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UV light Induces Transposition of *blaZ* gene Resulting in High Expression of β-lactamase in a Clinical Isolate of *Staphylococcus aureus* Sensitive to Ampicillin

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In the present study an ampicillin sensitive *Staphylococcus aureus* confirmed from the MIC results, was isolated from a patient and the nature of *S aureus* was confirmed by its ability to grow in modified Baird Parkar media. This strain when exposed to UV radiation of 280 nm for 10 seconds could grow even in the presence of ampicillin (100mg/ml) and nitrocefin (50mg/ml). The specific activity of β–lactamase in the extracellular secretions of UV exposed *S. aureus* showed the presence of β–lactamase enzyme indicating successful expression of the gene. Southern hybridization with PVGKS -1 probe and amplification of β-lactamase gene with primers designed from *blaZ* gene sequence of *S aureus* from the chromosomal DNA of UV exposed *S. aureus* indicated that *blaZ* gene was located on the chromosomal DNA. Amplification of transposase gene using specific primers upstream of *blaZ* gene of Tn552 indicated that this gene might have been transposed on to the chromosomal DNA of the *Staphylococcus aureus* and where the gene successfully expressed in the organism making the organism resistant to β-lactam antibiotics, such transposition of trnasposons on exposure to slight pressure and its cross infections among patients can be life threatening in hospitals.

Key words: *Staphylococcus aureus*, β-lactamase, Tn552, PVGKS-1, nitrocefin.

Staphylococcus aureus is a gram-positive pathogen and a leading cause of both nosocomial and it is one of the common causes of hospital and community acquired infections throughout the world. This organism is commonly found in the most of the anatomical locales of human beings and can colonize on any surface such as catheters, scalpels etc. The organism is capable of expressing several potentially virulent factors; thereby causing a diverse array of clinical manifestations, ranging from simple skin abscesses to life threatening pneumonia, endocarditis and toxic shock syndrome[1-3].

In growing bacteria the SOS response has been recognized as a critical component of the response to environmental stress, such as UV mutagenesis led to the development of several antibiotic resistant strains in laboratory conditions[4]. RecA forms filaments on the single-stranded DNA, and these nucleoprotein filaments

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facilitate recombinational repair[5,6] as well as bind the SOS gene repressor LexA, stimulating its autoproteolysis. This cleavage inactivates the LexA repressor and results in the induction of the SOS genes. Interestingly, in addition to the fluoroquinolones, e.g., ciprofloxacin, other antibiotics, such as trimethoprim[5], rifamycins[6], streptomycin[7] and b-lactams[8,9], have all been shown to induce LexA cleavage and the SOS response. In *E. coli*, almost all SOS-targeted UV mutagenesis results from the activity of *umuDC* operon is the only SOS locus that must be induced for SOS mutagenesis[10-12]. Development of such antibiotic resistance due to stress response has indeed made *S aureus* to acquire resistance to almost all the antibiotics. Thus, the treatment and management of *S. aureus* infections have become a major health problem with the isolation of various antibiotic resistant strains of *S. aureus* [13]. Several researchers have sequenced and annotated different antibiotic resistant strains of *Staphylococcus aureus*[14] to identify pathogenic islands which are linked to antibiotic resistance.

One of the earliest reports of antibiotic resistance was of a bacterium that produced a β-lactamase, an enzyme that hydrolyses the β-lactam rings in b-lactam antibiotics such as penicillin, ampicillin[15]. Plasmid-encoded or chromosomal encoded, β-lactamases (EC 3.5.2.6.) from *Staphylococcus aureus* are relatively small, single-chain exoproteins whose synthesis is inducible by a variety of, β-lactam compounds. The enzymes are well characterized both enzymologically and structurally[15-19]. They are extremely active, having both high substrate affinity and high turnover numbers. Moreover, they can be accurately assayed by a variety of very sensitive techniques[19-21].

This enzyme is introduced into Staphylococci through transposon Tn552, Tn4002, Tn3852 and Tn4201[22]. These are very similar to each other and probably have common origin. The transposons have shown to be active, in that both Tn4201 and Tn3852 have shown to translocate to a site on the chromosome thereby, making Staphylococci resistant to b-lactam antibiotics[22]. Thus, in the present study the *S. aureus* culture isolated from a patient was obtained from the Department of Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati and culture was established and was found to be totally sensitive to ampicillin. When the same culture was exposed to the UV rays of 280nm for 10 seconds, the culture showed resistance to ampicillin and to other b-lactam antibiotics. In the present study we have observed very high expression b-lactamase enzyme in the clinical isolate of *S aureus* and it may be due to the transposition of *blaZ* encoding transposon within the chromosome of *S aureus*.

MATERIAL AND METHODS

Culturing of *S aureus* strains

Staphylococcus aureus strain (sputum sample) was grown on blood agar plate at 37^{0}C. After overnight incubation, strains were examined by Gram stain and growing the strain in modified Baird Parkar media[24]. Single colony was picked up and was cultured in Brain heart infusion (BHI) broth[25].

Antibiotic Susceptibility tests

The susceptibility of the *S aureus* strains to antibiotics was measured *in vitro* using the disc diffusion method[26]. Susceptibility to nitrocefin, ampicillin was also tested by the agar dilution method[27].

UV induced mutagenesis in *S aureus* and b-lactamase production

The 10^{-9} diluted *S aureus* culture was exposed to UV radiation for 5 and 10 seconds then the culture was immediately plated on Baird Parkar agar plate contained 100µg/ml nitrocefin, ampicillin and incubated at 37^{0}C for 24h and the results were recorded[25, 27].

Preparation of the b-lactamase enzyme

β-Lactamase activity was determined in the extracellular secretions of nitrocefin resistance induced *S. aureus*[21]. The Bacteria in an exponentially growing culture (500 ml) were harvested by centrifugation and the supernatant thus obtained (crude enzyme) was stored at -20°C until use[28-31].

Enzyme Assay of β-lactamase

β-Lactamase activity was assessed spectrophotometrically by hydrolysis of nitrocefin. The assay mixture contained 83mg of nitrocefin, 167 mg of BSA, 10% glycerol and 0.33ml (0.6mg/ml of protein) of cell lysate that contained b-lactamase in a final volume of 1.5ml of 50mM phosphate buffer. β-Lactamase activity was

monitored by measuring the decrease in absorbance at 390 nm for 10 min at 28^{0}C. The enzyme activity was expressed as mmol of nitrocefin hydrolysed/min/mg of protein and the calculation was based upon the molar extinction coefficient of 15 000 M^{-1} cm^{-1} for nitrocefin[28,29,31].

The variable substrate concentration was taken from 10mM to 100mM and data from the kinetic studies were treated using the method of Hanes – Woolf where [S]/V vs [S] was plotted. The slope is equal to 1/Vmax and the intercept on the y-axis is Km/Vmax.

Thin-layer chromatography

The sample was spotted on a thin-layer chromatography plate (Silica gel 60 F-254, Merck), developed by an ascending method using the solvent composed of butanol-acetic acid-water (4:1:4v/v/v). The absorption at 254nm attributed to the, b-lactam ring of the respective antibiotics mentioned in Table 3 was observed by ultraviolet light (Vilber-Loumart gel documentation system)[28].

Protein determination

The protein content was estimated by Lowry method[32]. The protein of the crude enzyme solution used was 1.2 mg/ml.

Iodometric assay of β-lactamase

The iodometric assay was carried out at 37°C in 0.1 M phosphate buffer, pH 7.0 using antibiotics mentioned in Table 3. One micromole of b-lactam antibiotic hydrolyzed per hour was expressed as one unit[8,32,33].

Isolation of Chromosomal DNA from *S aureus*:

In order to identify the b-lactamase gene, chromosomal DNA was extracted from late log phase culture of ampicillin resistance induced *Staphylococcus aureus*[25]. Pure *S. aureus* culture showing resistance to ampicillin (selected after MIC test) was grown in BHI media that contained 50µg/ml of ampicillin and chromosomal DNA was isolated by the method described in Sambrook and Russell 2001[25]. The culture was centrifuged at 7000 rpm for 2 minutes. The pellet was suspended in 500µl of TEG buffer (TEG buffer = 50mM Tris-Cl, pH 8, 50mM EDTA and 20% glucose) and recentrifuged at 7000 rpm for 2 minutes. This step was repeated thrice. The pellet thus obtained was vortexed and suspended in 150 µl of 10mg/ml ice cold lysozyme prepared in TEG buffer and incubated at 37°C for 1½ hour. 30µl of 10mg/ml pancreatic RNAse was added and incubated for 30 min at 37°C. 0.1 ml of 10% SDS was added through the walls; entire solution was mixed and left for 1½ hour. The DNA was extracted from the above tube by adding equal volume of solution prepared by mixing 1M Tris-Cl pH 8 saturated phenol, chloroform and isoamylalcohol in 25:24:1 v/v/v and vortexed vigorously for 2-3 minutes. This solution was centrifuged at 10,000 rpm for 10 minutes aqueous phase was separated and DNA in the aqueous phase was precipitated by adding equal volume of ice cold isopropanol and centrifuged at 10,000 rpm for 10 min. The pellet was suspended in 70% ethanol and centrifuged at 10,000 rpm for 10 min. The pellet thus obtained was suspended in TE buffer (10mM Tris – Cl, 1mM EDTA pH 8) and the DNA was analysed by running 1% agarose gel. Thus, isolated DNA was digested with 3U/ml Hind III, EcoRI, Sal I and Pst I (Bangalore Genei Pvt Ltd) and the digested DNA was fractionated in 1% agarose gel and transferred on to nitrocellulose membrane by capillary method. After suitably blocking the nitrocellulose membrane with 100mg/ml calf thymus DNA the blot was probed with PVGKS-1(CATGTAATTCAAACAGTTCACA TGCCAAAGAGTTAAGAdUTPbiotin) probe whose 3'end was labelled with 11biotinylated dUTP using 1U/ml terminal nucleotidyl transferase (Bangalore Genei Pvt Ltd and Fermentas). The blot was treated with strepavidin conjugated with horse raddish peroxidase and dark brown bands were developed when the blot was treated with 0.09% H_2O_2 in the presence of diaminobenzidine hydrochloride[25, 34].

PCR amplification and identification of β-lactamase gene and transposase gene:

The primers for the amplification of *blaZ* gene: Forward primer (HP1) 5'--TCAAACAGTTCACATGCC-3'; Reverse primer (HP2) 5'-TTCATTACACTCTGGCG-3' and for the amplification of transposase gene Forward primer (LP1): 5' CATAGGTTATGCAAGA T3' Reverse primer (LP2): 5' TTAGTTAGATACCTT 3' were synthesized from Bangalore Genei and *blaZ* and transposase genes were amplified from the chromosomal DNA of *S. aureus*. The PCR conditions for *blaZ* gene amplification are 90^{0}C for 5 minutes and 46^{0}C for 30S and 72^{0}C for 45S and extension at 72^{0}C for 5 minutes and for

transposase gene are 90^0C for 5 minutes and 36^0C for 30S and 72^0C for 45S and extension at 72^0C for 5 minutes in Master cycler gradient (Eppendrof)[23,34, 35].

RESULTS AND DISCUSSION

The growth characteristics of the culture indicated that the isolated culture was pure, and its typical black shiny colour colony morphology with zone in modified Baird Parker (BP) medium indicated that the culture is pure *S aureus* only[24]. Fifteen-mm zone obtained at a minimum inhibitory concentration (MIC) of ampicillin (3.5mg/ml) indicated that the present clinical isolate of *S. aureus* was susceptible to ampicillin (27). 10^{-9} diluted mid log phase culture of *S. aureus* was exposed to UV-radiation of $1Jm^{-2}$ at 280nm for 5 seconds and for 10 seconds (27). 5 seconds UV exposed *S. aureus* culture could not grow on BP agar plate containing ampicillin while, 10 seconds UV exposed *S. aureus* culture could grow on BP agar plate containing ampicillin (100mg/ml) indicated that 10 seconds UV exposure was sufficient to induce ampicillin resistance in *S. aureus*. UV exposure of *S. aureus* for 5 seconds did not induce any changes because uvr repair mechanism in *S. aureus* and SOS

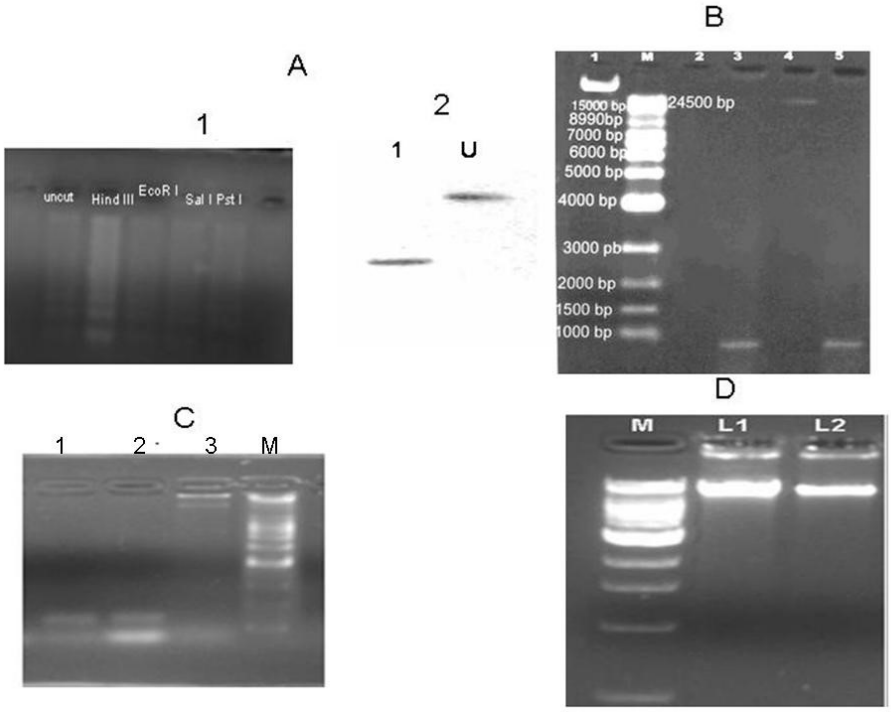

Fig. 1. A(1) Agarose gel electrophoresis showing restriction endonuclease digestion pattern of ampicillin resistant induced *S. aureus* chromosomal DNA. Lane Uncut *S aureus* chromosomal DNA, Lanes Hind III, EcoRI, Sal I and Pst I digested *S aureus* chromosomal DNA. A (2) Southern hybridization experiment of above digested *S aureus* chromosomal DNA with different restriction endonucleases was blotted on to nitrocellulose membrane (Pierce) and hybridized with PVGKS-1 probe and developed with H_2O_2 and diaminobenzidine tetrahydrochloride. Lane 1 Hind III digested *S aureus* chromosomal DNA and Lane 1 uncut *S aureus* chromosomal DNA B. Agarose gel electrophoresis showing PCR amplification of β-lactamase gene from ampicillin resistant induced *S. aureus* chromosomal DNA. Lanes: M: supermix molecular size ladder (Bangalore Genei); Lanes: 1 and 4 *S. aureus* chromosomal DNA; Lanes 3 and 5 PCR amplified product from chromosomal DNA of *S aureus*; lane 2 PCR amplified product from plasmid DNA of *S. aureus*. 4: *S. aureus* plasmid DNA. C: Agarose gel electrophoresis showing PCR amplification of transposase gene from ampicillin resistant induced *S. aureus* chromosomal DNA. Lanes1 and 2 PCR amplified products, lane 3 *S. aureus* chromosomal DNA, lane M: supermix molecular size ladder (Bangalore Genei). D: Agarose gel electrophoresis plasmid DNA from ampicillin resistant induced *S. aureus*. Lane M: supermix molecular size ladder (Bangalore Genei) lanes L1 and L2 Plasmid DNA

Table 1. Disk diffusion results of *S. aureus* against various β-lactam antibiotics

β-lactam antibiotics	Concentration in *m*g/ml	MIC - Minimum Inhibitory Concentration in mM
Cefaselin	2.0	10±0.2 mm
Nitrocefin	1.7	11±0.23 mm
Benzyl penicillin	3.0	12±0.18mm
Ampicillin	2.5	13±0.25mm

MIC- Minimum Inhibitory concentration,
mg –micrograms. mM- Millimetre in diameter of Zone of inhibition

Table 2. β-lactamase kinetics in the extracellular secretions of *S aureus*[31]

S. No	Source	Nitrocefin hydrolysed/min/mg	Km (mM)
1.	Extracellular fraction of *S. aureus* sensitive to nitrocefin	0.001	-
2.	Extracellular fraction of *S. aureus* clinical isolate resistant to nitrocefin after UV induction	23.02±0.042	6.4± 0.014

Table 3: Enzyme kinetics of β-lactamase present in the extracellular secretions of *S. aureus* sensitive to ampicillin and nitrocefin calculated from iodiomeric assay[28,33]

β-lactam antibiotics	Activity of β-lactamase mM β-lactam antibiotic hydrolyzed/mg/min
Cefaselin	0.0025
Benzyl penicillin	0.00155
Ampicillin	0.009

system must have repaired the changes induced by UV exposure hence, it could not grow on BP agar plate containing ampicillin. It is very well known that UV exposure induces heritable phenotypic changes in *S. aureus*, Thompson and Hart have induced Streptomycin resistance in *S. aureus* through UV exposure[7]. In the present high level b-lactamase activity was observed in the extracellular secretions of *S. aureus* when exposed to UV light[17,28,29,31] (Table 2). The expressed enzyme could show activity against b-lactam antibiotics listed in Table- 4 while the extracellular fraction of *S aureus* with out UV induction showed very poor activity or almost no activity at all (Table 3) thereby confirming the UV exposure of 10 seconds resulted in very high expression of β-lactamase enzyme in the clinical isolate of *S. aureus*.

Table 4: Enzyme kinetics of β-lactamase present in the extracellular secretions of UV induced *S. aureus* calculated from iodiomeric assay[28,33]

β-lactam antibiotics	Activity of β-lactamase mM β-lactam antibiotic hydrolyzed/mg/min	Km (mM)
Cefaselin	0.0325±0.0012	10.9±0.9
Benzyl penicillin	2.85±0.024	5.11±0.21
Ampicillin	5.1±0.12	2.55±0.23

The velocity of β-lactamase towards different β-lactam substrates and Km calculated from Hane's Woolf plot (Tables 3 and 4) indicated the expressed enzyme was highly active and the kinetics indicated that it is not only active against reported substrates (Table 2)[17,28,29,31]. In the present study iodometric method, UV-method and TLC method were used to corroborate the high expression of β-lactamase enzyme in the extracellular secretions of the UV induced *S. aureus*. In TLC distinct separation of hydrolysed β-lactam antibiotics over intact β-lactam antibiotics indicated the expression of β-lactamase in the extracellular secretions of UV induced *S. aureus*.

In order to locate the β- lactamase gene in *S. aureus* both chromosomal DNA and plasmid DNA were extracted from *S. aureus* and southern hybridization was performed with PVGKS–1. This probe was prepared from type A β-lactamase gene sequence and the 3' end was labelled with biotinylatedUTP using terminal transferase. The probe successfully hybridized with Hind III digested chromosomal DNA of *S aureus* but not with plasmid DNA (Fig. 1)[23,34,35]. This confirmed the location of *blaZ* gene on the chromosome. It is very well known that *blaZ* gene is part of transposons Tn4201, Tn3852 and Tn552 which are present in *S. aureus*[22]. The clinical isolate of *S aureus* showed very poor expression of *blaZ* gene before UV exposure (Table 3). However, upon UV exposure the expression of *blaZ* gene expression increased appreciably therefore, it is quite likely that this transposon was present in a place which resulted in the poor expression of *blaZ* gene.

In order to confirm that blaZ was transposed from its earlier position the *blaZ* and transposase genes were amplified by polymerase chain reaction. Polymerase chain reaction (PCR) was performed to amplify *blaZ* gene using the primers constructed from β-lactamase gene (34, 35) which could successfully amplify β-lactamase gene from the chromosomal DNA but not from plasmid DNA (Fig. 1). This further confirmed that b-lactamase gene was present on the chromosomal DNA of *S. aureus*. And successful amplification of transposase gene using the primers constructed from the upstream sequence of *blaZ* gene sequence in Tn552 DNA sequence of *S aureus* confirmed the assumption that *blaZ* gene was transposed to a different place in the *S aureus* genome which resulted in the active expression of β-lactamase enzyme[22,23] thereby, conferring resistance to various β-lactam antibiotics.

It is very well established that *S aureus* has an unique property of biofilm formation because of which it can colonize on any surfaces like catheters, scalpels, scissors etc used by the surgeons, and on all plastic surfaces including cell phones and if such mutated strains of *S. aurues* possessing drug resistance character infects patients they can cause very serious infections in both pre operative and post operative patients resulting in high mortality[6,8-12,36].

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