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EVALUATION OF PHENOTYPIC AND GENOTYPIC METHODS TO DETECT METHICILLIN RESISTANCE IN STAPHYLOCOCCUS AUREUS (MRSA)

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ABSTRACT

Background: SSIs are the second most common nosocomial (hospital-acquired) infection after urinary tract infection even with modern facilities, standard protocols of preoperative preparation and antibiotic prophylaxis. Increasing antibiotic resistance of pathogens associated with SSI also becomes a major therapeutic challenge for the physicians. **Methods:** A cross sectional hospital based observational study was conducted in the department of Microbiology, BIRDEM General Hospital from period of September, 2018 to August, 2019. A total of 187 study participants clinically suspected as a case of SSI were investigated during the study period from two tertiary care hospitals of Dhaka city. Out of 187, 84 samples were collected from patients of laparoscopic surgery and 103 samples from open surgery. Among 103 samples from open surgery 50 were collected from general surgery ward and 53 from obstetrics & gynecology ward. The isolated organism from samples were identified on the basis of colony morphology on MacConkey agar and Blood agar media, hemolytic criteria, pigment production, gram staining character, coagulase test, catalase test. Catalase negative Gram positive organisms were further identified by bile esculin and NaCl tests. Phenotypically confirmed MRSA strains were further tested for mecA and PVL gene by PCR. **Results:** Among different surgical specialties, 82.1% culture positive cases were found from laparoscopic surgery, 82% & 73.5% from general surgery and obstetrical-gynecological surgery respectively. In BIRDEM General Hospital 81.7% (103/126) isolates were gram negative and 18.3% (23/126) were gram positive bacteria. In DMCH 85.6% (113/132) were gram negative and 14.3% (19/132) were gram positive bacteria. *Staphylococcus aureas* was the predominant gram positive bacteria among the total isolates. Among the MRSA isolates, 72.4% were positive for mecA and 24.1% were positive for PVL gene. **Conclusion:** Among the isolated MRSA strain, 72.4% were positive for the *mecA* gene and 24.1% were positive for the PVL gene. The information obtained from this study will be helpful in understanding SSI in terms of aetiological agents, their antimicrobial resistance pattern, distribution of MRSA producers, in treatment and prevention accordingly.

KEYWORDS: Surgical Site Infection, Staphylococcus, Staphylococcus Aureus.

INTRODUCTION

Before the mid-19th Century, the majority of post-surgical patients developed SSI. The process began with fever followed by purulent pus discharge from the incision as well as sepsis and death. Despite considerable research on best practices and strides in refining surgical techniques, technological advances and environmental improvements in the operating room and the use of prophylactic preoperative antibiotics, infection at the surgical site remains the common adverse event occurring to hospitalized patients and a major source of morbidity following surgical procedures.[1,2]

SSI prolongs hospital stay and increases antibiotic prescribing and laboratory costs. Most of the infected patients (60%) are more likely to spend time in an ICU, 5 times as likely to be readmitted and their mortality rate is twice of non-infected patient. It is noteworthy that an estimated 40-60% of these infections are preventable.[3] The prevalence of hospital acquired infection is high in both developed and developing countries with attendant morbidity and mortality.[4,5]

SSI can be divided into three categories: superficial incisional, deep incisional and organ/space SSI. The first is the superficial incisional surgical site infection which

occurs within 30 days after surgery and is an infection involving the skin and subcutaneous tissue only. The second type of infection is the deep surgical site infection which may present after 30 days of surgery and involves the deep soft tissues including fascia and muscles deep to the incision. The third type of infection is organ/space infection in which the infection spreads to organs and spaces other than the incision which was opened or manipulated during surgery.[6]

Staphylococcus aureus (S. aureus) is perhaps the greatest concern of human pathogens because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections and its capacity to adapt to different environmental conditions.[7] It is one of the most common and important human pathogen associated with broad spectrum of diseases. S. aureus is a major cause of surgical wounds infections associated with open and laparoscopic surgery. Increasing drug resistance among S. aureus and the spread of methicillin resistant Staphylococcus aureus (MRSA) are global threat.

The resistance of MRSA to β-lactam antibiotics is associated with penicillin-binding protein 2a (PBP 2a), encoded by the *mecA* gene. Nowadays, MRSA is the leading cause of health-care associated infections globally and, as more patients are treated outside the hospital settings, is an increasing concern in the community.[8] *S. aureus* acquires methicillin resistance by insertion of staphylococcal cassette chromosome (SCC*mec*), carrying the *mecA* gene, into chromosome. This gene encodes an altered penicillin-binding protein, PBP-2a, which is not inhibited by existing ß-lactam antibiotics. There are several antimicrobial susceptibility methods for detection of MRSA, including oxacillin screening test, oxacillin and/or cefoxitin disk diffusion method and oxacillin minimum inhibitory concentration test.

There are many reports that these conventional antimicrobial tests are associated with false negative and positive results for MRSA identification. Therefore, it is necessary to use exact and specific methods, such as polymerase chain reaction (PCR) that is considered as a DNA-based assay. The pathogenicity of *S. aureus* is related to a number of virulence factors that allow the organism to adhere, avoid the immune system and cause harmful effects to the host. One of the important cytotoxins produced by some strains of *S. aureus* is the Panton Valentine leukocidin (*PVL*), encoded by two genes, *lukS- PV* and *lukF-PV*.[9]

In perspective of above discussion the present study was undertaken to detect the distribution of bacteria causing SSI, pattern of their antimicrobial susceptibility, genes for methicillin resistant *Staph aureus.*

OBJECTIVES

General objectives

Identify MRSA among isolated *Stahylococcus aureas* by phenotypic and genotypic method.

Specific objectives

The specific objectives of the study were to

1. Identify MRSA among isolated *Stahylococcus aureas* by phenotypic Cefoxitin disc diffusion method.
2. Detect mecA gene and Panton-valentine leukocidin (PVL) gene among Methicillin resistant *Staph aureas* by Polymerase chain reaction.

METHODOLOGY

Study design

Cross sectional hospital based observational study.

Study place

Department of Microbiology, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka.

Study period

01-year September, 2018 to August, 2019.

Sampling

Purposive

Study population

Clinically suspected surgical site infection patients with one or more of the following features of infection were included in this study.

Inclusion criteria

- All patients who developed 1. Spreading erythema 2. Localised pain (This is often different to the typical post-operative pain) 3. Pus/ discharge from the wound 4.Wound dehiscence 5.Unexplained persistent pyrexia.
- Wound deliberately opened by the surgeon for drainage.

  The study cases were selected by the surgeon and gynecologist working in the Department of Surgery, Obstetrics and Gynecology.

Exclusion criteria

- Patients not giving consent to participate in the study.

Sample collection

Wound swab samples were collected from 187 suspected post-surgical infected cases that fulfilled the inclusion criteria. Ninety four wound swab samples were taken from BIRDEM of which 44 swabs were taken from laparoscopic port site and 50 swabs were taken from open surgical wound site. Ninety three wound swab samples were collected from DMCH of which 40 swabs were taken from laparoscopic port site and 53 swabs were taken from open surgical wound site.

Samples were collected aseptically by sterile swab stick avoiding contamination from external sources. Swabs were collected using two sterile cotton tipped swab sticks one for culture and other for microscopy from clinically deep area of wound site prior to any dressing.

PHENOTYPIC DETECTION OF MRSA

Cefoxitin disc diffusion test

Cefoxitin disc diffusion test was performed on Mueller–Hinton agar plates using a bacterial suspension with the turbidity adjusted to a 0.5 McFarland standard. Disc containing 30 µgm of Cefoxitin were placed. Plates were incubated at 37˚C for 24 hour. *Staphylococcus aureus* strains were screened for MRSA by detection of resistance (zone of inhibition ≤21 mm) following the CLSI guidelines (CLSI, 2017).

Molecular study

Molecular analysis was done by polymerase chain reaction (PCR) assay. Isolated MRSA strains were carried out for detection of mecA and Panton valentine leukocidin (PVL) by polymerase chain reaction.

Separate Polymerase chain reaction was carried out with the specific primers. Four major steps of PCR include bacterial pellet formation, DNA extraction, DNA amplification in thermal cycler and visualization / documentation under UV light.

Formation of bacterial pellet

The colonies (about 5-6) of specific gram positive bacteria were subcultured into Muller Hinton agar media at 37 ˚C for 24 hours. A loop full of bacterial colonies was inoculated into eppendorf tube containing trypticase soy broth. After incubation at 37˚C for 24 hours eppendorf were centrifuged at 6000 rpm for 10 minutes (Mini spin plus). Then the supernatant was decanted.

Extraction of DNA by boiling method

Three hundred µL of distilled water (DW) was taken into the Eppendorf tube having bacterial pellet, mixed well and then boiling for 10 min. After boiling, the tubes were immediately placed on ice for cold shock for 5 minutes followed by centrifugation at 13,600 rpm for 6 min at 4°C. The supernatant was collected using micropipette in to another eppendorf tube which was further used as template DNA. Three microliters (3 µl) of the supernatant were used for the PCR. Extracted DNA was stored at -20°C until PCR amplification.

Mixing of mastermix and primer with template DNA

Sterile 0.2 ml micro centrifuge tubes / PCR tube were taken and the tubes were labeled with date and identification number. For each sample, a total 25 µl of mixture was prepared by mixing of 15 µl of master buffer composed of PCR buffer, MgCl2, deoxy nucleoside triphosphate / dNTP (New England Biolabs Inc, USA) and 1.25 µl Taq polymerase (Geneaid Biotech Ltd, Taiwan) and 2 µl of forward primer, 2 µl of reverse primer (Macrogen Korea), 3µl of DNA template (New England, Biolab, USA) and 3 µl nuclease free water were added. After a brief vortex, the PCR tubes were centrifuged in a micro centrifuge machine for few seconds. Primer was kept at -20℃.

DNA amplification of MRSA encoding gene in thermal cycler

Amplification was carried out in an automated DNA thermal cycler. The PCR tube were placed in thermal cycler and allowed to run by the following protocols:

Table 1: PCR thermal cycling condition of ESBL & MRSA encoding gene.

Gene	Preheating	Denaturation	Anneling	Extension	Cycle	Final elongation
mecA	5 min/94 ºC	50 s/94 ºC	50 s/58 ºC	50 s/72 ºC	32	10 min/72 ºC
PVL	4 min/94 ºC	45 s/94 ºC	45 s/56 ºC	30 s/72 ºC	30	2 min/72 ºC

Table 2: Selected primers with their sequences and product size.

Primers	Sequences, 5' -> 3'	Product size, bp	References
mecA F mecA R	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162	You et al., 2008
PVL F PVL R	ATCATTAGGTAAAATGTCTGGACATGA TCCA GCATCAAGTGTATTGGATAGCAAAAGC	433	McClure et al., 2006

Interpretation

The bands were identified to their molecular size, and by comparing with the molecular weight marker bp range of 25-766 for mecA and PVL gene. Samples were recorded as positive when a PCR product of approximately base pair (bp) was detected.

RESULT

One hundred eighty seven study participants were investigated during the study period from two tertiary care hospitals of Dhaka city. Out of 187, 84 samples were collected from patients clinically suspected as a case of SSI from laparoscopic surgery and 103 samples from open surgery. Among 103 samples from open surgery 50 were collected from general surgery ward and 53 from obstetrics & gynecology ward.

Table 3: Frequency of phenotypically determined Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin sensitive *Staphylococcus aureus* (MSSA) isolates causing SSI.

Types of surgery	No. of isolates (%)		
	MRSA	MSSA	Total S. aureas
Laparoscopic surgery (n=84) Open surgery	08 (9.6)	02 (2.4)	10 (12)
General Surgery (n=50)	12 (24)	01 (2)	13 (26)
Obstetrical & Gynecological Surgery (n=53)	09 (17)	02 (3.8)	11 (20.8)
Total (N = 187)	**29 (15.5)**	**5 (2.6)**	**34 (18.2)**

No. = Number
MRSA= Methicillin resistant *Staphylococcus aureus*
MSSA= Methicillin sensitive *Staphylococcus aureus*

Table 3 showed frequency of Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin sensitive *Staphylococcus aureus* (MSSA) isolates causing SSI. Among 187 cases from different surgery, 34 (18.2%) *Staphylococcus aureus* was detected. Out of 34 *Staphylococcus aureus*, 15.5% and 2.6% were Methicillin resistant *Staphylococcus aureus* (MRSA) strains & Methicillin sensitive *Staphylococcus aureus* (MSSA). The highest rate (24%) of MRSA was isolated from open surgery, followed by obstetrical & gynecological surgery (17%).

Table 4: Frequency of mecA and PVL gene detection among MRSA isolated from surgical site infection (N=29).

Types of surgery	Total MRSA isolates	Distribution of gene	
		mecA gene No. (%)	PVL gene No. (%)
Total	29	21 (72.4)	07 (24.1)
Laparoscopic surgery Open Surgery	8	5 (62.5)	2 (25)
General Surgery	12	10 (83.3)	3 (25)
Obs & Gynecological Surgery	9	6 (66.6)	2 (22.2)

PVL= Panton valentine leukocidin
MRSA= Methicillin resistant *Staphylococcus aureus*
No. = Number

Table 4 showed frequency of mecA and PVL gene detection among MRSA isolated from SSI. Among 29 isolated MRSA strain, 21 (72.4%) isolates were positive for the *mecA* gene and 7 (24.1%) isolates were positive for the PVL gene by PCR method. MRSA isolated in general surgery had the highest rate of mecA (93.3%) and PVL (25%) gene detection.

DISCUSSION

In the recent years there has been a growing prevalence of multidrug resistant organisms as a cause of serious infections in many hospitals. The problem gets more complicated in developing countries due to poor infection control practices, overcrowded hospitals and inappropriate use of antimicrobials. Early microbiological and molecular diagnosis is essential prerequisite to improve therapeutic outcome. As such, knowledge of actual pathogen with their antimicrobial drug resistance pattern can guide surgeon in treatment of SSI more correctly.

In present study, 187 eligible study participants were investigated during the study period from two tertiary care hospitals (BIRDEM General Hospital & Dhaka Medical College Hospital). Out of 187 samples, 84 (44.9%) and 103 (55.1%) samples were collected from patient with laparoscopic surgery and open surgery respectively. Ninety-four (50.3%) wound swab samples were taken from BIRDEM General Hospital, of which 44 (52.4%) swabs were taken from laparoscopic port site, 22 (44%) swabs were taken from open general surgical wound site and 28 (52.8%) swabs were taken from wound infection of patient undergone obstetrical & gynecological surgery. Among 93 (49.7%) wound swab samples taken from Dhaka Medical college Hospital, 40 (47.6%) samples were taken from laparoscopic port site, 28 (56%) samples were taken from open surgical wound site and 25 (47.2%) swabs were taken from patient undergone obstetrics-gynecological surgery.

In this study, 34 (18.2%) patient was infected with *Staphylococcus aureus* of which 29 (85.2%) were Methicillin resistant *Staphylococcus aureus* (MRSA) strains. MRSA strain was detected 24%, 17% and 9.6% in open general surgery, obs- gynecological surgery and laparoscopic surgery respectively.

In the present study, among 29 isolated MRSA strain 21 (72.4%) isolates were positive for the *mecA* gene and 7 (24.1%) isolates were positive for the PVL gene by PCR method. Among MRSA isolates, general surgery had the highest rate of mecA (93.3%) and PVL (25%) gene detection.

CONCLUSIONS

The findings of the present study on SSI in terms of MRSA will help the clinician in selecting proper antibiotics and the infection control committee to reinforce the infection control measures in their hospitals.

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