MOLECULAR DETECTION OF MACROLIDE AND LINCOSAMIDE-RESISTANCE GENES IN CLINICAL METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM BABOL AND AMOL, IRAN

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ABSTRACT

Introduction: *Staphylococcus aureus* (*S. aureus*) is one of the main causes of nosocomial infections and the increasing spread of antibiotic resistance in the last decades has led to an increase in mortality rates due to these infections.

Objectives: The aim of this study was to evaluate the antibiotic resistance pattern and the frequency distribution of *mecA, ermA, ermB, ermC and msrA* genes amongst *S. aureus* strains.

Methods: In this study, a total of 120 *S. aureus* strains were collected. The antibiotic resistance pattern, D-test and genotypic detection of *mecA, ermA, ermB, ermC and msrA* genes were carried out.

Results: From a total of 120 isolates, 71.66% were identified as methicillin-resistant *Staphylococcus aureus* (MRSA). The highest macrolide, lincosamide and streptogramin B resistance phenotype (MLS_B) belongs to constitutive resistance phenotype ($cMLS_B$) with 39.16%. Subsequently, the induction resistance phenotype ($iMLS_B$) and macrolides-streptogramin B resistance (MS_B) were 5.8% and 1.7%. The prevalence of *ermA*, *ermB*, *ermC* and *msrA* genes were reported 22.5%, 24.16%, 80.83% and 41.66%. There was a considerable correlation between the presence of different types of *erm* genes and MLS_B resistance phenotypes. Moreover, 39.16% of strains were identified as multi-drug resistant (MDR) strains.

Conclusions: The high prevalence of MRSA strains in our study indicates that influential and effective monitoring methods are needed for controlling *S. aureus* infections.

Keywords: *Staphylococcus aureus*, nosocomial infections, antibiotic resistance.

REZUMAT

Introducere: *Staphylococcus aureus* (*S. aureus*) este una dintre cauzele principale ale infecțiilor nosocomiale, iar creșterea nivelului de rezistență la antibiotice din ultimele decenii a condus la sporirea ratei mortalității cauzate de aceste infecții.

Obiective: Scopul acestui studiu a fost evaluarea profilului de rezistență la antibiotice și frecvența distribuției genelor *mecA, ermA, ermB, ermC* și *msrA* în tulpinile *S. aureus*.

Metode: În acest studiu, s-au colectat în total 120 de tulpini de *S. aureus.* S-a stabilit profilul de rezistență la antibiotice și s-au efectuat testul D și detectarea genotipică a genelor *mecA, ermA, ermB, ermC* și *msrA.*

Rezultate: Dintr-un total de 120 de izolate, 71,66% au fost identificate ca fiind *S. aureus* meticilino-rezistent (MRSA). Nivelul de rezistență cel mai ridicat (39,16%) la macrolide, lincosamid și streptogramină B (MLS_B) aparține fenotipului de rezistență constitutivă ($cMLS_B$). Rezistența inductibilă ($iMLS_B$) și rezistența la macrolide-streptogramină B (MS_B) au fost de 5,8% și 1,7%. Prevalența genelor *ermA, ermB, ermC și msrA* a fost de 22.5%, 24.16%, 80.83% și 41.66%. S-a observat o corelație semnificativă între prezența diferitelor tipuri de gene *erm* și fenotipul MLS_B. În plus, 39,16% dintre tulpini au prezentat multidrog rezistență (MDR).

Concluzii: Prevalența ridicată a tulpinilor MRSA din studiul nostru indică necesitatea unor metode eficiente de monitorizare pentru controlul infecțiilor cu *S. aureus.*

Cuvinte-cheie: *Staphylococcus aureus*, infecții nosocomiale, rezistență la antibiotice.

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INTRODUCTION

Steadily rising rate of methicillin-resistant *Staphylococcus aureus* strains and extensive changes in antimicrobial resistance pattern have been led to the use of macrolide, lincosamide and streptogramin B, which are called MLS_B antibiotics [1]. The same functional action is exerted against the bacteria, despite the different structures in these antibiotics. An alternative of choice for the treatment of skin and soft tissue infections caused by *S. aureus* are erythromycin as a macrolide and clindamycin as a lincosamide [2]. Clindamycin is the most commonly used lincosamide as an alternative treatment of *S. aureus* infections during penicillin intolerance or resistance to methicillin. In addition, clindamycin is an attractive drug for a variety of reasons such as ways of drug intake, a significant influence on skin tissue, and great effectiveness in treating the Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) strains. Moreover, it can inhibit the production of toxins and virulence factors in *S. aureus* strains by inhibiting the protein synthesis [3]. However, excessive use of these drugs can lead to bacterial resistance and failure to treat [4]. The presence of *msrA* gene in *S. aureus*, which is coding for a macrolide efflux protein, induces resistance to macrolides and streptogramin B antibiotics and creates the MS_B (macrolides-streptogramin B) phenotype [5]. On the other hand, the change of the ribosomal target site by *erm* (erythromycin ribosomal methylase) genes is one of the most common resistance mechanisms. Approximately nine varieties of *erm* genes have been identified in staphylococci, which can produce resistance to MLS_B drugs [6]. It should be mentioned that the MLS_B resistance phenotype is performed by using the D-test *in vitro* conditions [7].

The aim of this study was to evaluate the antibiotic resistance pattern and the frequency distribution of *mecA*, *ermA*, *ermB*, *ermC* and *msrA* genes amongst *S. aureus* strains.

MATERIALS AND METHODS

Collecting and identifying bacterial strains: In this study, a total of 120 non-duplicate *S. aureus* isolates were collected from 4 hospitals in Amol and Babol cities in the north of Iran. The studied strains were isolated from clinical specimens, including blood, wounds, sputum, etc. The strains were identified by using microscopy and other phenotypic microbiological methods, such as catalase production, coagulase, DNase, and also mannitol fermentation. Finally, *S. aureus* strains were preserved in Brain Heart Infusion (BHI) broth with 15% glycerol at -20°C.

Antibiotic susceptibility testing: The evaluation of antimicrobial susceptibility assay was carried out by using disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines for the following antibiotics: penicillin (10U), cefoxitin (30U), erythromycin (15 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), vancomycin (30 μg), rifampicin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), linezolid (30 μg), minocycline (30 μg), clindamycin (2 μg) and nitrofurantoin (300 μg) from Roscoe Company (Denmark). *S. aureus* ATCC 25923 was used as a quality control strain [8, 9]. In addition, the E-test method (Himedia, India) was used for determining minimum inhibitory concentration (MIC) of vancomycin-resistant *S. aureus* strains. *Enterococcus faecalis* ATCC 52199 and *S. aureus* ATCC 29213 strains were applied as controls [8].

D-test: In order to detect the resistance of erythromycin and clindamycin, according to the CLSI guidelines, erythromycin (15μg) and clindamycin (2μg) were used [8]. The bacterial suspension of 0.5 McFarland opacity standard was prepared from fresh cultures of each strain in normal saline and cultivated on a Mueller Hinton Agar medium (Merck, Germany). Then, erythromycin and clindamycin discs were placed on the plate, at 15 to 20 mm distance apart from each other, in edge-to-edge fashion, and incubated at 37°C for 18 hours. The results were reported as $cMLS_B$, $iMLS_B$, and MS_B phenotypes.

DNA Purification: DNA extraction was performed using Yekta-Tajhiz Azmakit (Iran), according to the manufacturer's instructions.

Polymerase Chain Reaction: Exclusive detection of methicillin-resistant *S. aureus* strains was carried out by cefoxitin disc diffusion and PCR molecular methods. Furthermore, *ermA*, *ermB*, *ermC*, and *msrA* genes were identified using specific primers according to previous studies.

Primers were purchased from Gen-Fanavaran Company (Iran). The sequences of primers and the PCR programs are presented in Table 1. The reaction was conducted in a final volume of 25 µL containing 12.5 µl of Super PCR Master Mix 2X (Yekta-Tajhiz Azma, Iran), 3 µL of total DNA, 0.4 µM of each primer and 7.5 µL of distilled water and amplified in thermocycler. 1.5% agarose gel electrophoresis and SYBR Safe DNA Gel Stain were used for DNA fragments analysis and visualized under UV light. Consequently, the *ermA*, *ermB*, *ermC*, and *msrA* positive strains were sequenced (Macrogen, Korea).

Multiplex PCR: Multiplex PCR was used to identify *ermC* and *ermB* genes by optimizing PCR conditions (Fig. 1). The reaction was conducted in a final volume of 50 µL containing 25 µL of Super PCR Master Mix 2X (Yekta-Tajhiz Azma, Iran), 10 µL of total DNA, 0.4 µM of each primer and 7 µL of distilled water and amplified in thermocycler (initial denaturation at 95°C for 3 min; denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extention at 72°C for 45 sec, repeated for 30 cycles; and a final extention at 72°C for 5 min). The PCR products were separated in 1.5% agarose gels stained and visualized under UV light.

Statistical analysis: Statistical analysis was performed using SPSS software version 24. P Values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

During the study, started in November 2017, 120 *S. aureus* strains were isolated from patients referred to four hospitals in the north of Iran (Amol and Babol, Mazandaran province), including 86 strains as MRSA and 34 strains as MSSA. The highest resistance belonged to penicillin (92.5%), followed by cefoxitin 71.66%, erythromycin 46.66%, clindamycin 42.5%, ciprofloxacin 23.3%, rifampicin 22.5%, trimethoprim/sulfamethoxazole 19.16%, minocycline 19.16%, levofloxacin 19.16%, and nitrofurantoin 4.16%. All strains were susceptible to vancomycin and linezolid. Forty-seven (39.16%) strains were distinguished as multiple drug resistant (resistant to more than three antibiotic classes). As a result of the D-test, resistance to erythromycin among 120 *S. aureus* strains involved 47 (39.16%) $cMLS_B$, 7 (5.8%) $iMLS_B$ and 2 (1.7%) MS_B. The frequency of *ermA*, *ermB*, *ermC*, and *msrA* genes was 22.5%, 24.16%, 80.83%, and 41.66%, respectively. There was a significant relationship between the presence of *erm* genes and erythromycin resistance phenotypes (P<0.05) (Table 2) and also a meaningful correlation between resistance genes and MRSA strains was observed (P<0.05) (Table 3). The correlation between MDR strains, resistance genes, and erythromycin resistance phenotypes are illustrated in Tables 4 and 5. Most of the erythromycin-resistant strains carried two or more resistance genes in which four strains harbored all four detected genes. A single resistance gene was shown by only 10 strains. However, four strains with an erythromycin resistance phenotype did not have any detected genes (Table 6).

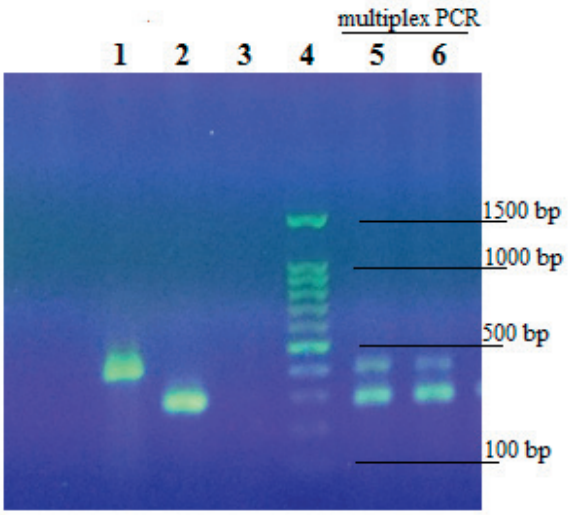

Fig. 1. Electrophoretic banding patterns of the amplified products of the two genes by multiplex PCR. Lanes 1 and 2: Positive control, *ermB* (416 bp), *ermC* (299 bp). Lane 3: Negative control (without template DNA). Lane 4: 100 bp DNA ladder. Lanes 5 and 6: Multiplex-PCR amplification of *ermB* (416 bp) and *ermC* (299 bp) genes.

Table 1. The sequence of primers and the PCR programs for detection of *mecA*, *ermA*, *ermB*, *ermC* and *msrA* genes

Reference	Gene	Amplicon size (bp)	Primer sequence (5′-3′)	Initial denaturation (C°–min)	Denaturation (°C –Sec)	Annealing (°C –Sec)	Extension (°C –Sec)	Final Extension (°C–min)
[9]	mecA	533bp	F:AGTTCTGCAGTACCGGATTTGC R: AAAATCGATGGTAAAGGTTGGC	94- 5	94 - 60	50- 30	72- 90	72-5
					30 cycles			
[43]	ermA	645bp	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAGT	95- 3	95 - 30	55- 30	72- 45	72-5
[44]	ermB	416bp	F: TAACGACGAAACTGGCTAAAA R: ATCTGTGGTATGGCGGGTAAG					
[45]	ermC	299bp	F: AATCGTCAATTCCTGCATGT R: TAATCGTGGAATACGGGTTTG		30 cycles			
[46]	msrA	940bp	F: GGCACAATAAGAGTGTTTAAAGG R:AAGTTATATCAGAATAGATTGTCCTGTT					

Table 2. The relation between the presence of *erm* genes and erythromycin resistance phenotypes

Genes	No. (%) in $cMLS_B$ (n=47)	No. (%) in $iMLS_B$ (n=7)	No. (%) in MS_B (n=2)	No. (%) in S (n=64)	P- value
ermA	23 (48.9)	1 (14.3)	1 (50)	2 (3.1)	P < 0.001
ermB	18 (38.3)	0	1 (50)	10 (15.6)	P = 0.01
ermC	43 (91.5)	5 (71.4)	1 (50)	48 (75)	P = 0.09
msrA	20 (42.5)	2 (28.6)	2 (100)	26 (40.6)	P = 0.3

$cMLS_B$ constitutive resistance phenotype (macrolide, lincosamide and streptogramin B resistance phenotype); $iMLS_B$ induction resistance phenotype; MS_B macrolides-streptogramin B resistance phenotype; S, susceptible; p-values were obtained from Fisher's exact test, where appropriate.

Table 3. The correlation between genes and both MRSA and MSSA strains

Genes	No. (%) in MRSA (n=86)	No. (%) in MSSA (n=34)	P- value
*er*mA	26 (30.2)	1 (2.9)	P = 0.001
ermB	24 (27.9)	5 (14.7)	P = 0.1
ermC	74 (86)	23 (67.6)	P = 0.037
*msr*A	38 (44.2)	12 (35.3)	P = 0.5

MRSA, methicillin-resistant *Staphylococcus aureus;* MSSA, methicillin-susceptible *Staphylococcus aureus* P-values were obtained from Fisher's exact test, where appropriate.

Table 4. The correlation between genes and MDR strains

Genes	No. (%) in MDR (n=47)	No. (%) in Non MDR (n=73)	P - value
mecA	44 (93.6)	(57.5) 42	P < 0.01
ermA	23 (48.9)	4 (5.5)	P < 0.01
ermB	18 (38.3)	11 (15)	P = 0.005
ermC	43 (91.5)	54 (74)	P = 0.018
msrA	21 (44.7)	29 (39.7)	P = 0.5

MDR, multidrug resistant; $cMLS_B$ constitutive resistance phenotype (macrolide, lincosamide and streptogramin B resistance phenotype); $iMLS_B$ induction resistance phenotype; MS_B macrolides-streptogramin B resistance phenotype; S, susceptible p-values were obtained from Fisher's exact test, where appropriate.

It should be mentioned that in this study the sequenced strains were selected by their certain characteristics, including MDR strains, having more than one resistance genes. Therefore, nine strains were registered at the NCBI GenBank with the following accession numbers: MG778115 and MG778116 for *ermA* gene, MG778119, MG778118 and MG778117 for *ermB* gene, MG787086 and MG787087 for *ermC* gene, MG787089 and MG787088 for *msrA* gene.

S. aureus is known as one of the most common nosocomial pathogen in recent decades, due to various causes, including the inappropriate use of antibiotics, which leads to the acquisition of resistance genes [10]. The ability of changing $iMLS_B$ to the $cMLS_B$ phenotype, occurring during treatment, as well as the easy transfer of resistance genes to MLS_B antibiotics, which creates cross-resistance (by mobile genetic elements such as transposons and plas-

Table 5. The correlation between erythromycin resistance phenotype and MDR strains

Phenotype (total=120)	No. (%) in MDR (n=47)	No. (%) in Non MDR (n=73)
cMLSB (47)	46 (97.8)	1
iMLSB (7)	0	7
MSB (2)	0	2
S (64)	1	63 (86.3)

MDR, multidrug resistant; p-values were obtained from Fisher's exact test, where appropriate.

Table 6. Distribution of the *erm* (*A, B, C*) genes and the *msrA* gene among 120 staphylococcal isolates

Genes	Erythromycin Resistant (n=56)						Erythromycin Susceptible (n=64)	
	$cMLS_B$(n=47)		$iMLS_B$ (n=7)		MS_B (n=2)		S	
	MRSA (n=44)	MSSA (n=3)	MRSA (n=4)	MSSA (n=3)	MRSA (n=2)	MSSA (n=0)	MRSA (n=36)	MSSA (n=28)
ermC	5	1	1	2			11	6
ermC + *msr*A	4		1	1			11	8
ermC + *msrA* +*ermB*	4	1					4	2
ermC + *msrA* + *ermA*	8						1	
ermC + *msrA* + *ermA* + *ermB*	3				1			
ermC + *ermB* + *ermA*	4							
ermC + *ermA*	7		1					
ermC + *ermB*	5	1					3	1
ermA	1							1
msrA					1			
None	3		1				6	10

$cMLS_{B,}$ constitutive resistance phenotype (macrolide, lincosamide and streptogramin B resistance phenotype); $iMLS_{B,}$ induction resistance phenotype; $MS_{B,}$ macrolides-streptogramin B resistance phenotype; S, susceptible; MRSA, methicillin-resistant *Staphylococcus aureus;*MSSA, methicillin-susceptible *Staphylococcus aureus*.

mids), have terminated to an increase of MLS_B antibiotic resistance among *S. aureus* strains [11, 12].

Regarding the importance of using clindamycin as a choice drug for the treatment of skin and soft tissue infections caused by *S. aureus* and other objects, an exact diagnosis of MLS_B resistance types is critical. The reason is that treating patients with $iMLS_B$ resistant strains with clindamycin leads to constitutive phenotype and treatment failure [13]. In our study, the high prevalence of MRSA strains was significant (71.66%), which is higher than most studies in Iran [14-16]. Conversely, the prevalence of MRSA strains in Zarei *et al.* (87.3%) and Goudarzi *et al.* (88.9%) was higher than the present research [17, 18]. Whereas, there are different reports on the incidence of MRSA in other countries such as Periera *et al*. in Brazil (21%), Stefanaki *et al*. in Greece (38.9%), Correa-Jiménez *et al.* in Colombia (47.46%), etc. [17-23]. The differences in the frequency of MRSA in geographical areas depend on various factors, for example, the monitoring of infection, health facilities and the pattern of antibiotic usage [24].

Our study is the first report on MLS_B resistance and frequency of the related genes in the north of Iran in which resistance to erythromycin was 46.6%, which is similar to, Sedaghat *et al.* in Isfahan and Mansouri *et al*. in Kerman [25, 26]. According to studies conducted in 2014, the lowest and highest prevalence of erythromycin resistance was reported in Colombia (13.04%), Canada (29%), Nepal (54.4%) and Turkey (56.7%) respectively [21, 27-29]. Consistent with the mentioned reports, the present rate of erythromycin resistance was close to Nepal's and Turkey's. In India in 2015, resistance to erythromycin was 39.41%, by including 71% MRSA strains [30]. Conversely, in Greece in 2017, this data was reported at 34.7% and MRSA strains were 60% [20]. It should be mentioned that in our isolates, 58% of the MRSA strains were resistant to erythromycin.

In the present study, our results show that the most common resistance phenotype to erythromycin is constitutive resistance phenotype ($cMLS_B$), which demonstrates concurrent resistance to erythromycin and clindamycin. The percentage of $cMLS_B$ phenotype in our report is more than the similar studies performed

in Iran, by Ghanbari *et al*. in Isfahan and Seifi *et al*. in Mashhad [14, 31]. The common phenotype in India was MS_B [32], while the $iMLS_B$ template phenotype was reported to be 27.5% and 31.5% in Turkey and Serbia [33, 34].

Our reported data had a similar pattern to Pereira *et al*. and Mokta *et al.* [19, 30]. In Brazil, $cMLS_B$, $iMLS_B$, and MS_B were 32.2%, 8.47%, and 5%, respectively, and the susceptibility of both erythromycin and clindamycin was 50.8%, which is close to our results. These differences in the pattern of MLS_B resistance can be attributed to various drug usage guidelines in Iran and other countries [13]. In comparison to different regions like Turkey and Japan, the inducible resistance was higher than constitutive resistance phenotype [33, 35].

In various studies, both *ermC* and *ermA* genes are the most common erythromycin resistance genes [36, 37]. In Fasihi *et al.* [38] and Sedaghat *et al.* [25] in Iran, the frequency of the *ermC* gene was 20.5% and 35.2%. In our report, the prevalence of the mentioned gene was considerably higher i.e. 80.83%, which indicates the high geographic diversity of macrolides resistance genetic support.

In Tunisia, the most abundant genes were *ermA* and *ermB* [39], in Turkey *ermA* and *ermC* [23], but in Brazil [19], similar to our study, the *ermC* gene was the highest one. Moreover, the frequency of *msrA* gene (40.83%) was higher than in other similar studies in Iran [18, 25, 38].

The *msrA* gene in *S. aureus* strains is responsible for the mechanism of the efflux pump, which leads to the resistance to macrolides and streptogramin B. The high prevalence of resistance genes can be attributed to the easy transfer of genes transmitted by extra-chromosomal genetic elements such as plasmids and transposons and this phenomenon has a critical role in the incidence rates of genes in diverse geographical regions. In this research, there was a significant relationship between phenotypic and genotypic resistance to erythromycin, accordingly, only 4 (7%) of isolates were erythromycin resistant, caused by other mechanisms than the presence of *ermA, ermB, ermC* and *msrA* genes. However, in a number of reported studies, no correlation between the presence of genes and resistance phenotypes was observed [38, 39]. In this report, two main hypotheses are described, the multi-gene resistance to erythromycin and small plasmids carriers. Despite the fact that they harbored the erythromycin resistance genes targeted in our study, 48 (40%) of strains did not show any resistance to erythromycin when tested by the phenotypic method. In the present research, all of the strains were examined but in similar studies, only erythromycin resistant strains were genotypically analyzed [19, 34, 40].

Attempts to cope with both bacterial infections and drug resistance encouraged scientists to revive the obsolete drugs. Recent studies have shown that penicillin susceptibility may be in a period of renaissance [41, 42].

In a study published by Cheng *et al.* in 2016, more than a quarter of patients with MSSA-related bacteremia could potentially be treated with injectable penicillin, which may have more drug benefits than other β-lactams. In this study, which was performed on MSSA clinical strains, susceptibility to penicillin was 28%. Concomitantly, penicillin-resistant strains showed greater resistance to other antibiotics, but statistically, the significant association was observed only with erythromycin [27].

In our study, 23.5% of the MSSA strains were susceptible to penicillin and penicillin-resistant strains were also resistant to other antibiotics, furthermore, there was a statistically significant relationship between resistance to penicillin, erythromycin (P=0.03) and also clindamycin (P=0.01). Therefore, penicillin offers more benefits in comparison to other first-line treatment antibiotics, especially in regard to the limited range of activity and the low risk of *Clostridium difficile* - associated diarrhea (CDAD) [27].

CONCLUSION

According to the high prevalence of MRSA strains and the relation between the existence of antibiotic resistance genes, adopting strategies for infection prevention and control are vital and necessary. In order to control the spread of antibiotic resistance, it is inevitable to obtain accurate information about the susceptibility

pattern; therefore, the D-test is a beneficial way to reach this aim. Also, the correct phenotypic and genotypic diagnosis of strains is a crucial approach to decrease the antibiotic resistance rates, especially in health care units.

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