

Comparison of Ferment Sugars, Produce Hemolysis and Measuring Growth in Methicillin-Resistant and Methicillin-Sensitive *Staphylococcus aureus* Isolates from Inpatients and Healthcare Workers in Gorgan Hospitals, North of Iran

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The *mec A* gene in *Staphylococcus aureus* leads to production of new penicillin-binding protein called PBP2a. This change may follow some changes in other phenotypes. The aim of this study was the comparison of Ferment Sugars, Produce Hemolysis and Measuring Growth in MRSA and MSSA isolates. 188 *Staphylococcus aureus* isolates separated from inpatients and healthcare workers (healthy carriers) were studied. Bacterial cultures in blood agar environment at 37°C during 24h and at 4°C during other 24h were applied for studying hemolysis. Sugar fermentation carried out in phenol red Broth medium, containing glucose, galactose, arabinose, fructose, xylose, ramnose, mannose, sucrose, trehalose, raffinose or maltose. For determining bacterial growth, bacterial concentration of 10³ was taken each hour during 12 cultured in MHA and colonies were counted after 24h. The mean amount of hemolysis diameter in MRSA isolates was rather more than that of MSSA isolates. The difference between MRSA and MSSA isolates were significant as to fermenting ramnose, trehalose, galactose and xylose. The mean rate of growth in MRSA were significantly different from that of MSSA isolates (p < 0.05). Resistance to methicillin in *Staphylococcus aureus* isolates accompanies the increase of ability to ferment sugars. This phenomenon may be one of reasons for increased pathogenicity of MRSA isolates; So results shows the logarithmic phase is longer in MRSA isolates, This may implicate that PBP2a production in methicillin-resistant isolates follows slowing down nutrients entrance into the bacterium that in turn may causes slow growth .

Key words: *Staphylococcus aureus*, MRSA, MSSA, Sugar Fermentation, Hemolysis, Generation Time.

Staphylococcus aureus is one of factors causing hospital and community-acquired infections. It can result in some important diseases

such as bacteremia, endocarditis, osteomalacia, toxic shock syndrome and dermatic infections (26). This bacterium lives generally on anteronasal section. About 20-40% of healthy ones can be carriers of the *Staphylococcus aureus* at any time. The possibility of being the carrier of this bacterium increases in some situations such as healthcare

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workers in hospitals; then the infection can transmit to others, especially patients, by hospital personnel¹⁷. This microorganism is the leader of agents resulting in bacteremia, surgical wounds, dermatic, soft tissue infections, and sometimes mortality³. The main features of the bacterium are its ability to cause disease, produce toxins and enzymes, and acquire resistance to antibiotics²². The discovery of methicillin resistant isolates during the 1960s increased the importance of this bacterium²³. The mechanism of resistance to methicillin involves mainly producing a new changed PBP (Penicillin Binding Protein) named PBP2a in bacterium cell wall which has a weak tendency to bind beta-lactam drugs and is not inhibited by these drugs⁸. Resistance to methicillin is coded by *mecA* gene which is located on SCCmec element. SCCmec is a mobile genetic element that can be transmitted among different *staphylococcus* species²¹.

Change in PBP may result in some change in bacterium wall structure which in turn accompanies other changes in some phenotypes. These changes have been well documented in related literature. It has been proved that the rate of enterotoxin B produced by *Staphylococcus aureus* is related to its resistance to methicillin²⁵. Studies on the presence of leukocidin panton valentine genes showed some differences between MRSA and MSSA isolates^{7,18}. In comparison with MSSA cells, MRSA cells have more lipids of all kinds¹⁹. Experiments showed that MSSA isolates have shorter generation time than MRSA isolates; Then, the former produce more cells per hour than the latter. In other words, growth logarithmic phase in the same conditions is longer in MRSA than MSSA isolates and it is possible that the time for the division of methicillin-resistant cells would be longer²⁰. Several clinical studies revealed that the costs, treatment duration, pathogenesis and mortality rate are higher in MRSA than MSSA²¹. The possible differences in pathogenesis and virulence between MRSA and MSSA are ever an open question.

Different views on the pathogenic role of MRSA have encouraged many researchers to investigate the role-playing factors in pathogenesis of MRSA and MSSA isolates. The current comparative study aimed to investigate the ability of methicillin-resistant and methicillin-

sensitive *Staphylococcus aureus* isolates to ferment sugars, hemolysis production in blood agar, and measure bacterial growth rate.

MATERIAL AND METHODS

This cross-sectional descriptive study was conducted on 188 isolates of *Staphylococcus aureus* separated from inpatients (0.59%) and healthcare workers (healthy carriers) (0.41%) in hospitals located in Gorgan city, Iran, during 2010-11.

Identification of Bacteria

Standard methods were used for identifying *Staphylococcus aureus* including gram staining, culturing in Mannitol Salt Agar (MSA), catalase, DNase and coagulase in forms of slide and tube procedures^{9,24}.

Polymerase Chain Reaction (PCR)

MRSA isolates were identified by PCR method as a standard test for detecting the existence or lack of methicillin-resistant gene (*mecA*). This was done by paired primers 5'-AAATCAGATGGTAAAGGTTGGC-3' and 5'-AGTTCTGCA GTACCGGATTTGC-3'¹⁴.

Sugars Fermentation

For testing sugar fermentation, Sugars that *Staphylococcus aureus* can or can't ferment based on diagnostic tables was used¹¹. The selected sugar solutions of 1% including glucose, galactose, arabinose, fructose, xylose, rhamnose, mannose, sucrose, trehalose, raffinose or maltose were added to tube containing 5 ml of phenol red broth medium³. Bacterium suspension of 100ml equal to concentration of 0.5 McFarland from 24h culture was added to each sugar contained phenol red broth⁴. All media were maintained under 37°C during 24 h. The stain change of medium from red to yellow shows the sugar fermentation. As all *Staphylococcus aureus* isolates consume glucose, the stain changes for this sugar were observed and recorded in three times: 4h, 8h and 24h¹¹.

Produce Hemolysis

Test for hemolysis in Blood Agar (BA) medium was done for each *Staphylococcus aureus* isolate. First, sinks with 5mm diameter were on blood agar culture. Second, bacterium suspension of 80 ml equal to the concentration of 0.5 McFarland was added to each sink. All media were incubated in 37°C for 24 h. Then, the hemolysis production

and its diameter were observed. In order to observe cold-hot hemolysin, they were maintained in freezer and after 24 h, the number and diameter of hemolysis produced in MRSA and MSSA isolates were observed again and compared⁴.

Measuring Growth Rate

For determining growth rate, turbidity of 0.5 McFarland equivalent was diluted as serial dilution until its ultimate concentration reached 10^3 CFU/ml (indicator tube); this was considered as zero hour of bacterial growth. Tube was incubated during 12 h in 37°C. Sampling, observing by graded slide and culturing in Mueller-Hinton Agar (MHA) were done each 2-3 h period. For this, bacterium suspension of 10 µl was distributed on MHA culture. Graded slide was applied for counting bacteria. Counted number was multiplied in inverse dilution coefficient for determining the number of bacteria in each ml of broth medium. Samples were diluted after fourth hour for counting bacteria. In the current study, plates with colony count in the range of 30-300 CFU/ml were considered as countable^{10, 28}. After repeating the

experiment, it was appeared that the best times for measuring bacterial logarithmic growth were between 4th-10th h and the growth enters stable phase after 10 h. Then, growth between 4-10 h was measured as growth rate.

RESULTS

PCR

Based on PCR method, 61 (32.4%) and 127 (67.6%) out of all 188 isolates of *Staphylococcus aureus* were resistance and sensitive to methicillin, respectively.

Sugars Fermentation

Glucose was fermented by all *Staphylococcus aureus* isolates during 24 h; that in 10 (16.4%) isolates fermentation was observable after 4thh, but in MSSA isolates, only 8 (6.3%) isolates fermented the sugar ($P < 0.05$). More than 94% of *Staphylococcus aureus* isolates (178<) fermented mannose, sucrose, fructose, terhalose and maltose; and 86.2% of isolates (= 162) fermented galactose. As table 1 shows, differences

Table 1. Comparing the abilities of MRSA and MSSA isolates to ferment sugars

Ability Sugar		Fermentation			P value
		MRSA (%)	MSSA (%)	Total (%)	
Glucose	(4 h)	10 (16.4%)	8 (6.3%)	18 (9.6%)	0.02
	(8 h)	29 (47.5%)	57 (44.9%)	86 (45.7%)	0.42
	(24 h)	61 (100%)	127 (100%)	188 (100%)	1
Mannose		59 (96.7%)	125 (98.4%)	184 (97.9%)	0.39
Terhalose		55 (90.2%)	126 (99.2%)	181 (96.3%)	0.005
Galactose		57 (93.4%)	105 (82.7%)	162 (86.2%)	0.03
Maltose		60 (98.4%)	118 (92.9%)	178 (94.7%)	0.1
Fructose		61 (100%)	121 (95.3%)	182 (96.8%)	0.09
Xylose		7 (11.5%)	3 (2.4%)	10 (5.3%)	0.01
Arabinose		8 (13.1%)	12 (9.4%)	20 (10.6%)	0.29
Sucrose		59 (96.7%)	125 (98.4%)	184 (97.9%)	0.39
Ramnose		11 (18%)	3 (2.4%)	14 (7.4%)	< 0.001
Raffinose		17 (27.9%)	24 (18.9%)	41 (21.8%)	0.11

Table 2. Comparing the frequencies of hemolysis layers of *Staphylococcus aureus* in 24h and 48h cultures

Hemolysis layers Culture time	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)
24 h (37 °C)	64 (34%)	81 (43.1%)	43 (22.9%)	0*	0*
48 h (4 °C)	50 (26.6%)	64 (34%)	24 (12.8%)	30 (16%)	20 (10.6%)

* No isolates in the two cultures had 3 or 4 hemolysis layers.

in fermentation for MRSA and MSSA were significant as to rhamnose [11 (18%) and 3 (2.4%), respectively, ($p < 0.001$)], xylose [7 (11.5%) and 3 (2.4%), respectively, $p = 0.01$], galactose [57 (93.4%) and 105 (82.7%), respectively, $p = 0.03$] and terhalose [55 (90.2%) and 126 (99.2%), respectively, $p < 0.005$].

Produce Hemolysis

64 (34%) of *Staphylococcus aureus* isolates could not create hemolysis in 24 h. After placing the plates in cold situation for other 24h (48 h culture), the amount decreased in 50 (26.6%)

isolates; this demonstrates a 7.4% increase in hemolysis production in cold situation.

The number of hemolysis layers around the bacterium in 24 h culture was more than those of 48h culture. In 48 h culture, 50 isolates had 3 or 4 hemolysis layer. In 24h culture, however, no isolate had more than 3 hemolysis layers (Figure 1 and Table 2). This difference was significant ($p < 0.001$). The mean amounts of hemolysis diameter in 24h culture (9.7ml) and 48h culture (14.05) were statistically significant ($p < 0.001$).

Table 3. Comparing the frequencies of hemolysis layers in 24h and 48h cultures in MRSA and MSSA isolate

Time & Layer	24 h (37 °C)			48 h (4 °C)				
	0 (%)	1 (%)	2 (%)	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)
MRSA	19(31.1%)	27 (44.3%)	15 (24.6%)	10 (16.4%)	22 (36.1%)	11 (18%)	12 (19.7%)	6 (9.8%)
MSSA	18 (35.4%)	13 (42.5%)	42 (22%)	40 (31.5%)	42 (33.1%)	13 (10.2%)	18 (14.2%)	14 (11%)
Total	64 (34%)	81 (43.1%)	43 (22.9%)	50 (26.6%)	64 (34%)	24 (12.8%)	30 (16%)	20 (10.6%)

Table 4. Descriptive statistic indicators for growth rate of *Staphylococcus aureus* isolates (in minutes)

Mean	35.35
Median	35.19
Mode	33.57
SD	3.84

observed in isolates of our region¹¹. All isolates in our study fermented glucose and the process began in 10% of the isolates during the first 4h and increased in 46% during 8h; this demonstrates the intensive need of the bacterium to rapid consume this kind of sugar for growth. Since the current study showed that less than 10% of these isolates had the ability to ferment xylose and arabinose,

Table 5. Means and SDs in the studied groups (in minutes)

Feature	MRSA	MSSA	Inpatient	Carrier
Generation time				
Mean	36.32	35.05	36.07	34.45
SD	2.99	4.11	3.81	3.75

There was no significant difference between resistance or sensitivity to methicillin and hemolysis layers in 24h culture and 48h culture ($p > 0.05$, Table 3).

Measuring Growth Rate

In this study, the mean growth rate of *Staphylococcus aureus* isolates was 35.35 minutes (table 4).

ANOVA test revealed that the times of bacterial division in MRSA and MSSA were significantly different ($p = 0.01$). So, the mean time for growth rate in methicillin-sensitive isolates and

methicillin-resistant were 35.05 and 36.32 minutes, respectively. Then logarithmic phase in MRSA isolates was longer (Table 5).

DISCUSSION

The ability of *Staphylococcus aureus* to ferment sugar as main food sources is important. Most isolates of *Staphylococcus aureus* have the ability to produce acid from various sugar types, such as glucose, mannitol, mannose, terhalose, maltose and sucrose. This phenomenon was

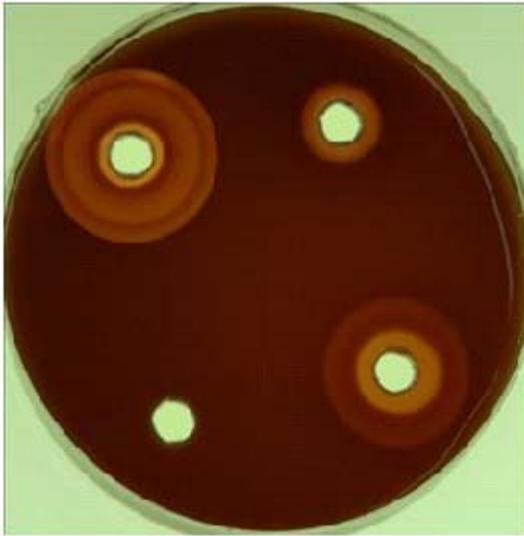


Fig. 1. Hemolysis layers of *Staphylococcus aureus*

some scientific resources such as Bergey's Manual of Determinative Bacteriology¹² and The Prokaryotes¹¹ in their diagnostic tables showed the inability of *Staphylococcus aureus* to ferment these sugars. The ability to ferment raffinose by isolates in this region was reported 23% which is higher than expected amount (<10%)³. However, Adegoke in 1982 (12) and Ajuwape in 2001², reported that 81.7% and 46.3% *Staphylococcus aureus* isolates fermented xylose, respectively. This difference may be the result of different features between domestic isolates and isolates in other regions; the deep study of the phenomenon in other regions of Iran can be effective in identifying Iranian domestic isolates. Also, it was identified that the ability of MRSA in fermenting each of above-mentioned sugars as well as rhamnose was more than MSSA. It is proposed that further studies should be conducted for studying the role of PBP2a in acquiring such ability. Studying 82 isolates of *Staphylococcus aureus* separated from goat, Adegoke found that all isolates fermented glucose during 24h. In addition, 98.8% and 98.7% of the isolates fermented maltose and sucrose with acid production, respectively¹. All 108 isolates in Atuwape's study fermented glucose, mannitol and sucrose and 98.1% and 89.1% of them fermented maltose and terhalose, respectively². The results of these two studies are in line with results achieved in our region.

Based on our findings, resistance to methicillin in *Staphylococcus aureus* isolates resulted in increasing their ability to ferment sugars, especially rhamnose, xylose and galactose. The glucose fermentation in MRSA isolates was more than that of MSSA ones. Based on the reality, it can be hypothesized that PBP2a may increase the permeability of sugars into bacteria that needs further studies.

The hemolysins and leukocidins released by *Staphylococcus aureus* are of the famous systolic toxins. The bacterium produces 4 different hemolytic toxins (α , β , γ and δ), but different isolates produce different amounts of these toxins²⁷. In our study, 37% of *Staphylococcus aureus* isolates had not the ability to produce hemolysis in blood agar medium. In the study by Coia et al., out of 210 isolates, only 12 (6%) were unable to produce hemolysin. This amount is definitely lesser than that of our region⁵.

Organisms that produce solution hemolysin in blood agar culture surface, create an area of beta hemolysis around their colonies²⁷. Alpha toxin is considered as a main factor for developing beta hemolysis area in blood agar at 37°C. This toxin is produced by 85% of *Staphylococcus aureus* isolates⁶. In Coia's study, its frequency was reported around 60% and as a result, however, if this toxin is the main factor for hemolysis in blood agar, around 40% of isolates lack it⁵, that this is relatively in line with our results. In a study in 2009, it was reported that alpha hemolysin coding gene (*hla*) in *Staphylococcus aureus* isolates of Austria, Hungary and Macedonia were 88%, 99.5% and 85%, respectively, that demonstrates the difference among hemolysin producing isolates in various European regions¹⁶, but the amount of expressed and alpha hemolysin-producing genes was not reported. This exotoxin is a secretory protein with hemolytic, systolic, dermonecrotic and lethal features.

Studies showed that the sensitivity of all cells to this toxin is not the same. For example, rabbit erythrocytes and human platelet have a high tendency to binding the toxin and some others such as human erythrocytes have a low tendency to do so⁶. Then, when culturing *Staphylococcus aureus* samples, it is better to use sheep or rabbit blood rather than human blood. No use of human

blood in some study settings may be a reason of hemolysis differences observed in previous studies.

In our study, the frequency of cold-hot hemolysis was analyzed by keeping the 24h bacterial culture in other 24h culture in freezer temperature and estimating the number of layers or diameters of hemolysis formed around bacterial colony. It was detected that 8.5% of isolates with no beta hemolysis in 37°C, beta hemolysis was observed at freezer temperature and its diameter reached up to 8-15 mm and it had one or two layers. In addition, keeping in cold situation resulted in the increase of the amount of hemolysis layers and diameters of 66 isolates. It is estimated that there is a beta hemolysin in 43.1% isolates of Gorgan city, and Coia found the frequency of this hemolysin in Scotland was 21.9%⁵. In accordance with our result, beta hemolysin coding gene (*hib*) of *Staphylococcus aureus* isolates in Macedonia and Hungary was about 33-62%¹⁶.

In line with our results, Coia found no significant differences between MRSA and MSSA isolates as to beta hemolysin production and the number of the producers of this hemolysin (14 out of 49 vs. 30 out of 152) ($p > 0.05$) (5). However, in the study by Kocsis in each three studied countries, the frequencies of beta hemolysin producing gene in the two isolate groups were significantly different. It is notable that beta hemolysin in MRSA isolates belonged to Macedonia and Hungary was higher, but the situation was the reverse in Austria case¹⁶. A possible reason for the difference between the results of this study and those of our study as well as Coia's is that we tested the phenotype and the two studies tested the genotype. Considering the results of the study, it is possible that some isolates in some regions contain more gene of this kind than isolates in other ones.

Beta toxin is a molecule with molecular weight of 35 KD and has Mg²⁺Sphingomyelinase (SMase) activity. As a result, the sensitivity of host cells depends on membrane SMase rate. This toxin is produced by many *Staphylococcus aureus* isolates and well possibly destroys tissue and causes abscess along with alpha toxin. Erythrocytes of sheep, goat and cow have more sensitivity to this toxin. As having the highest amount of SMase in outer row of the membrane of

their two cytoplasm layers, leucocytes are the most sensitive cells to this toxin. Beta toxin has a known role in the inflammation of cow breast. Aarestrup's study showed that beta toxin was produced in 72% of isolates from cow breast, 11% of isolates from healthy carriers and 13% of isolates from human septicemia¹³.

The frequency of hemolysin producing MRSA and MSSA isolates were compared in the current study. No significant difference was observed between the two isolate groups as to hemolysis diameter and number. The hemolysin lacking MSSA isolates, however, were more than hemolysin lacking MRSA isolates (30% vs. 17%); this demonstrates that methicillin resistant isolates have more tendencies to producing hemolysin. Coia found no significant difference between MRSA and MSSA isolates in producing alpha hemolysin ($p > 0.05$). However, MRSA isolates produced higher rate of this toxin than MSSA isolates (36 out of 49 vs. 86 out of 152). In this study, 12 isolates (7 MSSA and 5 MRSA) out of 210 ones were able to produce hemolysin. In addition, the rate of hemolysin production, especially multiple one was higher in MRSA than MSSA isolates⁵. Kocsis *et al.*, however, reported that the frequencies of alpha hemolysin producing gene between these two isolate groups were not significantly different in Austria and Hungary cases. But the situation was the reverse in Macedonia case, in which the difference was significant and 96% and 72% of methicillin-resistant and methicillin-sensitive isolates had this hemolysin, respectively. In the three studied countries, the frequencies of producing alpha hemolysin in methicillin-resistant were higher than that of methicillin-sensitive isolates ($p < 0.001$)¹⁶, this may be a cue for the fact that MRSA isolates are more pathogenic than MSSA isolates.

As Domingue reported, *Staphylococcus aureus* has two cell division times in logarithmic phase; one relates to in-vivo condition lasting 60 minutes and the other relates to in-vitro condition lasting about 24 minutes¹⁵. Based on 'Prescott's Microbiology' book, the growth of *Staphylococcus aureus* is 28.2 minutes. In another study, this rate is reported between 27-30 minutes²⁸. In our study, however, the generation time of the bacterium was 35.3 minutes.

Several experiments showed that the

generation time of MSSA is shorter than that of MRSA isolates and consequently, the former produce more cells than the latter per hour. In other words, logarithmic phase of growth in the same conditions are longer in MRSA isolates than MSSA ones. It is possible that the time of cell division in methicillin-resistant isolates is longer²⁰. Based on our results and in line with the above mentioned study, the bacterial division times of MRSA and MSSA isolates are different. The mean±SD of generation times in methicillin sensitive and methicillin resistant isolates were 35±4.11 and 36.3±2.99, respectively; this shows that the logarithmic phase of MRSA is longer than that of MSSA isolates. Karauzum reported the cell division time of MRSA and MSSA isolates 31±2.67 and 30.5±1.35, respectively¹⁵. The result does not accord ours as to generation time, and cell division time in methicillin-resistant was longer than methicillin-sensitive isolates. This fact may implicate that PBP2a production in methicillin-resistant isolates accompanies some change in nutrients' permeability into the bacterium. Slowing down nutrients' entrance into the bacterium causes slow growth. As the ability of many studied sugars to be fermented by MRSA is higher than that of methicillin-sensitive isolates, other factors, such as slowing amino acid, and vitamin entrance appear to involve in slowing down the growth of this bacterium. It is necessary to design studies for comparing the entrance and use of these substances in MRSA and MSSA isolates.

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