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Effect of Promoter Region Sequence Variations in Relation to Antibiotic Resistance of Methicillin- Resistant *Staphylococcus Aureus*

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ABSTRACT

Multidrug resistant bacterial strains are one of the concerns of healthcare sector. Among these, Methicillin-resistant Staphylococcus aureus (MRSA) is one of the major hospitals acquired multidrug resistant strains. MRSA shows it is resistant against methicillin and other penicillin like antibiotics by producing PBP2a (penicillin binding protein 2a) which is encoded by its mecA gene. In this process mecR1 and mecI gene act as regulator of mecA gene. The aim of this study is to identify sequence variance located in mecA, mecR1 and mecI promoter region and the effect of those changes in relation to pathogenicity of these MRSA strains. In this current research, work wound infections samples were collected from 90 patients who were infected during post-surgical mamagement. Samples were collected from Rajshahi Medical college hospital, Bangladesh. In antibiotic sensitivity test, MRSA was found 100% sensitive against only Choramphenicol and Bacitracin. It also shows partial resistance against Amikacin, Impenem, Doxycycline, Gentamycin and Neomycin. In HRM, analysis six types of genotypes (TT, AA, CC, GG, GA and CT) were identified in mecA, mecR1 and mecI genes. Two types of mutation (T>C and G>A) were found in current study. This HRM analysis was further correlate with antibiotic sensitivity test in terms of antibiotic resistance and sequence variance.

Keywords

Methicillin-resistant *Staphylococcus aureus* (MRSA), PBP2a (penicillin binding protein 2a), High resolution melting (HRM) curve analysis.

Introduction

Bacterial strains causing antimicrobial-resistant infections have increased in numbers in hospitals and communities. The increasing numbers of antibiotic-resistant strains are posing a serious threat to human health. Many antibiotics that were previously being used against bacterial infections are no longer effective because of new resistant strains. The emerging and re-emerging of resistant strains are now one of the biggest microbiological public health threats [1].

Almost 80 years ago, when antibiotics were first introduced it was a life-saving miracle. Since then antibiotics have saved millions of lives. But in recent years' unregulated use of antibiotics have raised a major concern threatening us towards a post-antibiotic era, where antibiotics won't work against infections; database lists the existence of more than 20,000 potential resistant genes (r genes) of nearly 400 different types, predicted in the main from available bacterial genome sequences [2]. *Staphylococcus aureus* is a gram-positive bacteria generally found on the skin and in the nose of 30% of healthy people. It is a major cause of human bacterial infections worldwide [3,4].

MRSA (Methicillin-resistant *Staphylococcus aureus)* is resistant to penicillin-like beta-lactam antibiotics [5]. Methicillin is a narrow spectrum *B*-lactam antibiotic of the penicillin class. Like all other *B*-lactam antibiotics it poses a *B*-lactam ring in its molecular structure [6]. Most *B*-lactam antibiotics work by inhibiting cell wall biosynthesis by preventing penicillin-binding proteins (PBPs) to act which catalyze cross-linking in the bacterial cell wall in the bacterial organism. These are the most extensively used group of antibiotics, constitute about 60% of total antibiotic usage [7].

Among gram-positive pathogens, a global pandemic of resistant *S. aureus* and Enterococcus species currently poses the biggest threat. MRSA kills more Americans each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined. Vancomycin-resistant enterococci (VRE) and a growing number of additional pathogens are developing resistance to many common antibiotics. The global spread of drug resistance among common respiratory pathogens, including *Streptococcus pneumonia* and *Mycobacterium tuberculosis*, is epidemic [8].

The resistance of staphylococci to methicillin and all β-lactam antibiotics is associated with the low affinity of a penicillin-binding protein, PBP2a, which is not present in susceptible staphylococci [9-13]. This protein is encoded by the *mecA* gene, which is located in the *mec* region and is DNA of foreign origin [14]. The expression of the *mecA* gene and the resulting production of PBP2a is regulated by proteins encoded by the penicillinase-associated *blaR1–blaI* inducer–repressor system and the corresponding genomic *mecR1–mecI* elements [15-17], identified in *Staphylococcus aureus* N315 the *mecR1–mecI* regulator element, which is located upstream of the *mecA* gene and is divergently transcribed from *mecA* [18].

The Drug-resistant and virulence of MRSA is variable. The degree of resistance and pathogenicity differ from one strain to another strain even the strains isolated from the same origin [19].

In this study, we aim to understand the genetic basis of methicillin-resistant in MRSA. In this regard, we checked the possible sequence variation in mecA, mecR1, and mecI genes using the HRM method and correlated them with drug-resistant and pathogenicity of the organism. High-Resolution Melt (HRM) analysis is a powerful technique in molecular biology, used for the detection of mutations, polymorphisms, and epigenetic differences in double-stranded DNA samples. It is useful because it simple, fast, effective, accurate, and cost-effective as well.

Materials and Method

Recruitment of the study subject

This study was performed in accordance with guidelines approved by the Institutional Animal, Medical Ethics, Biosafety, and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (Memo number: 58/320/IAMEBBC/IBSc), Institute of Biological Sciences (IBSc), University of Rajshahi, Rajshahi, Bangladesh. Wound infection samples were collected from 90 patients who were infected during post-surgical management. Samples were collected from the post-surgery unit, Rajshahi Medical College Hospital, Rajshahi. Verbal consent was taken from all participants.

Sample collection

Samples were collected from pus and used dressing materials of patients who caught post-surgical infections. Samples were collected using sterile swab sticks and cultured in MRSA selective agar media in a sterile condition. After culturing overnight MRSA was identified based on their characteristic blue colour colony.

ESBL Isolation

ESBL (Extended Spectrum Beta-lactamase) producing strains might also grow with MRSA. For the isolation of β-lactamase secreting ESBL from the collected samples, samples were cultured in ESBL selective media. ESBL positive samples were discarded.

Antibiotic sensitivity test

To test the degree of antibiotic resistance of collected samples an antibiotic sensitivity test was conducted using the disc diffusion method against 21 antibiotics of different classes.

DNA isolation

Bacterial samples were subjected to genomic DNA isolated using a Genomic DNA isolation kit (Promega, USA) as per the producer's protocol. Then the DNA samples were purified using a DNA purification kit (Promega, USA). Genomic DNA concentrations were equilibrated by ultra-violate spectrophotometry and agarose gel documentation. Salt removal of the purified genomic DNA was carried out by gel filtration using Sephacryl S-400 (GE Healthcare, USA).

Adjustment of PCR temperature and cycle for highest amplification

To obtain highest amplification templates were subjected to gradient PCR (Agilent Technologies Sure Cycler 8800). When amplification was sufficient for a specific temperature and cycle, template will be ready for HRM in that condition. For PCR, mecA, mecR and mecI genes, specific primers were used.

Table 1: List of Primers

		Amplicon size
***mecA* primers**	Forward primer: 5'- GCAATATTAACGCACCTCAC-3'	113
	Reverse primer: 5'- TACGACTTGTTGCATACCAT-3'	
***mecR1* primers**	Forward primer: 5'- TCGTCATTGGAATCGTCATA-3'	128
	Reverse primer: 5'- ACTAAACCAAATACCATCGG-3'	
***mecI* primers**	Forward primer: 5'-GCAGAATGGGAAGTTATGAA-3'	109
	Reverse primer: 5'- TACGAATGGTTTTTGGACTC-3'	

High resolution melting (HRM) analysis

Before performing HRM, we optimized the PCR condition for specific HRM primers (Table 1) by gradient PCR [20] and the optimized condition were confirmed by normal PCR. The concentration of DNA was again normalized based on the qPCR Cq value. HRM was performed according to the prior method [21] using the GoTaq® qPCR master mix at 55°C for 40 cycles in the Illumina Eco™ qPCR system (USA). The qPCR reaction mixture (10 μL) comprised of 5 μL (2x) GoTaq® qPCR master mix, 3 μL nuclease-free water, 0.5 μL each of HRM primer, and 1 μL template DNA. qPCR was performed with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 15 seconds by using Eco™ qPCR system.

Results

Isolation and identification of MRSA

On MRSA, selective media MRSA appear as a blue colony and other bacteria appear purple or cream white colour colony. A total of 90 samples were collected among them 32 samples were found to be MRSA positive after selective media isolation and separating ESBL producing strains.

Antibiotic susceptibility test of MRSA:

Total 21 antibiotics (Chloramphenicol, Ampicillin, Vancomycin, Imipenem, Cefepime, Amoxyclav, Levofloxacin, Azithromycin, Amikacin, Ampicillin/Sulbactam, Aztreonam, Bacitracin, Carbenicillin, Cefradine, Doxycycline, Gentamycin, Levofloxacin, Neomycin, Rifampicin, Cefotaxime, Cefuroxime) used for 32 samples. Results are summarized in table 2.

HRM Analysis

High-Resolution Melting (HRM) is a homogeneous and highly powerful method for SNP genotyping, mutation scanning, and sequence scanning in DNA samples.

Our melting curve shows that often 32 samples all together have a large spread and overlapping distribution of genotypes (Figure). Thus to increase our confidence, we excluded samples from plate layout in Eco Study software in post PCR analysis. This reduction or thinning of the melt curve allowed us to compare each sample with assurance, which revealed there was variation among them. The melting curve profile of mecA, mecR1, and mecI genes indicates possible mutation in respective genes.

We compared our findings with the above previous similar works [22] to identify the possible mutation in mecA, mecR1, and mecI genes.

Here are some examples of different HRM curves, which are found from different research work. Analyzing these curves, we can identify the different mutation types (Figure 4).

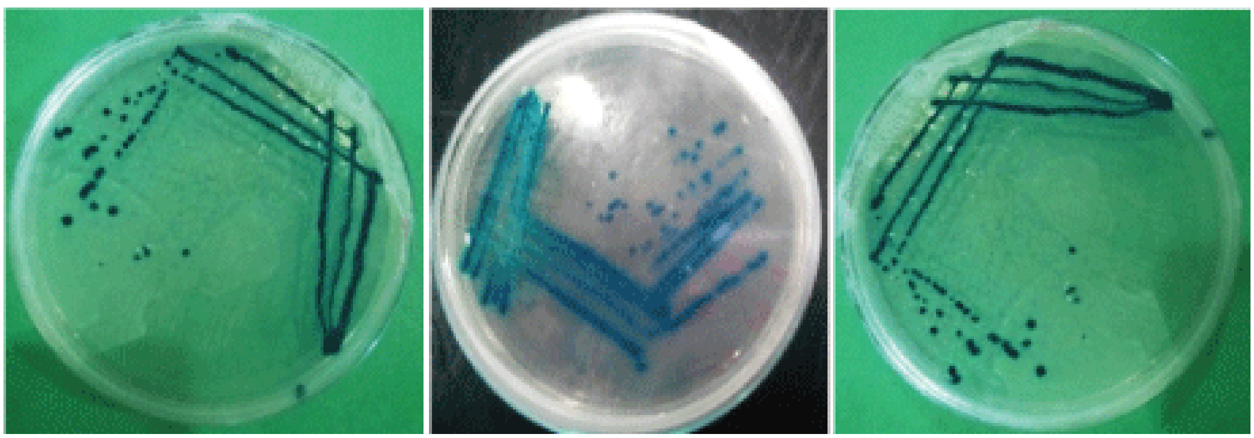

Figure 1: MRSA samples on selective media.

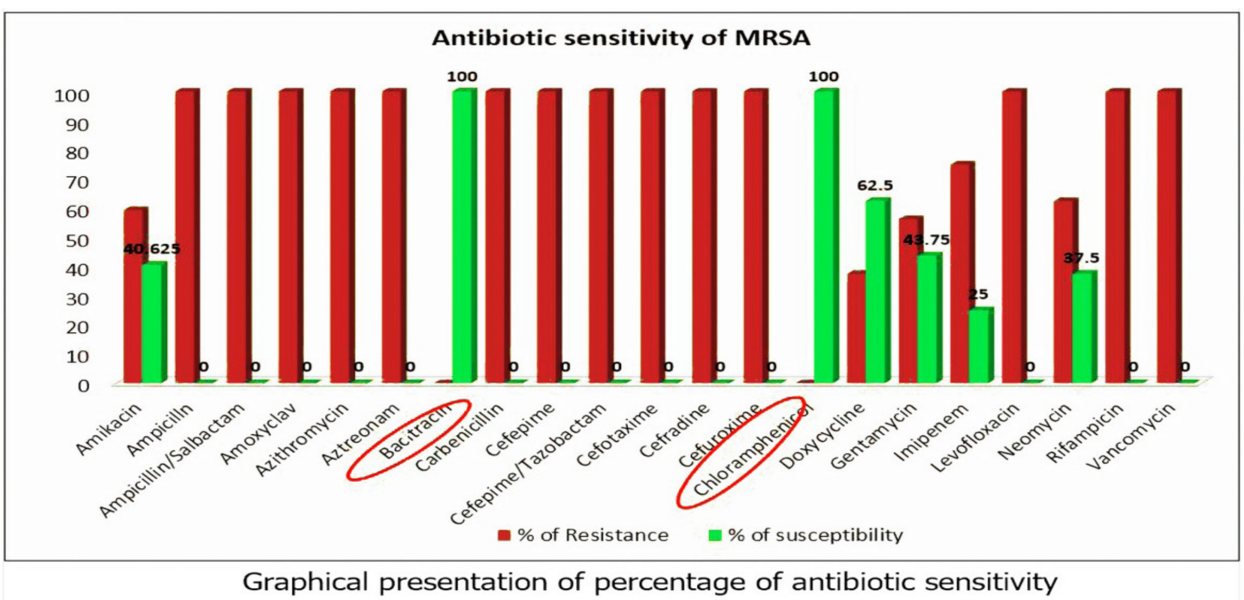

Figure 2: Graphical presentation of percentage of antibiotic sensitivity.

Table 2: Result of antibiotic susceptibility test of 32 samples.

Antibiotics	Required MIC (mm)	Resulted MIC (mm) of 32 samples																No. of resistant samples
Amikacin (30 µg)	18-24	17	14	18	18	13	15	18	18	19	16	12	19	12	18	12	18	19
		15	18	15	14	19	14	17	17	17	17	18	19	13	15	19	12	
Ampicillin (10µg)	27-35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ampicillin/Salbactam (10/10µg)	29-37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amoxyclav (30 µg)	28-36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Azithromycin (15µg)	24-30	11	-	10	12	11	9	8	12	10	9	9	8	11	-	-	10	32
		12	6	7	9	11	10	-	8	-	12	7	8	12	-	-	8	
Aztreonam (30 µg)	17-21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Bacitracin (10µg)	12-22	16	18	15	20	18	15	21	16	17	19	20	20	15	12	16	17	0
		20	18	15	15	21	20	16	17	19	21	21	15	16	19	20	21	
Carbenicillin (100µg)	13-17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cefepime (30 µg)	23-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cefepime/Tazobactam (80/10µg)	30-35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cefotaxime (30 µg)	25-31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cefradine (25µg)	29-37	-	9	-	10	6	8	8	9	-	10	6	-	8	-	10	9	32
		-	9	10	7	-	-	-	9	6	-	-	11	-	9	-	10	
Cefuroxime (30 µg)	27-35	-	-	12	10	-	8	9	10	12	13	-	7	12	19	12	18	32
		10	11	6	10	11	-	11	12	-	12	13	-	13	19	17	18	
Chloramphenicol (30 µg)	13-17	25	23	28	22	25	19	32	26	25	18	29	30	23	24	29	32	0
		29	19	21	21	26	25	24	19	21	26	29	27	27	25	20	30	
Doxycycline (30 µg)	23-29	24	24	15	19	25	18	25	24	19	25	22	23	21	24	23	25	14
		17	25	24	19	23	22	25	24	24	24	15	19	25	18	19	22	
Gentamycin (120µg)	19-27	17	21	26	19	15	20	18	16	19	17	21	16	17	24	23	25	15
		11	22	18	23	15	26	18	13	13	17	26	19	15	20	19	21	
Imipenem (10µg)	26-32	14	24	12	12	12	23	24	25	12	24	26	20	18	21	17	15	27
		29	12	21	27	23	27	24	19	19	14	12	12	12	23	12	26	
Levofloxacin (5µg)	25-30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Neomycin (30 µg)	18-26	23	17	26	12	15	24	9	16	12	24	11	23	10	10	28	16	19
		17	21	11	10	22	16	16	26	26	23	26	12	15	24	12	11	
Rifampicin (15µg)	23-26	20	7	6	8	-	16	10	8	-	7	10	19	21	-	-	13	32
		18	10	11	14	19	-	7	-	8	19	11	12	17	10	19	7	
Vancomycin (30 µg)	23-27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

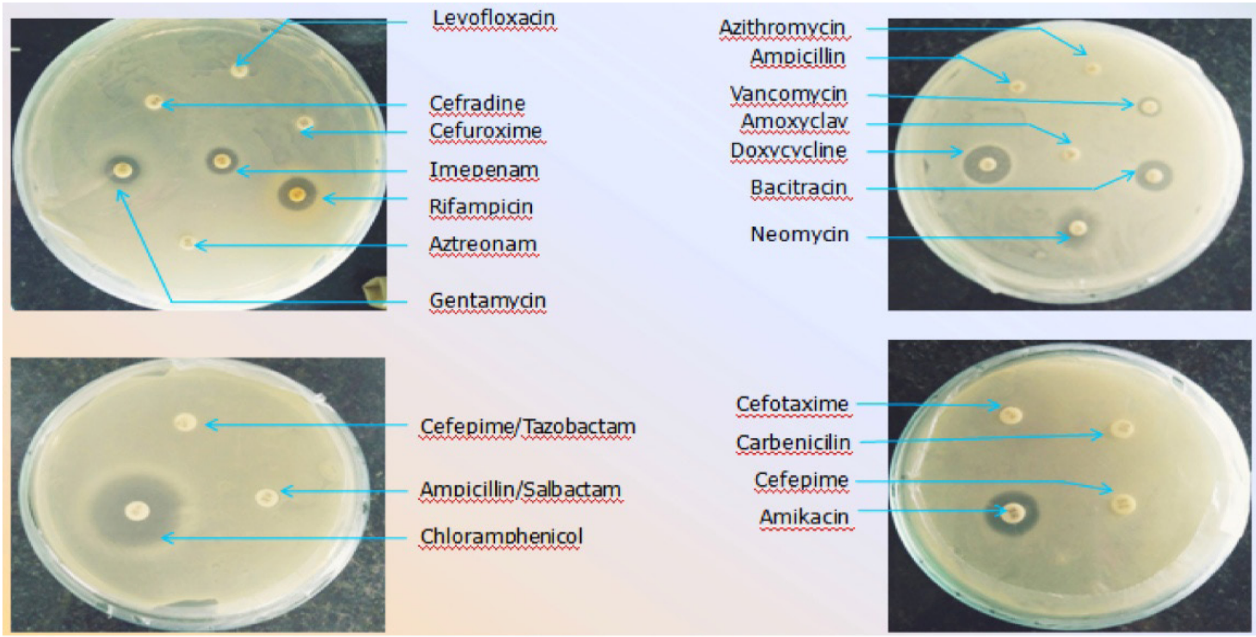

Figure 3: Examples of antibiotic sensitivity test of MRSA strains.

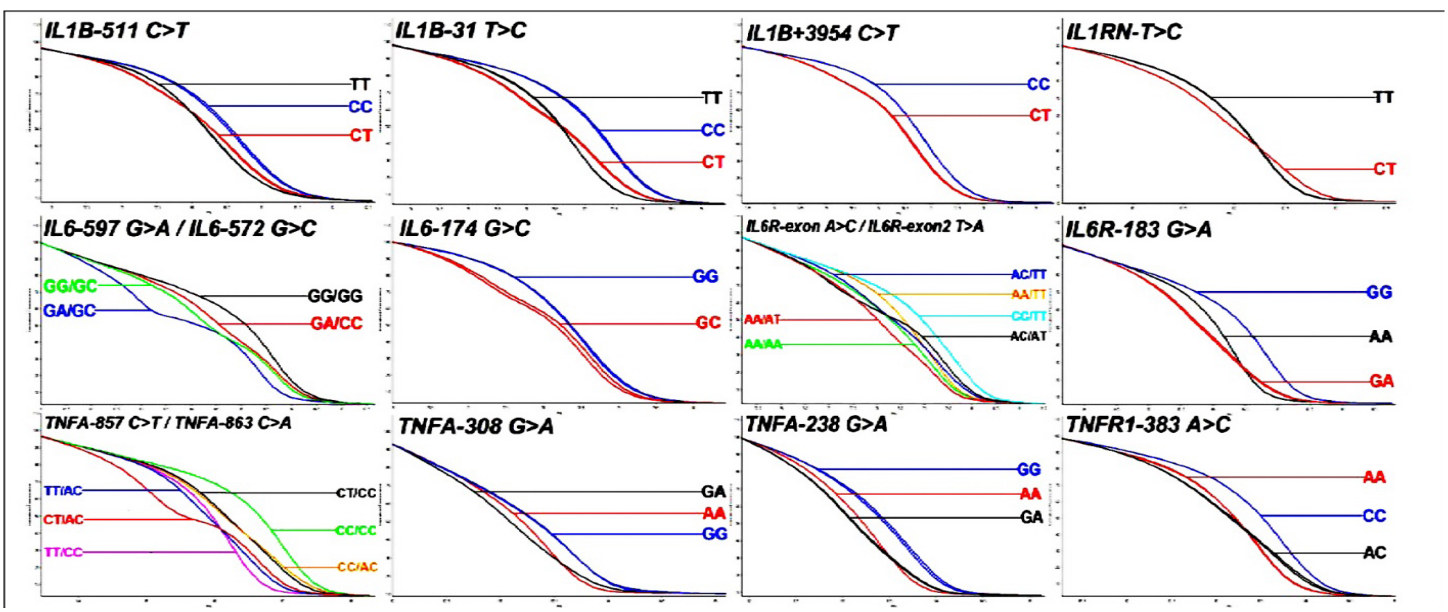

Figure 4: Examples of different polymorphisms showing the possibilities of different melting curves (Youet al., 2013).

HRM Analysis of mecA gene

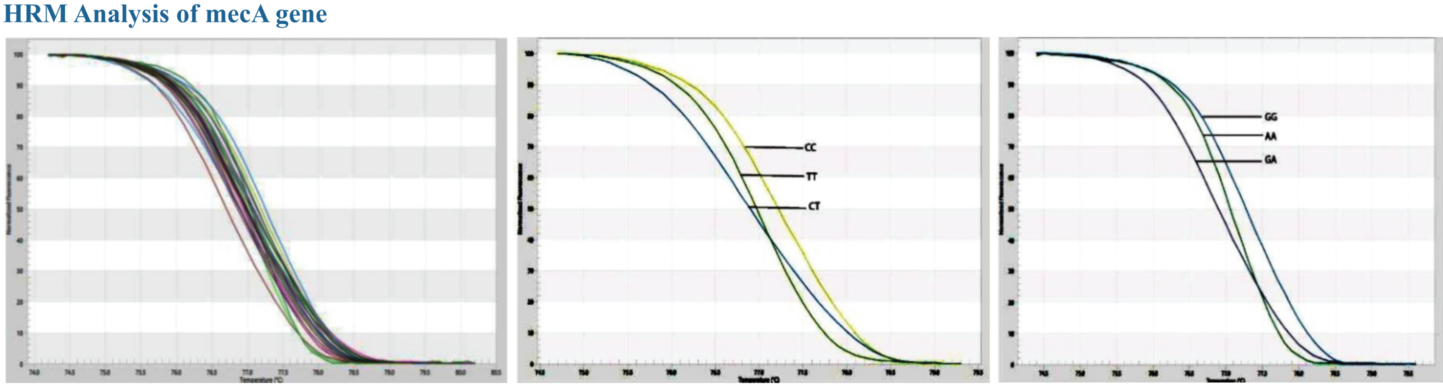

Figure 5: Normalized melt curve A. showing mecA gene of all 32 samples, B. showing T>C mutation, C. showing G>A mutation.

HRM Analysis of mecR1 gene

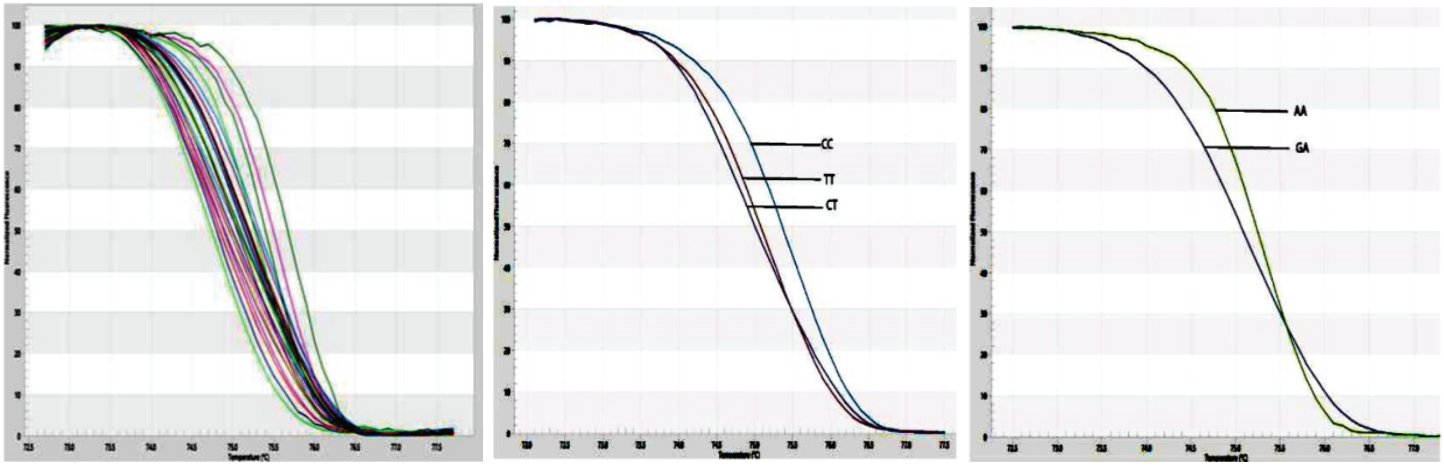

Figure 6: Normalized melt curve A. showing mecR1 gene of all 32 samples, B showing T>C mutation, C. showing G>A mutation.

HRM Analysis of mecI gene

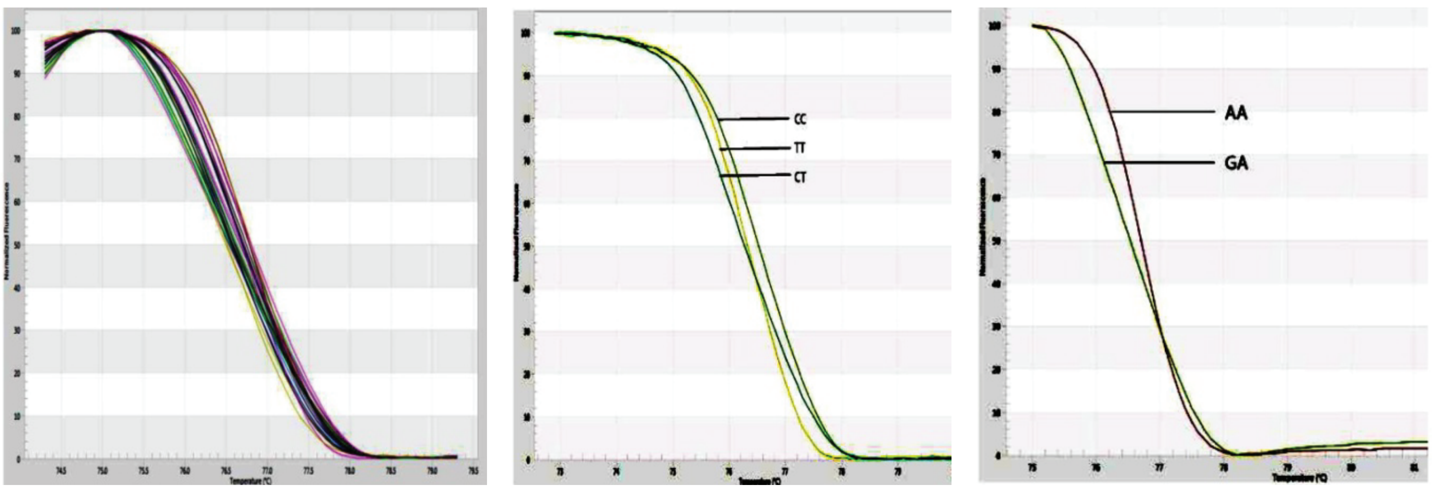

Figure 7: Normalized melt curve A. showing mecI gene of all 32 samples, B. showing T>C mutation,C. showing G>A mutation.

Discussion

Post-surgical hospital-acquired infections increase the risks of mortality and cause prolonged hospital staying which eventually increase medical costs. MRSA is one of the leading major causes of hospital-acquired post-surgical infections [8]. The occurrence of MRSA infections is also frequent in Bangladesh like in other countries [23]. In this current research work, samples were collected from both male and female patients irrespective of their ages.

In this study, among 21 antibiotics used only two antibiotics were found to be 100% effective against MRSA. MRSA is completely susceptible only against Bacitracin and chloramphenicol. MRSA is found to be completely resistant against antibiotics such as Ampicillin, Vancomycin, Cefepime, Amoxyclav, Levofloxacin, Azithromycin, Ampicillin/Salbactam, Aztreonam, Carbenicilin, Cefradine, Levofloxacin, Rifampicin, Cefotaxime, and Cefuroxime.
The study also revealed that MRSA is 59.38%, 43.75%, 46.88%, 84.38%, and 59.38% susceptible against Amikacin, Doxycycline, Gentamycin, Imipenem, and Neomycin respectively. Our current research is contradictory to the findings of Sachin and Anju [24] but similar to results of other workers where Vancomycin-resistant MRSA was reported [25-27].

To detect promoter region mutation of *mecA, mecR1,* and *mecI* genes, we performed a High resolution melting (HRM) analysis. In HRM analysis, we found six genotypes. these are TT, AA, GG, CC, GA and CT. We also found T>C and G>A types of possible mutation.

In *mecA* gene, we found T>C/ G>A type mutation in promoter region of samples 10, 21, 25; 7, 5, 11 and 14, 15, 17. In *mecR1* we found T>C/ G>A type mutation in samples 17, 19 23; 4, 9, 28 and 10, 15. In case of *mecI* gene we found T>C/ G>A type mutation in samples 10. 12; 2, 6, 29 and 3, 26.

In HRM analysis, we were able to detect mutation in *mecA, mecR1*, and *mecI* genes. In the antibiotic susceptibility test, we found variation in the inhibition zone that also indicates mutation in the samples.

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