ANTIBIOTIC SUSCEPTIBILITY AND R-PLASMID MEDIATED DRUG RESISTANCE IN STAPHYLOCOCCUS AUREUS

MOSTAFIZUR RAHMAN*
ABDUL HYE KHAN**
M. SHAHJAHAN*
DIPAK K. PAUL***
PERVEZ HASSAN****

SUMMARY: A total of twenty-eight Staphylococcus aureus strains were isolated from skin lesion samples. Results of antibiotic susceptibility test showed that these twenty-eight Staphylococcus aureus strains were resistant to ampicillin (72%), amoxycillin (72%), penicillin (72%), cotrimoxazole (15%), cloxacillin (50%) tetracycline (11%), cephradine (22%), cephalexine (7%) and nalidixic acid (18%). To understand whether this drug resistance phenomenon was plasmid mediated or not, plasmids were isolated from a chosen strain of Staphylococcus aureus (S_2) and a 23 KB plasmid was obtained. This 23 KB plasmid was then transferred to an antibiotic sensitive E. coli LE 392 and after which the sensitive E. coli LE 392 strain developed drug resistance. Plasmid analysis of the transformed E. coli LE 392 revealed that it contains a 23 KB plasmid corresponding to that of the donor Staphylococcus aureus strain which may harbor the gene(s) encoding multidrug resistance in the donor Staphylococcus aureus.

Key Words: Plasmid, multidrug resistance, staphylococcus aureus.

INTRODUCTION

Staphylococcus aureus is the major pathogen of the genus *Staphylococcus*. It may be found as commensals frequently on the skin of carriers without any infection. It is however an important pathogen which can be responsible for a great variety of pyogenic infections in man and animals. It is the causative agent of many suppurative processes ranging from localized abscesses which can occur anywhere of the body to fatal septicemias and pneumonia (1).

*From Department of Biochemistry, University of Rajshahi, Rajshahi, Bangladesh.
**From Department of Genetics and Breeding, University of Rajshahi, Rajshahi, Bangladesh.
***From Department of Applied Nutrition and Food Science, Islamic University, Khustia, Bangladesh.
****From Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh.

The staphylococci appears to become drug resistant more readily than most other bacteria. The appearance of drug resistant strains isolated from pathologic processes have followed the introduction of various antibiotics into general use and the proportion of resistant strains found have continuously increased. Penicillin was the first antibiotic used for Staphylococcal infections and penicillin resistance appeared shortly after its introduction. This was followed by the resistance to cotrimoxazole, ampicillin, amoxycillin and tetracycline. Resistance to erythromycin and chloramphenicol also occurs but to a lesser extent than other antibiotics. Strains resistant to more than one antibiotic are by now the rule (2).

Methicillin resistant strains of *Staphylococcus aureus* developed an intrinsic resistance to the "penicillinase

stable penicillin" like cloxacillin etc (3). This resistance to methicillin is often accompanied by resistance to many other antibiotics (4).

In Bangladesh reports of strains resistant to cotrimoxazole, cephradine, erythromycin, gentamicin, ampicillin, chloramphenicol and nitrofurantoin was documented in a recent investigation (5).

Plasmid mediated drug resistance in *Staphylococcus aureus* was reported by many workers. Reports about plasmid mediated resistance to chloramphenicol, gentamycin, tobramycin and kanamycin in *Staphylococcus aureus* were also documented (6). Similarly *Staphylococcus aureus* strains possessed plasmid copies of β-lactamase determinant was also documented (7). Moreover, besides plasmid mediated drug resistance, chromosomal DNA mediated multiple drug resistance phenomenon in *Staphylococcus aureus* was also reported by many workers (8-9).

In our study we have tried to evaluate antibiotic susceptibility of *Staphylococcus aureus* isolated from skin lesions samples collected from skin of out patients of Rajshahi Medical College Hospital and Medical Center of Rajshahi University. We have tried to establish the antibiotic susceptibility profile of *Staphylococcus aureus*.

Here we report on the isolation of a single 23 KB plasmid from a selected multidrug resistant strain of *Staphylococcus aureus*. We have also tried to present evidences that this 23 KB plasmid of multidrug resistant *Staphylococcus aureus* might harbor the gene(s) that endow multiple drug resistance in the selected *Staphylococcus aureus* and to find out the connection between the presence of plasmid and acquisition of drug resistance phenomenon in *Staphylococcus aureus*.

MATERIALS AND METHODS

Media and culture condition

Nutrient agar was used as general media. Blood agar, Gelatin agar, Muller-Hinton agar, Kligler's iron agar, Mannitol agar, Christensen's urea agar and DNAase agar were used in the isolation and identification. Bacterial culture was done at 37°C through out the experiment.

Collection of bacterial samples

About forty bacterial samples were collected from out door of Dermatology Department, Rajshahi Medical College Hospital and Pathological Laboratory of Rajshahi University Medical Center. Samples were collected from pus of wounds, abscesses and other skin lesions using sterile cotton swabs in sterile test tubes. In some instances blood, sputum and spinal fluid was collected depending upon the localization of infection processes. Samples were inoculated on to blood agar and nutrient agar plates. Well isolated colonies were picked up and stored in nutrient agar slant. Pure culture of the isolates were done by isolating single colonies from the stored bacteria.

E. coli LE 392 used in the transformation experiment was supplied by the Department of Biochemistry and Molecular Biology, Yamaguchi University, Japan.

Screening and identification of bacteria

All forty bacterial samples were subjected to hemolysis test on blood agar and β-hemolytic bacteria were isolated and purified. β-Hemolytic strains were further screened through Gram staining and catalase tests. All the primary screened strains were subjected to various morphological and biochemical tests (10).

In vitro antibiotic susceptibility test of *Staphylococcus aureus*

All the finally identified *Staphylococcus aureus* strains were subjected to *in vitro* antibiotic susceptibility test by antibiotic disc diffusion method (11). Ten commonly used antibiotics were used in the test.

Extraction, purification and estimation of plasmid DNA

A multiple drug resistant strain of *Staphylococcus aureus* was selected for plasmid extraction. Plasmid DNA was extracted from 100 ml over night broth culture of the selected multiple drugs resistant *Staphylococcus aureus* strain by a standard method (12). The plasmid DNA was purified with polyethylene glycol (PEG-8000) and estimated by spectrophotometric method (13).

Electrophoretic analysis of the plasmid DNA

Agarose gel electrophoresis of plasmid DNA of a selected multidrug resistant strain of *Staphylococcus aureus* was carried out on 0.8% agarose (14).

Plasmid DNA transfer to sensitive *E. coli* LE 392

As an attempt to transfer plasmid DNA to sensitive *E.coli* LE 392 competent cells were prepared by calcium chloride procedure and then transformation experiment was carried out (15).

Study of plasmid profile of the transformed *E. coli* LE 392

Transformed *E. coli* LE 392 was subjected to plasmid DNA extraction (12). The extracted plasmid DNA was purified with polyethylene glycol (PEG-8000) and subjected to agarose gel electrophoresis on 0.8% agarose (14).

RESULTS

Screening and identification of *Staphylococcus aureus* strain

All the forty bacterial strains were initially subjected to hemolysis test on blood agar, Gram staining and catalase test in an attempt to screen the β-hemolytic, Gram positive

and catalase positive strains and thirty-two samples were found to have such characters. These primarily screened strains were then subjected to various morphological and biochemical tests. It was found that out of thirty-two β-hemolytic, gram positive and catalase positive strains only twenty-eight strains were identified as *Staphylococcus aureus* on the basis of their gross morphology and biochemical reaction pattern. In respect to cultural characteristics and colony morphology it was found that on blood agar the colonies were circular, golden-yellow and white pigmented. On nutrient agar color of the colonies were yellowish-white. In nutrient broth the growth was turbid. On microscopic observation it was revealed that the cells were non-motile, arranged in pair or short chain and in grape like clusters. The cells were oval or spherical in shape.

In vitro* antibiotic susceptibility test of *Staphylococcus aureus

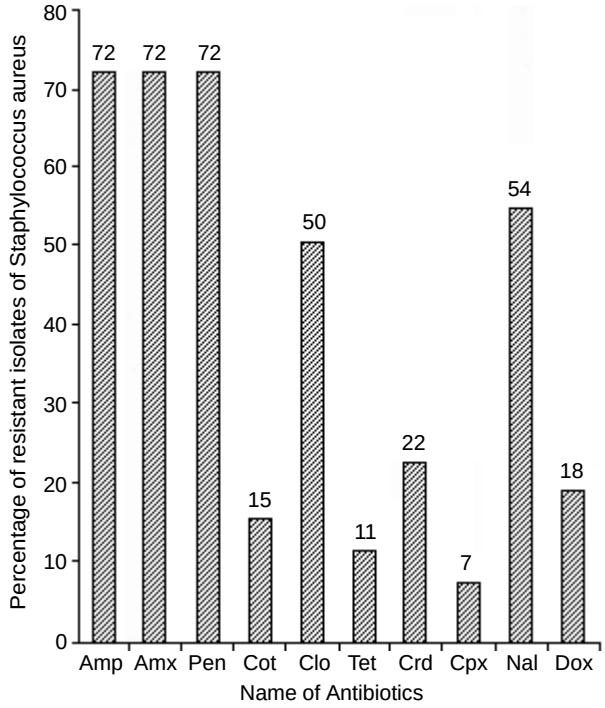
All the twenty-eight *Staphylococcus aureus* strains were tested *in vitro* to determine their antibiotic susceptibility pattern by antibiotic disc diffusion method (11). Majority of the strains showed multiple drug resistance to the drugs

Table 1: Biochemical reaction pattern of thirty-two β-hemolytic bacterial strains.

Strain No.	Coagulase test	DNAase test	Motility test	Uriase test	Gelatin Liquifaction test	Fermentation test		Comments
						Glu	Man	
S_1	+	+	-	+	+	+	+	*S. aureus*
S_2	+	+	-	+	+	+	+	*S. aureus*
S_3	+	+	-	+	+	+	+	*S. aureus*
S_4	+	+	-	+	+	+	+	*S. aureus*
S_5	+	+	-	+	+	+	+	*S. aureus*
S_6	+	+	-	+	+	+	+	*S. aureus*
S_7	+	+	-	+	+	+	+	*S. aureus*
S_8	+	+	-	+	+	+	+	*S. aureus*
S_9	+	+	-	+	+	+	+	*S. aureus*
S_{10}	+	+	-	+	+	+	+	*S. aureus*
S_{11}	+	+	-	+	+	+	+	*S. aureus*
S_{12}	-	+	-	-	+	+	+	*Not S. aureus*
S_{13}	+	+	-	+	+	+	+	*S. aureus*
S_{14}	-	-	-	+	+	+	-	*Not S. aureus*
S_{15}	+	+	-	+	+	+	+	*S. aureus*
S_{16}	+	+	-	+	+	+	+	*S. aureus*
S_{17}	+	+	-	+	+	+	+	*S. aureus*
S_{18}	+	+	-	+	+	+	+	*S. aureus*
S_{19}	+	+	-	+	+	+	+	*S. aureus*
S_{20}	-	-	-	+	+	+	+	*Not S. aureus*
S_{21}	+	+	-	+	+	+	+	*S. aureus*
S_{22}	+	+	-	+	+	+	+	*S. aureus*
S_{23}	+	-	-	-	+	+	-	*Not S. aureus*
S_{24}	+	+	-	+	+	+	+	*S. aureus*
S_{25}	+	+	-	+	+	+	+	*S. aureus*
S_{26}	+	+	-	+	+	+	+	*S. aureus*
S_{27}	+	+	-	+	+	+	+	*S. aureus*
S_{28}	+	+	-	+	+	+	+	*S. aureus*
S_{29}	+	+	-	+	+	+	+	*S. aureus*
S_{30}	+	+	-	+	+	+	+	*S. aureus*
S_{31}	+	+	-	+	+	+	+	*S. aureus*
S_{32}	+	+	-	+	+	+	+	*S. aureus*

Here '+' indicates positive test and '-' indicates negative test, Glu : Glucose and Man : Mannitol.

Figure 1: Antibiotic susceptibility profile of twenty-egiht *Staphylococcus aureus* strains. Here Amp: Ampicillin, Amx: Amoxycillin, Pen: Penicillin, Cot: Cotrimoxazole, Clo: Cloxacillin, Tet: Tetracycline, Crd: Cephradine, Cpx: Cephradine, Cpx: Cephalexine, Nal: Nalidixic acid and Dox: Doxicillin.



tested. From the overall resistance pattern it was observed that all the twenty-eight *Staphylococcus aureus* strains were resistant to ampicillin (72%), amoxycillin (72%), penicillin (72%), nalidixic acid (54%), cloxacillin (50%), cephradine (22%), doxicycline (18%), cotrimoxazole (15%), tetracycline (11%), cephalexine (7%).

Extraction, purification and estimation of the plasmid DNA

Single colony of a selected strain S_2 (sample no. 2) from twenty-eight multiple drug resistant isolates of *Staphylococcus aureus* was cultured for plasmid DNA extraction. The selected strain was resistant against at least seven antibiotics including cloxacillin ampicillin, amoxycillin, penicillin, tetracycline, cepheradine and chloramphenicol. Plasmid DNA was extracted by a standard method (12) and purified with polyethylene glycol (PEG-8000). Purified plasmid DNA was then estimated by spectrophotometric method (13). About 48 μg of plasmid DNA was obtained from 100 ml broth culture of the selected *Staphylococcus aureus* strain and the DNA was almost pure.

Electrophoretic analysis of the plasmid DNA

Electrophoretic analysis of the purified plasmid DNA was carried out by agarose gel electrophoresis on 0.8% agarose (14). The selected strain found to harbor a single plasmid DNA. On the basis of electrophoretic mobility on agarose gel the molecular size of the plasmid DNA was calculated to be 23 KB. In this experiment λ DNA (Hind III digested) was used as marker DNA.

Plasmid DNA transfer to sensitive *E. coli* LE 392

Plasmid DNA was transferred to sensitive *E. coli* LE 392 (15). After transformation experiment, when control and experimental plates were compared, bacterial growth was observed on experimental plates containing 30 μg/ml, 40 μg/ml and 60 μg/ml of ampicillin, 30 μg/ml, 40 μg/ml and 50 μg/ml of penicillin and 30 μg/ml, 40 μg/ml and 50 μg/ml of amoxycillin. No growth was observed on any of the control plates containing these three antibiotics in the same concentrations. Transformation experiment revealed that the 23 KB plasmid DNA of the donor *Staphylococcus*

Figure 2: Plasmid profile of drug resistant *S. aureus* isolates (S_2). Lane-1 shows the marker DNA (λ DNA Hind III digested). Lane-2 corresponds to plasmid DNA isolated from strain *S. aureus* (S_2).

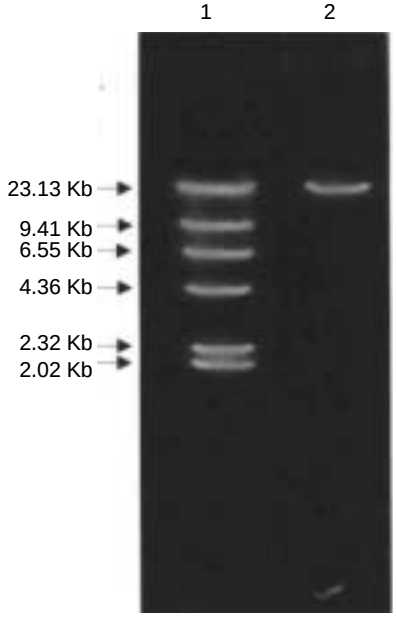
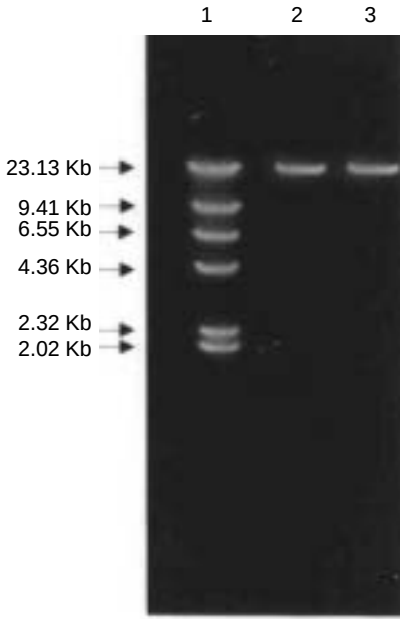


Figure 3: Plasmid profile of donar *S. aureus* isolates (S_2) and transformed *E. coli* LE 392. Lane-1 shows the marker DNA (λ DNA Hind III digested). Lane-2 and Lane-3 correspond to plasmid DNA isolated from strain *S. aureus* (S_2) and transformed *E. coli* LE 392.



aureus might harbor the gene(s) encoding multiple drug resistance phenomenon in the selected *Staphylococcus aureus* strain.

Study of plasmid profile of transformed *E. coli* LE 392

Plasmid DNA was isolated from transformed *E. coli* LE 392 and subjected to agarose gel electrophoresis with 0.8% agarose (14). From electrophoresis a single plasmid of 23 KB in molecular size has been calculated which resembled to that of the donor *Staphylococcus aureus* in molecular size.

The electrophoretic analysis of the transformed strain has further confirmed the fact that the 23 KB plasmid DNA isolated from the donor *Staphylococcus aureus* might harbor the gene(s) encoding multiple drug resistance phenomenon in the donor *Staphylococcus aureus* strain.

DISCUSSION

As an attempt to isolate β-hemolytic bacteria all these forty samples were subjected to hemolysis test on blood agar. When β-hemolysis was observed on blood agar after 24 - 48 hours incubation at 37°C, the β-hemolytic bacterial strains were collected and purified. All β-hemolytic bacterial strains were then screened through Gram staining and catalase test and thirty-two gram positive, β-hemolytic and catalase positive strains were obtained. The primarily screened strains were then subjected to different morphological and biochemical tests. It was found that twenty-eight out of thirty-two β-hemolytic strains were *Staphylococcus aureus* on the basis of gross morphology and biochemical reaction pattern. The *Staphylococcus aureus* strains were then subjected to antibiotic susceptibility test using then conventional and commonly used antibiotics. The results of strain identification and antibiotic susceptibility testing revealed that more than 50% of the skin lesion is caused by *Staphylococcus aureus* and penicillinase producing strains were available with multidrug resistance in most cases.

Table 2: Results of transformation experiment with *E. coli* LE 392.

Plasmid DNA Source	Recipient strain	Plate made with antibiotic	No. of transformed colony on the selection plates		Remarks
			Experimental	Control	Transformed
Staphylococcus aureus (S_2)	*E. coli* LE 392	Amp 30	50	---	Transformed
		Amp 40	35	---	Transformed
		Amp 60	15	---	Transformed
		Amx 30	45	---	Transformed
		Amx 40	30	---	Transformed
		Amx 50	20	---	Transformed
		Pen 30	55	---	Transformed
		Pen 40	50	---	Transformed
		Pen 50	40	---	Transformed

Amp: Ampicilline, Amx: Amoxycillin and Pen: Penicillin.

From the result of resistance pattern it was found that the strains were resistant to ampicillin (72%), amoxycillin (72%), penicillin (72%), and almost resistant to cloxacillin (50%) and nalidixic acid (54%). The isolated strains were mostly sensitive to cotrimoxazole (15%) and cephradine (22%) and highly sensitive to tetracycline (11%) and cephalexine (7%). So we should use these drugs in the treatment of skin lesions but must be cautious of their judicious use. Indiscriminate use of these drugs may lead to antibiotic resistance against them.

To reveal whether or not the multiple drug resistance phenomenon in the *Staphylococcus aureus* was plasmid mediated, a strain S_2 (sample No 2) from twenty-eight strains had been selected for plasmid isolation. On electrophoresis, a single plasmid of about 23 KB in molecular size was calculated. This 23 KB plasmid was then subjected to transformation to a sensitive *E. coli* LE 392 and from the results of transformation experiment it was found that *E. coli* LE 392 which was sensitive to ampicillin, amoxycillin and penicillin before transformation became resistant to these drugs.

The sensitivity of the transformed strains was further tested by antibiotic spread plate method using 30 μg/ml, 40 μg/ml and 60 μg/ml of ampicillin and 30 μg/ml, 40 μg/ml and 50 μg/ml of amoxycillin and 30 μg/ml, 40 μg/ml and 50 μg/ml of penicillin. In the case of ampicillin 50, 35 and 15, in the case of amoxycillin 45, 30 and 20 and in the case of penicillin 55, 50 and 40 drug resistant colonies were observed in the respective antibiotic plates. These findings indicated that multidrug resistance in the selected *Staphylococcus aureus* strain was plasmid mediated and the 23 KB plasmid of the donor multidrug resistant *Staphylococcus aureus* which was transferred to *E. coli* LE 392 was supposed to be carrying the gene(s) encoding multidrug resistance in *Staphylococcus aureus*.

For further confirmation, plasmid DNA from the transformed *E. coli* LE 392 was extracted, purified and subjected to gel electrophoresis on 0.8% agarose. From the result of electrophoresis a single 23 KB plasmid was calculated which corresponded to that of the donor *Staphylococcus aureus* (S_2) in molecular size. This result further confirmed that the 23 KB plasmid isolated from the *Staphylococcus aureus* might be carrying the gene(s) encoding multidrug resistance and the plasmid was transferable.

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Correspondence:
Abdul Hye Khan
House #W-71/C,
University of Rajshahi,
Rajshahi 6205, BANGLADESH.
e-mail: rajucc@citechco.net