International Journal of Drug Delivery Technology/ Elsiever journal

Mutagenicity Acridine orange mutagen on biological activity of *S.aureus* isolated from tonsillitis

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Abstract

Study design of cases are Cross-sectional study that describtive study design of 280 isolates collected from tonsillitis , the identification achieved by using Vitek2-GP.

The biological activity of antimicrobial sensitivity test of all 280 isolates against antibiotics including Ceftazidine 70(25%)resistance , 196(70%) sensitive, 14(5%) intermediate; Azithromycin 154(55%) sensitive , 28(10%) intermediate., 98(35%) resistance; Oxacillin 266(95%) sensitive, 14(5%) resistance; Cephalexin 238(85%) sensitive, 42(15%) resistance; Gentamycin 266(95%) sensitive, 14(5%) resistance; Ceftriaxone 238(85%) sensitive, 42(15%) resistance;Norfloxacin 252(90%) sensitive, 28(10%) resistance; Cefoxitin 266(95%) sensitive, 14(5%) resistance; Erythromycin 42(15%)sensitive, 238(85)% resistance; Cloxacillin 126(45%) resistance, 154(55%) sensitive; Imipenem 266(95%) resistance, 14(5%) sensitive; Vancomycin 266(95%) resistance, 14(5%) sensitive and Methicillin 280(100%) resistance, 0 (100%) sensitive) compared with CLSI(2013).

Radial caseinolysis assay and Fibrinolysis assay of Staphylococcal fibrinlysin achieved by quantitative screening of staphylokinase on plasma agar plate and semi-quantitative screening of staphylokinase on skimmed milk agar plate to limit fibrinolysis zone and caseinolysis zone around well on plate, the result showed variable degree of production staphylokinase some isolates positive for production, another negative for production staphylokinase (staphylococcal fibrinolysin).

Induced mutation achieved by chemical mutagenesis when exposition *S.aureus* to different mutage of Acridine Orange (AO) chemical mutagen in dose (10,20,30,40,50,60) mg, the results of mutagenesis in optimum mutage (dose of

mutagen) with increase production of staphylococcal fibrinolysin when occurs random mutagenesis was 75 colony (viable cells) viability in 30 mg.
Genotypic analysis of sak gene encoded for staphylokinase accomplished by using PCR, all results of *S.aureus* isolated from tonsillitis positive for possess sak gene (400bp).
Key words: Thrombolytic enzyme, Mutage, Mutant protein , Improvement, Genotoxicity.

Introduction

Mutagen is a physical or chemical agent that changes the genetic material DNA, leading to increase frequency of mutation, can cause cancer, there are types of mutations including spontenous mutations occurred by error repair and recombination when caused effect to DNA called genotoxic, also effect on transcription, replication the DNA which causes severe infection leading t cell death result in aberrant impaired or loss of function for a particular gene and accumulation mutations leading to cancers [1].

Acridine is an complex and a nitrogen heterocycle with the formulation $C_{13}H_9N$. Acridines are replaced derivatives of the origin ring. It is a planar molecule that is structurally linked to anthracene with one of the central CH groups substitute by nitrogen such the regarding molecule pyridine and quinoline, acridine is mildly basic. Acridine orange is a fluorochrome dye that can interchate into nucleic acid [2,3].

Staphylococcus aureus is a Gram-positive, round-form bacterium that is a member of the Firmicutes, organize in the upper respiratory tract and on the skin, is a facultative anaerobe that competence develop wanting the need for oxygen, it is an opportunistic pathogen, prevalent cause skin infections Impetigo, cellulitis, folliculitis, scalded skin syndrome , abscesses[4], respiratory infections(sinusitis), food poisoning, bacteremia, meningitis, endocarditis, osteomyelitis [5].

Staphylokinase (Sak), a protein excrete by many *S. aureus* strains [6] stimulate human plasminogen (h-plg) into plasmin. Plasmin in turn assimilate fibrin clots

causing proteolysis [7] . Sak (staphylokinase) induce staphylococcal get away from the fibrin clots and expedite systemic bacterial spreading from the infection location [8,9].

Study designs are the set of methods and execution used to stack and analyze data particular in a specific seek question, One of the headmost procedures in arranging a research study is the predilection of study design, There are two types of study design in global inclusive study designs observational and interventional, in the observational study designs out of the descriptive design is the simple, in order that permit the investigator to study with depict the allocation of one or more vary, wanting regard to any causal or other proposition [10].

Materials and Methods

Isolation and Identification of *S. aureus*

The isolation of *S.aureus* isolates were 500 samples from various provenance of humans assembled from hospitals in Baghdad in 2018 to 2019, all samples were cultivated in Nutrient broth, Nutrient agar medium and Mannitol Salt agar medium incubated at 37 ^{0}C for 24 hr. approve to Lemaire (2008), the gathering of samples were from tonsillitis[11].

The identification done of *S.aureus* by Vitek2-GP and by Genotypic revelation PCR , possessed 1ml of bacterial suspension from bacterial culture then centrifuged at 8000 rpm for 5 minutes, the pellet resuspended in 0.3 ml of normal saline and the turbidity compared with MacCfrland solution, the card position into Vitek-2 apparatus for identification of *S.aureus*, the results were revealed after 6 hr. (Biomeruex , 2010).

Antimicrobial Susceptibility test

The antimicrobial susceptibility test was accomplished by agar diffusion method of antibiotic discs to 500 isolates for 14 antibiotics shown in table(1) and compared with the commendation of CLSI [12].

Phenotypic of Fibrinolytic enzyme (staphylokinase) expression

1-Quantitative screening of staphylokinase by Plasma agar plate assay:

The medium was destined according to [13] by add up to pre- hated human plasma at 56°C for 20 minutes to nutrient agar medium in a percentage of 20% of the total volume , then media was mingled pleasantly and put on petriplates, preserved for time so as to solidification.

The plasma agar used to disclose expression of staphylokinase or thrombolytic enzyme activity for all bacterial isolates accomplished by inoculating 5ml of nutrient broth medium into 50µl of culture incubated at 37^{o}C plasma agar plates incubated at 37 ^{o}C for 24 hours, the positive result by formalization of clear zone of hydrolysis(Transparent lysis) nearly wells found on plasma agar plate to determine hydrolytic ability.

2- Semi-quanitative screening of staphylokinase by skimmed milk agar assay :

Skim milk agar medium used in order that reveal for staphylokinase for the medium have low fat milk destined 78.5 ml of distilled water sterilized by autoclave, subsequently cooled by dissolve nutrient agar in 12.5 ml of unsaturated skimmed milk . A bacterial strain of pure bacterial isolates was spotted in Nutrient broth for 18 hours at 37 ° C and done in the center of the milk agar accomplished 5mm by utilizing Cork borer. 0.1 ml possessed by micropipette layed in the hole of Casein agar plate incubated for 24-18 hours at 37 ° C. . the inhibition zone nealy the hole were metrical and observed with transparent areas around the hole (Casienolysis zone) [19].

Chemical mutagenesis :

The chemical mutagenesis accomplished by utilizing Acridine orange(AO) with some modification of methods

S.aureus mutagenesis according to [15] with some modification. The mutagens Acridine orange were used at different mutage (dose of mutagen) concentrations of (10,20,30,40,50,60) mg that is generality appropriate for mutagenesis in bacteria to induce mutation inoculated into Muller Hinton Agar, incubated at 37°C for 24 hr..

Results and Discussions

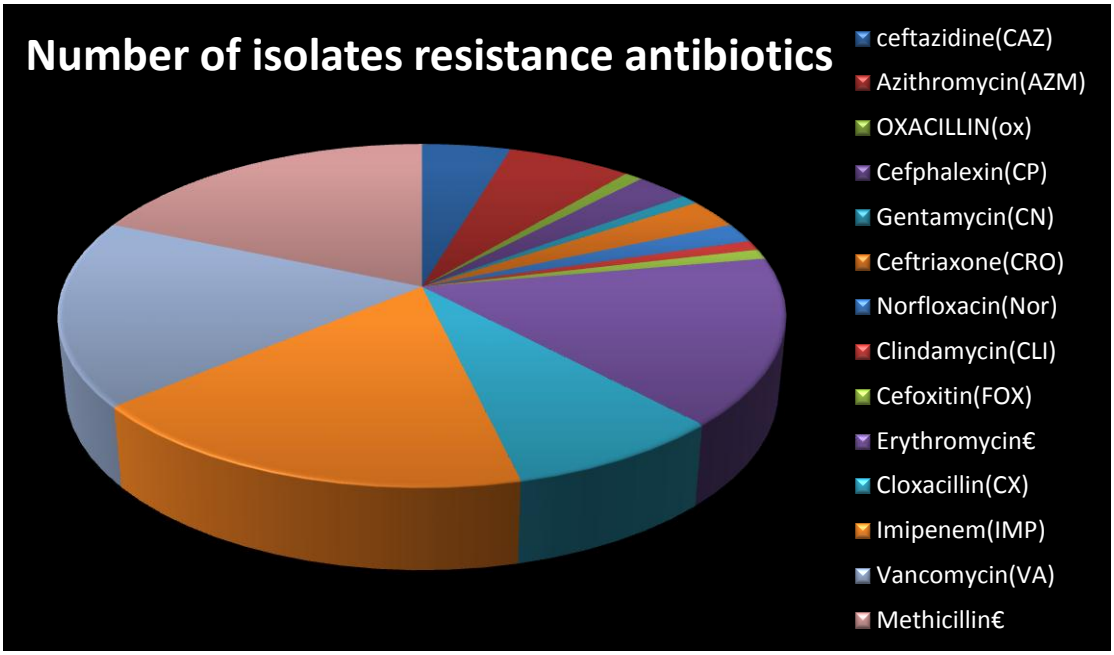
Isolation and Identification of *S.aureus*

The study design of cases are crosssectional study that describtive study 280 isolates of *S.aureus* isolated from tonsillitis were collected from hospitals in Baghdad . All specimen were cultivated in nutrient broth and nutrient agar medium at 37 ^{0}C for 24 hours . taken100 µl from the dilution that pervasion on mannitol salt agar medium (selective medium) incubated at 37 ^{0}C for 24 hours to pickout *S.aureus* according to [11]. The results of isolation from various provenance of human are 280 isolated from tonsillitis.

S. aureus colonize and doing symbiotically but competence cause disease when take over the tissues that colonized or pervade other tissues called pathobionts [16,17].

Antimicrobial susceptibility test of *S.aureus*

The biological activity of antimicrobial sensitivity test achieved of all 280 isolates against 14 antibiotics, by agar diffusion method on Muller Hinton Agar, consequences of test limited by liken with CLSI (2013), results of sensitivity and resistance exhibit in figure(1) were Ceftazidine 70(25%)resistance, 196(70%) sensitive, 14(5%) intermediate; Azithromycin 154(55%) sensitive, 28(10%) intermediate., 98(35%) resistance; Oxacillin 266(95%) sensitive, 14(5%) resistance; Cephalexin 238(85%) sensitive, 42(15%) resistance; Gentamycin 266(95%) sensitive, 14(5%) resistance; Ceftriaxone 238(85%) sensitive, 42(15%) resistance;Norfloxacin 252(90%) sensitive, 28(10%) resistance; Cefoxitin 266(95%) sensitive, 14(5%) resistance; Erythromycin 42(15%)sensitive, 238(85)% resistance; Cloxacillin 126(45%) resistance, 154(55%) sensitive; Imipenem 266(95%) resistance, 14(5%) sensitive; Vancomycin 266(95%) resistance, 14(5%) sensitive and Methicillin 280(100%) resistance, 0 (100%) sensitive).



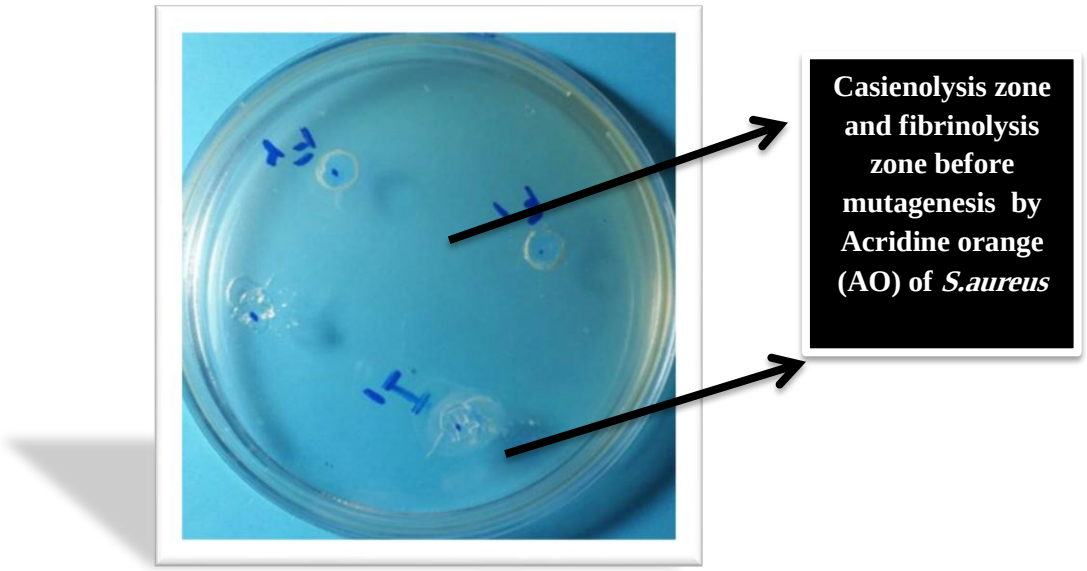
Figure(1): Number of *S.aureus* isolated from tosillitis resistance to 14 antibiotics.

Results of resistance of *S.aureus* represents Methicillin 280(100%), Vancomycin 266(95%),Imipenem 266(95%), Azithromycin 98(55%),Cloxacillin 126(45%) and Erythromycin 238(35%) , Cefatizidine 70(25%).

Phenotypic of Staphylococcal fibrinolysin (Staphylokinase) production

Staphylokinase is one of the substantial virulence factors generated by *S.aureus* to resist human immunity by react with α-Defensins a peptide excreted via host polymorphonuclear cells (PMNs) that grant antimicrobial protection intercede by disruption the safety of cell wall leading to bacteriocidal impact because interaction α-Defensins, as well, thrombolytic enzyme(staphylokinase) react with plasminogen subsequently transform plasmin that assimilate fibrin clots [18].

The expression of Staphylococcal fibrinolysin (Staphylokinase) acchieved on plasma agar plate and Casein agar plate to limit hydrolytic ability (hydrolysis areas) of plasma set up in the medium, result of phenotypic showed 260 isolates have expression of staphylokinase, including tonsils 100 of *S.aureus* exhibit in figure(3).



Figure(3): Caseinolysis zone and Fibrinolysis zone Hydrolysis zone on plasma agar medium and Casein agar medium by *S.aureus* before mutagenesis by Acridine orange (AO) chemical mutagen.

<u>Mutagenic effect of Acridine Orange (AO) Chemical Mutagen on *S.aureus*</u>

Chemical mutagenesis showed when expose *S.aureus* to different mutage (doses of mutagen) chemical mutagen of Acridine Orange (AO) in (10,20, 30, 40, 50, 60) mg have production of staphylokinase in concentrations 30 mg with viability 75 colony. Mutagenic effect of Acridine Orange (AO) chemical mutagen on *S.aureus* achieved inorder that increase production of staphylokinase in different mutage (doses of mutagen) to induction genetic mutation that induce raise staphylokinase expression.

Acridine Orange utilizing as a fluorescent staining agent to reveal the existence of bacteria in blood cultures and other bodily fluids Cerebral spinal fluid and buffy coat preparations at low pH under UV light, bacterial and fungal nucleic acid fluoresces orange whilist background mammalian nucleic acid fluoresces green [3,19].

Phenotypic detection of Staphylokinase after mutagenicity of Acridine orange (AO) chemical mutagen

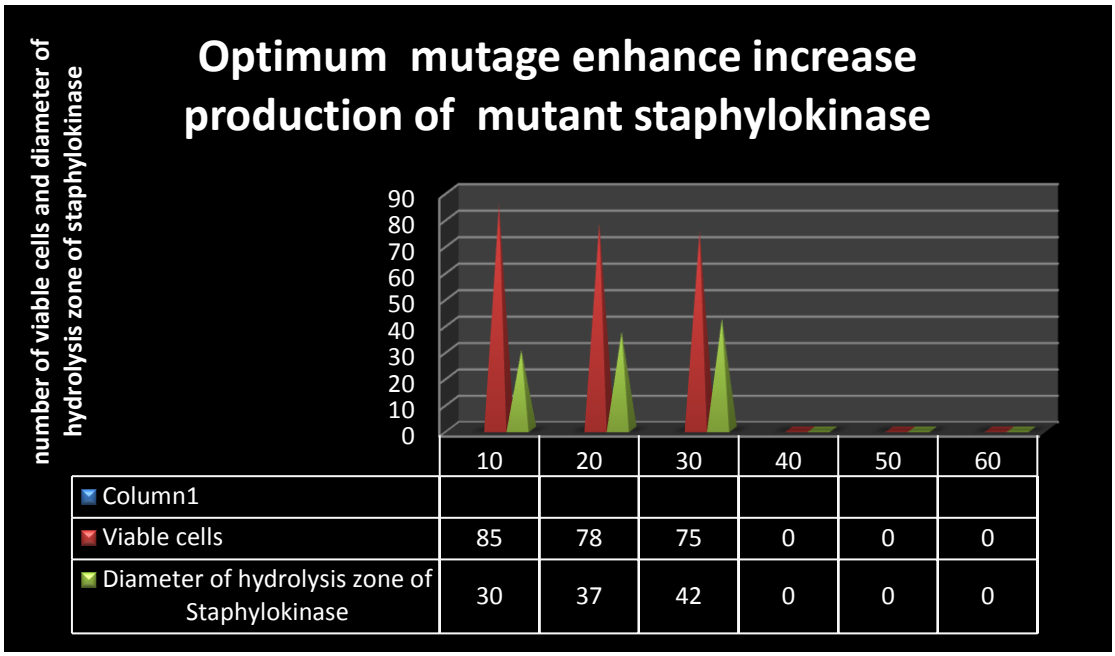
Staphylokinase take possession high affinity for fibrin that its thrombolytic agent leading to tissue damage, its support bacteria to spreading through human [20].

Results in figure (4) showed increase hydrolytic ability of fibrinolysis zone and caseinolysis zone area nearly well on plasma agar and nearly casein agar medium because production Staphylococcal fibrinolysin (Staphylokinase) when exposition *S.aureus* to Acridine Orange(AO) in mutage 30mg, the diameter of hydrolysis zone were variable degree from diameter of fibrinolysis zone and caseinolysis zone area before chemical mutagenesis from isolate to others ,production of staphylokinase occurreance because mutation in *sak* gene encoded for staphylokinase which leading to increase hydrolysis ability range into 42mm fibrinolytic enzyme (Staphylokinase) acts to fibrinolysis and caseinolysis found in plasma agar and skim milk agar.

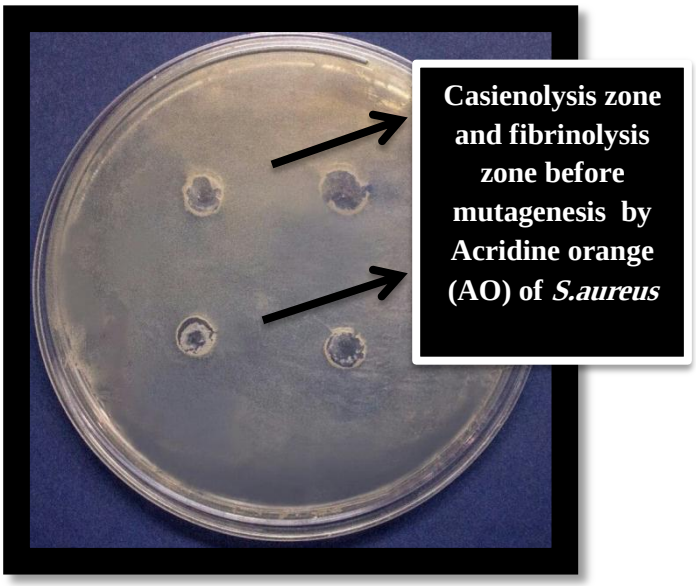
Table(1):Production of Staphylococcal fibrinolysin (Staphylokinase) by different mutage of Acridine Orange (AO) chemical mutagen.

	Chemical mutagen	Mutage (Dose Of mutagen)	Production Staphylococcal fibrinolysin	**Diameter of hydrolysis zone of staphylokinase /mm**	No.of Viable Colony of *S.aureus* isolated from Tonsillitis
	Acridine Orange (AO) /mg	10	+	30	Viability (85) colony
		20	+	37	Viability (78) colony
		30	+	42	Viability (75) colony
		40	-	0	No growth

	50	-	0	No growth
	60	-	0	No growth



Figure(4): Mutagenic effect of Acridine Orange (AO) chemical mutagen used to enhance mutagenicity of *S.aureus* isolated from Tonsillitis.



Figure(5): Caseinolysis zone and Fibrinolysis zone on plasma agar medium and Casein agar medium of *S.aureus* after mutagenesis by Acridine orange (AO) chemical mutagen.

Genetic analysis detection of *sak* gene encoded for thrombolytic enzyme(Staphylokinase)

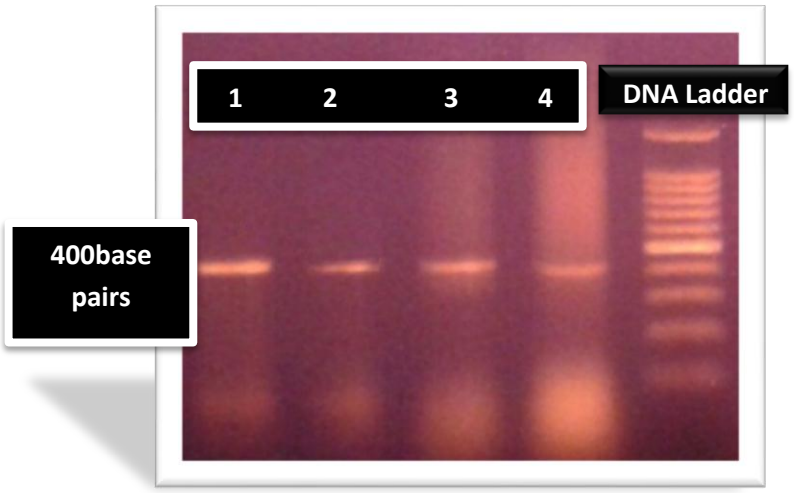


Figure (6) : Gel electrophoresis for amplified *sak* gene (400bp) on agarose gel (1%) , 50V for 1 hr. , Lane (1 – 6) represents *S.aureus* , (L) DNA Ladder (1500 bp) .

Various mutagens doing on the DNA differently, robust mutagens may result in chromosomal instability, cleading to chromosomal breakages and rearrangement of the chromosomes like translocation, deletion, and inversion. Such mutagens are cknown clastogens [21].

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