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RESEARCH ARTICLE

Cloning of staphylokinase gene produced from mutant *S.aureus* in *E.coli*

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Abstract

This study was aimed to cloning staphylokinase gene from locally isolated Staphylococcus aureus in E.coli and detection the gene expression in new host . Amplified Sak gene was then sequenced to determine the nucleotide sequence of the gene . Results of sequencing showed that there are three point mutation were occurred in the structural gene of staphylokinase according to base pair alignment with the same gene of *S.aureus* standard strain recorded in BLAST / NCBI . These mutations are miscense mutations doesn't affect the group of amino acid that was changed then doesn't affect enzyme tertiary structure and activity . Results of sequencing also showed that the complete nucleotide sequence of the gene doesn't have a restriction site for BamHI and XbaI that were used for gene cloning . Staphylokinase gene was then double digested with BamHI and XbaI to obtain restriction fragment with sticky ends came from the restriction sites for these two enzymes located in the specific primers designed for this purpose (forward primer containing restriction site for BamHI and reverse primer containing restriction site for XbaI). Then the restriction fragment of staphylokinase gene was ligated in pSP72 cloning vector previously double digested with BamHI and XbaI to get sticky ends compatible with the two sticky ends of Sak gene , then recombinant vector size was 2900 bp.

INTRODUCTION

Staphylokinase produced by Staphylococcus aureus is an ideal fibrin specific plasminogen activator, converts a precursor, plasminogen to active enzyme, plasmin to dissolve the blood clot during thrombolytic therapy and it also can to destroy the normal components of haemostatic system which leads to life threatening consequence and also death (Kotra et al., 2013) . Staphylokinase (SAK) is 136 amino acid extra cellular protein produced during the late exponential growth phase by lysogenic strains of S. aureus. SAK is a profibrinolytic agent that forms a 1:1 stoichiometric complex with plasminogen, which after conversion to plasmin, activates other plasminogen molecules to plasmin (Moussa , 2012).

Thrombolytic therapy is needing more clot specific third generation molecules to get maximum patency in a short time with fewer side effects like minimal bleeding risk and re occlusion . SAK (420 bp) is one of the bacterial proteins having relatively good clot specificity than t-PA, but production from native *S. aureus* poses a great risk in the protein production as it is pathogenic . Cloning of SAK gene to investigation protein production in the non pathogenic host would be useful for cost effective therapeutic protein production in the clinical practice (Pulicherla *et al*., 2011) .

Materials and Methods

Isolation of S. aureus from clinical samples

In order to isolate *S. aureus* , a total of 200 clinical samples were collected from hospitals in Baghdad governorate during the period between November and December 2013 . After identification by use selective media and biochemical tests according to (Harlly and Prescott, 2002) and confirmed by using VITECK-2 we got 54 isolates .

Assay of staphylokinase production

Assay of staphylokinase production on plasma agar plate was carried out according to Pulicherla et al. (2011) .

Genomic DNA extraction

Genomic DNA of S.aureus was extracted according to boiling method described by klingenberg et al.(2004) .

Amplification of staphylokinase (Sak) gene

Amplification conditions summarized in table (1) by use :

Forward primer: 5'- CGCGGATCCTCAAGTTCATTCGAC-3'

Reverse primer: 5'- GAATCTAGACCCAAGCTTTTTCCTTTCTATAACAAC-3'

The conditions of polymerase chain reactions indicated in (table 1) were optimized at different annealing temperatures between 50 an 60 ˚C by using gradient thermo cycler .

Table (1): Conditions for amplification of staphylokinase gene

Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
95 ˚C for 5 min.	35	94 ˚C for 1min.	51 ˚C for 1mins.	72 ˚C for 1 min.	72 ˚C for 10 min.
			52 ˚C for 1min.	72 ˚C for 1min.	72 ˚C for 10 min.
			53 ˚C for 1 min.	72 ˚C for 1 min	72 ˚C for 10 min.

PCR products were analyzed on agarose gel (1%) using horizontal electrophoresis unit, gel was immersed in 0.5X TBE buffer, then samples were loaded into the wells of the gel . Electrophoresis was carried out for one - two hours at 50V. After electrophoresis gel was stained with 10µl of ethedium bromide stock solution . DNA bands were visualized by using U.V transilluminator at 365 nm. in presence of 1500bp DNA ladders marker . (Maniatis et al., 1982) .

Sequencing of staphylokinase gene

PCR products from the amplification of staphylokinase gene were sent to NICEM/USA ABI3730XL Applied BIOSYSTEMS to determine the complete nucleuotide sequence of the gene .

Cloning of staphylokinase gene

Cloning of staphylokinase gene steps carried out according manufacturer company (Promega,2015) , transformation of Sak gene in E.coli JM 109(DE3) by use pSP72 cloning vector was carried out according to (Sambrook, and Russell,2001)

Results and Discussion

Isolation and Identification of *S.aureus*

In this study , and in order to isolate *S. aureus* , a total of 200 clinical samples were collected from three hospitals (Baghdad teaching hospital , Al-Imam Ali hospital and Al-Sadder hospital) includes urine samples (100 samples) and another 100 samples collected from skin infections . All sample were then cultured in nutrient broth medium and incubated at 37 °C for 24 hours . After incubation , serial dilution for each sample were carried out , then 100 µl from the appropriate dilution was spread on blood agar and mannitol agar medium and incubated at 37 °C for 24 hours to select S.aureus according to Lemaire (2008).

Results showed that bacterial isolates grown on blood agar were large, round, creamy white colonies, smooth translucent area surrounding the colonies as a result of β-haemolysin production (Morello et al .,2006). While on mannitol salt agar medium ,which was also considered a selective and differential medium for S. aureus , because it contains 7.5 % salt and phenol red as an indicator . On this medium , S. aureus appeared golden yellow surrounded with large yellow zone, round, smooth, raised, mucoid and glistening and color of medium was changed from pink to yellow due to ferment the mannitol and acid production (Atlas et al., 1995) .

Identification of isolate were confirmed by VITECK-2 tests . The identified isolates of S.aureus from skin infections and urinary tract infections were distributed as in table (2) . Results showed the there are 35 isolates obtained from samples collected from skin infections ,while only 19 isolates were obtained from samples collected from urinary tract infections. A study by Al-Marjani et al.(2015) identify 60 isolates of S.aureus from wound infections .

Table (2) Distribution of S.aureus isolates collected from clinical sources

Clinical Sample	Total samples	Number of isolates	Percentage (%)
Skin infections	100	35	35
Urinary tract infections	100	19	19
Total	200	54	27

In other study carried by Nandita and Stanley (2014), they collected 101 samples from urine infections and they identify 23 isolates of S.aureus from total samples in a percentage of 22.7 % . In locally study by Al -Marjani and Hadi(2013) , 86 Staphylococcus isolates from urine, blood and swaps from different hospitals in Baghdad , 64.1% of were identified as methicillin resistant Staphylococcus spp.

Staphylokinase production

In this study, ability of staphylokinase production by local isolates of S.aureus was examined by using well diffusion method . Results showed that all of the 54 isolates were able to produce staphylokinase on plasma agar medium according to formation of zone of hydrolysis around each well containing culture filtrate of each bacterial isolate . Results also showed that these local isolates differ in their abilities in staphylokinase production due to differences in size of zone of hydrolysis around each well . Results showed that the zones of hydrolysis were ranged between 25 mm and 36 mm while mutant S.aureus was 38mm . Hence the most efficient isolate in staphylokinase production was mutant *S.aureus* A15-M1 isolated from skin infections because the size of zone of hydrolysis around the well containing its culture filtrate was 38 mm .

These results are in consonance with other studied achieved by Pulicherla et al. (2011) and Yerasi et al .(2014) , Shagufta et al . (2014) , who detect staphylokinase production by S.aureus isolated also from skin infections and obtaining clear zones of hydrolysis on plasma agar medium after an overnight incubation at 37 ºC .

Genomic DNA of the (54) isolates S.aureus were extracted by boiling method and considerd as a templet DNA for amplification of staphylokinase gene . The purity of genomic DNA defined as the ratio of the absorbance at 260nm and 280nm (260 /280 ratio) was 1.7 which refers to pure DNA . It was clear that purity of DNA ranged between 1.8 to 2.0 due to the 260 /280 ratio that will vary somewhat with the relative amounts of G/C and A /T in the DNA sample (Nazina, 2001). On the other hand results also showed that the concentration of DNA was 50 µg /µl. From this DNA concentration, aliqoutes of DNA were taken and used for amplification of Sak gene . Amplification of Sak gene by PCR technique was carried out for all S.aureus isolates and mutatnt (*S.aureus* A15-M1).

Forward primer contain site restriction for BamHI , while reverse primer contain site restriction of XbaI . Reaction products of gradient PCR were analyzed on (1%) agarose gel to identify the amplified Sak gene in presence of 1500 bp DNA ladder marker . Results of amplification shown in figure (1) represents that there is a DNA fragment of about 400 bp obtained after electrophoresis on agarose gel .

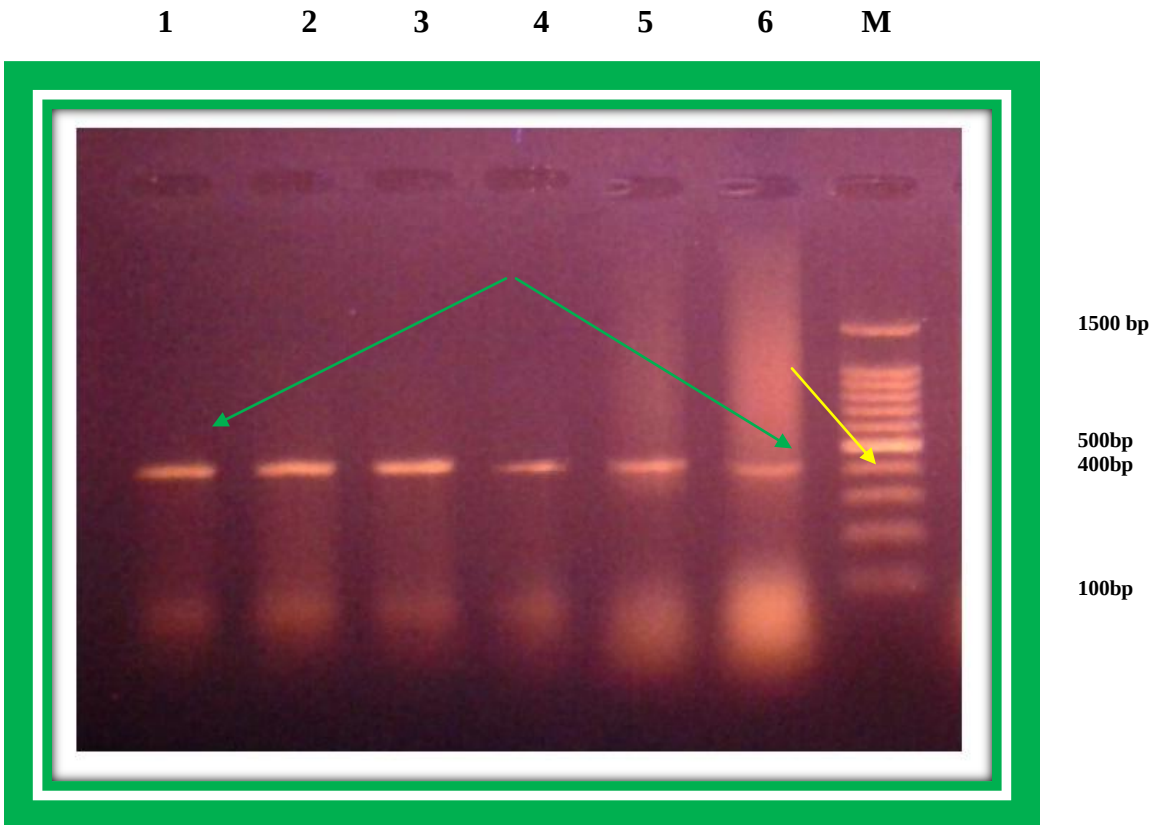

Figure (1) : Gel electrophoresis for amplified staphylokinase gene on agarose gel (1%) , 50V for 1 hour .
Lane (1 – 6) : *S.aureus* isolates (A15,A15-M1,A31,A34,A43,A49) .
(M) : DNA ladder (1500 bp) .

Nucleotides Sequencing of Sak gene

In order to cloning of Sak gene in E.coli JM 109 (DE3) polymerase chain reaction Product of Sak gene for *S.aureus* A15-M1 isolate was sent for determining the nucleotide sequence using NICEM/USA ABI3730XL Applied BIO SYSTEM . Sequencing was determined by an automatic sequencer , and the DNA sequences were analyzed and similarity were achieved with Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) . Results shown in figure (2) indicates the complete nucleotide sequence for Sak gene amplified from the local isolate of S.aureus A15-M1 compared with matchable sequence of the same gene taken from BLAST / NCBI on the web site .

Score	Expect	Identities	Gaps	Strand	Frame
678 bits(367)	0.0()	373/376(99%)	0/376(0%)	Plus/Plus	

Features:

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Query  10   AGGCGATGACGCGAGTTATTTTGAACCAACAGGCCCGTATTTGATGGTAAATGTGACTGG  69
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   AGGCGATGACGCGAGTTATTTTGAACCAACAGGCCCGTATTTGATGGTAAATGTGACTGG  95

Query  70   AGTTGATGGTAAAGGAAATGAATTGCTATCCCCTCGTTATGTCGAGTTTCCTATTAAACC  129
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  96   AGTTGATGGTAAAGGAAATGAATTGCTATCCCCTCATTATGTCGAGTTTCCTATTAAACC  155

Query  130  TGGGACTACACTTACAAAAGAAAAAATTGAATACTATGTCGAATGGGCATTAGATGCGAC  189
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  TGGGACTACACTTACAAAAGAAAAAATTGAATACTATGTCGAATGGGCATTAGATGCGAC  215

Query  190  AGCATATAAAGAGTTTAGAGTAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTA  249
           |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  AGCATATAAAGAGCTTAGAGTAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTA  275

Query  250  TTATGATAAGAATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT  309
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  TTATGATAAGAATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT  335

Query  310  TGTTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATTCAACTTAATTACAAAGGT  369
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  TGTTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATTCAACTTAATTACAAAGGT  395

Query  370  TGTTATAGAAAGGAAA  385
           |||||||||||| ||||
Sbjct  396  TGTTATAGAAAAGAAA  411
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Figure (2): Nucleotide sequence alignment of *S.aureus* A15-M1 staphylokinase gene and related sequence of *S.aureus* recorded in BLAST/ NCBI

The nucleotide sequence alignment between Sak gene of locally isolate *S.aureus* A15-M1 (Query) and standard strain (Sbjct) was carried out to ensure that there is no restriction site for BamHI and XbaI in Sak gene of standard strain . From this sequence it was found that there are mutations in locally isolate S.aureus A15-M1 compared with *S.aureus* standard strain .

This study consonance with Thi and Dinh (2012) and Pulicherla et al.(2013) in which Sak gene was isolated from *S.aureus* and amplified by PCR and analyzed on agarose gel in presence of 100bp DNA ladder marker . the size of PCR product (Sak gene) was 411bp .

Mutations in Sak gene

Mutation is a permanent change of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal genetic element. Mutation result from unrepaired damage to DNA or to RNA genomes typically caused by radiation or chemical mutagens, errors in the process of replication, or from the insertion or deletion of segments of DNA by mobile genetic elements (Aminetzach et al., 2005). Results of nucleotide sequence alignment of Sak gene for S.aureus A15-M1 and nucleotide sequence for the same gene for standard strain by using BLAST program in NCBI showed that there are three mutations in the coding region (open reading frame of the structural gene) of Sak gene for the locally isolated *S.aureus* A15-M1 as indicated in table (3). These mutations may be naturally occurring for the local isolate due to different environmental factors during growth and developing of this isolate, or may be occurred after mutagenesis by using UV-irrdiation . The type of these mutations was base pair substitution altering the codons for three amino acids in the structure of staphylokinase enzyme as shown in table (3). these mutation in the amino acids was missense mutation changing arginine (positively charged) to histidin (polar) in the site 105 of the Sak gene nucleotide sequence , and changing valine (hydrophobic) to alanine

(hydrophobic) in the site 204 , and the last was change arginine (positively charged) to lysine (positively charged) in the site 381 without altering enzyme activity .

Substitution mutation is a type of a mutation in which there is exchange between two bases . Such a substitution could change a codon to one that encodes a different amino acid and cause a change in the protein produced (Michael et al.,2010) .

Table (3) : Mutations in the nucleotide sequence of Sak gene for *S.aureus* A15-M1 and related changes in codons and amino acids

Mutated codon	Site of mutation (bp)	Amino acid change	Type of mutation
CGT → CAT	105	Arg → His	Missense
GTT → GCT	204	Val → Ala	Missense
AGG → AAG	381	Arg → Lys	Missense

Cloning of Sak gene in E.coli JM 109 (DE3)

Cloning technology has crucial impact in therapy development. Staphylokinase has major crucial role in thrombotic disorders and used as a drug against thrombosis (Yerasi et al., 2014) . So that , this study was carried out for cloning Sak gene in non-pathogenic organism well known as E.coli JM 109 (DE3) used for recombinant drug synthesis .

Restriction digestion of Sak gene

In order to cloning Sak gene from S.aureus in E.coli JM 109 (DE3) , two specific primers were used first used for amplification of S.aureus Sak gene . These primers were designed to be consist of two restriction sites for BamHI (in the forward primer) and for XbaI (in the reverse primer) . Hence the complete nucleotide sequence of Sak gene indicated in figure (2) doesn't have restriction sites for these two restriction enzymes to ensure that treatment of staphylokinase structural gene with BamHI and XbaI doesn't destroy the nucleotide sequence of the gene .

Results of restriction digestion of staphylokinase with BamHI and XbaI generates a restriction fragment of Sak gene with stick ends then purification of digested Sak gene was achieved with concentration 50 ng/µl and kept in -20 °C until use for further experiment .

Restriction digestion of pSP72 cloning vector

Results illustrated in figure (3) showed the linearized DNA molecule of the cloning vector after electrophoresis on agarose gel (1%) in presence of 10kb DNA ladder marker . This vector of 2462 bp by carrying AP^r as a selectable marker was purified for the cloning staphylokinase gene .

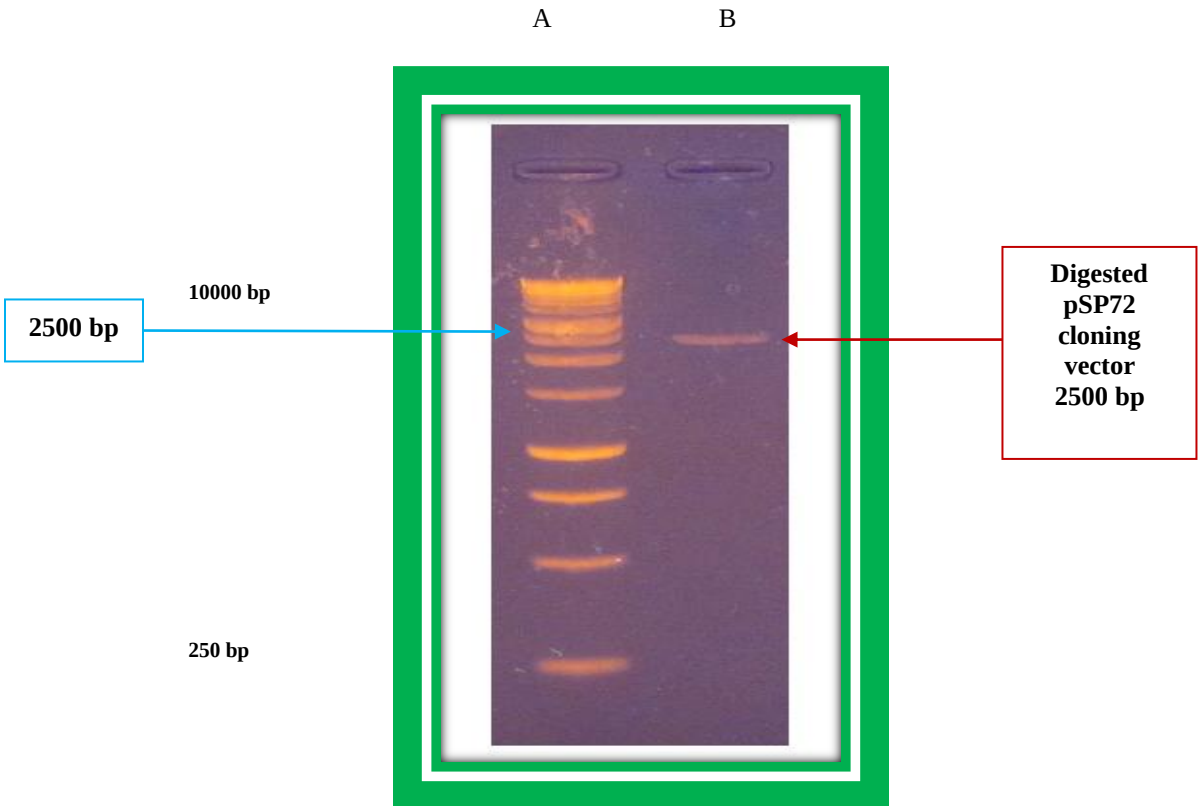

Figure (3): Gel electrophoresis on agarose gel (1%) for pSP72 cloning vector digested with BamHI and XbaI
Lane (A) : DNA ladder marker (10000 bp) .
Lane (B) : Digested pSP72 cloning vector .

Treatment of pSP72 cloning vector with BamHI and XbaI excise the fragment of DNA between these two enzymes within the multiple cloning site (MCS) in genetic restriction map of pSP72 cloning vector.

Cloning of staphylokinase gene in pSP72

Restriction fragment of staphylokinase gene treated with BamHI and XbaI was ligated with pSP72 treated with the same two enzymes , and inoculated at room temperature for five hours , then ligation reaction was terminated by heat inactivation at 70 °C for 15 minutes and cooled at room temperature , then used for transformation experiment .

Expression of Sak gene cloned into *E.coli* JM 109 (DE3) Transformants

Transformation of E.coli JM 109 (DE3) with recombinant vector pSP72 (cloning vector with Sak gene insert) was carried out according to Sambrook and Russell (2001) , aliquot of 200 µl of transformed cell suspension (DNA ligation mixture and competent cells) was plated on selective medium (plasma agar medium) containing 50µg/ml of ampicillin and 100µg/ml of IPTG , the plates were kept at 37 °C overnight to detect the expression of staphylokinase gene in transformed *E.coli* JM109(DE3) and positive transformants of E.coli JM 109 (DE3) containing recombinant pSP72 were selected . IPTG was caused induction of sak gene expression which its binds to the lac repressor and altering its conformation causing lac repressor and reducing its affinity to the lac operator and initiate protein synthesis .

Then gene expression of transformants was detected on plasma agar medium according to the formation of zones of hydrolysis around each clone . For conformation of the gene expression , well- difussion agar (specific method) was used and the degree of expression was detecting by measuring the diameters of zones of hydrolysis on plasma agar medium . Results showed that five transformants out of 83 (6%) were succefully expressed its own Sak gene according to the formation of zone of hydrolysis on plasma agar medium between 21-30mm.

Extraction of recombinant vector from *E.coli* JM 109 (DE3) transformnts

Extraction of recombinant vector (pSP72 cloning vector with Sak gene) from efficient transformant of *E.coli* JM109 (DE3) was carried out by using plasmid extraction kit , then analyzes on 1% agarose gel 50 V for two hours in presence of DNA ladder marker (10000bp) . Results illustrated in figure (5) showed the recombinant vector with molecular size of 2900 bp .

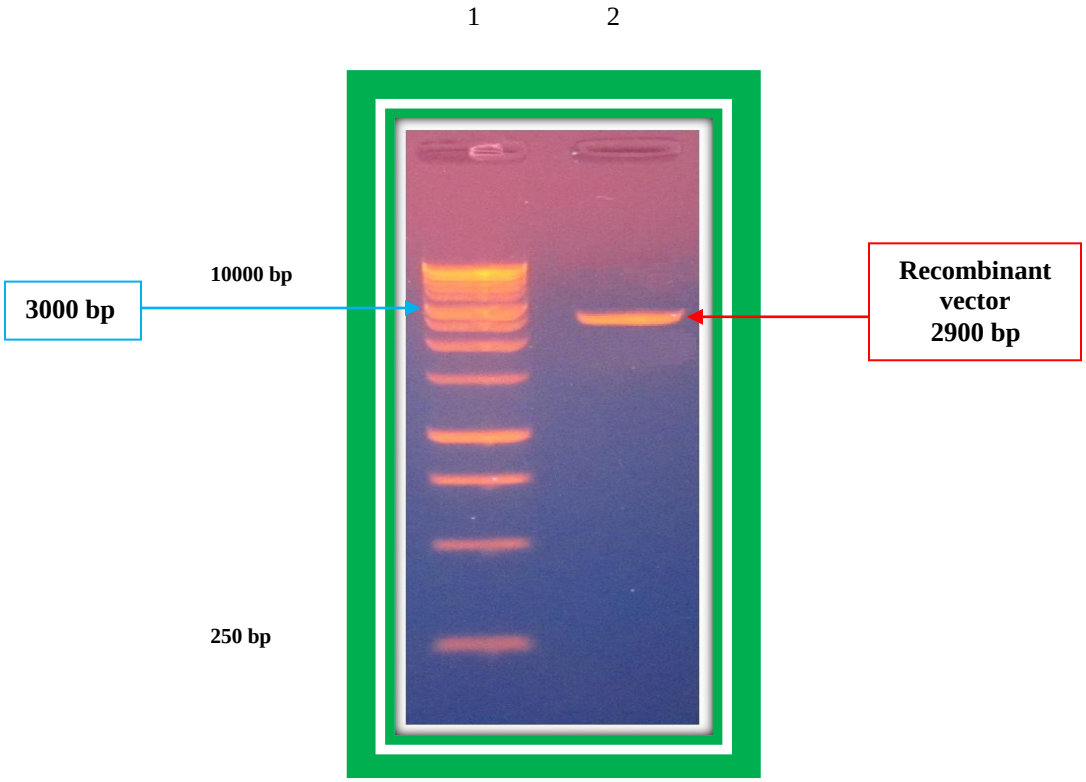

Figure (5) : Gel electrophoresis for recombinant pSP72 on agarose gel (1%) after extraction after extraction from transformant E.coli JM 109 (DE3) .

Lane (1) : DNA ladder marker (10000 bp) .

Lane (2) : Recombinant vector (pSP72 cloning vector with Sak gene).

Thrombotic disorders were one of the major impacts in human death owing to the thrombosis . Thrombi usually related to stroke, peripheral occlusive disease, pulmonary embolism, myocardial infarction and deep vein thrombosis . Therapy for these diseases was well recognized . Staphylokinase, plasminogen activator was widely used thrombolytic agents play major imperative role in fibrinolysis , So in clinical studies, Sak gene isolated from clinical sources was cloned into non-pathogenic *Escherichia coli* (DH5α) and it was produced recombinant staphylokinase (r-SAK) protein used for thrombolysis (Yerasi et al., 2014) .

A study achieved by Seetha et al.(2012) Sak gene from S.aureus was cloned in E.coli GJ1158 and gene expression in E.coli transformants was detected by formation of zone of hydrolysis on plasma agar medium . Staphylokinase is emerging as an important thrombolytic agent for the treatment of patients suffering from cardiovascular disease therefore this gene was isolated from S.aureus and cloned in *E. coli* BL21(DE3) and showed gene expression on plasma agar medium (Thi and Dinh , 2012).

Plant expression systems may be useful for the production of pharmaceuticals, as a large amount of protein can be produced with a relatively low cost, therefore expression of a staphylokinase, a thrombolytic agent in Arabidopsis thaliana carried out by transgene with planta Agrobacterium tumefaciens–mediated genetic transformation (Aneta et al.,2011) .

Conclusions

Molecular size of staphylokinase gene from locally isolated S.aureus was 411 bp with 99% nucleotide sequence similarity with gene sequence of standard strain . Gene expression and staphylokinase production in genetically engineered *E.coli* was in similar level of the gene from source microorganism (*S.aureus*) .

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