared and the MIC and MBC values were determined using the previously described method (29).

Data analysis

Data analysis was performed by SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's exact two-tailed tests were performed to assess any significant relationship (30, 31). Besides, p-value < 0.05 was considered statistically significant (32-34).

Results

MRSA distribution

Table 1 shows the MRSA distribution amongst examined raw and frozen meat samples. Thirty0two out of 200 meat samples (16%) were positive for MRSA.

MRSA distribution amongst the raw and frozen meat samples was 25% and 10%, respectively. Statistically significant differences were observed between type of samples and MRSA distribution (P< 0.05).

Table 1
MRSA distribution amongst examined raw and frozen meat samples

Meat samples	N. collected	N. positive for MRSA (%)
Raw	80	20 (25)
Frozen	120	12 (10)
Total	200	32 (16)

MTRSA antibiotic resistance

Table 2 shows the MRSA antibiotic resistance pattern. MRSA isolates had the highest resistance toward penicillin (100%), ceftaroline (100%), tetracycline (87.50%), erythromycin (68.75%), and gentamicin (46.87%). MRSA isolates of raw meat samples harbored the higher resistance rate toward tested antibiotics. (P< 0.05).

Table 2
MRSA antibiotic resistance pattern

Meat samples (N. positive)	Antibiotic resistance (%)						
	Pen*	Cft	Gen	Az	Ert	Tet	Cip
Raw (20)	20 (100)	20 (100)	10 (50)	8 (40)	15 (75)	18 (90)	10 (50)
Frozen (12)	12 (100)	12 (100)	5 (41.66)	3 (25)	7 (58.33)	10 (83.33)	4 (33.33)
Total (32)	32 (100)	32 (100)	15 (46.87)	11 (34.37)	22 (68.75)	28 (87.50)	14 (43.75)

*Pen: penicillin (10 µg/disk), Cft: ceftaroline (30 µg/disk), Gen: gentamicin (10 µg/disk), Az: azithromycin (15 µg/disk), Ert: erythromycin (15 µg/disk), Tet: tetracycline (30 µg/disk), Cip: ciprofloxacin (5 µg/disk).

MRSA genetic characters

Figure 1 shows the MRSA genotyping profile. Sea and etA were the most commonly detected virulence genes. Distribution of seA and eta genes amongst the MRSA isolates of raw and frozen meat samples were 75% and 58.33% and 50% and 33.33%, respectively.

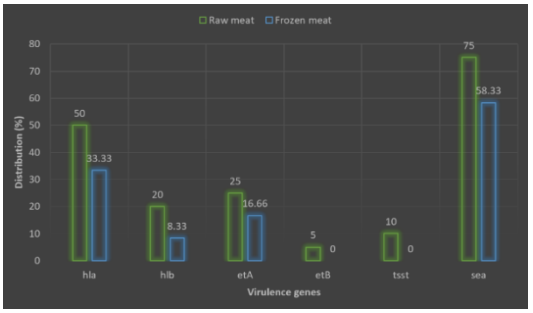

Figure 1. MRSA genotyping profile.

O. vulgare GC-MS

Table 3 shows the GC-MS analysis of O. vulgare essential oil. Ten chemical components (96.92%) were identified in the O. vulgare essential oil. β-Caryophyllene (31.10%), 1-Octen-3-ol (18.41%), 1,8-Cineole (15.19%), and Thujopsene (8.20%) were the most commonly identified chemical components in the O. vulgare essential oil.

Table 3
GC-MS analysis of the phytochemical compounds of the O. vulgare essential oil

No	Chemical compounds	Frequency (%)
1	β-Caryophyllene	31.10
2	1-Octen-3-ol	18.41
3	1,8-Cineole	15.19
4	Thujopsene	8.20
5	Borneol	7.14
6	p-Cymene	8.31
7	1,4-Terpineol	6.02
8	γ-Cadinene	1.55
9	Pinocarvone	0.61
10	p-Methylbenzaldehyde	0.39
Total		96.92

O. vulgare antimicrobial effects

Table 4 shows the growth inhibition zone of O. vulgare isolates. The diameter of the growth inhibition zones had the ranges between 6.86±0.52 to 10.72±1.47 mm. The dimeter of the growth inhibition zone of MRSA isolates treated with O. vulgare (1%) essential oil was 10.44±1.21 mm. The dimeter of the growth inhibition zone of MRSA isolates treated with O. vulgare (1%) essential oil was statistically higher than penicillin, gentamicin, ampicillin, and tetracycline ($P< 0.05$) and insignificantly lower than azithromycin ($P> 0.05$).

Table 4
Growth inhibition zone of MRSA isolates

Tested antimicrobial agents	Diameter of the growth inhibition zone of MRSA isolates (mm)
O. vulgare (1%)	10.44±1.21 a*
Tetracycline	7.19±0.29 c
Penicillin	7.20±0.31 c
Gentamicin	6.86±0.52 c
Azithromycin	10.72±1.47 a
Ampicillin	8.80±0.30 b

Table 5 shows the MIC and MBC of MRSA isolates treated with O. vulgare (1%) essential oil. The MIC and MBC of MRSA isolates treated with O. vulgare essential oil were 2 and 4 mg/ml, respectively.

Table 5
MIC and MBC values of O. vulgare against MRSA isolates

Treatment	MIC (mg/ml)	MBC (mg/ml)
Z. multiflora	2	4

Discussion

Despite all advances in medicine (35-45), some diseases remains life threating (46-50). MRSA infections are considered one of the most widespread globally with high morbidity and mortality (51). The main idea of the present study was to show the role of raw and frozen meat in transmission of MRSA strains into the human population and also their role as a vector of antibiotic resistance from animals to humans. Our findings showed the higher prevalence of MRSA in raw meat samples. It is probably due to the inactivation of MRSA in frozen meat samples. Bhargava et al. (2011) reported that the prevalence of MRSA in beef, chciken and turkey meat samples were 20.5%, 25.0% and 24.6%, respectively (52). Findings of Febler et al. (2011) (53) showed that MRSA strains was most prevalent in turkey (35.3%), followed by chicken (16.0%), veal (15.2%) pork (10.7%), and beef (10.6%) which was in contrast with our results.

Detected genes in this survey are mainly responsible for the adhesion and invasion of the MRSA strains into the host cells. Our findings showed that presence of virulence strains of MRSA isolates in raw and frozen meat samples may show an important public health threat regarding the consumption of raw or undercooked meat. As Sea is the most important enterotoxin A encoding genes, thus its presence here supported the hypothesis of the role of meat in transmission of MRSA food poisoning. Antimicrobial analysis showed that all isolates were resistant to penicillins and cephalosporins. This finding may be due to the nature of the MRSA strains which pose complete resistant toward all types of peniciliins and cephalosporins. Momtaz et al. (2013) (5) revealed that the S.

aureus strains harbored the highest levels of resistance against tetracycline (97.5%), methicillin (75.6), sulfamethoxazole (31.7%), trimethoprim (31.7%), streptomycin (31.7%), gentamicin (29.2%), enrofloxacin (28.0%), ampicillin (26.8%), chloramphenicol (20.7%), and cephalothin (17.0%).

Fowoyo and Ogunbanwo (54) revealed that the *S. aureus* bacteria recovered from ready-to-eat foodstuffs exhibited the boost incidence of resistance toward trimethoprim–sulfamethoxazole (74.90%), ampicillin (86.70%), cefotaxime (3.50%), amoxicillin–clavulanic acid (52.50%), ciprofloxacin (23.90%), oxacillin (35.70%), gentamicin (11.40%), erythromycin (15.70%), and ofloxacin (7.10%) which was relatively similar to our findings. O. vulgare essential oil harbored high antimicrobial compounds. Thus, it is not surprising that all isolates had considerable susceptibility against it. High antimicrobial effects of the O. vulgare essential oil against S. aureus bacteria was also reported from Brazil (55), and China (56). In keeping with this, only few studies use this medicinal plant as an antimicrobial and preservative agent in foodstuffs (57). The impact of veterinary and food hygiene in medical sciences has been determined before (58-66).

Conclusion

In conclusion, this survey confirmed that meat, especially raw and frozen cattle meat samples, can transmit virulent and resistant MRSA strains into the human population. In this regard, isolates had high resistance toward routinely used antimicrobials. However, isolates were susceptible to O. vulgare essential oil which pose its use as a preservative or edible antimicrobial agent in food matrix to diminish the growth and proliferation of foodborne bacteria and extend the shelf life of targeted products. However, more researches should perform in this regard.

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