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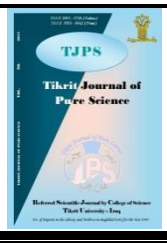

Detection of S*ak* Gene and Expression of Staphylokinase in Different Clinical Isolates of *Staphyloccocus spp.*

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Abstract

This study had investigated the presence of Staphylokinase (SAK) phenotypically and genetically. A total of 100 *Staphylococcus spp.* samples were isolated from different clinical cases. Morphological, biochemical tests and for further conformation; Viteck2 technique were adopted to isolate and diagnose the staphylococcal samples. Phenotypic assay tests including hydrolysis of casein and plasma agar were performed to check for the production of Staphylokinase from producing *Staphylococcus spp.* Out of 100, only 32 isolates had exhibited (SAK) activity whereby 29 of them represented the species *S. aureus* while 3 belonged to others. All isolates were subjected to Polymerase Chain Reaction (PCR) to determine the possessed *sak* gene. This investigation certainly had shown that not all isolates of *S. aureus* has the sak gene, only the lysogenic one has it and that it is not species committed but also other staphylococcus species could have it.

Introduction

The genus Staphylococcus considered to be a Gram-positive bacteria that colonize human or animal skin and mucosal membranes. They were basically subdivided into groups depending on novobiocine susceptibility/resistance and coagulase activity, whereby *S. aureus* is novobiocine-resistant and coagulase-positive. Nowadays, due to large differences in pathogenicity, the staphylococci are often categorized into *S. aureus* and CNS, the latter being a combined group of all other species. Also *S. aureus* is an opportunistic pathogen and cause a wide range of diseases. Among staphylococci, it is the most invasive species and an etiological agent of diverse human and animal maladies [1, 2, 3]. It has a unique character of producing an extracellular enzyme which is called Staphylokinase or " spreading factor" that has a role in anti-clotting functioning thereby avoiding the body's fibrinous Reactions (as a wall-off barrier) [4]. This extracellular protein (136 amino acid) aids to disrupt blood clotting throughout altering plasminogen to plasmin. The kinase enzyme also has proteolytic acitivity which helps to degrade the fibrin clot, a major constituent of thrombus. The blockage of blood vessels can cause myocardial disorders due to blood clot eventually leading to death [5, 6]. Staphylokinase; the single chain protein with 15.5 kDa is considered as a bolus thrombolytic that aids in chasing or partial digesting of the plasminogen to an inactive pro-enzyme; plasmin with high fibrin specificity thus acting as a clot dissolver [7, 8 , 9 ,10].

The aim of this study was to isolate *staphylococcus aureus* and screen for Staphylokinase (SAK) from pathogenic staphylococci species phenotypically and genetically using specific PCR.

Materials and Methods

Sample collection and growth conditions

Samples were collected from different clinical cases at Tikrit teaching hospital, outside patients of dermatology and E.N.T clinics and emergency wards. All sample isolates were grown at 37°C for 24 hours in the incubator on mannitol salt agar medium (samples that did not show any growth were excluded and not counted). The bacterial cultures from the mannitol salt agar were taken and the isolates were submitted for biochemical and other tests which included: Gram's staining, Coagulase tube and slide tests, Catalase test culturing on DNAase agar and pigmentation to differentiate the pathogenic *Staphylococcus* spp. following procedures conducted by [11, 12, 13, 14].

Automated identification and characterisation of *Staphyloccocus spp.* by VITEK2

Further identification (confirmation) was also carried out by VITEK2 system (BioMérieux VITEK, USA) to characterize the *Staphylococcus spp.* This system accommodate colorimetric reagent cards that are incubated and interpreted automatically. According to the manufacturers' instructions. Micro plates for Gram positive organisms (GP) were used [15].

Phenotypic assay for staphylokinase producing *Staphylococcus aureus* and other species

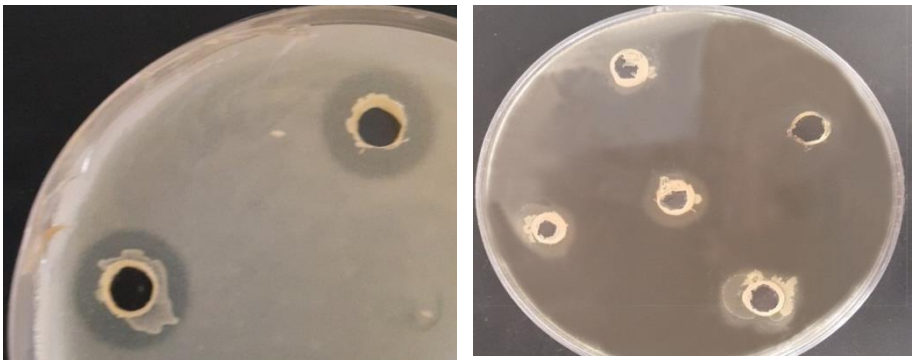
Screening the enzyme phenotypically was conducted using skimmed milk and heated plasma agar assays and the result was shown as a clearance zone around the wells**.**

1- Skimmed Milk hydrolytic test

This casein- agar lytic assay was conducted by preparing nutrient agar then adding non- fat milk regularly to the media followed by the addition of fresh human serum percent as a supplement (2 ml percent). Well diffusion plate technique was used to see the hydrolysis of casein (proteolytic) activity since the produced enzyme utilizes the casein present in the media [16].

2- Human-heated plasma agar test

This test was performed by collecting freshly human blood obtained in tubes with an anti-coagulant agent (EDTA). The tubes of blood were then centrifuged at 10,000 rpm for 10 min. supernatant (plasma) was gathered out and heated in the water bath for 20 min at 56°C. The heated plasma was then cooled to optimum temperature and added to nutrient agar and mixed well by shaking (15ml of nutrient agar medium was prepared, 10 ml of plasma was added) and poured into petri dishes. Well diffusion was also used in this technique [17].



(1) Casein hydrolysis assay **(2) Heated plasma assay**

Figure (1) screening For Enzyme Production

Genomic DNA Extraction

A simple and rapid method was used for the preparation of staphylococcal genomic DNA following a procedure presented by Hoffman *et al* and Moore *et al* [18, 19] with minor modification. Purity and concentration of DNA samples were estimated by nanodrop, at a wavelength of A260/A280 nm and to check the quality of the total DNA, agarose gel electrophoresis was determined. Samples were mixed with loading buffer (loading: DNA 2/7 v/v) and loaded into the wells of the gel and visualized by ethedium bromide following procedure of Al- Noaami [20].

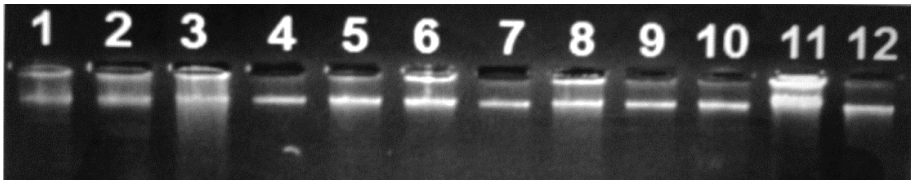


Figure (2) Electrophoresis gel for genomic DNA of some *Staphylococcus spp.* isolates by electrophoresis on 1% agarose gel stained with ethidium bromide

Identification of S*ak* Gene from Isolated Sample

The nucleotide sequences of the full length *sak* gene primers (24 nt forward primer: CGCGGATCCTCAAGTTCATTCGAC and 27 nt reverse primer: CCCAAGCTTTTTCCTTTCTATAACAACwas used in this study (their respective amplified products was 489 bp) [21].

The reaction mixture were prepared according to the instruction of AcuuPower PCR PreMix from (BiONEER, Korea), the primer used was at optimum concentration of 10 pmol. Deionized sterile water was used to adjust the volume of this mix was adjusted to 25 µL. DNA amplification was carried out in a Labnet thermocycler for 35 cycles following profile: an initial step 95°C for 5 min, 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, ending with a final extension at 72°C for 5 min. PCR product (7 µl) was observed in a 2% agarose gel in 1x TBE buffer 45 V, 60 Am for 10 min at the first run followed immediately with 120vol, 200Am for 65 min to separate the different amplification products efficiently. The gel was stained via ethidium bromide and photographed using gel documentation system. Molecular characterization of the *sak* gene was conducted with by running against a 100bp DNA ladder [22].

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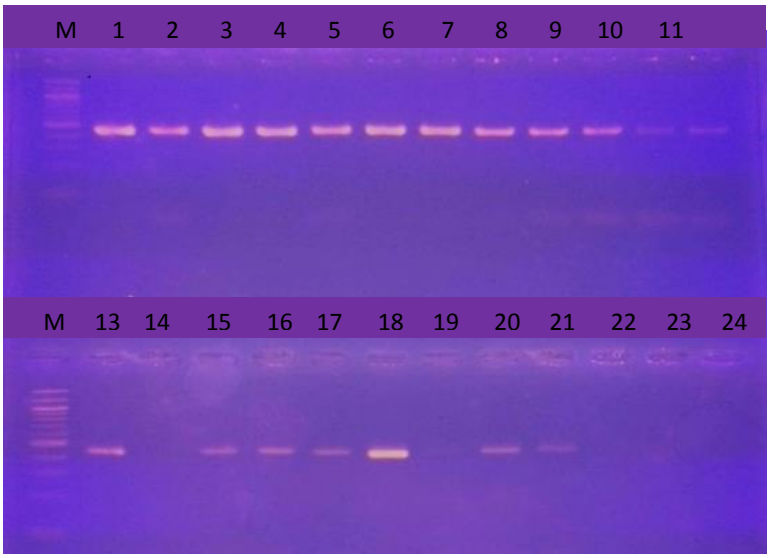

Figure (3) specific PCR detection of sak gene on 2%agarose gel electrophoresis, lane M referred to 100bp. DNA ladder

Results and Discussion

All isolates included in this research were subjected to check for the production of staphylokinase and they were categorized according to their source of infection after they had been identified and diagnosed at the species level based on their micro and macro characteristics and Vitek technique. To study the capability of *Staphyloccoucs spp*. strains on producing staphylokinase two in vitro methods had been undertaken followed by molecular verification.

Table (1) Results for The screening of staphylokinase

	S. aureus	Other species	Total No.
Sak. positive	28	4	32
Sak. negative	52	16	68
Total No.	80	20	100

Table (2) presence of staphylokinase concerning with source of Staphylococcal spp. infection

Source / Staphylokinase	Skin	Ear	Wounds	Burns	Urine	Total No. (%)
Presence	11	8	3	1	9	32
Absence	19	18	10	7	14	68
Total Number	30	26	13	8	23	100

Results illustrated in table 2 above showed that skin source infection had scored high level of staphylokinase (11 out of 32) comparing with others, agreed with Bokarewa and Tarkowski [23] who demonstrated high rate of staphylokinase expression in their clinical samples isolated from skin and other external mucosal origin and relative low levels in isolates invading internal organs. Also the result agreed with what was published by Hameed [24] about staphylokinase production.

The plasmolytic activity and casein hydrolysis were both identified by a halo zone around the growth of colonies matching results between those tests were close favoring the heated plasma agar test observing larger clear zone ≤ 30 mm and the highest ratio of positive results in plasma test was quite a bit more than in casein hydrolysis asaay and this almost agreed with Jasim *et al* [25]. The gel electrophoresis of genomic DNA had revealed a good size band knowing that all concentration of DNA samples were uniformed on 50. The quantity obtained with this protocol ranged between 350 ng-500 ng/µl, the variation in amount depended up on the growth of cells. The gene *sak* was detected from the total DNA (genomic) of a lysogenic *S. aureus* and other species (*S. scuri* and *S. xylosis*). Expression of *Staphylokinase* from clinical samples to detect it's presence using skimmed milk agar and plasma agar hydrolysis assays had occurred followed by the detection of the possessed *sak* gene. The comparative results between Phenotypic and genotypic characteristics of staphylokinase were compatible in terms of the presence and absence and also the intensity (strong positive) and the very sharp band had referred the copy number of this gene. Remarkably, figure 3 illustrate that samples (1-10, 18) showed clear obvious thick band while others were less thought they harbored the gene. Jasim and his partners [26] declared in their work that bacteriophages which invade many bacterial species contribute to control the expression of virulence factors and evolutions of pathogenicity which might explain the current results. On the other hand, taking in consideration all screened isolates, two isolates belonging to the *S. sciuri* species had recorded the position of staphylokinase and that agreed with the study achieved by Zeman and others [27] who posted that the genome of phage φ575 harbors genes for

staphylokinase and phospholipase in the bacterial host (*S. sciuri*). Staphylokinase, which is a plasminogen activator and a good promising agent for clot dissolving by forming a 1 : 1 stoichiometric complex with inactive human plasminogen (hPg) circulating in the blood that is followed by cleavage of 10 amino acids from N-terminal by hydrolysis of Lys^{10}-Lys^{11} peptide bond and finally forms catalytically active SAK. This catalytically active SAK - plasminogen complex later forms a ternary complex with another molecule of plasminogen and transform this plasminogen to plasmin (hPm) after the cleavage of the Arg^{561}-Val^{562} peptide bond in hPg for that, this resultant SAK-plasmin complex functions as a plasminogen activator for the conversion formation in the clot [28, 29].

The staphylococcal lysogenic conversion literature includes two well-documented examples of negative conversion, where staphylococcal phages (in many cases, phages carrying additional lysogenic conversion genes) insertionally inactivate chromosomal genes that encode important exoproteins. Staphylokinase may also contribute to bacterial colonization by interacting with the immune system of the host [30] Cloning of the Sak gene in a controlled host microorganisms such as *E. coli* helps to increase production of the recombinant enzyme. Many biophysical and chemical modifications are about to be used to proceed its half-life in the human circulatory system [31].

This investigation certainly had shown that not all isolates of *S. aureus* has the *sak* gene, only the lysogenic one (transformed) has it and that it is not species committed but also other staphylococcus species could have it.

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الكشف عن الجين (ساك) والتعبير عن أنزيم ستافيلوكاينيز في عزلات سريرية مختلفة لأنواع من بكتيريا المكورات العنقودية

سهى ماهر عبد ، عدنان فاضل العزاوي

قسم علوم الحياة ، كلية العلوم ، جامعة تكريت ، تكريت ، العراق

الملخص

اجريت هذه الدراسة لأجل التحقق من وجود انزيم ستافيلوكاينيزالمحلل مظهريا ووراثيا حيث اشتملت على 100 عزلة لبكتيريا المكورات العنقودية تم جمع العينات من مصادر سريرية مختلفة. وقد اعتمدت الاختبارات المظهرية والكيميائية الحيوية تلاها للتأكيد استخدام تقنية Viteck2 لتشخيص أنواع المكورات المستحصلة. أجريت اختبارات الفحص المظهري التي تضمنت اختبارين هما فحص تحلل كازائين الحليب و فحص تحلل أجار بلازما الدم المسخن للتحقق من إنتاج انزيم ستافيلوكيناز من قبل المكورات العنقودية. ومن أصل 100 عزلة، أظهرت 32 عزلة نشاط (ساك) فقط، كان 29 منها يمثل النوع الذهبي *S. aureus* بينما 3 منها تنتمي إلى أنواع اخرى. تم إخضاع جميع العزلات إلى تفاعل سلسلة البلمرة المتعدد (PCR) لتحديد ما اذا كانت تمتلك الجين. وقد أثبت هذا البحث بالتأكيد أنه ليس كل عزلات المكورات العنقودية الذهبية لديها الجين (ساك *sak*)، إلا أنه فقط البكتريا المصابة بالعاثي تمتلك الانزيم المنتقل اليها من خلاله وأنه ليس بالضرورة وجوده بهذا النوع فقط ولكن أيضا الأنواع المكورات العنقودية الأخرى يمكن أن تحتويه.