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RESEARCH ARTICLE

Genotypic and Phenotypic Detection of Mutant Thrombolytic Enzyme (staphylococcal fibrinolysin) Expressed by Mutant *Staphylococcus aureus* Vancomycin Sensitive S.aureus (VSSA) and Methicillin Sensitive S.aureus (MSSA) by Hydroxylamine Chemical Mutagen

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Abstract

The aim of this study in order to determine mutagenesis of virulence S.aureus MRSA, VRSA then make it non virulent VSSA, MSSA by Hydroxylamine (HA) chemical mutagen. The study design of cases are Cross-sectional study so as to limit types of epidemiologic studies including descriptive study of 500 isolates collected from different sources of human infections from Tonsils, Nose, Tumors, Urine, Blood and Skin(Acne), all identification by Vitek2-GP. The antimicrobial sensitivity test done of all 500 isolates against 14 antibiotics, the resistance of antibiotics including Vancomycin 475(95%), Methicillin 500(100%), Imipenem 475(95%), Erythromycin 175(35%), Cloxacillin 225(45%), Azithromycin 275(55%), Ceftazidine 125(25%), Ceftriaxone 75(15%), Cephalexin 75(15%), Norfloxacin 50(10%), Cefoxitin 25(5%), Gentamycin 25(5%), Oxacillin 25 (5%) and Clindamycin25 (5%) compared with CLSI(2013).The expression of fibrinolytic enzyme (Staphylokinase) done on plasma agar plate to locate hydrolysis around well of plasma on medium, the result showed 180 (69%) isolates of Vancomycin resistance *S.aureus* (VRSA) and Methicillin resistance *S.aureus* (MRSA) produces Staphylokinase. The chemical mutagenesis was done by exposure VRSA *S.aureus* and MRSA *S.aureus* to different doses of chemical mutagen including Hydroxylamine(HA).The study of antibiotics after chemical mutagenesis accomplished on Muller Hinton Agar plate and *in vitro* to human serum to Vancomycin , Methicillin that have high resistance before mutagenesis, the result showed after chemical mutagenesis by Hydroxylamine (HA) of *S.aureus* (VRSA) and *S.aureus* (MRSA) become sensitive to Vancomycin called Vancomycin Sensitive *S.aureus* (VSSA) and sensitive to Methicillin called Methicilline Sensitive *S.aureus* (MSSA) with possess higher productivity of Staphylokinase (thrombolytic enzyme).The genetic analysis of *sak* gene of mutant VSSA, MSSA *S.aureus* done by genotypic PCR after DNA extraction with amplification of mutant VSSA, MSSA *S.aureus sak* gene to determine each isolates possess *sak* gene of VSSA ,MSSA *S. aureus* with high expression of staphylokinase, the results showed all isolates of mutant VSSA MSSA *S.aureus* possess *sak* gene with 420bp compared with DNA Ladder Marker.

Key words: *Fibrinolytic enzyme, Mutagenesis, Mutagenic specificity.*

Introduction

Staphylococcus aureus is a great bacterial human pathogen that gives rise to a wide variety of clinical symptoms [1] due to the prpocession of multi-drug resistant strains Methicillin-Resistant *Staphylococcus aureus* (MRSA) [2, 3].Study designs are the series of methods and procedures used to accumulate and anatomize data particular in a specific seek question. A descriptive study is one of type of study destined to depict the allocation of one or more variables, without consideration to any causal or other hypothesis involve several kinds case reports, case series, cross-sectional studies and ecological studies, in the first three types data are composed on personage whilst the last (ecological studies) one utilize assembled data (groups) [4]. Staphylokinase (SAK, Staphylococcal fibrinolysin and Müller's factor) is a protein created by *Staphylococcus*

aureus. It includes 136 amino acid residues and has a molecular mass of 15kDa [5]. Staphylokinase is positively regulated by the agr gene regulator.

It stimulates plasminogen to form plasmin, which assimilate fibrin clots. This deactivate the fibrin network which forms to keep infections localized by interacts with plasminogen to form complex that disclose the active site of the plasminogen molecule. Staphylokinase split IgG and complement component C3b, block phagocytosis [6]. Mutagen in genetics is a physical or chemical factor and biological mutagen that may done directly on DNA occasion damage, some act replication error that influenced by replication mechanism, almost effect on chromosomal partion[7].

Mutagenesis is a significant technique to DNA in laboratory to make mutant genes, proteins, strains of bacteria or genetically modified organisms (GMOs).The mutation may produce mutant proteins with new induced properties or novel assignment that may be significant in of mercantile use [8].

Materials and Methods

Isolation and Identification of *S. aureus*

Isolation of *S.aureus* from different sources of humans infections collected from Baghdad hospitals in 2018 - 2019, all samples were cultivated in Nutrient broth, Nutrient agar medium and Mannitol Salt agar medium incubated at 37 ^{0}C for 24 hr. identification by using Vitek2-GP and by genotypic PCR for detection 16srRNA of *S.aureus* according to [9,10].

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test was done by agar diffusion method of antibiotic discs of 500 isolates for 14 antibiotics that Vancomycin, Methicillin, Imipenem, Erythromycin, Cloxacillin, Azithromycin, Ceftazidine, Ceftriaxone, Cephalexin, Norfloxacin, Cefoxitin, Gentamycin, Oxacillin and Clindamycin, compared with the recommendation of [11].

Phenotypic Detection of Thrombolytic Enzyme (staphylokinase) Expression

Plasma Agar Plate Assay

The medium was prepared according to [12] by adding 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 mixed kindly and put on petriplates, preserved for time so as to to solidification. The plasma agar utilized to reveal expression of staphylokinase or thrombolytic enzyme activity for all bacterial isolates achieved by inoculating 5ml of nutrient broth medium into 50µl of fresh culture incubated at 37^{o}C inoculated into plasma agar plates incubated at 37 ^{o}C for 24 hours, the positive result by forming of zone of hydrolysis around wells on plasma agar plate.

Chemical Mutagenesis by Hydroxylamine (HA):

The chemical mutagenesis done by using Hydroxylamine (HA) with modification Vancomycin Resistance *S.aureus* (VRSA) and Methicillin Resistance *S.aureus*(MRSA) mutagenesis according to [13] with some modification. The mutagens, hydroxylamine were used at concentrations of (10,20,30) mg which is most suitable for mutagenesis in bacteria to enhance mutation inoculated into Muller Hinton Agar, incubated at 37°C for 24 hr.

Results and Discussions

Study Design

In a cross-sectional study design reliant the consequence and the exposition at the same time but in case-control studies based the result situation; cohort study design relying on the exposition situation. In a cross-sectional study design are merely chosen reliant the inclusion and exclusion criterion for the study, these study design faster and are inexpensive [14].

Isolation and Identification of *S.aureus*

The study design of cases are cross-sectional study design that descriptive study design of 500 isolates of *S.aureus* were collected from hospitals in Baghdad from 280(56%) tonsils , 100(20%) nose, 40(8%) tumors, 17(3.4%) urine, 27(5.4%) blood and 36(7.2%) skin(Acne).

All samples are culture in nutrient broth and nutrient agar medium at 37 ^{0}C for 24 hours [9, 10]. Results of isolation exhibited in Figure (1), also results of identification displayed all isolates are *S.aureus* when identified by using Vitek2-GP.

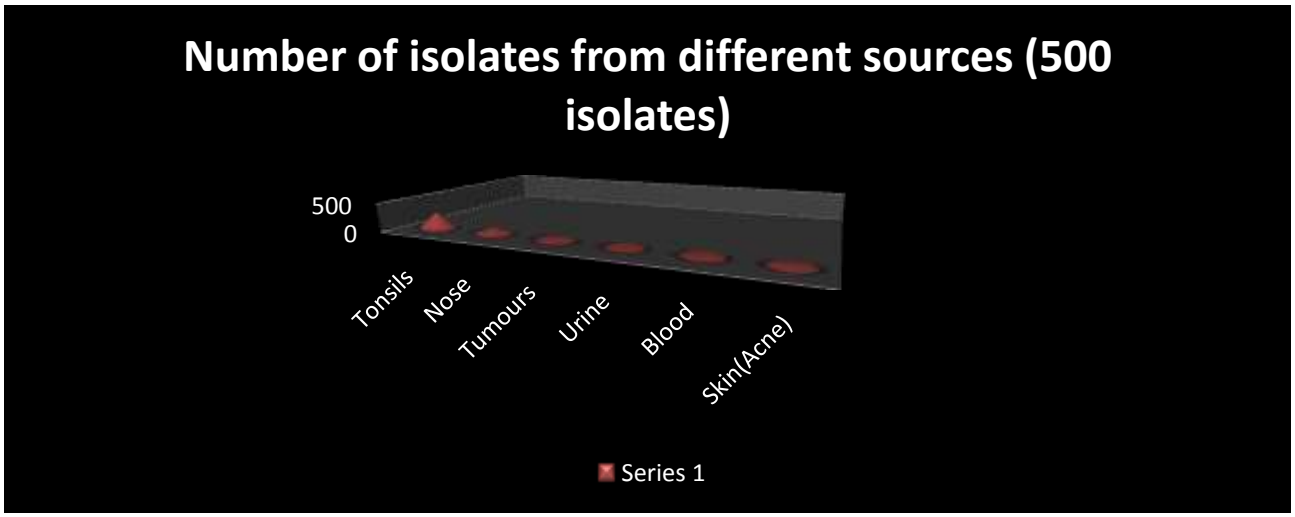

Figure 1: Cross-sectional study design of cases in order that limit of prevalence (Epidemiologic studies) of *S.aureus* from different clinical sources

Antimicrobial Susceptibility Test of *S.aureus* before Chemical Mutagenesis

The antimicrobial sensitivity test done of all isolates (500) isolates against 14 antibiotics, by agar diffusion method on Muller Hinton Agar, results of test compared with[15], results of sensitivity and resistance showed in figure (2) were Ceftazidine 125(25%)resistance , 350(70%) sensitive, 25(5%) intermediate; Azithromycin 275(55%) sensitive, 50(10%) intermediate., 175(35%) resistance; Oxacillin 475(95%) sensitive, 25(5%) resistance; Cephalexin 425(85%) sensitive, 75(15%) resistance; Gentamycin 475(95%) sensitive, 25(5%) resistance; Ceftriaxone 425(85%) sensitive, 75(15%) resistance; Norfloxacin 450(90%) sensitive, 50(10%) resistance; Cefoxitin 475(95%) sensitive, 25(5%) resistance; Erythromycin 75(15%)sensitive, 425(85)% resistance; Cloxacillin 225(45%) resistance, 275(55%) sensitive; Imipenem 475(95%) resistance, 25(5%) sensitive; Vancomycin 475(95%) resistance, 25(5%) sensitive and Methicillin 500(100%) resistance, 0 (100%) sensitive). Resistance of the antibiotics that are the higher to lower of Six antibiotics including Methicillin 500(100%), Vancomycin 475(95%), Imipenem 475(95%), Azithromycin 275(55%), Cloxacillin 225(45%), Erythromycin 175(35%), Ceftazidine 125(25%), Ceftriaxone 75(15%), Cephalexin 75(15%), Norfloxacin 50(10%), Cefoxitin 25(5%), Gentamycin 25(5%), Oxacillin 475 (5%).

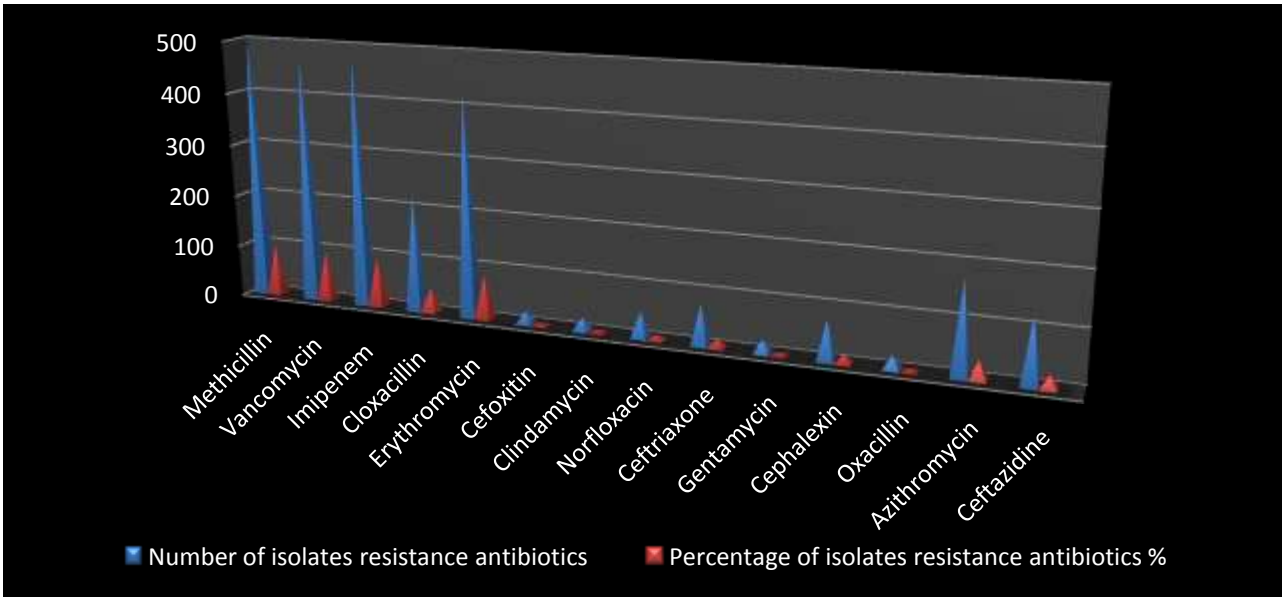

Figure 2: Number of resistance isolates of *S.aureus*, with percentage of resistance isolates

Results of resistance S.aureus before chemical mutagenesis represents Methicillin 500(100%), Vancomycin 475(95%), Imipenem 475(95%), Azithromycin 275(55%),Cloxacillin 225(45%) and Erythromycin 175(35%).

Antimicrobial Sensitivity Test after Chemical Mutagenesis by Hydroxylamine (HA)

Study susceptibility of antibiotics after chemical mutagenesis also done to Vancomycin, Methicillin to find out each isolates resistance or sensitive for Vancomycin and Methicillin after mutagenesis high resistance before mutagenesis, result in Figure (3) showed antibiotic sesnsitivity after chemical mutagenesis of *S.aureus* (VRSA) and *S.aureus* (MRSA) become sensitive to Vancomycin and Methicillin with higher productivity (overproduction) of Staphylokinase, thrombolytic enzyme. One of the kinds of chemical mutagen act by chemical alter to nitrogen base of DNA it sympathize to base such as nitrous acid that occasion an oxidative deamination in amino groups that modify into keto groups also modify cytosine into uracil, uracil have capability to pairing with adenine that leading to cause alteration from a C-G pair to T-A, as well happen deamination of adenine that forming hypoxanthines base that base-pair with cytosine [16]. Consequence of antimicrobial susceptibility of VRSA and MRSA accomplished for six antibiotics including Vancomycin, Methicillin, Imipenem, Azithromycin, Ceftazidine and Erythromycin on Muller Hinton Agar medium after chemical mutagenesis by Hydroxyleamine (HA), results of mutant *S.aureus* become sensitive for Vancomycin and Methicillin called VSSA and MSSA have overproduction of staphylokinase showed in Figure (3).

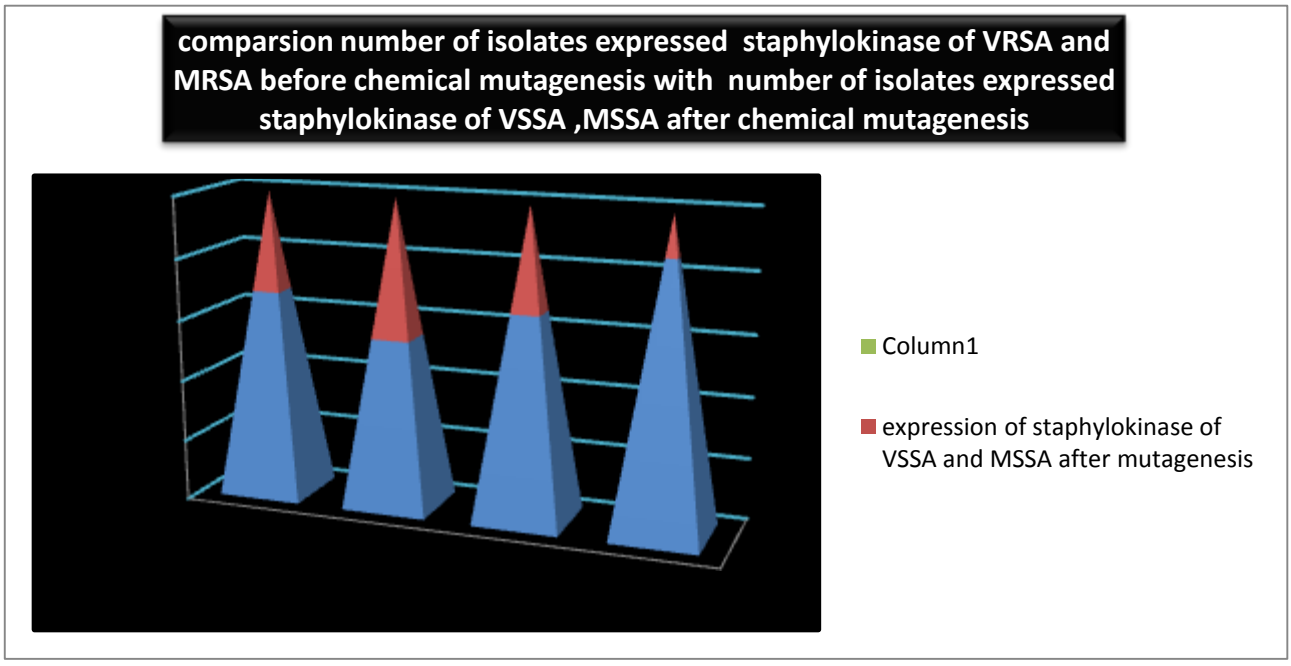

Figure 3: Comparison of number of VRSA and MRSA isolates before chemical mutagenesis and VSSA and MSSA after chemical mutagenesis have high productivity of staphylokinase (fibrinolytic enzyme)

Results comparison number of isolates of Vancomycin resistance *S.aureus* isolates and Methicillin Resistance *S.aureus* isolates before exposure to chemical mutagenesis of 180 isolate were resistance to Methicillin and Vancomycin have production staphylokinase (thrombolytic enzyme) were from tonsils 96 isolates(53%),tumors 36(20%),blood 30 isolates (17%) and skin 18 isolates (10%) have expression of thrombolytic enzyme when study phenotypic on plasma agar of MRSA and VRSA , either results of the expression of staphylokinase were overproduction to staphylokinase(fibrinolytic enzyme) of Vancomycin sensitive *S.aureus* (VSSA) and Methicillin Sensitive *S.aureus* (MSSA) because of chemical mutagenesis have been selected 100 isolates (56%) possess sensitive to Vancomycin and Methicillin antibiotics with overproduction of staphylokinase from tonsils 46 isolates (46%), tumors 29 isolates (29%), blood 15 isolates (15%) and skin(Acne)10 isolates (10%) have overproduction of staphylokinase , leading to convert Vancomycin Resistance *S.aureus* (VRSA) into Vancomycin Sensitive *S.aureus* (VSSA) and convert Methicillin Resistance *S.aureus* (MRSA) into Methicillin Sensitive *S.aureus* (MSSA) become non-virulent that converted from virulent into non virulent that latter used to other experiment in order to use in medical applications.

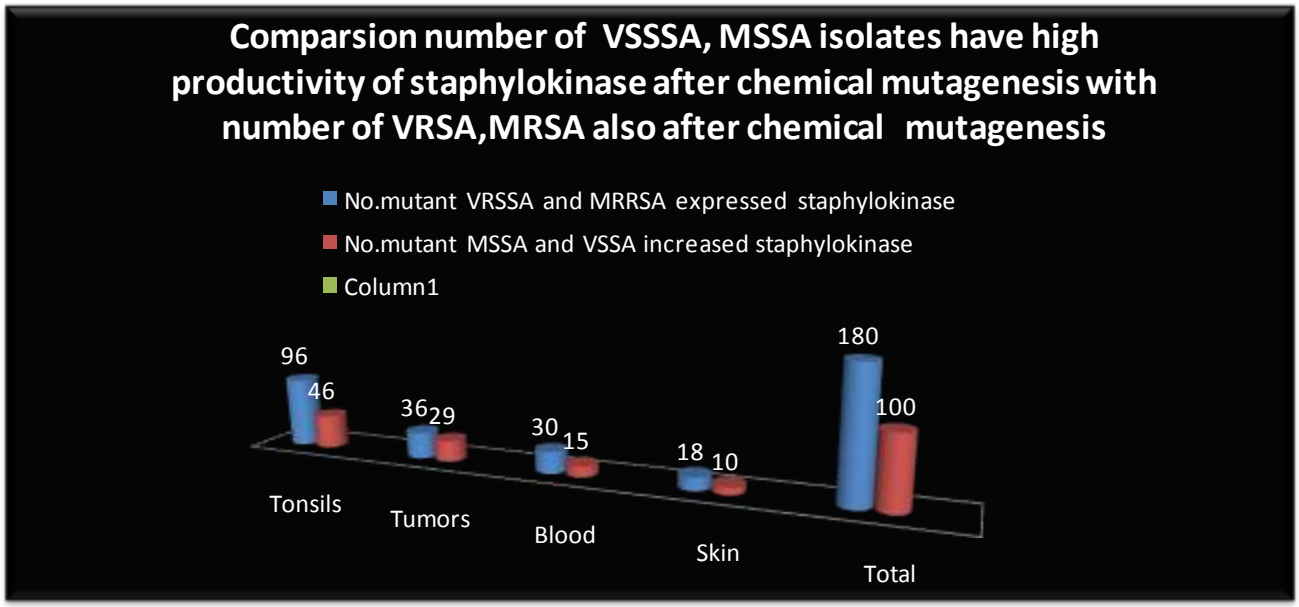

Figure 4: Comparsion number of mutant VSSSA, MSSA isolates have high productivity production staphylokinase with number of mutant VRSA, MRSA Vancomycin Resistance *S.aureus* (VRSA) and Methicilline Resistant *S.aureus* (MRSA) after chemical mutagenesis by Hydroxylamine (HA) of 180 mutant *S.aureus* isolates(total)

Phenotypic Detection of Thrombolytic Enzyme (Staphylococcal fibrinolysin) before Chemical Mutagenesis

The expression of thrombolytic enzyme (Staphylokinase) done on plasma agar plate in order to determine hydrolysis areas of plasma around well found in the medium, result of phenotypic showed 260 (56%) isolates have expression of staphylokinase, including tonsils 100 (38%), Skin(Acne) 36(14%), Tumors 38(15%), Blood 27(10%) and Nose 59(23%) production staphylokinase; 180 (69%) isolates from have been expression of staphylokinase are resistance to Vancomycin and Methicillin Vancomycin resistance *S.aureus* (VRSA) and Methicillin resistance *S.aureus* (MRSA) showed in Figure(5).

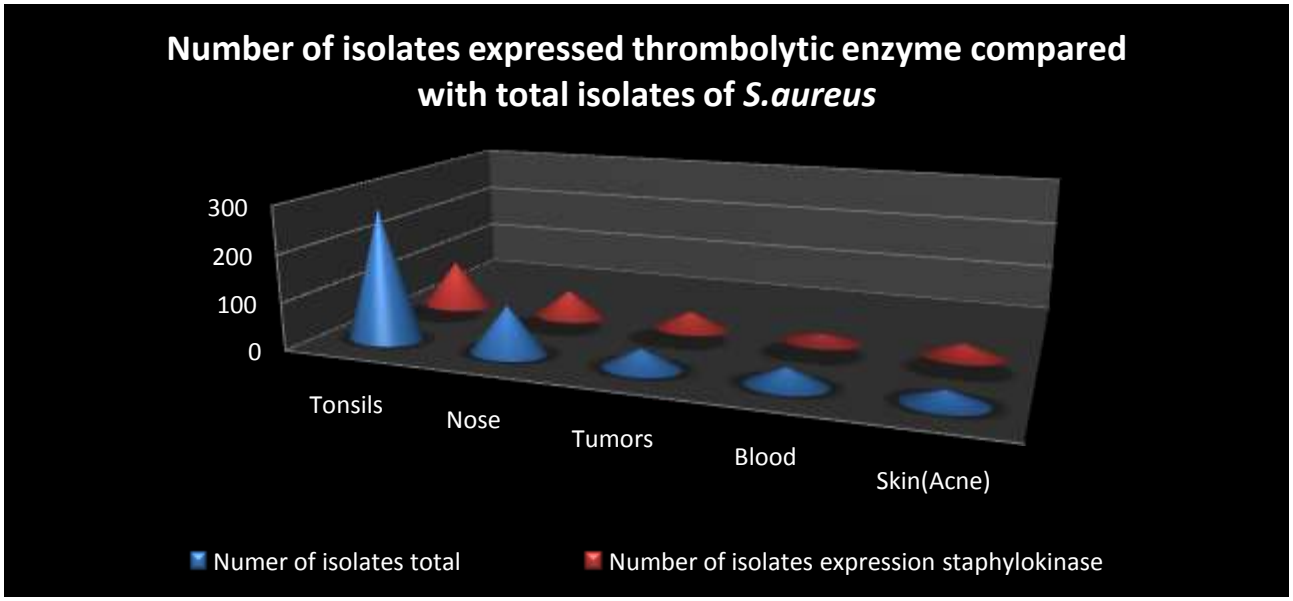

Figure 5: Number of isolates expression thrombolytic enzyme by phynotypic on plasma agar comparison with total isolates from different sources

Results the expression of thrombolytic enzyme (staphylococcal fibrinolysin) on plasma agar medium showed 260 (56%) isolates expression staphylokinase enzyme from *S.aureus*, of which 180 isolates (69%) have the expression of staphylokinase resistance for Vancomycin, Methicillin, Imepinem, Erythromycin and Azithromycin.

Phenotypic Detection of Staphylokinase after Chemical Mutagenesis

Staphylokinase possess high affinity for fibrin that its thrombolytic agent leading to tissue damage, its help bacteria to invasiveness through infect human [17].

Table1: Overproduction of staphylokinase VSSA and MSSA by Hydroxuleamine (HA) chemical mutagen with different concentrations

	Chemical mutagen	Concentrations Of mutagen	Overproduction of staphylokinase of VSSA and MSSA isolates	No.of Viable Colony
	Hydroxyleamine (HA)/mg	10	+	Viability 85 colony
		20	-	No growth
		30	-	No growth

The chemical mutagenesis done in order to improvement expression of staphylokinase by using Hydroxylamine (HA) in different doses with different methods by induce genetic mutation that alters staphylokinase expression. Results the chemical mutagenesis of VRSA *S.aureus* and MRSA *S.aureus* to different doses of chemical mutagen including Hydroxylamine(HA) in (10,20,30) mg showed the viability of VSSA and MSSA with overproduction (increase production) of staphylokinase of after to exposed Hydroxylamine in 10 mg are 85 colony(viable cells), the results explained in Table(1). Staphylokinase has very substantial effectiveness as a thrombolytic agent generated from *S.aureus* in order to its activity and cheap cost as compared to another thrombolytic agent. Overproduction of staphylokinase achieved after exposure *S.aureus* to random mutagenesis by UV radiance [18].

Genetic Analysis of *sak* Gene Encoded for Thrombolytic Enzyme before and After Chemical Mutagenesis of Mutant VSSA, MSSA *S.aureus* by Genotypic PCR

Genomic DNA of 100 isolates of mutant VSSA, MSSA *S.aureus* were extracted by PCR DNA extract kit and amplified by Go Taq master mix for amplification of *sak* gene that encoded for thrombolytic enzyme (staphylokinase). The purity of genomic DNA defined as the ratio of the absorbance at 260nm and 280nm (260 /280 ratio) was 1.8 pure DNA. Amplification of *Sak* gene by genotypic PCR technique of mutant VSSA, MSSA *S.aureus* carried out with annealing temperatures 52 °C, denaturation DNA 95°C and extension 72°C by using specific primers, forward primer and reverse primer for leading strand and lagging strand DNA respectively.

Forward Primer: 5'-CGCGGATCCTCAAGTTCATTCGAC -3'

Reverse Primer: 5'-GAATCTAGACCCAAGCTTTTTCCTTTC TATAACAAC-3'

The genotypic method for identification of bacteria was preferable than phenotypic because PCR technique modern is a golden standard appliance to assure bacteria inclusive S. *aureus*. The fast diagnostic of pathogen in the clinical samples substantial to progress care of patient and therapy the diseases [19].

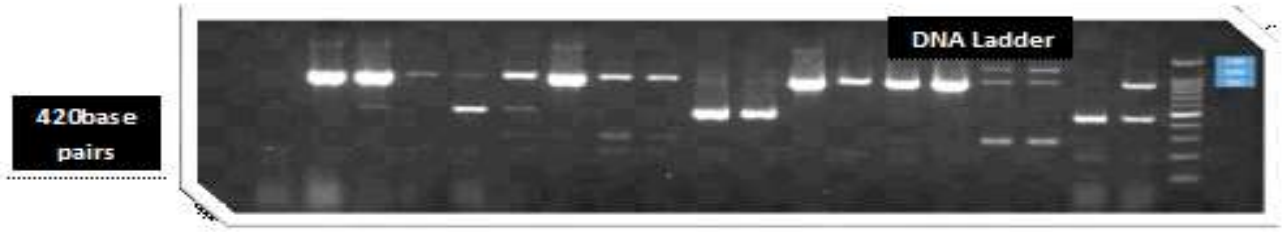

Figure 6: Gel electrophoresis for amplified sak gene (420bp) on agarose gel (1%) , 50V for 1 hr., Lane (1 – 15) represents VSSA, MSSA *S.aureus* , (L) DNA Ladder (1500 bp)

Results of genotypic *sak* gene encoded for staphylococcal fibrinolysin showed all mutant VSSA, MSSA *S.aureus* possess *sak* gene after chemical mutagenesis by Hydroxylamine (HA) compared with control before mutagenesis when accomplished agarose gel electrophoresis of *sak* gene (420bp) compared with DNA Ladder (1500bp). Products of PCR analyzed on (1%) agarose gel electrophoresis to locate prescence or not prescence *Sak* gene (400bp) compared with DNA Ladder Marker (1500bp). Results of genotypic amplification in Figure (15) showed all isolates (100) mutant VSSA, MSSA *S.aureus* have sak gene encoded for high expression of staphylokinase when genotypic detection of PCR. There are several PCR protocols to enhance sensitivity, especially when dealing with small numbers of bacteria as the target, Nested PCR is one of detection of only a few bacteria in clinical specimens [20, 21].

References

1. Rasigade JP, Vandenesch F (2014) Staphylococcus aureus: a pathogen with still unresolved issues. Infect. Genet. Evol., 21: 510-4.
2. Chambers HF (2005) Community-associated MRSA--resistance and virulence converge. N. Engl. J. Med., 07: 352(14):1485-7.
3. Boucher HW, Corey GR (2008) Epidemiology of methicillin-resistant Staphylococcus aureus. Clin. Infect. Dis., 01: 46 (5):S344-9.
4. Aggarwal R, Ranganathan P (2019) Study designs: Part 2-Descriptive studies. Perspect clin. Res., 10(1): 34-36.
5. Bokarewa MI, Jin T, Tarkowski A (2006) "Staphylococcus aureus: Staphylokinase". Intern J. Biochem. Cell Biol., 38(4): 504-509.
6. Rooijakkers SH, Wamel WJ, Ruyken M, Kessel KP, Strijp JA (2005) "Anti-opsonic properties of staphylokinase". Microbes and Infection. 7 (3): 476-84.
7. Kim D, Guengerich FP (2005) "Cytochrome P450 activation of arylamines and heterocyclic amines". Annual Review of Pharmacology and Toxicology, 45: 27-49.
8. Hsu PD, Lander ES, Zhang F (2014) "Development and applications of CRISPR-Cas9 for genome engineering". Cell J., 157 (6): 1262-78.
9. Holt JG, Krieg NR, Sneath PHA, Staley JT, William ST (2004) Bergey's Manual of Determinative Bacteriology. 9th Edn., Williams and Wilkins, Baltimore, MA., USA.
10. Lemaire S (2008) Intracellular Staphylococcus aureus, an emerging links to persistent and relapsing infections: factors influencing the activity of antimicrobials against intracellular S. aureus. Doctor at Thesis. Universite Catholique de Louvain, 14: 766-777.
11. CLSI, Clinical and Laboratory Standard Institute (2013) Analysis and presentation of cumulative antimicrobial susceptibility test data. 3rd ed. Approved guideline M39-A3. Wayne PA USA.
12. Pulicherla KK, Gadupudi GS, Rekha VPB, Seetharam K, Anmol K, Sambasiva Rao KRS (2011) Isolation, Cloning and Expression of Mature Staphylokinase from Lysogenic Staphylococcus aureus Collected from a Local Wound Sample in a Salt Inducible E.coli Expression Host. International Journal of Advanced Science and Technology. 30:35-42.
13. Kodym A, Afza R (2003) Physical and chemical mutagenesis. Methods. Mol. Biol., 226:189-204.
14. Setia MS (2016) Methodology Series Module 3: Cross-sectional Studies. Indian J. Dermatol., 61(3): 261-264.
15. CLSI, Clinical and Laboratory Standard Institute (2013) Analysis and presentation of cumulative antimicrobial susceptibility test data. 3rd ed. Approved guideline M39-A3. Wayne PA USA.
16. Jeremy W Dale, Simon F (2010) Molecular Genetics of Bacteria, 5th edition. A John Wiley and Sons, Ltd., Publication.
17. Chen CJ, Unger C, Hoffmann W, Lindsay JA, Huang YC, Gotz F (2013) Characterization and comparison of 2 distinct epidemic community-associated methicillin-resistant Staphylococcus aureus clones of ST59 lineage. PLoS One, 8(9): e63210.
18. Mohanasrinivasan V, Subathra CD, Monidipa B, Jannatul FS, Pragya L, Jemimah NS, Vaishnavi B (2014) Production of fibrinolytic Staphylokinase from UV Mutated Staphylococcus aureus VITSDVM7. International Journal of Chem. Tech. Research, 6: 8.
19. Croxatto A, Prodhom G, Greub G (2012) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. Fems. Microbiol. Rev., 36: 380-407.
20. Madico G, Quinn TC, Boman J, Gaydos CA (2000) Touchdown enzyme time release-PCR for detection and identification of Chlamydia trachomatis, C. pneumoniae, and C. psittaci using the 16S and 16S-23S spacer rRNA genes. J. Clin. Microbiol., 38:1085-1093.
21. Apfalter P, F Blasi, J Boman, CA Gaydos, M Kundi, M Maass, A Makristathis, A Meijer, R Nadrchal, K Persson, ML Rotter, CY Tong, G Stanek, AM Hirschl (2001) Multicenter comparison trial of DNA extraction methods and PCR assays for detection of Chlamydia pneumoniae in endarterectomy specimens. J. Clin. Microbiol., 39:519-524.