Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 1728 - 1736
Received 15 April 2021; Accepted 05 May 2021.

Comparative Study for Biofilm Formation between Clinical Methicillin-Resistant and Methicillin Susceptible *Staphylococcus Aureus* Isolates

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Key words :*Staphylococcus aureus*, Biofilm ,*ica ABC* , PCR , MRSA ,MSSA

Abstract

One hundred seventy five samples were collected, included, blood ,urine,nasal,ear,sputum and burn from patients in Teaching General Hospital in Baquba city during the period (March to July 2018).The phenotypic and biochemical tests showed that 55 isolates were *Staphylococcusaureus* bacteria ,21(38.18%) MRSA and 34(61.82%) MSSA.The result of antibiotic resistant were resistance to Pencillin 55(100%),Azithromycin 42(76.36%) and Gentamycin 32(58.18%),followed by Tetracycline 29(52.72%),Ceftaroline 26(47.27%), Ciprofloxacin 11(20%) , 6(10.90%) for each of Rifampin and Chloramphenicol ,the least resistance was to Doxycycline 2(3.63%) followed by Linezolid 3(5.45%). We found that all of *S. aureus* isolates were produced slime layer by CRA plates 55(100%),but 17 (80.95 %)of MRSA isolates were black colonies , (9.52%) for each of very black and weak black.In MSSA isolates 3(8.82%)were very black ,21(61.76%)black,9(26.47%)weak black and 1(2.94%)was red. The results of microtiter plates assay showed that all of *S. aureus* isolates 55(100%) were attached but in several amount . Attachment of MRSA isolates were 3(14.28%)strong,15(71.42%) of isolates were moderate and 3(14.28%) isolates were weak,butinMSSA isolates 5(14.70%)strong,19(55.88%)moderate and 10(29.41%)weak. The frequency of *icaA,icaB,icaC* genes in MRSA and MSSA isolatesisolateswere (100%)

Introduction

Staphylococcus aureus is one of the pathogenic bacterial species belonging to the family of Staphylococcaceae, which is divided into two main groups depending on the ability to produce the coagulase enzyme [(1)].*S. aureus* is a facultative anaerobes bacteria ,that is commonly involved in community- acquired infection diseases and nosocomial infection diseases , including abscesses, impetigo, folliculitis, cellulitis, boils and a number of rarer but more serious diseases such as infective endocarditis , necrotizing fasciitis ,pyomyositis, necrotizing pneumonia,osteomyelitis, septic arthritis [(2)].*S.aureus*is gram –positive bacteria cause wide domain of bacterial infections in humans, This related to their ability to produce many virulence factors include toxins and extracellular enzymesand involved in the way of giving this bacterium the chance to attache to surfaces and tissues anddisease develops , leading to harmful toxic effects in the human[(3)].

Virulence factors include :capsular polysaccharides, cell-surface factors such as microbial surface components recognizing adhesive matrix molecules, and staphyloxanthin and secreted factors assuperantigens, pore-forming toxins exoenzymes such as lipases, nucleases, proteases, hyaluronidase, and staphylokinase[(4)].

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many of human infections are caused by the ability ofS.aureus to develop biofilms, Biofilm is the growing of bacteria on the different surfaces and enclosed within a DNAandseveral proteins,exopolysaccharide (EPS)[5].

Biofilm formation requires the adhesion of bacteria to a surface followed by cell-cell adhesion, forming the several layers of the biofilm ,The intercellular adhesion (*ica*)locus consists of *icaADBC* operon which contains four genes encoding to main proteins that required for the generation of PIA [6].

The *icaA* gene is encoding the N-acetylglucosamyltransferase. Co- expression of the *icaD* gene with this gene increases the activity of this enzyme. The *icaB* is the deacetylase responsible for the de-acetylation of PIA. The *icaC* encodes the transmembrane protein, which hypothetically plays a role in secretion and elongation of the growing extra- cellular polysaccharide[7].

The aim of current studywas to compare the accurancy of biofilm formation detection methods whether it is phynotypic or genotypic and the extent ofanumberof antibiotics on MRSA and MSSA.

Material and methods

Bacterial isolation and identification

One hundred seventy five samples were obtained from different clinical sources from (urine (45),blood(40), ear(30),nasal(25),burns(24),and sputum(11) from Cosultant Clinic of BaqubaEducational Hospital from March 2018 till July 2018.Isolated bacteria were identified as S.aureus by using conventional microbiological tests ,gram stain , oxidase,catalase,growth on mannitol salt agar ,coagulase assay ,DNase test .Biochemical tests were performedby using API 20 Staph system and Vitek 2 compact which provides a typicalidentification.All isolates were stored at -20 °C for subsequent studies.

Antibiotic susceptibility testing and detection of MRSA

For MRSA detection all of the identified S.aureus bacteria were screened by test their resistance to Oxacillin(1µg) discs, S.aureus ATCC 25923 strain was used as the control in the testing of antibacterial susceptibility test[8].

The test of antibiotic susceptibility was performed as recommended by Clinical LabboratoryStandared Institute (CLSI, 2018).Different disks were used in this test ,included ,Azthromycin (15 µg),Ciprofloxacin (5µg),Rifampin (5µg),Linezolid (30µg),Chloramphenicol (30µg),Penicillin (10µg),Gentamicin (10µg),Cetaroline(30µg),Tetracycline(30µg) andDoxycycline (30µg).Oxacillin (1µg).

Phenotypic detection of biofilm production

A-Congo Red Method

Detection of qualitative of biofilm formation was performed byculturing the isolates on Congo Red Agar (CRA) plates containing 0.8 g/L of Congo Red dyeHimedia(India) and 36 g/L saccharoseOxoid (England). Isolates were inoculated on CRA plates and incubated aerobically for 24 h at 37 ◦C. Growth of black colonies was slime producing isolates, while non-slime (red colonies producers)[9].

A-Microtiter plate (MTP) method

As previously described this method was followed . Briefly, the wells of microtiter plate were filled with 180 µltrypticase soy broth (TSB) supplemented with 1% glucose. 20 µl of the bacterial culture with turbidity equal to 0.5 McFarland standards was added to individual wells of sterile polystyrene

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96-well, flat- bottomed tissue culture plates. After incubation to 24 h at 37ºC, the cells were decanted and the wells was washed three times with phosphate buffer saline solution. biofilms formed cells fixedby(methanol)for20min,then added150µl of(0.1%) Crystal violet(CDH India) to each well. After 15 min, the plate was washed and left to dry. TheCrystal violetdye bound to the adherent cells was dissolved with 1mL of 95% ethanol per well, and the optical densities (OD) of plates were read at (490) nm by using a microtiter-plate reader. Each test was performed in triplicate. As a negative control, TSB medium was used to determine background OD. Optical density cut-off (ODc) was determined.The adherence ability of tested isolates and classified to four categories:,strongadherent(OD>0.33296),(4×ODc<OD),moderate adherent (OD≤0.33296)(ODc<OD ≤4×ODc), weak adherent (OD ≤0.16648)(ODc< OD≤ 2×ODc)and non-adherent (OD≤0.08324) (non biofilm formation(OD ≤ ODc) (negative control)[10].

Molecular Detection of biofilm formation

Polymerase chain reaction (PCR)

All isolates of bacteria were molecular screening of *icaABC* genes using PCR method. PCR amplification was performed by using an ABIO pure,USA . Amplification program consisted of initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec and extension at 72 °C for 30 sec with a final step of 72 °C for 7 min. Amplification program for icaB consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealingat52°Cfor30secandextensionat72°C for 90 sec with a final step of 72 °C for 10 min .The PCR products were analyzed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide and visual inspection compared 100 base plus DNAmarker. For PCR amplification ,The primers and expected sizes of the amplification product are listed in(table 1).

DNA Extraction

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure Extractionas the following steps: For pellet calls, 1ml of overnight culture for 2min at 13000 rpm. Supernatant then discarded. For gram-positive bacteria,l00µ l Nuclease-free water plus 10 solution added to pellet and vortex.Incubated in the water bath for 30min at 37oC.After incubation, samples centrifuge for 2min at 13000 rpm. Supernatant then discarded.For protein digestion and cell lysis, 20µl of Proteinase Buffer BL was added to sample then the tube mixed vigorously using vortex andIncubatedat 56˚C for 30 min, for fart .From absolute ethanol addedtothesample, 200µlpulse-vortex tomixthe samplethoroughly.All of the mixtures were transferred to the mini column carefully, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replace with a new one.From Buffer BW 600µladdedwastotheamini column, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm) and the collection tube was replaced with a new one.From Buffer TW 700µl was applied. Cent rpm. The pass-through was discarded and the mini column was reinserted back into the collection tube.The mini-column was Centrifuge at full speed (>13,000 x g) for 1 min to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube.100µl addedandcubated for 1min at room temperature, then centrifuge at 5,000 rpm for 5min[11].

Table 1: primers and expected sizes of the amplification product

gene	primer	sequences	size	Ref.
icaA	*icaA*-F	ACACTTGCTGGCGCAGTCAA	188	12

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	icaA-R	TCTGGAACCAACATCCAACA		
icaB	*icaB*-F *icaB*-R	TCCTTATGGCTTGATGAATGACG CTAATCTTTTTCATGGAATCCGT CC	190	7
icaC	*icaC*-F *icaC*-R	ATGGGTTATAACTACGAACGTG CGTGCAAATACCCAAGATAAC	192	7

Results and Discussion

Identtification of *Staphylococcus aureus*

After collection ofsamples we diagnosed the *Staphylococcusaureus*that it gave a positive grown on mannitol salt agar ,in microscopy it appeared as cocci shape ,clusters (like grape),non motile,positive gram stain, biochemical tests showed positive result to catalase and coagulase,negative to oxidase and andol,in addition to adopting API20 Staph and Vitek 2 .

In the 55(31.42%)clinical isolates studied of *S. aureus*from 175 samplewere, 13(23.63%)fromear,12(21.81%)from blood,10(18.18%)fromnasal,9(16.36%)from urine,6(10.90%)from sputum and 5(9.09%) from burns as shown in table 2 .Astudy in Iraq found that *S. aureus* isolation from various clinical origin was(43.7%)[13].But an other study in Mexico city they recorded a lees isolation percent of *S. aureus*,it has been just 18.6% [14].

Table 2 Distribution of S.aureus isolates according to source of specimens

Source of specimens	MRSA N(%)	MSSA N(%)	Total
Urine	5(9.09%)	4(7.27%)	9(16.36%)
Blood	3(5.45%)	9(16.36%)	12(21.81%)
Ear	6(10.9%)	7(12.72%)	13(23.63%)
Nasal	3(5.45%)	7(12.72%)	10(18.18%)
Burn	1(1.81%)	4(7.27%)	5(9.09%)
Sputum	3(5.45%)	3(5.45%)	6(10.90%)
Total	21(38.18%)	34(61.82%)	55(31.42%)

The result of MRSA screening were 21(38.18%)of MRSA isolates and 34(61.82%) MSSA .In an other study MRSA isolation recorded (70.83%)and the resistsnce of Linezolid was (4.5%) (Hassan *et.al*.,2017). Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common pathogen that affect in hospitalized patients[15].

Suscepyibility of *S. aureus* isolates to antibiotics

In this study we used 10 antibiotics for 55 clinical isolates of *S.aureus*.The highest resistance was to Pencillin 55(100%),Azithromycin 42(76.36%) and Gentamycin 32(58.18%),followed by Tetracycline 29(52.72%),Ceftaroline 26(47.27%),Ciprofloxacin 11(20%) ,6(10.90%) for each of Rifampin and Chloramphenicol ,the least resistance was to Doxycycline 2(3.63%) followed by Linezolid 3(5.45%).the most of MRSA isolates were multidrug resistant (MDR) as shown in(table 3).
The resistance of *S. aureus*against antibiotics developsand in the same time when the mortality andmorbidity rates increase or the duration of treatment is prolonged[16].

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Table 3 Antibiotic profiles of MRSA and MSSA resistant by disk diffusion method

Antibiotics	MRSA n(%)	MSSA n(%)	P value
pencillin	21(100)	34(100)	0.08
ceftaroline	10(47.61)	16(47.05)	0.23
gentamicin	21(100)	11(32.35)	0.07
azithromycin	21(100)	21(61.76)	1.00
tetracycline	15(71.42)	14(41.17)	0.83
doxycycline	5(23.80)	0	0.00
ciprofloxacin	10(47.61)	1(2.94)	0.007**
rifampin	6(28.57)	0	0.00
linezolid	0	3(8.82)	0.00
chloramphenicol	6(28.57)	0	0.00
Total	21(100)	34(100)	0.08

** ***P<0.05,0.01***

Phenotypic detection of biofilm formation

Biofilm formation by *S.aureus* play an important role in the adhesion activity and cause a various infections in human [(17)].CRA was used as a phenotypic test of biofilm forming detection . We found that all of *S. aureus* isolates in phenotypic methods by CRA plates 55(100%) were produced slime layer,but17 (80.95 %)of MRSA isolates were black colonies in 24 h,2(9.52%) for each of very black and weak blackIn MSSA isolates 3(8.82%)were very black ,21(61.76%)black,9(26.47%)weak black and 1(2.94%)was red(table3).In Abbasi and Zamanzad article they found that 12 (46.1%) isolates from 26 of MRSA were produce black colonies and the other strains of*S.aureus*were 1(3.8%) red,7(26%)almost black and 6(23%)very black[(18)],while astudy in India about MRSA isolated from pharyngitis patients they found that 28.6% were very black ,36.5% black ,12.7% red black and 22.2% were red.A study in Turkey they found that 15(46.9%)were black colonies [(19)].

The results of microtiter plates assay showed that all of*S. aureus* isolates 55(100%) were attached but in several amount . Attachment of MRSAisolates were 3(14.28%)strong,15(71.42%)of isolates were moderate and 3(14.28%) isolates were weak,but inMSSA isolates 5(14.70%)strong,19(55.88%)moderate and 10(29.41%)weak(table 3).In another study found that 3(6%)of *S.aureus* isolates were strong ,15(30%) were moderate and 32(64%) were weak[(5)].In study of Mirzaee*et al.*the result of microtitre plate assay showed that 38.7% of the isolation were able to form biofilm strongly[(7)],butthestudy ofRajabi*et.al*. revealed that (9%) of the *S.aureus* isolates forms biofilm strongly ,(26%) moderate and (48%) weak[(20)]. That differences between various studies might be due to heterogeneity in the origins of the isolates such as environmentalconditionand source of isolation[(21)].

Table 4:Slime production ,adhesion formation by MRSA and MSSA

Method	Slime/adhetion	MRSA n(%)	MSSA n(%)	p.value
Congo red agar	Very black	2(9.52)	3(8.82)	0.655
	black	17(80.95)	21(61.76)	0.516
	Weak black	2(9.52)	9(26.47)	0.035*
	red	0	1(2.94)	0.00
Microtiter plate	Strong	3(14.28)	5(14.70)	0.48
	modirate	15(71.42)	19(55.88)	0.493
	weak	3(14.28)	10(29.41)	0.05*

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	none	0	0	0.00
Total		21	34	0.00

**** P<0.05***

Molecular detection of biofilm formation genes (*ica ABC*)

We used PCR method to identify biofilm formation genes(*ica A ,icaB, icaC*). The size of products of the three geneswere(188, 190,192)bp respectively . All of the primers that used in our study showed specificity with a single band.The frequency of *icaA,icaB,icaC* genes in MRSA and MSSA isolatesisolateswere (100%)(Figure 1,2,3),this finding agreement with Uribe-Garcia *et.al*.stady, they recorded a saim percent for *icaA,B* gene in MRSA isolated from periodontal lesions[14], another study in America came out with a similar result as it was reported last year that the *icaAB* genes were present in all isolates ,but *icaC* was 99%[16]. In Contreras *et.al*.they found that*icaA* gene frequency was 96.3%.. On the other hand[22], Hassan *et.al*.reported a lower prevalence in *icaA* gene it was only81.78%[3]. A In a somewhat close proportion recorded by astudy compared between MRSA and MSSA isolates the result in MRSA of the*icaA*were (76%),*icaB* (69%) and *icaC* was(64%) , in MSSA *icaA* (71%) , *icaB* (54%) and *icaC* (69%) [6]. In this study, we found that the ability to form biofilm is higher in bacteria with multiple resistance to antibiotics, which increases the likelihood of treatment failure in chronic infection with bacteria[11].Although,the isolates of *S. aureus* has multipil mechanisms to invasive host cells and It has been shown that *S.aureus* possess many mechanisms and means of antibiotic resistance in addition to forming a biofilm Regardless of it being MRSA or MSSA[23,24] .

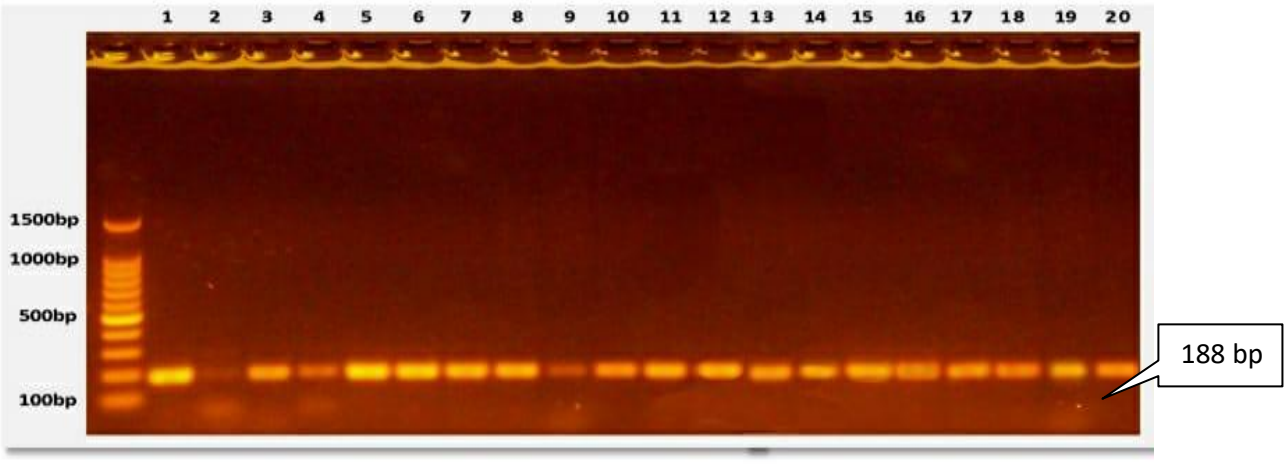

Figure 1:Gel electrophoresis of amplified products of *icaA* gene. First Line isDNA ladder 100bp line(**1,2,3**, **4**,**56**,**7**, **8**, **9**, **10) MRSA** isolates, (**11,12,13,14,15,16,17,18,19,20) MSSA** isolatesall of them are positive resalt

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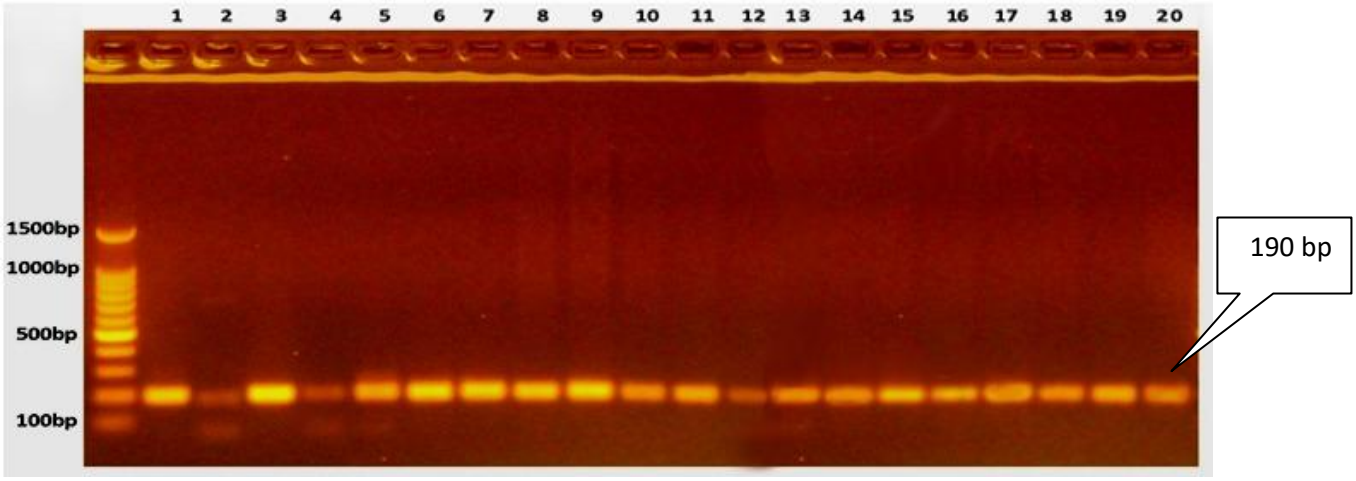

Figure 2:Gel electrophoresis of amplified products of *icaB* gene. First Line isDNA ladder 100bp line(**1,2,3**, **4**,**56**,**7**, **8**, **9**, **10)MRSA** isolates, (**11,12,13,14,15,16,17,18,19,20) MSSA** isolatesall of them are positive resalt

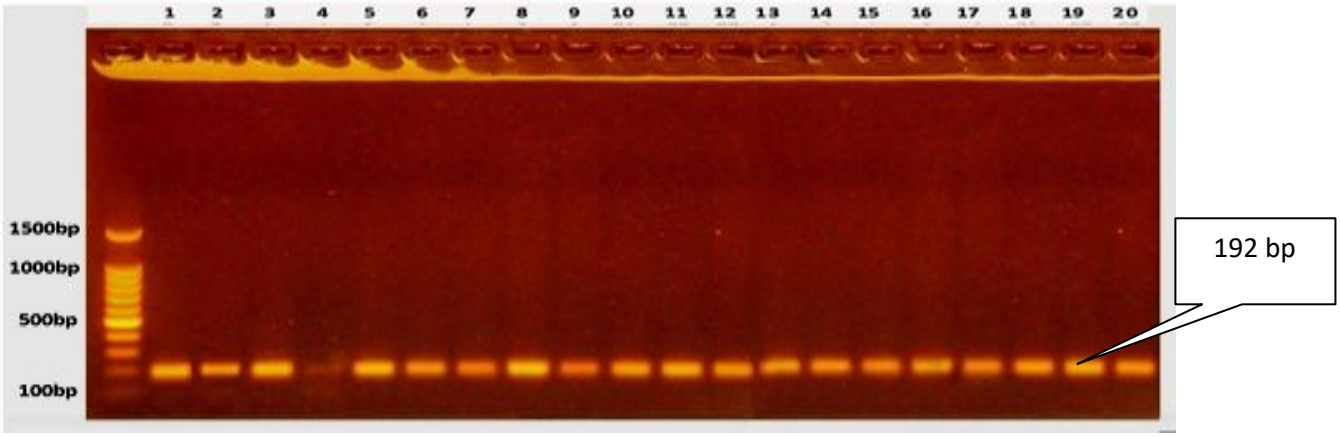

Figure 3:Gel electrophoresis of amplified products of *icaC* gene. First Line isDNA ladder 100bp line(**1,2,3**, **4**,**56**,**7**, **8**, **9**, **10**)**MRSA** isolates,(**11,12,13,14,15,16,17,18,19,20)MSSA** isolates all of them are positive resalt

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