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Original Article

Healthcare-associated (HA) and community-associated (CA) methicillin resistant *Staphylococcus aureus* (MRSA) in Bangladesh – Source, diagnosis and treatment

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been a common pathogen in healthcare facilities, but now, it has emerged as a problematic pathogen in the community setting as well. This study reported source, diagnosis and treatment of HA-MRSA and CA-MRSA.

A total of sixty-five clinical samples (urine, pus, wound swab) were collected from clinical origin of Dhaka city, Bangladesh. All the isolates were tested phenotypically by conventional methods and genotypically by PCR targeting *nuc*, *pvl* and *mecA* genes. Finally sequencing was carried out for *pvl* gene to know the mutagenic variation or any amino acid changes in *pvl* gene. Chi square test was employed for statistical analysis. Patients of age group 51–60 years are more susceptible (46.15%) to MRSA, CA-MRSA or HA-MRSA infection. Female are (32.30%) more susceptible to MRSA infection. Among 65 isolates 53 isolates identified phenotypically as *S. aureus.* These were positive for amplification of *nuc* (270 bp) gene of *S. aureus*. Moreover, among 53 isolates 33 phenotypically considered as MRSA and 38 (72%) showed positive amplification for *mecA* (162 bp) gene. Among 38 MRSA isolates 22 (57.89%) confirmed as CA-MRSA and 16 (42.10%) as HA-MRSA. Finally, sequence analysis for *lukS/F-PV* genes from 4 representative isolates detected a new single nucleotide polymorphism in comparison with the control sequence. However, no amino acid changes were found. Statistical analysis showed HA-MRSA isolates were more commonly found in urine sample and CA-MRSA in pus and wound swab. CA-MRSA isolates were more resistant to tested antibiotics than HA-MRSA.

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1. Introduction

Staphylococcus aureus isolates are resistant to methicillin, termed methicillin-resistant *S. aureus* (MRSA). They are usually found to be resistant to other β-lactam antimicrobial drugs [1]. Methicillin-resistant *S. aureus* (MRSA) is one of the most frequent cases of hospital and community-associated [2,3]. According to previous report, in Bangladesh indiscriminate and incomplete uses of antibiotic made MRSA more alarming [4].

Recent study in Bangladesh shows, prevalence rate of methicillin-resistant *S. aureus* (MRSA) in clinical samples is 63%, which is a high incidence comparable to the United States (60%) [5,6]. Approximately 75% were also found to be resistant to methicillin, in burn wound infection [7], where 8.5% multi-drug resistant *S. aureus* were also found in burn units [8]. Traditionally, MRSA has been considered a major nosocomial pathogen in healthcare facilities, also been observed as emerging pathogen in community. Therefore, the current susceptibility patterns of local strains are essential for the judicious use of antibacterial agents as well as to become aware of the MRSA in hospitals and community areas in Bangladesh.

Healthcare-associated MRSA infection, labeled as HA-MRSA if patients were hospitalized >48 h prior to the current infection (i.e., patient was not MRSA infected with MRSA at the time of hospitalization but culture and infection were identified >48 h after admission).

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Community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains have appeared as a significant pathogen in community-associated settings as well as healthcare-associated ones [8]. A systematic review by Yusuf et al. revealed that Bangladesh is very vulnerable to develop CA-MRSA due to irrational use ofantibiotics [9].

Most of the CA-MRSA associated with skin and soft tissue infection (SSTI) and bacteremia respectively. But previous data showed that CA-MRSA also responsible for 25% of UTI infection [10]. These infections are acquired by persons who have not been hospitalized nor have had certain medical procedures such as dialysis, surgery, or catheterization before admission to hospital. They carry genes for Panton-Valentine leukocidin (PVL) [11–13]. CA-MRSA strains often originate from isolates picked up in healthcare facilities on previous visits [14]. There is only study that has been performed on CA-MRSA in Bangladesh [10]. In that study it has been reported that 25.0%. CA-MRSA was present in the tested community.

Detection pattern can be categorized into conventional detection (such as biochemical identification, antibiogram) and molecular detection (such as PCR, sequencing). Antibiotic susceptibility pattern is a valuable tool for the detection of methicillin resistance pattern by using cefoxitin disk (30 μg) ($\leq$ 21 zone diameter known as MRSA) (NCCL, 2014). But it is not well enough for the characterization of CA-MRSA and HA-MRSA.

Molecular testing is now used as a gold standard for detection of *S. aureus*(*nuc*gene), MRSA (*mec*A gene) [15] and distinguishing amongst CA-MRSA and HA-MRSA [16]. However, this is the most intensive way to detect CA-MRSA and HA-MRSA. CA-MRSA is distinguishable from HA-MRSA based on the production of Panton-Valentine leukocidin (PVL) [17]. This PVL Encoded by *lukS-PV-lukF-PV*, a component of the phage genome inserted in bacterial chromosome. The PVL is a bicomponent cytotoxin that is preferentially linked to furuncles, cutaneous abscess, and severe necrotic skin infections [18,19].

Prevention of *S. aureus* infection and reduction of the spread of virulent and resistant strains are therefore of great importance [20]. Rapid diagnostic surveillance is becoming necessary in health care institutions to reduce infection rates and to better understand the disease.

The purpose of this study was to determine the prevalence and molecular characterizations of pathogenic *Staphylococcus aureus*that associated with health care and community settings in Dhaka, Bangladesh by conventional method and molecular method.

Table 1
Sample size and type.

Sample size (No. of samples collected)	Sample type (urine, pus, wound swab)
36	Urine
18	Pus
11	Wound swab (w/s)

2. Materials and methods

2.1. Sample collection

Sixty-five clinical samples (Table 1) were collected during the months from August to November, 2015 from clinical origin of Dhaka city. A demographic profiling of patients was done.

2.2. Isolation and identification of S. Aureus

Sixty-five bacteria was isolated from sixty-five samples& identified by cultural characteristics, colony morphology, Gram's stain and biochemical tests.

2.3. Antimicrobial susceptibility test

A standard disc diffusion technique for antimicrobial susceptibility testing was determined *in vitro* by using the standardized agar-disc-diffusion method known as Kirbey Baur method [21]. Cefoxitin (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), gentamicin (10 μg), tetracyclin (30 μg), oxacillin (1 μg) and vancomycin (30 μg) disks were used for this purpose.

2.4. Determination of minimal inhibitory concentration (MIC)

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a chemical that prevents visible growth of a bacterium. Broth micro dilution was used for determination of MIC) against three antibiotic pure powder: gentamicin ceftriaxone, vancomycinin sterile flat-bottom 96-well polystyrene plates [22].

2.5. DNA Extraction and PCR assay

Extraction of DNA from bacterial cell by boiling or heating method is an efficient method. DNA from bacterial cell was extracted by boiling method according to Queipo-Orthuno et al. [23]. Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler.

PCR was performed in a thermal cycler (ApliedBiosystem, Veriti 96 well thermal cycler, USA). The reaction mixtures consisted of 2 μl of the extracted DNA template, 4 μl 5x PCR buffer, 2 μl $MgCl_2$ (25 Mm), 0.4 μldNTPs (10 mM), 0.1 μl (250 U GoTaq® Hot Start DNA Polymerase), 1 μl from the forward and reverse primers. The volume of the reaction mixture was completed to 20 μl using nuclease free water. PCR parameters are listed in Table 2.

2.6. Detection of amplicons by agarose gel electrophoresis

The amplified products were visualized by horizontal electrophoresis in 1.3% (for *nuc* gene), 2% (for *mecA* gene) and 1.2% (for *PVL* gene) of agarose gel with a 100 bp DNA ladder (Promega, USA) was prepared in 1X TAE buffer. The gel was viewed by UV-Transilluminator at 254 nm and 365 nm wavelength.

Table 2
PCR thermocycling conditions.

Gene	Temperature (°C)/time cycle					Cycle No.
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
Nuc	95/10 min	94/1 min	55/30 sec	72/1.30 sec	72/3.30 sec	37
MecA	94/4 min	94/30 sec	53/30 sec	72/1 min	72/4 min	35
PVL	94/4 min	94/45 sec	56/45	72/30 sec	72/2 min	30

2.7. Sequencing of PCR products (PVL Gene)

Amplicon off our representative isolates and respective primers (Table 3) were sent to icddr, b (International Centre for Diarrhoeal Disease Research, Bangladesh) (www.icddrb.org) for single-pass DNA sequencing by Sanger method.

Table 3
Oligonucleotide primers used for the simultaneous amplification of *nuc*, *mecA*, *PVL* genes of *Staphylococcus aureus*.

Primer	Primer sequence (5′-3′)	Product size (bp)	Reference
nuc-F	GCGATTGATGGTGATACGGTI	270	[24]
nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
*mec*A-F	TCCAGATTACAACTTCACCAGG	162	[25]
*mec*A-R	CAATTCATATCTTGTAACG		
Luk-PVL-F	ATCATTAGGTAAAATGTCTGGACATGATCA	433	[26]
Luk-PVL-R	GCATCAAGTGTATTGGATAGCAAAAGC		

Table 4
Age and gender distribution of patients.

Age	Male (%) n = 14	Female (%) n = 51
≤10	0 (0)	0 (0)
11–20	0 (0)	0 (0)
21–30	0 (0)	10 (20)
31–40	1 (7)	10 (20)
41–50	3 (21)	9 (18)
51–60	10 (71)	13 (26)
61–70	0 (0)	7 (14)
71–80	0 (0)	2 (4)
≥81	0 (0)	0 (0)

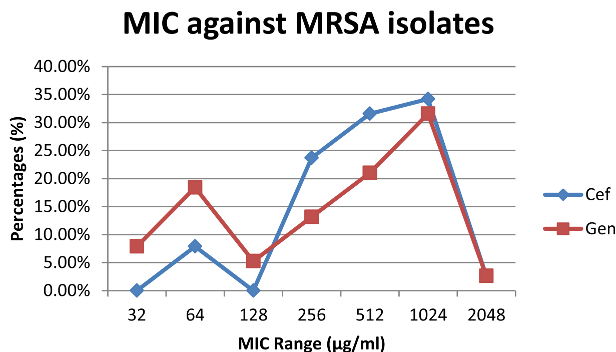

Fig. 1. Different resistant pattern against Ceftriaxone and Gentamicin.

3. Results

Sixty-five clinical samples (urine, pus, wound swab) were collected from patients (14 male and 51 female) (Table 4). Age and sex distribution pattern of their samples showed that most of the samples were obtained from age between 51–60. Female were more susceptible to MRSA infection then male.

3.1. Isolation and identification of S. Aureus

Among 65 isolates 53 confirmed as *S. aureus*by cultural characteristics, colony morphology, Gram's stain and biochemical tests.

Reference strain of the of *Staphylococcus aureus ATCC* 25923 was used in this study. This reference strain was collected from Microbiology lab of Shishu Hospital Dhaka, Bangladesh.

3.2. Antibiotic susceptibility pattern of S. Aureus

A total of 53 morphologically and biochemically characterized isolates were tested against nine commonly used antibiotic.

All isolates were sensitive to vancomycin. Isolated strain showed highest resistance to oxacillin (97%) followed by gentamicin (85%) and ceftazimide (76%). Furthermore, isolates were found to be resistant tocefoxitin (72%), tetracylcin (68%), ciprofloxacin (53%), chloramphenicol (66%), clindamycin (34%).

According to NCCLs, (2013) *S. aureus* isolates with zone of inhibition of cefoxitin disk ≤21 mm were phenotypically considered MRSA worldwide. In this study resistance pattern provides 63% of MRSA infection associated with patients.

3.3. Determination of minimal inhibitory concentration (MIC) using microdilution tray

MIC breakpoint was recorded according to NCCL, (2013). All MRSA isolates showed highest resistance to ceftriaxone and gentamicin (Fig. 1). But all MRSA isolates were sensitive to vancomycin (MIC: 0.25 µg/ml-8 µg/ml).

3.4. PCR for detection of nuc gene specific for S. aureus, mecA and pvlgenes

All the 53 strains showed positive amplification of 270 bp fragments specific for *nuc*gene of *S. aureus* (Fig. 1). All the 38 (72%) strains showed positive amplification of 162 bp fragments specific for *mec*A gene of MRSA (Fig. 2). Out of 38 isolates of MRSA 22 (58%) isolates showed positive amplification for 433 bp fragments specific for *PVL* gene (Fig. 3).

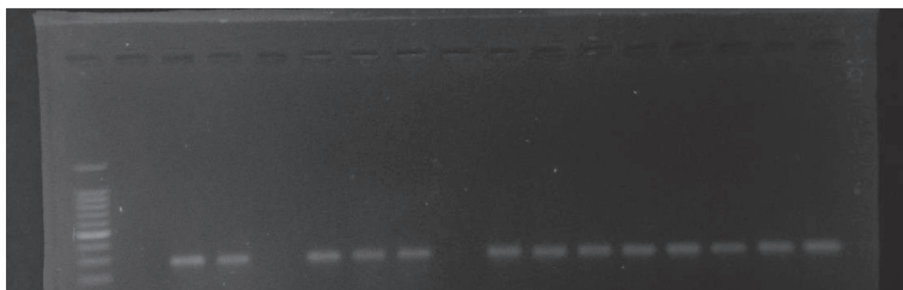

Fig. 2. PCR showing positive amplification of 162 bp fragments specific for *mecA* gene of *S. aureus*. Lane 1 showing 100 bp ladder. Lane 2 showing negative control *S. aureus ATCC 25923*. Lane 3 showing positive control.

3.5. In silico analysis

3.5.1. Sequence comparison and alignment of amino acids

Comparison of the sequences done with the other sequences from Genebank of National Biotechnology Information Centre (https://www.ncbi.nlm.nih.gov) by means of the basic local alignment search tool BLAST [24] revealed the identity of sample.

Homology analysis of *PVL* gene was performed comparing with control sequence (Accession No. KP896298.1). The result of translation and multiple amino acids sequences alignment revealed that the change at position 33 (GCC/TCC) gives the same type of amino acid which is serine (Figs. 4 and 5).

3.6. Data analysis

SPSS version 23.0 software was used for performing the statistical analysis. Chi square test was employed to compare the categorical variables between the two groups. $P < 0.050$ was considered statistically significant.

A total of 38 MRSA strains consisting of 22 CA-MRSA and 16HA-MRSA were studied. There was no significant difference with regard of sex distribution of patients and infection with CA-MRSA and HA-MRSA (Table 5).

A statistical analysis performed with type of infection that showed association between type of infection and MRSA (CA-MRSA and HA-MRSA).

Statistical analysis results significant association between clinical samples and type of infection (Table 6).

For the detection HA-MRSA urine sample is most suitable where for CA-MRSA, pus and wound swab is found to be good one (Table 6).

Antibiotic resistant pattern of CA-MRSA and HA-MRSA was analyzed. CA-MRSA isolates were significantly more susceptible to clindamycin. All isolates were sensitive to vancomycin (Table 7).

This statistical analysis results that CA-MRSA became more resistant to commonly used antibiotics.

Table 8 showed that the PCR was found to be the best method for detection of HA-MRSA and CA-MRSA.

Table 5
Gender distribution of patients infected with CA-MRSA and HA-MRSA.

Sex	CA-MRSA (n = 22)	HA-MRSA (n = 16)	*P* value
Male	6	5	0.92
Female	16	11	0.92

$P \leq 0.05$ considered statistically significant.

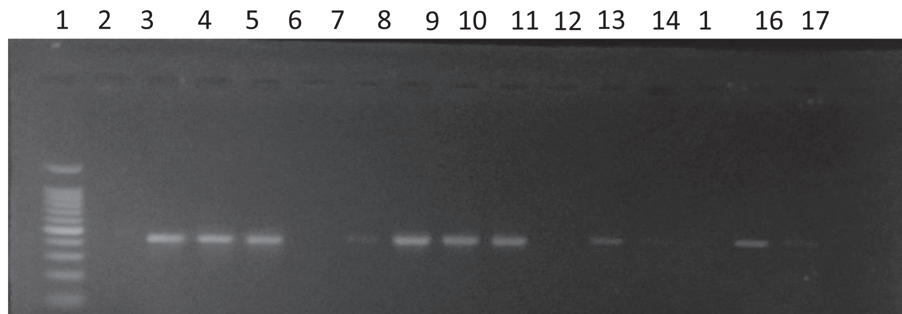

Fig. 3. PCR showing positive amplification of 162 bp fragments specific for *pvl* gene of *S. aureus*. Lane 1 showing 100 bp ladder. Lane 2 showing negative control. Lane 3 showing positive control. Lane 7 and 17 showing relatively faint band.

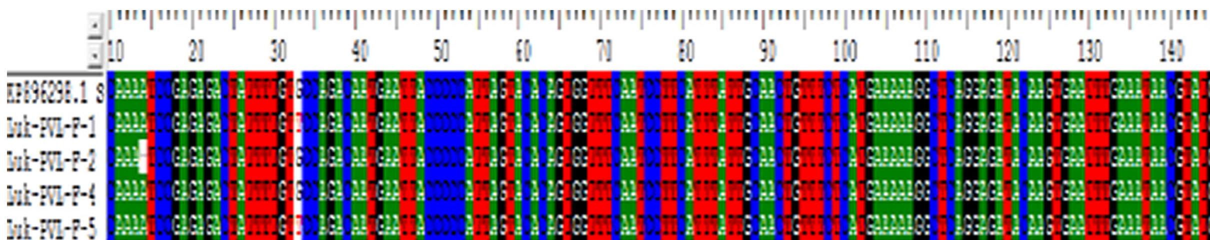

Fig. 4. Sequence pattern (snapshot) of 4 isolates those are compared with a control sequence (Accession No. KP896298.1.

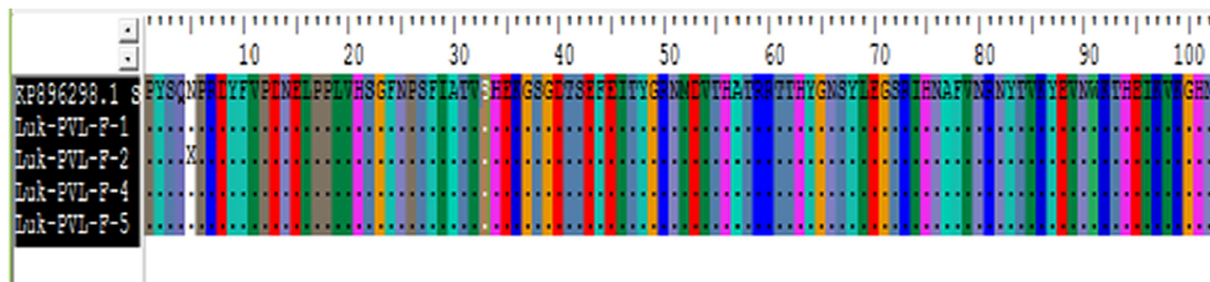

Fig. 5. Amino acids multiple sequence alignment of *pvl* gene. The alignment was performed using ClustalW sequence alignment.

Table 6
Clinical samples and infections caused by CA-MRSA and HA-MRSA.

Type of sample	CA-MRSA (n = 22)	HA-MRSA (n = 16)	*P* value
Urine	6	13	<0.005
Pus	10	2	<0.005
Wound swab (w/s)	6	1	<0.005

$P \leq 0.05$ considered statistically significant.

Table 7
Antibiotic resistance pattern of CA-MRSA and HA-MRSA.

Antibiotics	Antibiotic resistance pattern		*P* value
	CA-MRSA (n = 22) Number (%)	HA-MRSA (n = 16) Number (%)	
Cefoxitin	22	16	–
Ciprofloxacin	10	10	0.375
Chloramphenicol	18	10	0.175
Clindamycin	04	10	<0.01
Oxacillin	22	16	–
Tetracyclin	15	15	0.075
Gentamicin	14	14	0.100
Ceftazidime	21	15	0.925
Vancomycin	0	0	–

$P \leq 0.05$ considered statistically significant.

Table 8
Comparison of different methods for detection of HA-MRSA and CA-MRSA.

Type of detection	HA-MRSA	CA-MRSA
Antibiogram	Good (11/16)	Poor (12/22)
PCR (molecular method)	Extremely good (16/16)	Extremely good (22/22)

4. Discussion

Methicillin resistant strains of *Staphylococcus aureus* (MRSA), is no longer limited to the hospital, but also occurs among otherwise healthy communities. Such community-acquired MRSA is an emerging pathogen that primarily causes skin and soft tissue infections. Report has also been showed that CA-MRSA was present in the community in 25.0% causing the UTI [10].

In the present study, prevalence of CA-MRSA was higher compared to HA-MRSA strains. Age between 51–60 years are more prone to cause MRSA infection. Female might be more susceptible to MRSA infection. But data analysis results no significant difference with regard to age and gender distribution of patients to cause HA-MRSA and CA-MRSA.

According to chi square test we observed that most of the CA-MRSA were found in pus and wound swab. This observation is consistent with the results of previous studies [25]. Most of the UTI infections were more commonly caused by HA-MRSA which is comparable to the results of previous studies.

Among 65 isolates 53 confirmed as *S. aureus*by cultural characteristics, colony morphology, Gram's stain and biochemical tests along with reference strain of the of*Staphylococcus aureus ATCC* 25923.

The antibiotics selected for this study were cefoxitin, ciprofloxacin, chloramphenicol, clindamycin, oxacillin, tetracylcin, gentamicin, ceftazidime, vancomycin. All isolates were sensitive to vancomycin. Isolated strain showed highest resistance to oxacillin (97%) followed by gentamicin (85%) and ceftazimide (76%). Furthermore, isolates resistant tocefoxitin (72%), tetracylcin (68%), ciprofloxacin (53%), chloramphenicol (66%), clindamycin (34%).). Results showed that prevalence of MRSA (72%) increasing over time, where Haq*et al.* (2005) reported 63% MRSA range in Dhaka city, Bangladesh [5].

HA-MRSA normally exhibits multidrug resistance whereas CA-MRSA is mostly susceptible to antibiotics [26,27]. But data analysis results a high percentage of antibiotic resistance with CA-MRSA. So drug susceptibility test is not well enough for detection of HA-MRSA and CA-MRSA.

MIC test was also performed against 38 MRSA along with *S. aureus ATCC* 25923. Only vancomycins found to be susceptible to all clinical isolates. That would be a drug of choice of CA-MRSA and HA-MRSA infection.

Now-a-days molecular detection providing excellent result for detection of *S. aureus,* MRSA, CA-MRSA and HA-MRSA. Phenotypically confirmed fifty-three isolates were confirmed as *S. aureus* by presence of *nuc* gene. Thirty-eight isolates were confirmed as MRSA by both phenotypic and molecular method. Molecular detection of CA-MRSA and HA-MRSA was performed by PCR.

Molecular detection pattern revealed 22/38 (57.90%) CA-MRSA isolates and 16/38 (42.10%) HA-MRSA isolates among 38 MRSA isolates. This isolation of CA-MRSA and HA-MRSA considered the higher prevalence of CA-MRSA than HA-MRSA in Bangladesh. Iqbal et al. also showed the high prevalence of CA-MRSA in Dhaka, Bangladesh with increasing antibiotic resistance pattern [10].

After detection of CA-MRSA and HA-MRSA cycle sequencing of *pvl* gene was performed for four isolates at icddr, b (International Centre for Diarrhoeal Disease Research, Bangladesh). A neucleotide mismatch was found in two isolates in comparison with control sequence (Accession No. KP896298.1) without any change in amino acid (serin).

Our tested 4 CA-MRSA sequences of *PVL* gene all of were showed phylogenic similarity with seqences isolated from Japan, India, UK, USA, Manila and other country might be an indicator of major epidemiological burden. So this organism causing CA-MRSA that confined not only in Bangladesh but also all over the world which is a major epidemiological burden.

The study provides information about MRSA, HA-MRSA and CA-MRSA prevalence and their resistance pattern among patients. MRSA colonization and infection bring large financial burdens to health care facilities and patients. Every strain of bacteria is susceptible to a specific antibiotic; hence, it is important to identify the antibiotics before prescribing to the infected person. As a measure to identify and to eradicate MRSA, hospitals have begun screening inpatients for the presence of MRSA and decontaminating them with prophylactic antibiotic treatments. Vancomycin remains the drug of choice for treatment of infections caused by MRSA.

Finally, for reduction of infection caused by MRSA, CA-MRSA, HA-MRSA and to better understand the disease and their efficient treatment, early and rapid diagnosis is of prime concern. Molecular detection by PCR might be useful for this purpose in Bangladesh.

5. Conclusions

The results of this thesis revealed the prevalence of MRSA strain (72%) in Dhaka, Bangladesh. People, age between 51–60 and female are most strongly associated with MRSA infection. This study also revealed that CA-MRSA infections appear to be an emerging phenomenon in Bangladesh. PCR is found to be the gold standard for the detection of MRSA, CA-MRSA and HA-MRSA. The majority of clinical samples carried *PVL* gene (twenty-two out of thirty-eight isolates 57.90%) that indicates the prevalence of CA-MRSA increasing day by day. Prevalence of HA-MRSA was 42.10% (16/38). The *pvl* locus represents a stable genetic marker of these CA-MRSA strains. Attention should be paid to the emergence of PVL-positive CA-MRSA on Bangladesh. To have a substantial impact on the burden of HA-MRSA and CA-MRSA disease and to prevent increasing incidence, risk factors for development of HA-MRSA and CA-MRSA infection must be identified.

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