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CLONING AND TRANSFORMATION OF MecA GENE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM HOSPITAL SAMPLES

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ABSTRACT

Antibiotic resistance is common among bacterial pathogens associated with both community acquired and nosocomial infections. In view of the present problem of drug resistance, we investigated the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and amplified the mecA gene in the isolates from the hospital samples. The *S. aureus* isolates were analyzed for their susceptibility to different classes of antibiotics using Gentamycin and it shows resistance to Kanamycin, Methicillin, Amphotericin and Tetracyclin. MIC test was conducted for *S. aureus* for antibiotics Ampicillin and Gentamycin. Different samples show different MIC of antibiotics. Plasmid DNA was isolated from the sample 1 and mecA gene was amplified by suitable primers by PCR. This amplified product was sequenced and sequenced size was determined to be 1320 bp. Further it was cloned to plasmid pUC18 vector and transformed to DH5α strains of *E. coli.* This study highlights the emerging trend of multiple drug resistance in *S. aureus* samples isolated from hospitals.

Key words: mecA gene, Cloning, Transformation**,** *S.aureus,* MIC, Blast.

INTRODUCTION

Drug resistance is the reduction in effectiveness of a drug such as an antimicrobial or an antineoplastic in curing a disease or condition. When the drug is not intended to kill or

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inhibit a pathogen, then the term is equivalent to dosage failure or drug tolerance. More commonly, the term is used in the context of resistance acquired by pathogens. When an organism is resistant to more than one drug, it is said to be multidrug resistant[1].

Drug or toxin or chemical resistance is a consequence of evolution and is a response to pressures imposed on any living organism. Individual organisms vary in their sensitivity to the drug used and some with greater fitness may be capable of surviving drug treatment. Drug resistant traits are accordingly inherited by subsequent offspring, resulting in a population that is more drug resistant. Unless the drug used makes sexual reproduction or cell-division or horizontal gene transfer impossible in the entire target population, resistance to the drug will inevitably follow. This can be seen in cancerous tumors where some cells may develop resistance to the drugs used in chemotherapy. A quicker process of sharing resistance exists among single-celled organisms, and is termed horizontal gene transfer in which there is a direct exchange of genes, particularly in the biofilm state. A similar sexual method is used by fungi and is termed parasexuality. Some processes that destroy 100% of pathogens make use of high sugar concentrations as in fruit canning or high salt and vinegar concentrations as in pickling. Strong sugar and salt solutions have a high osmotic gradient, destroying pathogens by dehydration through their permeable cell walls.

Other methods include irradiation, or high temperatures as in canning and pasteurization. Examples of drug-resistant strains are to be found in microorganisms such as bacteria and viruses, parasites both endo and ecto plants, fungi, arthropods, mammals, birds, reptiles, fish and amphibians[2].

S. aureus is a Gram-positive, spherical bacterium (coccus) with a diameter of 1-1.3 μm. When viewed microscopically, *S. aureus* appears in clusters, like bunches of grapes. Growing in food, some strains can produce toxins which cause acute gastro-intestinal diseases if ingested. The enterotoxin produced by *S. aureus* is a heat-stable protein, which survives heating at 100°C for 30-700 minutes[3]. The main reservoirs of *S. aureus* are humans and animals. Healthy people carry the organism in their nose and throat (50%), on their hands (5-30%), and in wounds. *S. aureus* can also colonize food contact surfaces, and it can become a persistent organism in slaughterhouses. *S. aureus* can contaminate foods through contact with contaminated hands, materials and surfaces, but also via the air (coughing)[4]. *S. aureus* can grow both aerobically and anaerobically in various foods. It is characteristic that *staphylococci* can grow at low water activity (approx 0.86), corresponding with a salt content of about 14%[5].

EXPERIMENTAL

Materials and methods

Sample collection and chemicals

Staphylococcus aureus Source: Sample 1, 2, 3 and 4 from government hospital, Gulbarga, Sample 5 and 6 from a testing lab, Bagalkot. Pure culture of *Escherichia coli* from NCCS, Pune, India. Antibiotics like Ampicillin, Amphotericin, Gentamycin, Tetracyclin, Kanamycin, and Methicillin obtained from Himedia chemicals, India. Other chemicals of analytical grade also obtained from Himedia chemicals, India

Revive of cultures

Peptone broth of 60 mL was prepared in 100 mL conical flask. The 60 mL of broth was equally distributed into 6 different sterile test tubes. These test tubes were covered by aluminum foil properly and the test tubes were autoclaved at a temperature of 121ºC, 15 lbs per square inch pressure for 15 minutes. Then the test tubes were cooled to room temperature. The given 6 different strains were inoculated into 6 different test tubes and they were labeled properly and the tubes were incubated at 45ºC for 24 hours. All collected samples were subjected for antibiotic sensitivity test as explained by[6-7] and MIC of antibiotics was determined[8].

Plasmid isolation

The terrific broth containing appropriate antibiotic in micro tubes was inoculated with a single bacterial colony picked with a toothpick and Incubated at 37ºC on with vigorous shaking. Then the broth was centrifuged for 3 min at 10,000 rpm and aspirated the medium completely, leaving pellet as dry as possible. The cells were resuspended in 200 µL of lyses buffer as quickly as possible, preferably while vortexing the tube, 15 µL of lysozyme solution was added. The tubes were placed in boiling water bath for exactly 40 seconds. Then immediately centrifuged for 10 minutes at room temperature. Immediately 200 µL of lyses buffer was added, contents were mixed by inverting tube rapidly 2-3 times. 150 µL sodium acetate was added and make up the volume by adding isopropanol and mix quickly by tapping the bottom of the tube. Centrifuged for 1minute, sticky pellet were removed with toothpick. 1 mL alcohol was added and mixed by inverting the tubes, stand at room temperature for 2 minutes. Centrifuged at 10,000 rpm for 15 min at 4ºC to pellet DNA. DNA was dried and resuspended in 50 µL of TE, pH 8.0 containing DNAse free RNAse. Incubated at 37ºC for 10 minutes. Plasmid DNA was digested with appropriate restriction enzyme and analyzed by gel electrophoresis. A freshly isolated plasmid DNA was taken in vial and 2.5 µL of RNAse was added. Then the mixture was incubated at 37ºC for 1 hour

exactly in boiling water bath. Again the mixture was incubated by rising temperature to 60ºC for 10 minutes exactly and maintained at cold condition[9].

Molecular identification

Molecular identification was carried out by using the PCR technique. The amplification was carried out by using the PCR master mix (Invitrogen). The amplified product was subjected to Agarose gel electrophoresis as per the instruction given by the kit provider[10].

Gel elution

A vial containing DNA was taken and 132 µL of gel binding buffer was added. The mixture was incubated at 55ºC to 60ºC for 7 minutes. The contents were mixed after every 2-3 mins so that agarose was dissolved. Lysate was loaded in Hielute miniprep spin column and volume was made to 700 µL with gel binding buffer mixture. And centrifuged at 12,000 rpm for 1min at room temperature. The flow through was discarded and column was placed back into the same collection tube. And another 300 µL of gel binding buffer was added into column and centrifuged at 12,000 rpm for 1 min and flow was discarded.

Wash

Diluted gel buffer was prepared as indicated above then column was placed into same collection tube and 700 µL of dilutes gel wash buffer was added. Then centrifuged at 12,000 rpm for 1min at room temperature and flow through was discarded. The empty column was centrifuged at 14,000 rpm for 2 min to dry the column membrane.

DNA Elution

The column was placed into a new 2 mL collection tube and 30-50 µL of elution buffer was added (10 mM tris-Cl, pH8.5) directly on to the column membrane. This was incubated at room temperature for 1 min. Then centrifuged for 1 min at maximum speed greater than 14,000 rpm to elute DNA[7].

Restriction enzyme treatment

Restriction enzyme treatment was carried out by taking the amplified product and a standard pUC18 vector purchased from Invitrogen. The restriction enzyme mix containing pUC18 vector was incubated at 37^{o}C for 1 hour. Then incubated at 60^{o}C for 5-10 minutes to kill Hind III present in the mixture. The restriction enzyme mix containing amplified product was incubated at 37^{o}C for 1 hour. Then incubated at 60^{o}C for 5-10 minutes[8]. The restriction enzyme mix treated with pUC18 vector and amplified product DNA was mixed and 2.5 µL

ligase buffer was added and 0.4 µL of T_4 DNA ligase enzyme was added. The volume was making up to 10 µL by adding 2.1 µL of HPLC water and incubated at 16°C for overnight.

Preparation of competent *E. coli* cells

LB media of 5 mL was inoculated by colony of *E. coli* and incubated at 37°C for overnight with shaking[11]. From the overnight culture 300 µL was taken and inoculated into fresh 30 mL LB media and incubated at 37°C with shaking until the absorbance reaches to 0.4-0.5 at 550 ηm. Then the culture was centrifuged at 8,000 rpm for 10 min at 4°C in pre cooled rotor. The supernatant was discarded and cells were resuspended in 300 µL ice cold, sterile 50 mM $CaCl_2$ and left in ice for 15 min with occasional shaking. Then the cells were centrifuged at 8000 rpm for 10 min at 4°C in pre cooled rotor and $CaCl_2$ was discarded. The cells were resuspended in 3 mL ice cold sterile 50 mM $CaCl_2$[12].

Uptake of DNA by competent cells

The suitable amount of DNA was added into a vial contained 300 µL of competent cells and vial containing 50 µL of competence cells was referred as blank. These vials were left on ice for 30 min. The vials are incubated at 42°C for 2 min in boiling water bath. Then vials are returned to ice and left for 10 min and 1 mL of fresh media was added and incubated at 37°C for 1 hr[12].

Recombination selection with pUC18

The one LB-Ampicillin plates and one LB plate was prepared and dried. The overnight culture of host *E. coli* strain in 5 mL LB was prepared. After expressing the cells a short dilution series in microfuge tubes was made.

- Tube 1: 200 µL cells
- Tube 2: 20 µL cells + 180 µL LB
- Tube 3: 2 µL cells + 198 µL LB

A 200 µL of 2% X-gal in dimethylformide was prepared and 50 µL of this was added to each tube along with 10 µL 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Immediately the content of each tube was spreaded to an LB-Ampicillin plate. Controls:

(a) 200 µL of transformed cells were spreaded on to an LB plate.

(b) 200 µL of overnight culture was spreaded on to an LB-Ampicillin plate.

All the plates were incubated at 37°C for overnight and then growth in the plates was observed[10].

RESULTS AND DISCUSSION

Antibiotic sensitivity test

The inhibition zones were observed only in Ampicillin and Gentamycin to the given samples of *S. aureus* (Fig 1).

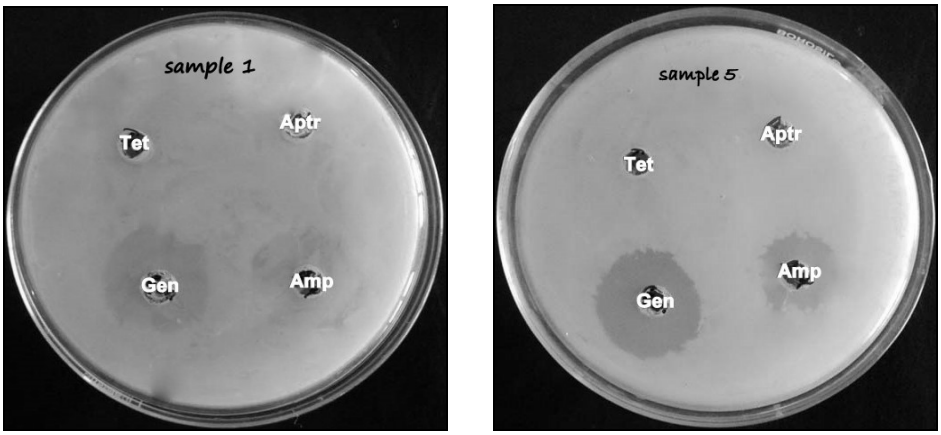

Fig. 1: The effect of antibiotic sensitivity test for different samples of *S. aureus* against different antibiotics.

Minimal inhibitory concentration test (MIC)

The minimum inhibitory concentration was found to be 25 µg. And the antibiotic sensitivity against different antibiotics was given in Table 1 and 2 (Fig 2).

Table 1: Diameter (cm) of inhibition zones of different antibiotics on samples of *S. aureus* at 25 µg concentration.

Antibiotics	Samples					
	S1	S2	S3	S4	S5	S6
	Diameter in cm					
Ampicillin	1.8	1.9	1.4	2.0	1.1	1.0
Gentamycin	1.8	1.8	1.9	1.8	1.8	1.5

Cont…

Antibiotics	Samples					
	S1	S2	S3	S4	S5	S6
	Diameter in cm					
Tetracycline	0.0	0.0	0.0	0.0	0.0	0.0
Amphotericin	0.0	0.0	0.0	0.0	0.0	0.0
Kanamycin	0.0	0.0	0.0	0.0	0.0	0.0
Methicillin	0.0	0.0	0.0	0.0	0.0	0.0

Table 2: Diameter (cm) of inhibition zones for Ampicillin and Gentamycin at different concentration for given samples of *S. aureus*

Samples	Ampicillin			Gentamycin		
	2.5 µg	5 µg	10 µg	2.5 µg	5 µg	10 µg
	Diameter in cm					
S1	0.0	0.0	0.0	0.0	0.0	0.0
S2	0.0	0.0	1.0	1.9	2.5	2.8
S3	0.0	0.0	1.1	0.0	0.0	0.9
S4	0.8	1.6	1.9	1.8	2.0	2.3
S5	0.0	0.0	0.0	0.0	0.0	0.0
S6	0.8	1.5	1.8	2.0	2.2	2.5

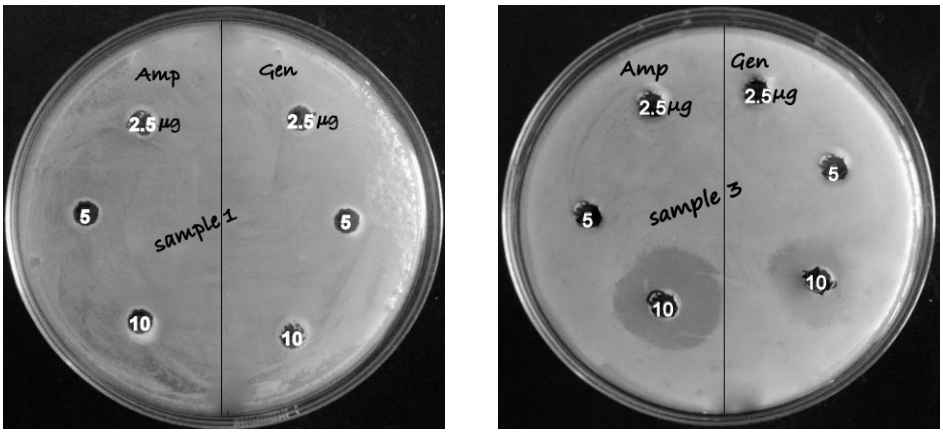

Fig. 2: The effect of MIC test for different samples of *S. aureus* against Ampicillin and Gentamycin

PCR Result

The amplified product was obtained at an annealing temperature of 59.2^{o}C (Fig 3).

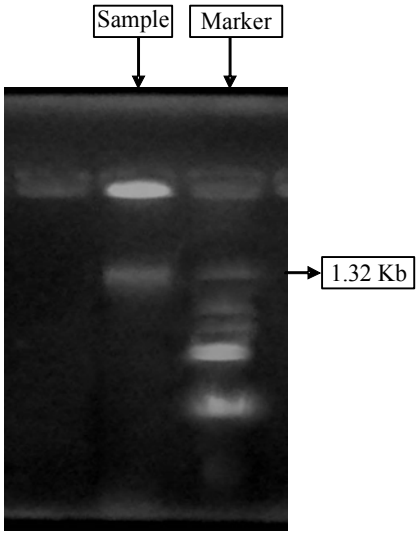

Fig. 3: The band formation after gel electrophoresis of amplified product

Sequence of the amplified product

atgaaaaagataaaaattgttccacttattttaatagttgtagttgtcgggtttggtatatatttttatgcttcaaaagataaagaaattaata
atactattgatgcaattgaagataaaaatttcaaacaagtttataaagatagcagttatatttctaaaagcgataatggtgaagtagaaat
gactgaacgtccgataaaaatatataatagtttaggcgttaaagatataaacattcaggatcgtaaaataaaaaaagtatctaaaaata
aaaaacgagtagatgctcaatataaaattaaaacaaactacggtaacattgatcgcaacgttcaatttaattttgttaaagaagatggta
tgtggaagttagattgggatcatagcgtcattattccaggaatgcagaaagaccaaagcatacatattgaaaaattaaaatcagaacg
tggtaaaattttagaccgaaacaatgtggaattggccaatacaggaacagcatatgagataggcatcgttccaaagaatgtatctaaa
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ccttcgttccacttaaaaccgttaaaaaaatggatgaatatttaagtgatttcgcaaaaaaatttcatcttacaactaatgaaacagaaag
tcgtaactatcctctagaaaaagcgacttcacatctattaggttatgttggtcccattaactctgaagaattaaaacaaaaagaatataaa
ggctataaagatgatgcagttattggtaaaaagggactcgaaaaactttacgataaaaagctccaacatgaagatggctatcgtgtca
caatcgttgacgataatagcaatacaatcgcacatacattaatagagaaaaagaaaaaagatggcaaagatattcaactaactattga
tgctaaagttcaaaagagtatttataacaacatgaaaaatgattatggctcaggtactgctatccaccctcaaacaggtgaattattagc
acttgtaagcacaccttcatatgacgtctatccatttatgtatggcatgagtaacgaagaatataataaattaaccgaagataaaaaaga
acctctgctcaacaagttccagattacaacttcaccaggttcaactcaaaaaatattaacagcaatgattgggttaaataacaaaacatt
agacgataaaacaagttataaaatcgatggtaaaggttggcaaaaagataaatcttggggtggt

Source: BioServe Biotechnologies Pvt. Ltd, Hyderabad

Sequence analysis and BLAST result

Cloning and transformation

Clones of pUC18 plasmid were obtained with genes of our amplified product (mecA *S. aureus* genes) Transformed colonies appeared in white color (Table 3).

Table 3: Sequences producing significant alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
GU301105.1	Staphylococcus aureus strain M600 penicillin binding protein 2a (mecA) gene, partial cds	2438	2438	100%	0.0	100%
GU301106.1	Staphylococcus aureus strain M16 penicillin binding protein 2a (mecA) gene, partial cds	2433	2433	100%	0.0	99%

The diameter of inhibition zones of Gentamycin was larger compared to the Ampicillin, so *S. aureus* showed sensitivity to Gentamycin than Ampicillin and *S. aureus* showed more resistance to the antibiotics like Kanamycin, Methicillin, Tetracycline and Amphotericin.

For Ampicillin, the samples 1 and 5 doesn't shows inhibition zone, so these samples require more than 10 µg concentration. The samples 4 and 6 show the inhibition zone at 2.5 µg concentration but the sample 2 shows the inhibition zone at 10 µg concentration. For Gentamycin, the samples 1 and 5 doesn't show inhibition zone, so these samples require more than 10 µg concentration. The samples 2, 4 and 6 show the inhibition zone at 2.5 µg concentration.

The plasmid DNA was isolated from sample 1 of *S. aureus* and further subjected to PCR for amplification of mecA gene. We came to know mecA gene of *S. aureus* requires the annealing temperature of 59.2^{o}C for amplification.

After transformation cells having gene of our interest integrated in the multiple cloning site (MCS) of plasmid pUC18 vector, So the LacZ gene become inactivated and

does not produces the β-galactosidase enzyme. Therefore X-gal in the media does not degraded and colonies appear in white color on the LB Ampicillin media.

After transformation cells having plasmids without gene of our interest produces the β-galactosidase enzyme by activation of LacZ gene, here IPTG in the media induces the lac operon and activates the LacZ gene. X-gal is naturally colorless, structurally similar to lactose and acts as a substrate for β-galactosidase enzyme, so β-galactosidase enzyme degrades the X-gal and produces blue color complex in cells, so cells appear blue in color on the LB Ampicillin media when exposed to light.

The competent cells are normal *E. coli* cells without plasmid, so Ampicillin in the media (LB Ampicillin media) inhibits the growth of *E. coli.* The white color conies appeared on the plate confirms the successful transformation of mecA gene into *E. coli.*

CONCLUSION

In our project *S. aureus* showed resistance against the antibiotics like Kanamycin, Methicillin, Gentamycin, and Amphotericin. We could isolate the gene which gives resistance against Methicillin, it has been also reported that there are more than one gene responsible for Methicillin resistance. The other gene of Methicillin resistance and resistance against other antibiotics has to be studied further.

The present study adds to the knowledge of molecular reason for MDR (multi drug resistance). The outcome of work can be further useful to find a solution for the challenge of facing MDR pathogen.

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