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ORIGINAL ARTICLE

A Molecular Detection of Drug Resistance Genes of Methicillin-Resistance *Staphylococcus Aureus* (MRSA) from Different Clinical Specimens

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Abstract

Background: *The rise of drug-resistant virulent strains of Staphylococcus aureus, particularly methicillin resistant S. aureus (MRSA) is a serious problem in the treatment and control of staphylococcal infections.*

Objective: *The aim of this study was to detect antimicrobial susceptibilities and the presence of drug resistance genes of MRSA from tertiary care hospitals.*

Material methods: *This study was carried out in the Department of Microbiology, Mymensingh Medical College during the period from Jan, 2015 to Dec, 2015. Clinical samples, including wound swab, pus, exudates from diabetic ulcer and burn ulcer, aural swab, blood and urine were collected. Standard microbiological procedure & biochemical tests were carried out to detect S. aureus. Oxacillin disk diffusion test was done by Kirby-Bauer disk diffusion method. Results: Total 69 isolates of S. aureus were selected for the study. The isolates were collected from three different tertiary care hospitals, of which 33, 27 and 9 were from MMCH, BIRDEM hospital and SSMCH respectively. Among the 69 isolates, 17 (24.6%) and 52 (75.3%) were distinguished as MRSA and MSSA respectively by ODDM (Oxacillin disk diffusion method). In contrast, detection of presence and absence of mecA gene by PCR identified 20 (28.9%) and 49 (71.01%) isolates as MRSA and MSSA respectively. All of the S. aureus (MRSA and MSSA) isolates were sensitive to vancomycin and gentamicin. All MRSA isolates (100%) showed resistance to Penicillin and Oxacillin. Among the MRSA isolates about 88.2% were resistance to Ceftazidime, 64.7% were resistance to Erythromycin and Ciprofloxacin, 11.7% were resistance to Tetracycline. Among the MSSA isolates about 94.2% were resistance to Penicillin and 9.6% resistance to Ciprofloxacin. The MSSA were less resistance for non-beta lactam drugs than MRSA. Regarding drug resistance genes, the blaZ genes were present in 47 out of 49 (95.8%) MSSA and in 18 out of 18 (100%) MRSA. The erythromycin resistance gene ermB was found in 8.69% isolates, of which highest 20% in MRSA and 4.08% in MSSA. The ermA was not found in any isolates. Among tetracycline resistance genes, tetK were detected in 10.1% and tetL were found in 2.8% of MRSA. The highest tetK genes were found in 20% of MRSA and in 6.1% of MSSA. Regarding, the gentamicin*

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drug resistance, the aac(62)-Iaph(22 2)-Ia gene was not found in any isolates.

Conclusion: The relatively high proportion of MRSA and the associated antibiotic resistance seen in this study emphasizes the need for country based surveillance of MRSA to develop strategies that will improve MRSA treatment and control.

Key Words: *Methicillin resistant Staphylococcus aureus, Antibiotic sensitivity.*

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Introduction:

The *Staphylococcus aureus* strains that are resistance to penicillinase resistant penicillins (Methicillin) are referred to as Methicillin Resistant *Staphylococcus aureus* (MRSA)[1] and has been a major cause of nosocomial infection around the world. The MRSA is potentially a great threat to medical therapy.[2] With the introduction of penicillin in the early 1940s, after two years of clinical use, penicillin-resistant *Staphylococcus aureus* isolates began to appear[3]. HA-MRSA isolates carry one of the three types of SCC*mec* (types I, II or III) or occasionally types IV and V, and are generally multidrug resistant[4]. MRSA organisms generally are resistant to multiple antibiotics, including aminoglycosides, macrolides, fluoroquinolones, clindamycin, chloramphenicol, and beta-lactams. A knowledge of the prevalence of MRSA and their antimicrobial susceptibility pattern becomes necessary for the selection of appropriate treatment.

Methods:

This Cross sectional observational study was carried out in the Department of Microbiology, Mymensingh Medical College during the period from July, 2014 to Dec, 2015. Ethical permission was taken from the institutional ethical review committee and the clinical samples including wound swabs, pus, exudates from diabetic ulcer and burn ulcer, aural swab, blood and urine were collected from Mymensingh Medical College hospital, BIRDEM hospital and Sir Salimullah Medical College hospital. Standard microbiological procedure & biochemical tests were carried out for identification of *S. aureus*. Specimens were inoculated into blood agar. The plates were incubated at 37^0 C for 24 hours. The catalase, tube coagulase and mannitol fermentation tests were performed for the identification of *S. aureus*. Finally, a total of 69 isolates of *S. aureus* were selected for the study. Resistance to methicillin was determined by the oxacillin disc-diffusion assay according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). MRSA strains were further tested for resistance to other antimicrobials using commercial discs (Oxoid); Penicillin-G, Oxacillin, Erythromycin, Tetracycline, Gentamicin, Ceftazidime,

Primer sets for Multiplex PCR 1:

Multiplex PCR oligonucleotide primers were used. The sequences from 5 2 to 3 2 ends of these oligonucleotide primers were as follows[6]

Staphylococcus genus-specific 16S *r*RNA, product size: 756bp
Staph756F : 5 2 - AAC TCT GTT ATT AGG GAA GAA CA-3 2
Staph750R : 5 2 - CCA CCT TCC TCC GGT TTG TCA CC-3 2

lukS/F-PV genes, product size : 433bp
Luk-PV-1 : 5 2 - ATCTTTAGGTAAAATGTCTGGACATGATCCA-3 2
Luk-PV-2 : 5 2 - GCATCAAGTGTATTGGATAGCAAAAGC-3 2

ACME-*arc*A gene, product size : 513bp
*arc*A-F : 5 2 - GCAGCAGAATCTATTACTGAGCC-3 2
arcA-R :5 2 - TGCTAACTTTTCTATTGCTTGAGC-3 2

nuc gene, product size 297bp
Nuc-1 : 5 2 - GCG ATT GAT GGT GAT ACG GTT-3 2
Nuc-2 : 5 2 - AGC CAA GCC TTG ACG AAC TAA AGC-3 2

*mec*A gene, product size : 157bp
MecA147-F: 5 2 - GTGAAGATATACCAAGTGATT-3 2
MecA147-R : 5 2 - ATGCGCTATAGATTGAAAGGAT-3 2

Multiplex PCR 2 (drug resistance genes of MRSA and MSSA) [7]

Primer pairs :	gene	product size (bp)	resistance by the gene
AME-1, AME-2 :	*aac(6)-Ie-aph(2)-Ia*	675	high-level resistance to aminoglycosides (gentamicin, etc)
AME-3, AME-13 :	*aph(3)-IIIa*	354	aminoglycosides (Kanamycin, etc)
AME-7, AME-14 :	*ant(4)-Ia*	266	aminoglycosides (dibekacin, etc)
tetM-1, tetM-2 :	*tet(M)*	435	tetracycline

Multiplex PCR 3 (drug resistance genes of MRSA and MSSA)[8]

Primer pairs :	gene	product	resistance by the gene size (bp)
ermB-N1, ermB-N2 :	*erm(B)*	379	microlide (erythromycin)
ant6-N1, ant6-N2 :	*ant (6)-Ia*	548	aminoglycosides (streptomycin, etc)
tetK-1, tetK-2 :	*tet(K)*	615	tetracycline
blaZ-1, blaZ-2 :	*blaZ*	173	beta-lactams (beta-lactamase)

Multiplex PCR 4 (drug resistance genes of MRSA and MSSA)[9]

Primer pairs :	gene	product	resistance by the gene size (bp)
ermA-1, ermA-2 :	*erm(A)*	135	macrolide (erythromycin)
tetL-1, tetL-3 :	*tet(L)*	740	tetracycline

Master mixture (50µl) for each reaction was prepared and Program in thermal cycler was as follows (DLin DL9700 TOUCH, China):
Initial denaturation at 94^0C for 1 min, followed by
30 cycles in an automated DNA thermal cycler and each cycle consists of:-
Denaturation at 94^0C for 30 sec
Annealing at 55^0C for 30 sec — 30 cycles[10]
Extension at 72^0C for 60 sec
Final extension at 72^0C for 5 min.

Ciprofloxacin and Vancomycin by the disc diffusion technique and according to the CLSI guidelines in the same manner used for the oxacillin disc testing. For molecular study, DNA was extracted by heat mehod 100°C for 10 mintues[5]. Multiplex PCR was performed by using standard protocol with specific primers for detection of 16S *r*RNA gene for *Staphylococcus*, *nuc* gene for *Staphylococcus aureus*, *mec*A gene for MRSA, PVL gene as a virulence factor and ACME (arginine catabolic mobile element) cassette gene-*arc*A gene for worldwide spreading USA 300 MRSA clone[6]. Also, the drug resistance genes *erm(A)* and *erm(B)* for erythromycin, *tet(K)* and *tet(L)* for tetracycline, *blaZ* for beta-lactam drugs and *aac(62)-Iaph(22 2)-Ia* gene for gentamicin were used[7].

The PCR products were analyzed by 1% agarose gel (Alpha Imager, Germany) electrophoresis and photographed using a gel documentation system (Alpha Imager, Germany).

Results:

A total of 69 *Staphylococcus aureus* isolates were investigated. The isolates were collected from three different tertiary care hospitals, of which 33, 27 and 9 from MMCH, BIRDEM hospital and SSMCH respectively. Among the 69 isolates, 17 (24.64%) and 52 (75.36%) were distinguished as MRSA and MSSA respectively by ODDM. In contrast, detection of *mecA* gene by PCR identified 20 (28.98%) and 49 (71.01%) isolates as MRSA and MSSA respectively (Table-I). A higher rate was detected from diabetic ulcer 4 (57.1%). Among the collected clinical specimens the MRSA were found predominantly in pus 4 (33.3%) followed by wound swab 8 (22.8%) (Table-II).

Table-I

Detection of MRSA and MSSA by Phenotypic (oxacillin disk diffusion Method and MIC) and Genotypic method by PCR (n=69)

Phenotypic detection (ODDM and MIC)	Genotypic PCR (*mec*A gene) Positive (%)	Negative (%)
MRSA (n=17)	17 (100)	0
MSSA (n=52)	3 (5.7)	49 (94.2)

Values in the parenthesis indicate percentage.

Table-II

Distribution of MRSA among the S. aureus isolates obtained from different clinical specimens by PCR (n= 20)

S. aureus isolates from different specimens	MRSA (%)
Wound swab (n=35)	8 (22.8)
Pus (n=12)	4 (33.3)
Exudates from diabetic ulcer (n=7)	4 (57.1)
Exudates from burn ulcer (n=3)	1 (33.3)
Aural swab (n=3)	1 (33.3)
Urine (n=5)	1 (20)
Blood (n=4)	1 (25)
Total (n= 69)	20 (28.5)

Values in the parenthesis indicate percentage.

Based on the disc diffusion results, the antibiotic resistance pattern was as follows: 88.2% MRSA isolates were resistance to Ceftazidime, 64.7% were resistance to Erythromycin and Ciprofloxacin, 11.7% were resistance to Tetracycline. All MRSA isolates (100%) showed resistance to Penicillin and Oxacillin. All MSSA isolates showed 94.2% resistance to Penicillin and 9.6% resistance to Ciprofloxacin. Both MRSA and MSSA showed 100% sensitivity to Vancomycin and Gentamicin (Table-3). Among the 69 isolates of *S. aureus blaZ* genes were found 97.01%. The *blaZ* genes were present in 47 (95.8%) out of 49 MSSA and in 18 (100%) out of 18 MRSA. The erythromycin drug resistance genes *ermB* were found in 5.7% isolates, of which highest 15% in MRSA than 4.08% in MSSA. The *ermA* was not found in any isolates. Among the tetracycline drug resistance genes, *tetK* was detected in 10.1% and *tetL* was found in 2.8%. The highest *tetK* genes were found in 20% of MRSA, followed by in 6.1% of MSSA. Regarding, the gentamicin drug resistance, the *aac(62)-Iaph(22 2)-Ia* gene was not found in any isolates (Table-IV).

Table-III: Pattern of antimicrobial resistance among MRSA (n=17) and MSSA (n=52) isolates against commonly used antibiotics

Name of antibiotics	**Number (%) of resistant isolates among**	
	MRSA n=17	MSSA n= 52
Penicillin	17 (100)	49 (94.2)
Oxacillin	17 (100)	0 (0)
Erythromycin	11 (64.7)	29 (55.8)
Ceftazidime	15 (88.2)	41 (83.7)
Ciprofloxacin	11 (64.7)	5 (9.6)
Tetracycline	2 (11.7)	0 (0.0)
Gentamicin	0 (0)	0 (0.0)
Vancomycin	0 (0)	0 (0.0)

Values in the parenthesis indicate percentage.

Table-IV

Detection of drug resistant genes among genotypically detected MSSA (n=49) and MRSA (n=20)

Name of antibiotics	Drug resistant genes	MSSA N=49	MRSA N=20	Total N=69
Penicillin	*blaZ*	47 (95.8)	20 (100)	67 (97.01)
Erythromycin	*ermB*	2 (4.08)	4(20)	6 (8.69)
	ermA	None	None	None
Tetracycline	*tetK*	3 (6.1)	4 (20)	7 (10.1)
	tetL	1 (2.04)	1 (5)	2 (2.8)
Gentamicin	*aac(62)-Iaph(22 2)-Ia*	None	None	None

Values in the parenthesis indicate percent

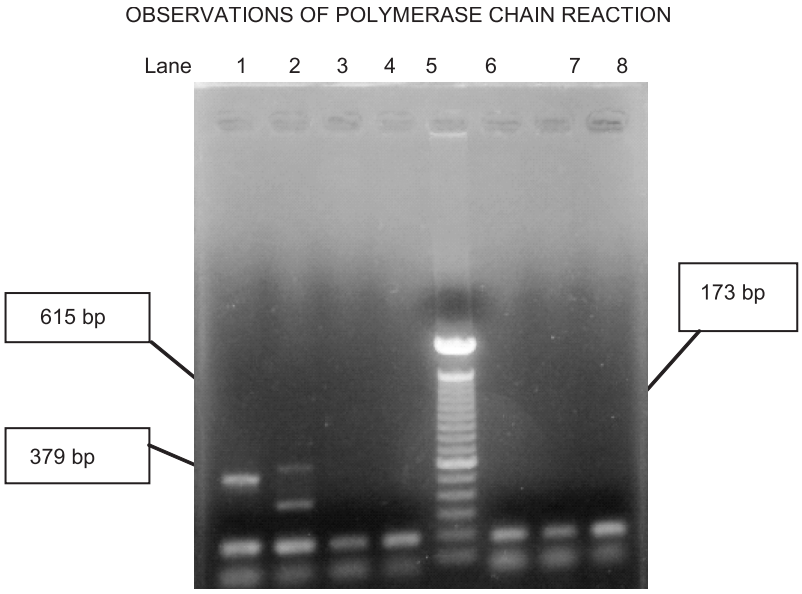

Fig.-1 : *Multiplex PCR was done to detect the ermB (379 bp), tetK (615bp) and blaZ (173bp) genes. Lane 1-4 and 6-8 showing bands of the amplified product of blaZ, and lane 2 showing band of ermB and tetK genes*

Discussion:

The prevalence of MRSA varies strongly among the countries of the world[11]. In the present study we found the isolation rate about 28.5% of MRSA among hospitalized and outdoor patients (Table II). A study in the same institute found MRSA rate about 26%[12] was similar with this study. But two other studies from Bangladesh reported isolation rates of MRSA as 46.0% by Dutta[13] and 53.2% by Masud[14], that were not in agreement with the present study. In 2010, a CDC published report showed that invasive (life-threatening) MRSA infections in healthcare settings are declining. Invasive MRSA infections that began in hospitals declined 28% from 2005 through 2008[15].

The prevalence rate of MRSA was found to be 29.1% in a study in India[16] and an another study in Nepal (26.14%)[17] which is in accordance with this study.

Some studies from developed countries reported prevalence as 56.8% in Hong Kong, 49% in Portugal, 50% in USA[18], 50.5% in Turky[19] and in developing countries like Pakistan and India, it was 51% and 51.9% respectively[20].

Table II shows that, the highest number of MRSA was detected from diabetic ulcer 57.1%. This finding was in agreement with the study by Hanan E Mohamed and Ayman H Al-Gadaa in 2012, where predominant organisms from diabetic ulcer was *S. aureus* and among *S. aureus* 48.8% were MRSA[21]. The high prevalence may be due to the fact that this microorganism is a skin colonizer that becomes opportunistic in immunocompromised people such as diabetic patients.

All MRSA isolates (100%) showed resistance to Penicillin and Oxacillin. About 88.2% MRSA isolates were resistance to Ceftazidime, 64.7% were resistance to Erythromycin and Ciprofloxacin, 11.7% were resistance to Tetracycline (Table-III). All MRSA isolates encountered in this study were sensitive to vancomycin and Gentamicin. A recent previous study on MRSA in MMCH by Masud had shown the resistance to Erythromycin was 75%, ceftazidime 65.5% and Ciprofloxacin 71.4%. These findings were similar to this study. But there were different findings from other Asian countries 2011[22] where their study showed the drug resistance to Erythromycin was 90.4%, Ciprofloxacillin 77.6% and Gentamicin 78.6%. Antibiotic sensitivity pattern varies country to country, region to region, district to district even hospital to hospital. It also depends on the antibiotic prescription pattern of the locality or institute.

Table-IV shows that among 69 *S. aureus blaZ* genes were found 97.01%, followed by *tetK* 10.1% and *ermB* 8.69%. The number of *blaZ* genes detected in this study were very high than *tetK* and *ermB* genes. These findings reveals that in our country, the *S. aureus* has high resistance to beta- lactam drugs than non beta lactam drugs. In a similar study on the different antibiotic resistance genes in Turkey[23] also found *blaZ* were 93.5%, *tetK* 13.1% and *ermB* 5.8% which is in agreement with our study.

No study about the drug resistance genes of *S. aureus* had been done in Bangladesh. In our study, both the *ermB* and *tetK* present in 20%, *tetL* 5% and no *ermA* gene were found in MRSA . A previous study by Sekiguchi[24] in Japan found 92.7% MRSA isolates with both *ermB* and *tetM* genes positive and no *ermB* gene positive MRSA. Another study in Shenyang by Sun also showed drug resistance genes on Hospital acquired MRSA of *ermA* 86.9%, *ermB* 45.8%, *tetK* 45.8% and *tetL* 11.2%[25]. It seems that the drug resistance pattern of our country is different from that of other countries.

Conclusion:

We find that infections, especially wound infections, caused by MRSA are quite high in this region. So, we recommend a wise, cautious and rational antibiotic policy, particularly in *Staphylococcus aureus* infections. Since in this study, vancomycin resistant strains were not yet isolated from this area, we suggest to keep it reserved, whenever a sensitive alternative is available.

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