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Methicillin-resistance *Staphylococcus aureus* in Southeast Iran: Herbal Control and Detection Methods Comparison

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Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a type of staph bacteria that has developed resistant to certain antibiotics such as beta-lactams. Rapid and sensitive detection of MRSA strains in patients, of course, improves the global health status. In the present study we compared the sensitivity and specificity of a rapid Polymerase Chain Reaction (PCR) assay with a traditional empiric therapy in MRSA detection, using a literature-derived model. Furthermore, we tested Aloe Vera (AV) and *Trachyspermum ammi* L. (TAL) extracts for evaluating their antibacterial potency against MRSA. Twenty five *S. aureus* isolated from the nose and throat of totally 160 samples in 3 different groups, including 80 healthy subjects, 40 hospital staffs and 40 inpatients. The results of disk diffusion method showed that phenotypic antibiotic resistance percent in *S. aureus* isolates were entirely classified as cloxacillin (100%), methicillin (100%), penicillin (88%), cefixime (72%), azithromycin (56%), kanamycin (48%), clarithromycin (48%), ampicillin (48%), erythromycin (48%), amoxicillin (32%) and tetracyclin (8%). The distribution of antibiotic-resistant mecA gene according to PCR was 40%. Moreover, the best Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) were 0.3 and 0.62 for both AV and TAL. Totally, 76% of MICs observed as cloxacillin-resistant. We concluded that the mecA gene-PCR assay is a rapid, sensitive and clinically useful test for early detection of MRSA. It is also recommended that more chemical investigations on AV and TAL require for introducing these extracts as antibacterial agents for control of MRSA.

Key words: *S. aureus*, mecA gene, medicinal plants, antibiotic resistance

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a global health concern and commonly manifest as minor skin infection and toxin-mediated diseases, including gastroenteritis, staphylococcal scalded-skin syndrome, staphylococcal food poisoning and toxic shock syndrome (Grundmann *et al*., 2006; Morrison-Rodriguez *et al*., 2010). An increasing proportion of *S. aureus* infections has become resistant to antibiotics such as methicillin, penicillin and cephalexin. These infections are known as MRSA and often referred to as a superbug in the media (Sattler *et al*., 2002). Community-associated MRSA infections were first reported in the early 1980s and often associated with the spread of strains from hospitals into the community (Crum *et al*., 2006; Chambers, 2001; Rybak and LaPlante, 2005; Palavecino, 2004). Carriage rates vary by geographic location and the specific population being sampled (Rim and Bacon III, 2007; Salgado *et al*., 2003). In addition, according to a long-term study, there has been a dramatic increase in community-associated MRSA infections since 2002 (Crum *et al*., 2006). A relative high frequency of MRSA outbreak has been found in some regions of Iran, including Isfahan and Tehran provinces. Methicillin-Sensitive *S. aureus* (MSSA) is naturally found within the environment while MRSA is predominantly seen in hospital settings. They usually infect humans only through cuts and open wounds, not through inhalation or ingestion. Contact can lead to a mild skin infection which often heals on its own. Rapid and accurate discrimination of the MSSA and MRSA strains is essential for appropriate therapeutic management and timely intervention for infection control. Further, MRSA has been reported to be associated with higher bacteraemia outbreaks than MSSA species and, in this respect, applying suitable antibiotics may reduce their morbidity, mortality and health care costs (Lodise *et al*., 2007; Cosgrove *et al*., 2003). Since, differentiate MRSA from MSSA by traditional methods is a time-consuming process, researchers looked at other potential and novel methods to access optimal treatment in a timely fashion. As a Semi-Synthetic Penicillin (SSP) covers MSSA but not MRSA, vancomycin is an appropriate choice because covers both MSSA and MRSA. However, SSP is superior to Vancomycin against MSSA (Cosgrove *et al*., 2003). Methods for detection of methicillin resistance species and related published data are contradictory with regard to the recommendation. One of the rapid and sensitive methods for discriminating MRSA from methicillin-susceptible *S. aureus* (MSUSA) is Polymerase Chain Reaction (PCR).The aims of the present study in the comparison of methods for detection of MRSA. Moreover, we determine the MIC and MBC of Aloe Vera (AV) and *Trachyspermum ammi* L. (TAL) extracts against isolated MRSAs.

MATERIALS AND METHODS

***S. aureus* isolates:** Twenty five nose and throat sample from 160 persons, including 80 healthy subjects, 40 hospital staffs and 40 inpatient were collected in the Amir Al-Momenin Hospital (Zabol, south-eastern Iran) and screened during years 2010-2011. The Samples were quarterly collected from infected men. Ten microliters of each sample were cultured over blood agar (Merck, Germany) (pH = 6.5) and incubated at 37°C for 24 h. Isolated Gram and catalase positive cocci were further tested for biochemical characterization. Carbohydrate fermentation was analyzed by urease, ONPG, vogues-proskauer, arginine ultilization, lysostaphin sensitivity, coagulase, clumping factor thermonuclease and haemolysin tests (Well and Stood, 1989).

Antibiotics susceptibility: The susceptibility of for antibiotics was carried out using disc-diffusion method on Muller-Hinton agar as recommended by Clinical and Laboratory Standards Institute (CLSI, 2003) with minor modification. Briefly, *S. aureus* isolates were grow overnight on blood agar and colony suspension was prepared using a sterile saline water equivalent to a 0.5-Mc Farland standard. Suspension (100 μL) was spread over the media plate and antibiotic disc aseptically transferred on the surface of inoculated media plate. Then the plates were incubated for 24 h at 30 and 35°C for cloaxacillin and other antibiotic, respectively and zone of grow inhibition recorded. Isolates were tested with different antibiotics with different concentration as shown in parenthesis viz., cloxacillon (5 μg), difloxcain (25 μg), amoxicillin (25 μg), kanamycin (30 μg), azithromycin (15 μg), Cefixime (5 μg), clarithromycin (15 μg), tetracyclin (30 μg), penicillin (10 μg), ampicillin (10 μg), erythromycin (15 μg) and methicillin (15 μg).

MIC of antibiotics: The MIC of antibiotics was determined using bacterial broth dilution method according to the method used by Baron and Finegold (1990). To study the effect of antibiotics, the Nutrient broth (Merck, Germany, pH = 6.5) containing known concentrations (512, 256, 128, 64, 32 to 2 μg mL^{-1}) of each antibiotic were prepared (Cloxacillin; Farabi, Iran). Nutrient broth without antibiotic was used as the control media. The MIC was defined as the lowest drug concentration which prevented visible growth of bacteria (Baron and Finegold, 1990).

Amplification of mecA gene: Following the biochemical test, Staphylococcal isolates were identified using species- specific gene amplification (16S-rDNA). The total volume of the reaction mixture (25 μL), including 2 μL of dNTPs (200 μm μL^{-1}, 2.5 μL of 10×Taq buffer containing 15 mM Mgcl2, 1 μL of each oligonucleotide forward and reverse primers (10 pm μL^{-1}), 0.35 μL of Taq DNA polymerase (3 U μL^{-1}), 1 μL DNA (30 ng μL^{-1}) and distilled water (17.2 μL). Forward and reverse primers were designed for the mecA gene of *S. aureus* as forward: 5'-AAAATCGATGGTAAAGGTTGGC-3' and reverse: 5'-AGTTCTGCAGTACCGGATTTGC-3'. These primers amplified a 532 bp PCR product in the PCR reaction. The 16s rDNA was used as the internal control gene based on previously reported primers (Lovseth *et al*., 2004) that amplify a 228 bp PCR product. The Master-cycler (Eppendorf) was programmed to as follows for mecA: initial denaturation for 3 min at 95°C and 30 cycles, including; denaturation step at 94°C for 30 sec, annealing at 55°C for 1 min, initial extension 72°C for 1 min and final extension at 72°C for 4 min. The PCR products and 50 bp DNA ladder (Cinagen-Iran) were separated on 1.5% agarose gel and visualized by ethidium bromid staining.

Plant material: The leaf of Aloe Vera (AV) and seeds of *Trachyspermum ammi* L. (TAL) were collected from the local regions of Iran (Zahedan and Kerman, Southeastern, Iran). The collected samples were dried at room temperature, crashed and stored until extraction step.

Preparation of extracts: Plants were properly dried and pulverized into a coarse powder (Najafi *et al*., 2010). Twenty grams ground powders of each sample were soaked in 60 mL ethanol 95%, separately, for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered (Whatman No. 1 filter paper). Then the filtrates were evaporated using a rotary evaporator. Finally, the extracts were stored at 40°C in air tight screw-cap tube.

MIC and MBC of plant extracts: The broth microdilution method was used to determine MIC and MBC according to Yu and colleagues procedure (Yu *et al*., 2004). Briefly, serial doubling dilutions of the extract in Mueller Hinton broth containing 0.5% (V/V) Tween 80 over the range 0.3-10.0 mg mL^{-1} were prepared and added to a 96-well microtiter plate. To each well, 10 μL of indicator solution (prepared by dissolving a 10 mg extract in 2 mL of DMSO) and 10 μL of Mueller Hinton Broth were added. Finally, 10 μL of bacterial suspension (10^6 CFU mL^{-1}) was added to each well to achieve a concentration of 10^4 CFU mL^{-1} of the bacteria. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates and placed in an incubator at 37°C for 18-24 h. The lowest concentration at which the color and turbidity changes occurred was taken as the MIC value. The average of three values was calculated to provide the MIC and MBC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not exhibit a visible growth according to the turbidity assessment. The MBC was defined as the lowest concentration of the extract at which the incubated microorganism was completely killed.

Statistical assessment: All experiments and measurement were repeated at least three times. Statistical analyses were performed using SPSS and Excel 2010 software. All experimental results were analyzed using mean descriptive statistics and the correlation-coefficient. A value of $p<0.05$ was regarded as statistically significant.

RESULTS

Antibiotic susceptibility: Antibiotic susceptibility of *S. aureus* nose and throat isolates was evaluated for 12 antimicrobial agents (Table 1). Overall, *S. aureus* were resistance to eleven agents, including cloxacillin (100%), methicillin (100%), amoxicillin (32%), kanamycin (48%), cefixime (72%), clarithromycin (48%), tetracyclin (8%), penicillin (88%), ampicillin (48%), erythromycin (48%) and azithromycin (56%). In inpatients resistance pattern were amoxicillin (8%), kanamycin (20%), cefixime (28%), clarithromycin (20%), tetracyclin 0%), penicillin (32%), ampicillin (16%), erythromycin (16%) and azithromycin (20%). In hospital staffs resistance pattern were amoxicillin (8%), kanamycin (8%), cefixime (8%), clarithromycin (0%), tetracyclin (0%), penicillin (8%), ampicillin (8%), erythromycin (0%) and azithromycin (8%). In healthy subjects resistance pattern was observed as below; amoxicillin (16%), kanamycin (20%), cefixime (36%), clarithromycin (28%), tetracyclin (8%), penicillin (48%), ampicillin (24%), erythromycin (32%) and azithromycin (28%).

Table 1: Percentage of antimicrobial susceptibility of 25 strains of *S. aureus*

AZM (%)	DIF (%)	CLR (%)	CFM (%)	ME (%)	CL (%)	TE (%)	AMX (%)	E (%)	AM (%)	K (%)	P	Quality
8	72	16	8	-	-	80	28	32	32	36	4	S
36	28	36	20	-	-	12	40	20	20	16	8	I
56	-	48	72	100	100	8	32	48	48	48	88	R

P: Penicillin, K: Kanamycin, AM: Ampicillin, E: Erythromycin, AMX: Amoxicillin, CFM: Cefixime, CLR: Clarithromycin, AZM: Azithromycin, DIF: Difloxcain, ME: Methicillin, CL: Cloxacillin, TE: Tetracyclin, S: Sensitive, I: Intermediate, R: Resistant

Table 2: Antibacterial effect of ethanol extract from selected plant against *S. aureus* isolate and antibiotic resistant

Bacterial code	mecA gene	MIC for cloxacillin	*Trachyspermum* MIC/MBC (mg mL^{-1})	*Aloe vera* MIC/MBC (mg mL^{-1})	Position	Resistance pattern
1	-	8	10-May	0.3/0.62	C	K-M-P
2	-	16	0.3/0.62	1.25/2.5	B	A1-A2-A3-C1-K-M-P
3	-	2	2.5/5	1.25/2.5	C	A1-C1-K-M-P
4	-	2	2.5/5	1.25/2.5	C	A3-C2-E-M-P
5	-	8	2.5/5	1.25/2.5	B	A1-A2-A3-C1-K-P
6	-	2	1.25/2.5	1.25/2.5	C	A1-A2-C1-K-P
7	+	512	2.5/5	2.5/5	C	A3-C2-E-M-P
8	-	2	2.5/5	10-May	C	A3-C1-C2-E-M
9	-	64	1.25/2.5	0.62/1.25	C	A3-C1-C2-M
10	-	2	1.25/1.25	1.25/2.5	C	A1-A2-C1-K-M-P
11	-	32	1.25/2.5	0.3/0.62	C	A1-C1-K-M-P
12	+	512	2.5/5	N	C	A3-C1-C2^{-}
13	+	64	2.5/5	N	A	A2-C1-K-M-P
14	-	2	2.5/5	N	A	A1-A2-C1-E-K-M-P-T
15	+	128	2.5/2.5	2.5/5	A	A3-C1-M-P
16	+	256	2.5/5	2.5/5	A	A1-A2-C1-K-M-P
17	-	64	2.5/5	10-May	A	A1-A2-A3-C1-E-K-M-P-T
18	-	32	1.25/1.25	2.5/2.5	A	A1-C1-C2-E-M-P
19	+	512	2.5/5	2.5/5	A	A1-A3-C2-E-M-P
20	+	64	1.25/2.5	10-May	A	C2-M-P
21	-	16	0.3/0.62	1.25/1.25	A	A1-C1-K-M-P
22	-	8	2.5/5	0.62/1.25	A	A3-C1-C2-E-M-P
23	+	256	10-May	0.3/0.62	A	A3-C2-E-M-P
24	+	512	2.5/2.5	N	A	A3-C1-C2-E-M
25	+	512	1.25/2.5	1.25/1.25	A	A3-C2-E-M-P

A: Healthy subjects, B: Hospital staffs, C: Inpatient. A_1: Ampicillin, A_2: Amoxicillin, A_3: Azithromycin, C_1: Cefixime, C_2: Clarithromycin, D: Difloxcain, E: Erythromycin, K: Kanamycin, M: Methicillin, P: Penicillin, T: Tetracycline and N: None accrued

Assessment of MIC for antibiotic: The MIC of the cloxacillin against 25 clinical isolates of *S. aureus* are shown in Table 2. Strains of *S. aureus* that growth in ≤2 or ≥4 μg mL^{-1} concentrations of cloxacillin consider as sensitive and resistant for this antibiotic, respectively (NCCLS, 2001). Accordingly, seventy 6% of *S. aureus* was found to be resistant to cloxacillin (19 sample) and 24% of *S. aureus* was found to be susceptible to cloxacillin (6 sample).

Amplification of mecA gene: Amplification of 16S-rDNA confirms that all the 25 Staphylococcal isolates are *S. aureus species*. The amplification of mecA gene (Fig. 1) in different species revealed that 40% of the isolates harbored this gene while in other isolates the gene was not found (Table 2).

Assessment of MIC for plants: Different inhibitory effects of the alcoholic extract of AV and TAL plants on the most *S. aureus* isolates were demonstrated in Table 2. The results show that alcoholic extract of the AV and TAL plants had an inhibitory effect on the most isolates. For alcoholic extract of AV higher and lower MIC was 5 and 0/3 mg mL^{-1}, respectively and only 3 of 25 *S. aureus* isolates were inhibited. Strains of 12, 13, 14 and 24 of *S. aureus* growth in all concentration of AV alcoholic extracts. For alcoholic extract of AV higher MBC was 10 mg mL^{-1} and only 3 of 25 *S. aureus* isolates were inhibited. For alcoholic extract of TAL higher and lower MIC was 5 and 0.3 mg mL^{-1}, respectively and only 2 of 25 *S. aureus* isolates were inhibited.

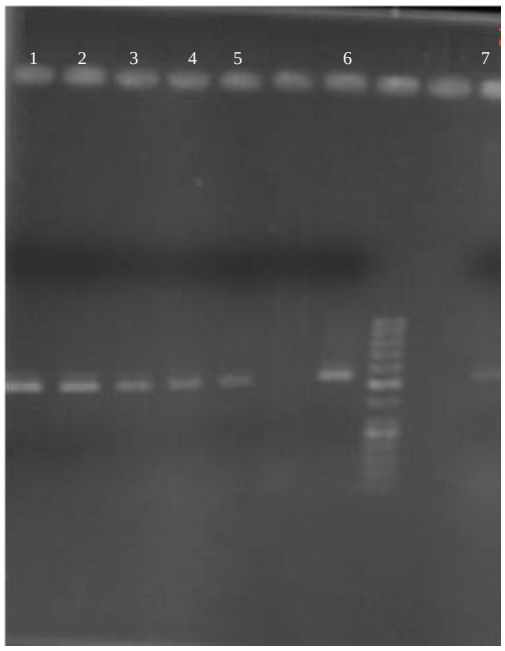

Fig. 1: Polymerase chain reaction results for mecA gene, lane No. 1-6: 532 bp fragment of mecA gene and lane 7: 50 bp DNA size markers

DISCUSSION

In the healthcare setting, yearly over 150,000 individuals are estimated to suffer from infections by MRSA in the European Union (Kock *et al*., 2010). In the United States, over 90,000 invasive infections are estimated to occur per year, translating to an incidence rate of over 30/100,000 (Klevens *et al*., 2007). Distribution of a particular antibiotic-resistant species in some regions might be due to the frequent and long-term uses of same antimicrobial agents for therapeutic applications (Sabour *et al*., 2004; Moon *et al*., 2007). The investigation was carried out on distribution of antibiotic resistance MIC cloxacillin and gene mecA in isolated *S. aureus* from different population groups. Diverse biochemical patterns were observed among recovered *S. aureus* isolates so that some of those showed unusual characteristics. The isolated *S. aureus* population showed a considerable resistance to some of antibiotics which are frequently used in clinical applications (Table 1). Restistance to cloxacillin and penicillin were detected in isolated more than other antibiotics. Our data have been supported with high proportion of penicillin resistance among the Brazilian (Rabello *et al*., 2005) and Austrian (Gonano *et al*., 2009) herds. In the case of other antibiotic such as tetracyclin, the results have been shown that resistant was observed in a higher frequency than those found (2) for *S. aureus* isolates from wound. The majority of the MRSA screening methods focus on detecting the mecA gene that is present within a transposon-encoded genetic region known as SCCmec (Wolk *et al*., 2009). This structural gene is responsible for methicillin resistance via., the production of an altered penicillin-binding protein which maintains staphylococcal cell-wall integrity due to its low affinity to β-lactam antibiotics. In this study 100% isolates of *S. aureus* were methicillin-resistant by disc-diffusion method, 76% isolates were methicillin-resistant to MIC method and the amplification of mecA revealed 40% isolates harbored the gene. Similarly, high prevalence (44.1%) of the MRSA strains in north India hospitals has been reported (Tyagi *et al*., 2008). In Tehran, MRSA isolates were constituted 49% of all isolates (Saderi *et al*., 2009). The study in Kermanshah, 2 isolates (36.8%) being resistant to methicillin (MRSA) (Mohajeri *et al*., 2013). Among MRSA, five isolates could not amplify the mecA gene. These isolates appear to show a low expression of the mecA gene or production methicillinase (alteration of PBP subtype) and/or overproduction of the beta-lactamase. The phenotypic expression of resistant could vary due to growth conditions or might be limitation in detection in microbiological methods (Zmantar *et al*., 2008). It also showed that the alcoholic extracts of the AV and TAL plants had potent antimicrobial activity against *S. aureus* isolates. In the present study, the MIC of the AV plant extract was determined in the ranges 0.3-5 mg mL^{-1} against the strains of *S. aureus*. The study of Thiruppathi, the result show ethyl acetate and ethanol extract of Aloe vera give the best result against *E. coli*. Ethanol shows the maximum inhibition (7-12 mm) and ethyl acetate (1-9 mm) (Thiruppathi *et al*., 2010). The study of Abraham, the result show 0.5 mg mL^{-1} concentration extract of Aloe vera did not produce any observable zone of inhibition while 1.0-2.0 mg mL^{-1} produced different range of zone of inhibition against *S. aureus* (Abraham *et al*., 2012). The study of Prashar, the volume of extract AV at which zone of inhibition was observed was 300, 350, 400 and 200 μL for *P. aeruginosa*, *S. aureus*, *E. coli* and *A. niger*, respectively (Prashar *et al*., 2011). The study of Agarry, antimicrobial susceptibility test showed that ethanol extract both the gel and the leaf of AV inhibited the growth of *S. aureus* (18.0 and 4.0 mm, respectively) (Agarry *et al*., 2005). In our study the MIC of TAL ranged from 0.3-5 mg mL^{-1} against the strains of *S. aureus* and the most frequent number of isolates showing inhibitory effect were seen in MIC of 2.5 mg mL^{-1} for *S. aureus* isolation. Acording to the study of Wadhwa and colleague, the maximum activity of essential oil of *Trachyspermum ammi* was on *B. subtilis* (16-17 mm) and minimum activity was on *S. aureus* (11-12 mm) (Wadhwa *et al*., 2010). The study of Usha, crude ethanol extract of ajowan produced zone of inhibition 7 mm against both *Bacillus subtilis* and *S. aureus*. Crude ethanol extract produced zone of inhibition of 8 and 9 mm against *E. coli* and *Pseudomonas* sp., respectively (Usha *et al*., 2012).

CONCLUSION

Rapid MRSA PCR testing appears to reduce mortality rates and can be expected to be cost effective in the country across a wide range of MRSA prevalence rates and test costs. To avoid a false-positive result and fast detection, we recommend mecA gene PCR method for the screening of MRSA in nasal and throat swab specimens in contrast with disk diffusion method. Medicinal plants could be sources of compounds which might be useful in managing antibiotic resistant *S. aureus*. AV and TAL extract is one of the potent agents to stop the antimicrobial activity, especially for the gram positive bacteria on the lesser extent. Further its activity is much better than as compared to the traditional antibiotics.

However, further studies about the isolation of active compounds and the absence of toxicity of plant extracts are necessary to propose these plants as alternative approaches to resistance management.

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