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Full Length Research Paper

Prevalence of β-lactamase-producing and non-producing methicillin resistant *Staphylococcus aureus* in clinical samples in Bangladesh

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Staphylococcus aureus **has been reported to be a major cause of community and hospital acquired infections. Indiscriminate use of antibiotics resulted in the development of multi-drug resistant *S. aureus* throughout the world. Development of multi-drug resistant strains of *S. aureus* is increasingly alarming in Bangladesh. We attempted to study the current prevalence of β-lactamase-producing and non-producing methicillin-resistant *S. aureus* (MRSA) in clinical samples and to find out the correlation of antimicrobial resistance pattern with their plasmid profiles. Twenty three clinical isolates of *S. aureus* were evaluated during the study period (2009). The isolates were identified by conventional methods. Antibiotic susceptibility of the isolates was performed by disk diffusion method. Plasmid profiles were observed by agarose gel electrophoresis. In the present investigation, 43·48% isolates were ensured methicillin resistant while the remaining 56·52% isolates were found to be methicillin sensitive by disk diffusion method. β-lactamase test which was performed by acid formation method showed that 50% of the MRSA isolates produced β-lactamase. Our studies of resistance pattern to commonly prescribed antimicrobials showed that MRSA isolates were highly sensitive to vancomycin (100%), fusidic acid (90%), chloramphenicol (80%), neomycin (80%), rifampin (80%), gentamycin (70%), ceftriaxone (60%), cephalexin (60%), ciprofloxacin (60%), and cloxacillin (60%). Plasmid profiling of the selected resistant isolates of *Staphylococcus* revealed clear and distinct bands of plasmid DNA. These isolates showed severe resistance to amoxicillin (70%), co-trimoxazole (90%) and erythromycin (80%).**

Key words: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), resistance, β-lactamase, Bangladesh.

INTRODUCTION

Staphylococcus aureus, a spherical aerobic gram-positive, catalase positive, oxidase positive, non-motile, -spore-forming coccus, is an opportunistic pathogen in human and animal, and is one of the most frequent sources of hospital- and community-acquired infections. Generally, *S. aureus* is responsible for superficial infections and toxic epidermal necrolysis, systemic infections such as endocarditis inflammation of bone or bone marrow, pneumonia and toxinoses such as food poisoning or toxic shock syndrome. However, among gram-positive cocci, only β-lactamase of major clinical significance is *Staphylococcal* β-lactamase, which rapidly hydrolyses benzylpenicillin, ampicillin, cephalosporins, and related antimicrobials (Foster, 1996; Francis et al., 1997; Brumfit and Hamilton, 1989; Sampathukumar, 2007; Daini and Akano, 2009; Hotu et al., 2007). Methicillin-resistant *S. aureus* (MRSA) is a special strain of *S. aureus* that is resistant to the antibacterial activity of methicillin and other related antibiotics of the penicillin class. MRSA have acquired genes encoding antibiotic resistance to all penicillins, including methicillin and other narrow-spectrum β-lactamase-resistant penicillin antibiotics (O'Brien et al., 1999; Maltezou and

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Giamarellou, 2006; Boyce, 1994; Chambers, 2001; Maltezou and Giamarellou, 2006). Although, MRSA has traditionally been seen as a hospital-associated infection, community-acquired MRSA strains have appeared in recent years, notably in the USA and Australia (Okuma et al., 2002). Several new strains of MRSA have been found showing antibiotic resistance even to vancomycin and teicoplanin; these new evolutions of the MRSA bacteria are called Vancomycin Intermediate-resistant *Staphylococcus aureus* (VISA) (Sieradzki and Tomasz, 1997; Schito, 2006). MRSA is relatively ubiquitous and is the cause of many community, endemic and epidemic nosocomial colonization and infections (Hsueh et al., 2005; Marples and Reith, 1996; Chambers, 2001). Community-acquired MRSA infections in the absence of identified risk factors have been reported. Many outbreaks of infections due to MRSA have occurred and it has now become endemic in several centers in the world (Brumfit and Hamilton-Miller, 1989; Boyce, 1994; O'Brien et al., 1999). The emergence of community-acquired MRSA that is capable of causing infections in otherwise healthy people has also been reported (Diep et al., 2008; Daini and Akano, 2009). Staphylococcal antibiotic resistance has been associated with resistant plasmids that have the ability to mediate the production of drug inactivating enzymes such as β-lactamases (Adeleke and Odelola, 1997) and other functions (King et al., 2006; Diep, 2006). MRSA also differ in their resistance to antibacterial agents and in the genetic location of these resistance determinants. Studies have shown that the genetic determinants for antibiotic resistance reside on plasmids, chromosomal DNA, or on transposable elements (Lyon and Skurray, 1987; Udo, 1993).

In Bangladesh, as reported previously, the frequency of MRSA was alarming due to indiscriminate and incomplete uses of antibiotics (Khan et al., 1991; Rahman et al., 2002). In 1991, 62.61% MRSA was reported in a situation when methicillin was not yet introduced in Bangladesh market (Khan et al., 1991). However, in 2002 47.2% MRSA was reported in an investigation on clinical *S. aureus* isolates (Rahman et al., 2002). Both of these prevalence rates of MRSA were higher than the rate in some developed countries like Austria 21.6%, Belgium 25.1%, Spain 30.3%, and France 33·6% (Herwaldt and Wenzel, 1996). Therefore, the current situation of the susceptibility patterns of local strains is essential for the judicious use of antibacterial agents as well as to become aware of the MRSA in hospitals and community arenas in Bangladesh. Based on this previous study, we took further initiation to look into the recent prevalence of MRSA isolates in clinical samples collecting from two largest pathological centers at Dhaka city of Bangladesh.

The patterns of antibiotic susceptibility of methicillin-sensitive and -resistant isolates to the commonly used antimicrobial agents were studied. β-lactamase production and plasmid profiles of these bacteria were also investigated.

MATERIALS AND METHODS

MRSA isolates

Twenty three isolates of *S. aureus* were obtained from the two largest pathological centers: Medinova Medical Services and Popular Diagnostic Center in Dhaka City, Bangladesh during our study in 2009. The isolates were identified as *S. aureus* by gross and microscopic morphology, and by biochemical tests such as coagulase test, catalase test and oxidase test following established methods. All isolates were collected from patients in whom *S. aureus* was the sole causative infectious agent. The staphylococcal infection was ensured by clinical and para-clinical correlations. Mixed specimens were obtained from pus, blood, cerebrospinal fluid (CSF), urine, throat swab, umbilical swab, sputum, prostatic semen, etc.

Antibiotic susceptibility test

The pattern of antibiotic sensitivity of *S. aureus* to 17 antimicrobials was determined by disk diffusion method (National Committee for Clinical Laboratory Standards, 1997). The antimicrobial disks were sourced from the HiMedia Laboratories Ltd., Mumbai, India. All tests were performed on Mueller-Hinton agar (Oxoid Ltd. Basingstoke, Hampshire, England) and zones of inhibition were measured after incubation at 37°C for 24 h. The zone diameters measured around each disk were interpreted on the basis of guidelines by the NCCLS 1985 (Bauer et al., 1966).

β-Lactamase test

β-Lactamase production was assayed by the acid-formation method. A piece of Whatman No.1 filter paper (5×6) was briefly placed in a sterile Petri dish. The bluish penicillin solution was added drop wise to saturate the paper. Thick masses of bacterial colonies of the test organism were transferred with a bacteriological loop from the test culture to the filter paper and spread over an area of 5 mm diameter. The paper was then incubated at 37°C for 30 min with the Petri dish covered. The paper was examined and yellow zones formed by β-lactamase producing strains were noted.

Plasmid profile analyses

Plasmid was isolated by miniprep methods and analyzed by agarose gel electrophoresis using 1.5% agarose gel.

RESULTS AND DISCUSSION

We have investigated the current prevalence and pattern of MRSA isolates in clinical samples collected from two renowned pathological centers in Dhaka city, Bangladesh. *S. aureus* was also examined for the relationship of antimicrobial resistance with plasmid profiles.

Table 1. *In vitro* sensitivity pattern of *Staphylococcus aureus* to different antimicrobials.

Sample No.	Specimen	Amoxycillin	Ceftriaxone	Cephalexin	Cephradine	Chloramphenicol	Ciprofloxacin	Cloxacilline	Co-trimoxazole	Erythromycin	Fusidic Acid	Gentamicin	Neomycin	Oxacillin	Penicillin G	Rifampin	Tetracycline	Vancomycin
1.	Pus	R	S	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S
2.	P/S (24/M)	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S
3.	Rt. Eye	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S
4.	P/S	R	S	S	S	S	R	S	R	R	S	S	S	R	R	S	R	S
5.	W/S (5/M)	R	S	S	S	S	R	S	S	S	R	S	R	S	R	R	S	S
6.	Rt. eye (36/M)	S	S	S	S	R	S	S	R	R	R	S	S	S	R	R	S	S
7.	Pus 887	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	S
8.	P/S (58/M)	R	S	S	S	S	R	S	R	R	S	S	S	S	R	S	R	S
9.	Urine C-13	R	R	S	R	S	S	R	R	R	S	R	S	R	R	S	S	S
10.	Urine (35/F)	R	S	R	S	S	R	S	R	S	S	S	S	R	R	S	R	S
11.	T/S (2/M)	R	S	S	R	R	R	S	R	S	S	R	S	S	R	S	R	S
12.	CSF (22/M)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S
13.	Urine C-40	R	R	S	R	S	S	R	R	R	R	S	S	R	R	S	M	S
14.	Pus (70/M)	R	S	S	S	S	R	S	R	R	R	S	R	S	R	S	S	S
15.	Sputum	S	S	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S
16.	Pus 472	R	S	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S
17.	Blood	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S
18.	Urine C-29	S	S	S	S	S	S	R	S	R	S	S	S	R	R	S	S	S
19.	T/S C-54	S	S	S	S	S	S	S	R	R	S	S	S	R	R	S	S	S
20.	Pus 787	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S
21.	U/S (15D/M)	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S
22.	18E	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R	S
23.	8E	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S

Observation of *in vitro* antibiotic sensitivity pattern and β-lactamase pattern

In vitro sensitivity patterns of 23 *S. aureus* isolates to different antimicrobials are shown in Table 1 and sensitivities to these isolates to oxacillin / methicillin are shown in Table 2 and β-lactamase production patterns of same staphylococcal isolates are shown in Table 3.

In this investigation, among the 23 clinical isolates of *S. aureus*, 43.48% isolates were classified as methicillin-resistant and 56.52% isolates were found to be methicillin-sensitive (MSSA) by disk diffusion method using 1 µg oxacillin disk. Most of the isolates, both MRSA and MSSA were sensitive to ceftriaxone (82.60%), cephalexin (82·60%), cephradine (82.60%), fusidic acid (82·60%) and gentamycin (82.60%). Methicillin-resistant isolates were resistant to all β-lactam antibiotics. Among the isolates (both MRSA and MSSA), high percentage of isolates were resistant to co-trimoxazole (65.21%), erythromycin (56.52%) and amoxicillin (56.52%), but the resistance were higher in case of MRSA isolates, and 90% were resistant to co-trimoxazole, 80% to erythromycin, and 70% to amoxicillin. Virtually, all *S. aureus* were susceptible to vancomycin. In this study, no isolates have been found susceptible to penicillin G. On the other hand, all the isolates were susceptible to vancomycin. These findings are similar to the findings of Supriya et al., 1999 [33]. But they observed less percentage of MRSA (19·56%) which is much lower than the present study.

Test for β-lactamase production revealed that 43.48% isolates produced β-lactamase. The highest number of isolates was from pus (Table 4) and 80% of these produced β-lactamase. Of the isolates from pus samples, 40% isolates were resistant to oxacillin and both of them have produced β-lactamase and the remaining 60% isolates was sensitive to oxacillin of which, only 33.33% isolates produced β-lactamase. The second highest number of isolates was obtained from urine, of which all the isolates were oxacillin- resistant and of the oxacillin-resistant isolates, 25% produced β-lactamase while the remaining isolates obtained from urine were found to be

Table 2. *In vitro* sensitivity pattern of MRSA to different antimicrobials

Sample No.	Specimen	Amoxycillin	Ceftriaxone	Cephalexin	Cephradine	Chloramphenicol	Ciprofloxacin	Cloxacilline	Co-trimoxazole	Erythromycin	Fusidic Acid	Gentamicin	Neomycin	Oxacillin	Penicillin G	Rifampin	Tetracycline	Vancomycin
1.	Rt. Eye	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S
2.	P/S	R	S	S	S	S	R	S	R	R	S	S	S	R	R	S	R	S
3.	Pus 887	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	S
4.	Urine C-13	R	R	S	R	S	S	R	R	R	S	R	S	R	R	S	S	S
5.	Urine (35/F)	R	S	R	S	S	R	S	R	S	S	S	S	R	R	S	R	S
6.	CSF (22/M)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S
7.	Urine C-40	R	R	S	R	S	S	R	R	R	R	S	S	R	R	S	M	S
8.	Pus 472	R	S	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S
9.	Urine C-29	S	S	S	S	S	S	R	S	R	S	S	S	R	R	S	S	S
10.	T/S C-54	S	S	S	S	S	S	S	R	R	S	S	S	R	R	S	S	S

Table 3. Number of isolates from different specimens and their sensitivity to methicillin / oxacillin.

Specimen	No. of isolate	MSSA	MRSA
Pus	5	3	2
Urine	4		4
Prostatic semen (P/S)	3	2	1
Right eye (Rt. eye)	2	1	1
Throat swab (T/S)	2	1	1
Wound swab (W/S)	1	1	
Umbilical swab (U/S)	1	1	
Cerebrospinal fluid (CSF)	1		1
Blood	1	1	
Sputum	1	1	
18E	1	1	
8E	1	1	
Total (%)	23 (100)	13 (56·52)	10 (43·48)

oxacillin-sensitive and non-β-lactamase producing. Among the isolates obtained from prostatic semen, 33·33% showed oxacillin resistance but did not produce β-lactamase enzyme. Our data indicate that the isolates obtained from pus and urine samples showed more resistance to MRSA and also retained β-lactamase production capacity.

Plasmid profile observation and antimicrobial resistance

To look into the plasmid profiles in MRSA, we selected 13 multi-drug resistant strains, isolated the plasmid DNA by alkaline lysis miniprep method, and analyzed by agarose gel electrophoresis (Figure 1). We also furthermore, investigated the resistant patterns of these isolates using 17 different antimicrobials to correlate among these in terms of plasmid presence and multi-drug resistance (Table 5). From our data, we observed that the isolates which showed resistance to more than three antimicrobials possessed very distinct and clear plasmid band(s) whereas, the isolates that showed resistance to two or less of the tested antimicrobials possessed no plasmid bands. Interestingly, isolate S5 (Pus 887) showed resistance to 14 antimicrobials including penicillin G, amoxycillin, gentamicin, ceftriaxone, cephalexin, cephradine, co-trimoxazole, erythromycin, ciprofloxacin,

Table 4. *In vitro* β-lactamase production by S. aureus isolates.

Sample no.	Specimen	β-Lactamase production
1.	Pus	(+)
2.	P/S (2/M)	(+)
3.	Rt. Eye	(-)
4.	P/S	(-)
5.	W/S (5/M)	(+)
6.	Rt. Eye (36/M)	(-)
7.	Pus 887	(+)
8.	P/S (58/M)	(-)
9.	Urine C – 13	(+)
10.	Urine (35/F)	(-)
11.	T/S (2/M)	(+)
12.	CSF (22/M)	(-)
13.	Urine C - 40	(-)
14.	Pus (70/M)	(-)
15.	Sputum	(+)
16.	Pus 472	(+)
17.	Blood	(+)
18.	C-29 Urine	(-)
19.	T/S C-54	(+)
20.	Pus 787	(-)
21.	U/S (15D/M)	(-)
22.	18E	(-)
23.	8E	(-)

(+) = β-Lactamase producer, (-) = non-β-lactamase producer, Rt. Eye = Right eye, P/S = Prostatic Semen, T/S = Throat Swab, W/S = Wound Swab, U/S = Umbilical Swab, CSF = Cerebrospinal Fluid, D = Day, M = Male, F = Female.

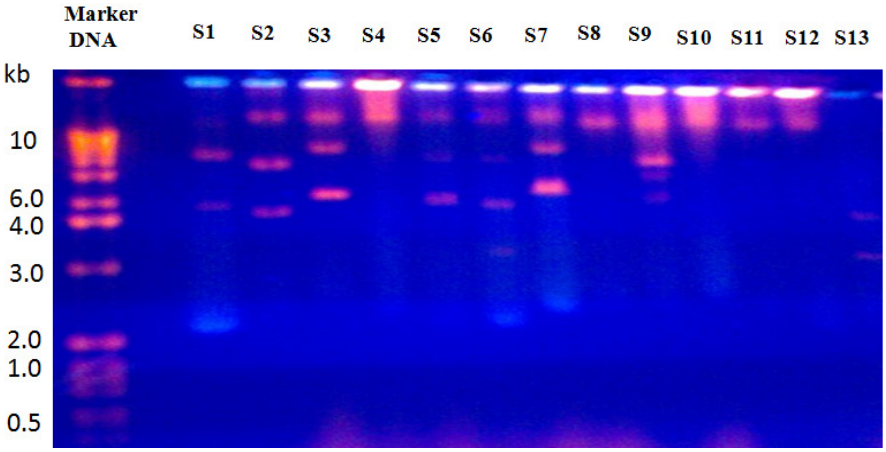

Figure 1. Gel electrophoresis result of 13 selected clinical isolates of *S. aureus*.

tetracycline, chloramphenicol, neomycin, fusidic acid and oxacillin; and sensitivity to only 3 antimicrobials and revealed light bands of plasmid DNA in the gel electrophoresis analysis (Table 5, Figure 1). Isolate S7 (CSF, 22/M), showed resistance to 15 antimicrobials including penicillin G, amoxycillin, co-trimoxazole, erythromycin, ciprofloxacin, tetracycline, gentamicin, ceftriaxone, cephalexin, cephradine, chloramphenicol, cloxacillin, neomycin, rifampin and oxacillin and the revealed clear and distinct band of plasmid DNA. On the other hand, isolate S10 (Pus 787) showed resistance to only two antimicrobials namely penicillin G and tetracycline, and revealed no bands of plasmid DNA in the gel electrophoresis. Whereas, isolate S12 (blood)

Table 5. *In vitro* sensitivity pattern of 13 selected (plasmid-examined) clinical isolates of *S. aureus* to different antimicrobials.

Sample no.	Specimen	Amoxycillin	Ceftriaxone	Cephalexin	Cephradine	Chloramphenicol	Ciprofloxacin	Cloxacilline	Co-trimoxazole	Erythromycin	Fusidic Acid	Gentamicin	Neomycin	Oxacillin	Penicillin G	Rifampin	Tetracycline	Vancomycin
S1.	Pus	R	S	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S
S2.	Rt. Eye	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S
S3.	P/S	R	S	S	S	S	R	S	R	R	S	S	S	R	R	S	R	S
S4.	W/S (5/M)	R	S	S	S	S	R	S	S	S	R	S	R	S	R	R	S	S
S5.	Pus 887	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	S
S6.	P/S (58/M)	R	S	S	S	S	R	S	R	R	S	S	S	S	R	S	R	S
S7.	CSF (22/M)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S
S8.	Pus (70/M)	R	S	S	S	S	R	S	R	R	R	S	R	S	R	S	S	S
S9.	Pus 472	R	S	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S
S10.	Pus 787	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S
S11.	U/S (15D/M)	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S
S12.	Blood	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S
S13.	T/S C-54	S	S	S	S	S	S	S	R	R	S	S	S	R	R	S	S	S

showed resistance to two antimicrobials namely penicillin G and cephradine and also revealed no bands of plasmid DNA. The data as depicted in Figure 1 and Table 5 also revealed that most of the plasmid containing isolates showed resistance to co-trimoxazole, which predict the presence of co-trimoxazole-resistant gene in the plasmid because none of the co-trimoxazole sensitive isolates showed plasmid bands.

In this study, investigation was carried out to know the prevalence of multiple-drug resistant (MDR) gene-carrying plasmids in the MRSAs but no vivid result was found. However, multi-drug resistant isolates showed more plasmid bands and all the isolates which did not show any plasmid were sensitive to almost all the antimicrobials. Our studies showed a 43·48% prevalence of MRSA in the tested clinical samples which was almost similar to that reported by Rahman et al. (2002). Such high rates of MRSA have also been reported in India (Anupurba et al., 2003; Vidhani et al., 2001). However, Udaya et al. (1997) reported 20% MRSA and Mehta et al. (1998) 32.8% MRSA in some regions of India. In Nepal, Mulligan et al. (1993) reported 26.14% in its eastern part. In summary, the prevalence of MRSA seems to be higher in Bangladesh, India and Nepal as compared to other parts of the world (Udo et al., 1993; Herwaldt and Wenzel, 1996; Mulligan et al., 1993; Mansouri and Khaleghi, 1997) except in Africa (Olukoya et al., 1995; Adeleke and Odelola, 1997).

In this present study, most (70%) of the isolates which showed plasmids were found to be resistant to amoxicillin. On the other hand, no correlation was observed between tetracycline resistance and plasmid profiles. Most of the erythromycin-resistant isolates showed prominent bands of plasmid DNA. However, no inter-relation was found between the 2nd and 3rd generation cephalosporin-resistance used (in this investigation) and plasmid profiles. All the isolates were found to exhibit resistance to penicillin G.

Although in the present study, it was observed that there is a tendency that multi-drug resistant isolates contain plasmids but no solid evidence could be provided. In order to clarify this issue, further studies are to be initiated. Abuse and irrational use of antibiotics will lead to development of drug resistance. In a developing country like Bangladesh, there is lack of guidelines in the practice of antibiotic prescriptions. However, our studies might provide a platform for physicians to choose and prescribe rational antibiotics in the treatment of MRSA in hospital and community infections.

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