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Gene expression of agr system in Staphylococcus aureus and it is relation with antibiotic resistance

Jilan Ahmed Shakur[1*], Rasmiya Abd Aburesha[2]

[1]Collage of science, microbiology, university of Baghdad /Iraq
Email: jelianahmed5@gmail.com

[2] Collage of science, microbiology, university of Baghdad /Iraq
Email: Rasmiaabd8@gmail.com

***Correspondence author:** Jilan Ahmed Shakur (jelianahmed5@gmail.com)

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Abstract

Background: A commensal bacterium has ability to cause a wide range of infections is Staphylococcus aureus device-related infections by regulator system called accessory gene regulator (agr). Methods: Identification S. aureus that isolated from urine clinical sample has been done according to the cultured on the mannitol salt agar and biochemical tests, then confirm the identification by amplification 16srRNA gene through conventional PCR and detection agr (I, II, III, IV) gene by multiplex PCR then expression of agr gene with antibiotic and without antibiotic by real time (qPCR). Result: VThirty isolates of S. aureus diagnosed and (76,70%) percentage of this isolates resistance to amoxicillin and found all this isolates have agr gene that related with antibiotic resistance. Conclusion: 16sRNA polymerase chain reaction is confirmation method for distinction S. aureus strain from other type of bacteria and agr gene have important role in antibiotic resistance.

Keywords:

S. aureus, 16srRNA gene, agr system, antibiotic resistance

Staphylococcus aureus is a commensal bacterium has ability to cause different type of diseases like skin-soft tissue and endocarditis, regulator for these diseases is the accessory gene regulator (agr) the main quorum sensing system of S. aureus cells (Miller and Gilmore, 2020).

The agr system size is 3.5 kb activates number of toxins and degradative enzymes, components of co-regulated through the cross-activation of divergent promoters P2 generate RNAII and P3 generate RNAIII. RNAII contains of four genes include agrII, agrIV, agrIII and agrI, agrII gene product is a transmembrane endopeptidase responsible of the introduction of the thiolactone modification and C-terminal cleavage and agrIV transcript encodes a peptide precursor of the extracellular quorum signal of agr called auto inducing peptide (AIP) where as agrIII and agrI genes encoded a two-component to signal transduction system to histidine kinase sensor and agrIII a transmembrane protein phosphorylated upon the binding of AIP its associated response regulator agrI , Upon being activated by agrIII-dependent phosphorylation, agrI binds to the P2 promoter region for RNAII and the P3 promoter region for RNAIII, as well as the promoters controlling expression of the PSMα and PSMβ peptides, RNAII covers the agrI,II,III,IV operon involves in secretion, maturation, export of auto-inducing peptide (AIP) that activates a classical two-component signal transduction system (TCSTS) and is regulated by P2 promoter. RNAIII encodes δ-hemolysin (hld) is the effector molecule of agr operon and is regulated by P3 promoter agr can also be activated by a variety of other regulators, such as SarA and environmental factors such as glucose concentration or pH (Le and Otto, 2015: Paulander et al ., 2018). agr gene a part of human diseases and S. aureus resistance to antibiotic treatment, this is associated with antibiotic resistance genes spread (Saedi et al., 2020).

Material and method

Sample collection

Collected 150 clinical samples during the period (February to May, 2022) used sterile swap for taken sample from tonsil and sterile cup for collected urine sample all they from the people return to Tuz general Hospital in the Salah al-Din Governorate and from patient broadcasts in hospitals.

Bacterial identification

Used two methods for S. aureus identification, first methods , primary identification it is cultured all isolates on the a selective medium (Mannitol salt agar) that differentiate S. aureus through the fermentation of mannitol , microscopic examination by Gram's stain, taking smears of samples and investigated through an oil immersion microscope lens the cells appears under light microscopes as Gram positive cocci clustered mostly in grape-like irregular clusters circular in clusters , single, paired or short chains because of S. aureus bacteria divided in three planes during binary fission (Gnanamani et al., 2017 :Chotigarpa et al., 2018). A biochemical test (catalase test , coagulase test , oxidase, motility test and hemolysis test), Catalase test was distinguished by transport some pure colonies of bacterial isolates taken from mannitol salt agar media to clean glass slide by sterilized loop, then add few drops of 3% H_2O_2 on it air bubbles production is refer to positive result that evidence enzyme production (Omran & Hussein, 2019). In coagulase test evaluated to distinguished free plasma coagulase enzyme that lead to coagulation product from interaction between blood and S. aureus bacteria, which indicates positive result While the clotting did not emerge at room temperature that refer to negative result (Rakotovao-Ravahatra et al., 2019).

Second methods confirmed primary identification by detection 16srRNA through PCR (Polymerase chain reaction) include two steps.

1-Extraction and determine DNA of Staphylococcus aureus:

Genomic material (DNA) of S. aureus extraction according to Geneaid Biotech kit instruction with add some modification include uses agarose gel electrophoresis then stained it by ethidium bromide that resolved for DNA extracted presence , to keep the extracted DNA must be conserved at - 20 C° until uses in PCR process (Onasanya et al., 2003) .

According to (Al-Noaami, 2014) the extracted DNA for all one of S. aureus isolates were mixed with the loading buffer together (DNA: loading 7/2 v/v) then loaded inside specific wells of the gel.

2-16srRNA gene amplification

16srRNA gene usually used as identify, classify and quantify unites that was coded for the small subunit (SSU) of the bacteria species ribosome have many factors make it importance target for diagnosis different type of prokaryotes microorganism like bacteria and archaea (Cox et al., 2013).

Used specific primer (Gumaa et al., 2021) in PCR program according the TRANS protocol to detection 16srRNA

Table (1) PCR reaction components for detection 16srRNA according to Trans protocol

Primer	Type of reaction	Master mix volume	Volume of F-primer (10pmol/ml)	Volume of R-primer (10pmol/ml	Nuclease free water	DNA	Final volume
16s rRNA	uniPCR	10	1	1	6 ml	2 ml	20 ml

Table (2) PCR program to amplify 16srRNA

Steps	oC	Minute -Second	Cycle	Product band
premier denaturation	95 °C	4 Minute	1	257
Denaturation	95 °C	4 Minute	30	
Annealing	53 °C	45 Second		
Extension	72 °C	45 Second		
Final extension	72 °C	7 Minute	1	

Antimicrobial resistance test

Detection and determination S. aureus antimicrobial resistance of each isolate, antimicrobial resistance testing was executed by the disc diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2022 on (MHA) Muller Hinton agar (CLSI,2022; Sastry et al., 2021). Amoxicillin (AMC 30) was used to determine the antibiotic resistance its obtained from Bioanalyse company.

Detection S. aureus agr (I,II,III,IV) gene and detected the relation between agr expression and antibiotic resistance

Agr gene amplification:

Detection S. aureus agr gene that were encoding for regulate virulence factor was carried out by the amplification the extraction DNA of S. aureus with target gene by used PCR that include using various specific set of primers encoding for each target gene mixed with the template (DNA sample) and master mix reagent (PCR buffer, MgCl2, Taq polymerase and dNTPs) the end constituent was the deionized water, then the mixture were mixed and centrifuged for 3second to collect the drops from walls for ensure the final volume of 25ml, transferred mixture to a thermal cycler to start reaction according to the steps of the specific program (Fadhil and Mohammed, 2022).

Table (3) PCR reaction components of agr according to (Trans protocol)

Primer	Type of reaction	Master mix volume	Volume of F-primer (10pmol/ml)	Volume of R-primer (10pmol/ml	Nuclease free water	DNA	Final volume
Agr type I, II,III,IV gene	Multiplex PCR	10	1	1ml	6ml	2 ml	20 ml

PCR program to amplify agr gene using specific primer (Zhang et al., 2018).

Table (4) PCR program to amplify agr gene

Steps	oC	Minute-Second	Cycle	Product band
Initial denaturation	95 °C	4minute	1	1.441 2.575 3.323 4.659
Denaturation	95 °C	30 second	30	
Annealing	54 °C	45second		
Extension	72 °C	1minute		
Final extension	72 °C	10min	1	

Gene expression of agr system include many steps:

First Step - RNA extraction: RNA

Extraction according to Transzol Up Plus RNA kit controls provides powerful lysis and easy column based purification. Used many reagents for isolation RNA from S. aureus cell which is a number of steps are lysis the cell and centrifugation to get some solution separates to the three layer, the first layer in upper colorless layer containing RNA, second layer is intermediate layer separated between up and down layer , third layer is and pink layer was organic layer.

Second Step-Transfer RNA to cDNA

cDNA synthesis super mix kit (Easy Script@ first strand) controls synthesis cDNA was stored at -4C° until used for qPCR protocol.

Third Step-Real time reaction (qPCR)

Designed specific primer of agrI,II,III,IV gene for gene expression according to Humanizing genomic macrogen control.

Table (5) sequence of agr gene and housekeeping gene

Gene name	Forward nucleotide	Revers nucleotide
AgrIIII	CACAGGAATGGGCTTCTTTT	TGCACATGCACCTTCTTCTT
recAS	AAGTACGTCGTGCAGA	TGACCCATTCGTTCGC

recAS= housekeeping gene

Table (6) qPCR program of agr gene and housekeeping gene (recAS)

Gene name	Steps	oC	Minute-Second	Cycle	Product band
Agr	Initial denaturation	95 °C	4 Minute	1	181
	Denaturation	95 °C	30 Second	30	
	Annealing	57 °C	45 Second		
	Extension	72 °C	45 Second		
	Final extension	72 °C	7 Minute	1	
recAS	Initial denaturation	95 °C	4 Minute	1	229
	Denaturation	95 °C	30 Second	30	
	Annealing	55 °C	45 Second		
	Extension	72 °C	45 Second		
	Final extension	72 °C	7 Minute	1	

Result and discussion

First method of identification: Isolated 30 strains of S. aureus from 150 clinical sample were growth on the mannitol salt agar plates fermenter mannitol and gave a golden yellow colony, positive for gram stain's, cocci shape under light microscope, positive for catalase and coagulase tests but negative to oxidase and motility also more strain of S. aureus hemolysis because it is lysis the red blood cell

Antimicrobial resistance test

Found 76,70% percentage of S. aureus isolates has high ability to grow on the MHA media that are refer to bacterial resistance to 30µg of amoxicillin, all the resistance percentage return to amoxicillin affected on the cell wall synthesis process of S. aureus bacteria (AL-Zoubi, 2015).

Second methods confirmed first methods identification by 16srRNA genes amplification through PCR technique:

In molecular detection of S. aureus includes two steps the first one extraction DNA from S. aureus bacteria that have thick peptidoglycan layer makes DNA extraction difficult. Extracted DNA and purified from all S. aureus using the manufacturer's DNA purification kit (DNA extraction kit) used agarose gel electrophoresis and stained by adding ethidium bromide for determined DNA extracted presence and integrity the optimum DNA concentration, figure (1) showed the result the result showed.

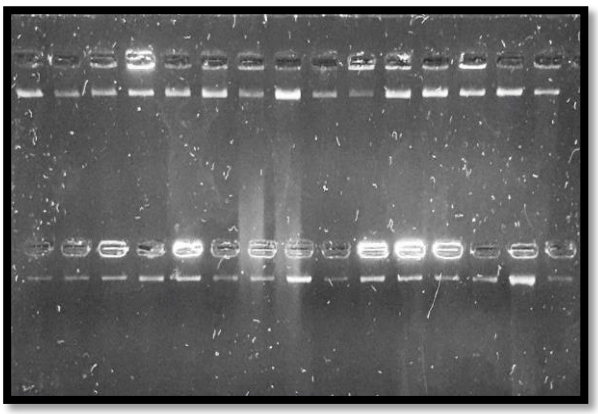

Figure (1) showed Staphylococcus aureus DNA extraction used 1 % of agarose gel electrophoresis to visualized that stained with ethidium bromide by 1xTBE and used UV light transilumiator at 350nm.

The second step used PCR technique to amplifying the 16srRNA gene that helped to identify bacterial genome it was developed and effective tool helped to recognize the specific bacterial strains this step applied on the 30 isolates taken from urinary tract after primary identification and found all isolates are S. aureus bacteria. figure (2) clarify 16srRNA amplification

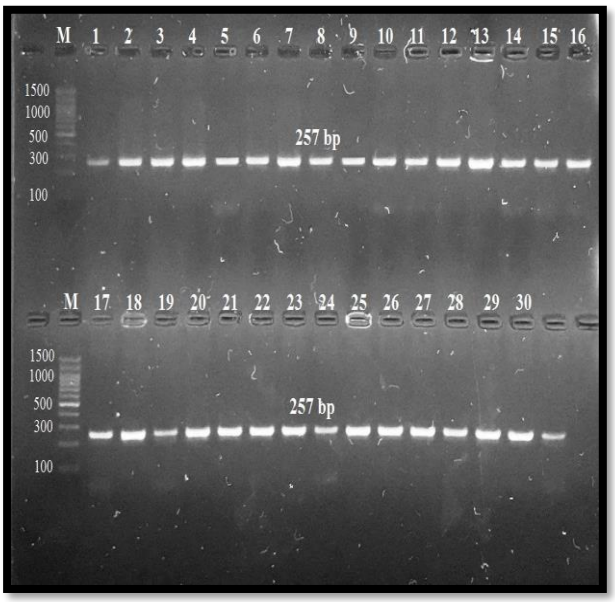

Figure (2) PCR amplification of Staphylococcus aureus 16srRNAgenes (257bp) used 1 % of agarose electrophoresis to visualized that stained with ethidium bromide by using TBE for 30min at 90 Volt. Vertical lane M =Marker and horizontal Lane number (1-30): Urinary tract sample from people return to Hospital.

The increasing multidrug-resistant (MDR) staphylococcal infections lead number of study research and discuss about the essential questions that is how the variations in genetic material of bacteria cause antibiotic resistance therefore in modern years the16srRNA gene amplification become moistly genetic marker to confirm S. aureus taxonomy identification.

The researcher increases use to16srRNA amplification technique to identify S. aureus because of their interfamilial transmission incorporated with local communities and another cause detect the presence of mutations in 16srRNA (Gumaa et al., 2021).

Detection S. aureus agr gene and detected the relation between agr expression and antibiotic resistance

All isolated showed has agr gene with percentage 100%. figure (3) showed agr gene in all isolated. agr gene has important function in S. aureus pathogenesis and resistance to antibiotic (Bernabe et al., 2021).

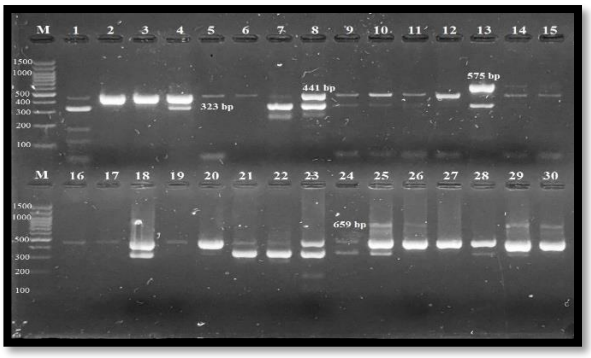

figure (3) PCR amplification of Staphylococcus aureus agr system (323bp, 441bp, 659bp) used 1 % of agarose electrophoresis to visualized that stained with ethidium bromide by using TBE, for 30 minute at 90 Volt.

Relation between agr gene expression and antibiotic resistance

The expression level of agr gene that is involved in S. aureus virulence factor regulated was investigated by quantitive PCR assay (qPCR) and designed specific primer for agr gene and housekeeping expression showed in table (1) for five S. aureus isolates (30,23,16,21 and 15) selected based on resistance to amoxicillin , indicates the expression agr gene of sample when strain growth with Amoxicillin and growth without Amoxicillin, figure (4-12) showed the amplification curve of agr gene expression , table (7) Relation between agr gene expression and antibiotic resistance. Accessory gene regulator of S. aureus bacteria used for controlling the expression of number genes that code for bacterial virulence factors regardless of sample type and have important role in bacterial resistance to antibiotic it has various mechanisms to resistance antibiotic like remove the entered drug through an efflux pump (Bibalan et al., 2018: Kalia et al., 2019).

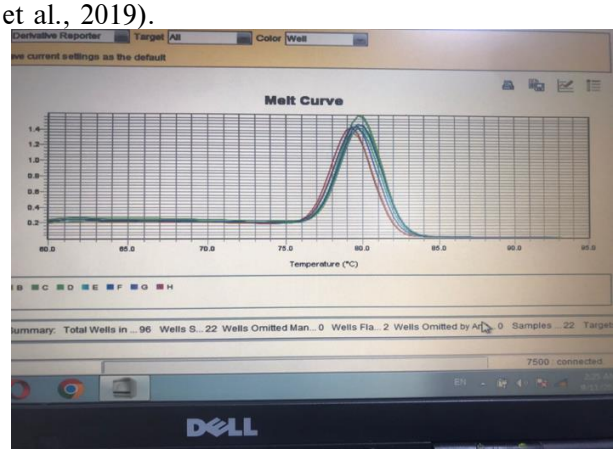

Figure (4) Amplification curve of Staphylococcus aureus agr gene expression

Table (7) Relation between agr gene expression and antibiotic resistance

Sample	CT Treatemnt	CT Untreated	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2^-ΔΔCt
C	0	17.029	0	0	0	1
1	18.079	17.029	3.226	3.401	-0.175	1.128964405
2	16.733	10.737	-0.378	-0.424	0.046	0.968618189
3	26.445	24.086	0.508	1.696	-1.188	2.278366754
4	15.412	13.401	1.024	1.212	-0.188	1.139183377
5	22.504	19.863	-2.302	-0.104	-2.198	4.588428097

Conclusion

1 The diagnosis method 16sRNA polymerase chain reaction method was a confirmation method for distinction S. aureus strain from other type of bacteria
2 Agr gene have important role in antibiotic resistance.

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