

ORIGINAL ARTICLE

Isolation and Identification of Vancomycin Resistant *Staphylococcus aureus* from Post Operative Pus Sample

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Abstract: *Staphylococcus aureus* is most frequently isolated pathogen causing bloodstream infections, skin and soft tissue infections and pneumonia. Recently, *S. aureus* have evolved resistance to both synthetic and traditional antibiotics. This study was carried out to isolate pathogenic *S. aureus* from post-operative pus sample, and VRSA was identified by evaluation of resistance patterns using conventional antibiotics. Thirty post operative pus samples were collected from nearby Hospital and species identification was confirmed by Gram staining, standard biochemical tests and PCR amplification of the *nuc* gene. Antibiotic susceptibility tests were carried out by MIC, MBC, DAD test and BHI vancomycin screening agar. VRSA were confirmed by PCR amplification of the *vanA* and *vanB* genes. From this study, it was observed that isolated *S. aureus* strains are pathogenic; 30% of strains were resistant to penicillin G, ampicillin and erythromycin; 26.67% strains were resistant to cephotaxime, gentamycin, streptomycin, tetracycline, chloramphenicol, norfloxacin, methicillin and vancomycin.

Key-words: *Staphylococcus aureus*, vancomycin, antibiotic emergence, *vanA* and *vanB* gene, VRSA.

Introduction

Staphylococcus aureus, a Gram positive cocci, is major human pathogen causing large variety of infections worldwide and predominates in surgical wound infections with prevalence rate ranging from 4.6% - 54.4% [1-5]. *S. aureus* causes superficial skin infections to life-threatening diseases such as endocarditis, sepsis and soft tissue, urinary tract, respiratory tract, intestinal tract, bloodstream infections [6-7]. The species is identified on the basis of physiological or biochemical characters [8], by detection of *eta* and *etb*, staphylococcal enterotoxin genes and the Sa442 DNA fragment [9-11]. *S. aureus* has developed resistance to most classes of antimicrobial agents. Penicillin was the first choice of antibiotics to treat staphylococcal infection. In 1944, by destroying the penicillin by *penicillinase*, *S. aureus* become resistant [12]. More than 90% *S. aureus* strains are resistant to penicillin [13]. Methicillin, a semi synthetic penicillin was used to treat Penicillin Resistant *Staphylococcus aureus* but resistance finally emerge in 1962 [14-15]. MRSA is mediated by the presence of PBP-2a which is expressed by an exogenous gene, *mecA* [16]. High prevalence of MRSA in hospitals has been reported from many states of India [17]. MRSA isolates has reached phenomenal proportions in Indian hospitals, with some cities reporting 70% of the strains are resistant to methicillin [18].

Vancomycin, a glycopeptide antibiotic continues to be an important antimicrobial agent to treat MRSA but resistance finally emerge. In 1996, a *S. aureus* strain with intermediate resistance to vancomycin (VISA) (vancomycin MIC= 8µg /ml) was first isolated from a patient in Japan [19]. Shortly afterward, VISA strains were isolated in USA, Europe and other Asian countries [20-21]. Characterization of these VISA strains indicates that the mechanisms of resistance are complex and involve changes in cell wall content and composition [22-23]. In June 2002, the World's first reported clinical infection due to *S. aureus* with high resistance to vancomycin (VRSA) (vancomycin MIC>128 µg /ml) was diagnosed in a patient in the USA [24]. This isolate contain the *vanA* genes from enterococci and the methicillin-resistance gene *mecA*. Till today only five VRSA have been found all over the world. First in USA in 2002 [24], second in Michigan in 2002 [25], third in Pennsylvania in 2002 [26], fourth in New York in 2004 [27], fifth in New York in 2005 [28], and the sixth in Kolkata (India) in 2005 [29]. The present study was aimed to (i) isolate the pathogenic *Staphylococcus aureus* from post operative pus sample, (ii) observe antibiotic emergence pattern of isolated *S. aureus* strains against some conventional and traditional antibiotics to identify VRSA.

Materials and Methods

Culture media and Chemicals: Luria broth, nutrient agar, tryptic soy broth, agar powder, beef extract, pancreatic digest of casein, DNase agar, mannitol salt agar, blood agar, Mueller-Hinton broth, brain heart infusion broth, crystal violet, lugol's iodine, safranin, N, N, N', N'-tetramethyl-*p*-phenylenediamine dihydrochloride, rabbit plasma, latex agglutination reagent, chloroform:isoamyl alcohol, phenol:chloroform:isoamyl alcohol, antibiotic discs, penicillin G, ampicillin, cephotaxime, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin, lysostaphin, agarose and bromophenol blue were purchased from Himedia, India. Tris-Hcl, Tris buffer, Sodium chloride (Nacl), hydrogen peroxide (H₂O₂), sucrose, potassium dihydrogen phosphate (KH₂PO₄), di potassium hydrogen phosphate (K₂HPO₄), ethylene diamine tetra acetate (EDTA), Sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium acetate, ammonium acetate, potassium chloride (Kcl), hexadecyltrimethylammonium bromide, 2-mercaptoethanol, isopropanol, ethanol were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Proteinase K, RNase A, ethidium bromide (ETBR) were purchased from Sigma Chemical Co., USA. Oligonucleotide primers were purchased from Ocimum Biosolutions, India. PCR grade nucleotide mixture, MgCl₂, dNTP and Taq DNA polymerase were purchased from Roche applied science, USA. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

Collection and transport of sample: Thirty (30) post operative pus samples were collected from patients admitted to Burn and Wound section of Midnapore Medical College and Hospital, Midnapore, West Bengal, India during a three month period from December 15, 2008 to June 15, 2009. Samples were obtained using cotton tipped swabs from the pus of deep-seated wounds of patients. Swabs were transported to the laboratory in autoclaved Luria broth (LB) within 30 minutes of collection [30].

Culture of microorganisms: Pus containing swabs, kept in LB were shaken in a shaking incubator at 37°C for overnight. Bacterial cultures were grown on Nutrient agar (NA) media and purified by a single colony isolation technique on NA containing 10% sodium chloride [29]. Isolates were sub-cultured from primary media to tryptic soy agar plates containing 5% sheep blood agar and incubated at 35°C in 5 to 7% CO₂ for 16 to 24 hr. A thick suspension of each strain was then prepared by transferring three to six isolated colonies into approximately 0.5 ml of sterile 0.85% NaCl solution at room temperature. This suspension was used to perform the latex agglutination test and the slide coagulase test [31]. *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213 were obtained from Microbiology laboratory of Calcutta University and *S. epidermidis* ATCC 12228, *S. epidermidis* NCTC 5866, *E. coli* ATCC 23509, *E. coli* ATCC 25922, *E. faecalis* ATCC 51299 were obtained from Microbiology laboratory of Midnapore Medical College and Hospital. These strains were stored in agar slants at 4°C for further studies as reference strain.

Species Identification: Identification of the clinical isolates was performed by traditional biochemical tests, including Gram staining; oxidase, catalase, coagulase, latex agglutination, motility, thermonuclease (DNase), haemolysis and mannitol fermentation tests; and lysostaphin susceptibility [1, 31-40]. PCR amplification of the *nuc* gene was performed for all isolates.

a) Genomic DNA isolation: Genomic DNA of isolates was isolated according to Rallapalli et al. 2008 [41]. Bacterial culture was grown overnight in Mueller Hinton broth and 2 ml of the culture was transferred into a micro centrifuge tube and spun at 4000 rpm for 20 minutes. The pellet was re-suspended in 567 µl of TE buffer to which 30 µl of 10% SDS and 3 µl of 20 mg/ml *proteinase* K and 2 µl of 10 mg/ml *RNase* A were added, mixed gently and incubated for 1 hour at 37°C. Following this, 100µl of 5M NaCl was added and mixed thoroughly. After addition of 80µl of 10% CTAB (50 mM Tris, pH 8.0, 0.7 mM NaCl, 10 mM EDTA, 2% hexadecyltrimethylammonium bromide, 0.1% 2-mercaptoethanol)-0.7M NaCl solution and the tubes were incubated for 10 minutes at 65°C. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube and 0.8 volumes of isopropanol were added, mixed gently until the DNA was precipitated. The DNA was washed with 70% ethanol and re-suspended in 50 µl TE buffer.

b) Detection of *nuc* gene by PCR: PCR amplification was performed according to Saha et al. 2008 [29] with some modification. A partial *nuc* gene was amplified using *nuc* gene primers (*nuc* F 5' GCGATTGATGGTGATACGGTT 3' and *nuc* R 5' AGCCAAGCCTTGACGAACATAAAGC 3') which were selected on the basis of the published nucleotide sequence of the 966 bp *nuc* gene derived from the *S. aureus* Foggi strain [42]. Bacterial genomic DNA (aliquot of 2 µl containing 0.1 µg of genomic DNA) was added at template DNA to PCR mixture consisting ten fold

concentrated reaction buffer (500mM KCl, 100mM Tris-HCl, pH 8.3), with final concentrations of 1.5 mM MgCl₂, 200 μM dNTP, 2 μM of each primer. This mixture was supplemented with 1.25 U of Taq DNA polymerase. The final reaction volume for PCR was 25 μl. PCR amplification was performed with an Eppendorf thermal cycler. The cycling parameters consisted of 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min 30 s. *S. aureus* ATCC 25923 was used as quality control. The PCR products were mixed with 2 μl bromophenol blue, electrophoresed in 1.2% agarose gel with 0.1% ETBR and visualized by using UV transillumination.

Antibiotic susceptibility testing

1. *Determination of Minimum Inhibitory Concentration:* The MIC values of penicillin G, ampicillin, cephalexin, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin were determined by a broth dilution method using Mueller-Hinton broth (MHB), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [43]. About 5×10^4 cells in MHB were treated with different concentrations of antibiotics and shaken for 16 hr at 37°C. The minimum concentration at which there was no visible turbidity was taken as the MIC of that antibiotic.

2. *Determination of Minimum Bactericidal Concentration:* The MBC value of antibiotics was determined according to Okore 2005 [44] with some modification. This is an extension of the MIC Procedure. Antibiotics treated bacterial culture showing growth or no growth in the MIC tests were used for this test. Bacterial culture used for the MIC test were inoculated onto the Mueller-Hinton agar and incubated at 37°C for 24 hr. Microbial growth or death were ascertained via no growth on Mueller-Hinton agar plate. The minimal concentration of the antibiotic that produced total cell death is the MBC.

3. *Disc Agar Diffusion (DAD) test:* Susceptibility of isolates to penicillin G, ampicillin, cephalexin, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin was determined by the disc agar diffusion (DAD) technique according to Acar 1980 and Bauer et al. 1966 [45-46]. The test bacterium taken from an overnight culture (inoculated from a single colony) was freshly grown for 4 hr having approximately 10^6 CFU/ml. With this culture, a bacterial lawn was prepared on Mueller-Hinton agar. Filter paper discs of 6-mm size were used to observe antibiotic susceptibility patterns against 11 antibiotics [amount of antibiotic per disc in microgram (μg); penicillin G (10 units), ampicillin (10), cephalexin (30), gentamycin (10), streptomycin (10), tetracycline (30), erythromycin (15), chloramphenicol (30), norfloxacin (10), methicillin (5) and vancomycin (30)]. Antibiotic discs were obtained commercially from Himedia. The diameter of zone of bacterial growth inhibition surrounding the disc (including the disc), was measured and compared with a standard for each drug. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance [46]. *S. aureus* ATCC 25923, an all-sensitive reference strain, was used as a quality control strain for the DAD test.

4. *Inoculation on BHI vancomycin screen agar*: Isolates were inoculated on Brain Heart Infusion screen agar according to Tiwari and Sen 2006 [47]. 6 µg/ml vancomycin containing BHI agar screen plates were prepared. Inoculum suspensions were prepared by selecting colonies from overnight growth on nutrient agar plates. The colonies were transferred to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. The final inoculum concentration of 10^5 to 10^6 CFU per spot was prepared by adding the sterile saline to the bacterial suspension. These suspensions were inoculated onto BHI screen agar plates and were incubated for 24 hr at 35°C in ambient air. Any visible growth indicated the vancomycin resistance. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 51299 were used as vancomycin susceptible control strains and vancomycin resistant control strain, respectively.

5. *Confirmation of VRSA by PCR- based detection of vancomycin- resistance genes*:

a) *Plasmid DNA isolation*: Cells from *S. aureus* were lysed, and plasmid DNA was isolated as described by Clewell et al. 1974 [48]. *S. aureus* plasmid DNA was prepared by suspending cells from 100 ml of an exponential culture in 2 ml of TE buffer (0.05 M Tris-HCl, 0.05 M EDTA; pH 8.0) containing 20µg of lysostaphin per ml. This suspension was incubated for 20 min at 37°C, and the cells were lysed by the addition of 16 ml of lysing solution composed of 1% SDS in TE buffer (pH 12.4). This solution was heated in a water bath at 55°C for 20 min, and 20% SDS was added to make the final concentration 3%. Immediately, 5 M NaCl was added rapidly to a final concentration of 1 M, centrifuged at 17,000 rpm with an Eppendorf cold centrifuge to remove the majority of chromosomal DNA. The supernatant was decanted into glass and made 10% (wt/ vol) polyethylene glycol (molecular weight, 6,000) and then held at 4°C overnight. Resulting DNA was pelleted at 10,000 rpm, suspended in a small volume of TE buffer.

b) *Detection of vanA and van B gene by PCR*: Oligonucleotide primers for *van A* (*van A* F5' ATGAATAGAATAAAAAGTTGC 3' and *van A* R5' TCACCCCTTT AACGCTAATA3') and *van B* (*van B* F5' GTGACAAACCG GAG GCGAGGA 3' and *vanB* R5' CCGCCATCCTCCTGCAAAAAA 3') genes were selected according to Saha et al. 2008 and Tiwari and Sen 2006 [29, 47]. Clinical isolates that was suspected to be VRSA, by MIC, MBC, DAD and vancomycin screening agar test; was used for this study. The PCR amplification mixture contained 1X Phusion GC buffer containing 1.5 mM MgCl₂, 200 µM dNTP, 2 µM each primer, 0.1 µg template DNA, 3% (v/v) DMSO and 1 U Phusion DNA polymerase. The amplification conditions were initial denaturation at 98°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 s; annealing at 50°C for 1 min; polymerization at 72°C for 1 min 30 s for *vanA* gene and initial denaturation at 94°C for 10 min; 30 cycles with a 30 s denaturation step at 94°C, a 45 s annealing step at 50°C and a 30 s extension step at 72°C and 10 min extension step at 72°C and a holding step at 4°C for *vanB* gene. The PCR products were mixed with 2µl bromophenol blue, electrophoresed in 1.2% agarose gel with 0.1% ETBR and visualized by using UV transillumination.

Results

Species identification:

The clinical isolates were identified using standard biochemical tests. Purification of bacterial culture by a single colony isolation technique on NA containing 10% sodium chloride exhibited several types of colony. Table 1 illustrates, 73.17% isolates were Gram positive and 26.83% isolates were Gram negative; 100% of gram positive isolates are oxidase positive, catalase positive and coagulase positive; all isolates were non-motile and gave positivity in latex agglutination test; 100% of gram positive isolates had thermonuclease activity, mannitol fermentation activity, haemolytic activity (α haemolysis-45% and β haemolysis-55%) and were susceptible to lysostaphin. PCR amplification of the *nuc* gene of isolates using the gene-specific primers and the genomic DNA preparation yielded a 230 bp amplicon (Fig. 1).

Table-1: Results of standard biochemical tests of clinical isolates, collected from pus sample of patient. ND=Tests are not done, + ve = tests are positive, -ve = tests are negative.

Sample	Isolates	Gram Staining	Oxidase	Catalase	Coagulase	Motility	Latex agglutination	Thermo nuclease	Haemo-lysis	Growth on MSA	Lysostaphin susceptibility
S1	S1a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S2	S2a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
	S2b	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
S3	S3a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S3b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S4	S4a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S4b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S5	S5a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S6	S6a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
	S6b	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
S7	S7a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S8	S8a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S9	S9a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S9b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S10	S10a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S11	S11a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S12	S12a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
	S12b	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
S13	S13a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S13b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S14	S14a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S15	S15a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S16	S16a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S17	S17a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S18	S18a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S19	S19a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S20	S20a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve

Sample	Isolates	Gram Staining	Oxidase	Catalase	Coagulase	Motility	Latex agglutination	Thermo nuclease	Haemolysis	Growth on MSA	Lysostaphin susceptibility
S21	S21a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
	S21b	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
S22	S22a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S22b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S23	S23a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S24	S24a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
	S24b	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
S25	S25a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S26	S26a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S27	S27a	-ve	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S27b	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S28	S28a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S29	S29a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve
S30	S30a	+ve	+ve	+ve	+ve	Non motile	+ve	+ve	+ve	+ve	+ve

Figure-1: Agarose gel electrophoresis of PCR-amplified *nuc* genes of clinical isolates

- (a) Lane 1: 100 bp ladder; 2: *S. aureus* ATCC 25923, as positive control; 3-22: twenty clinical isolates.
- (b) Lane 1: 100 bp ladder; 2: *S. aureus* ATCC 25923, as positive control; 3-12: ten clinical isolates.

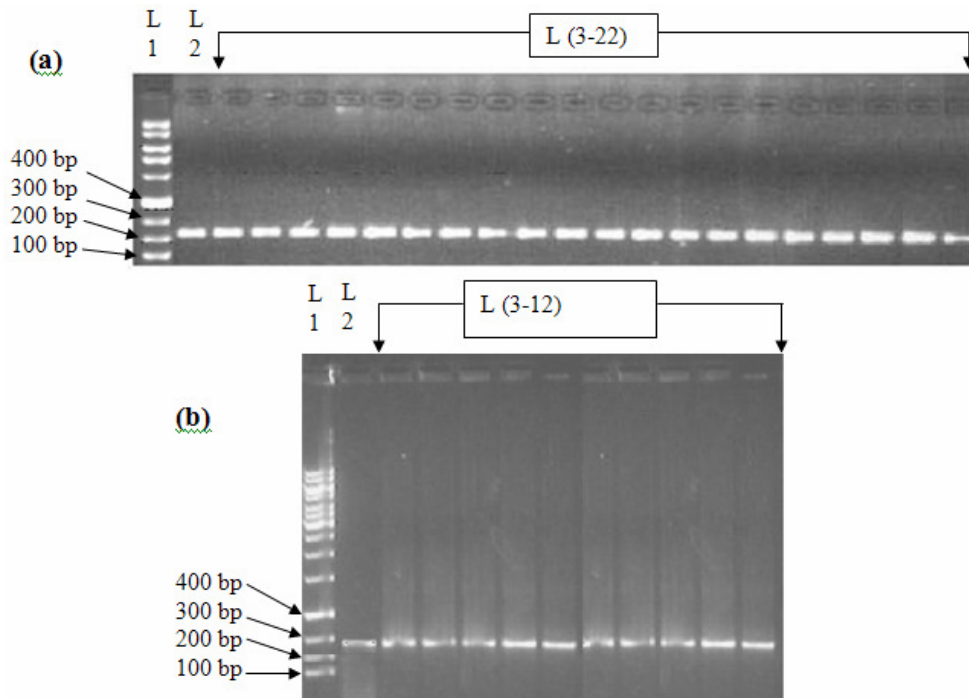
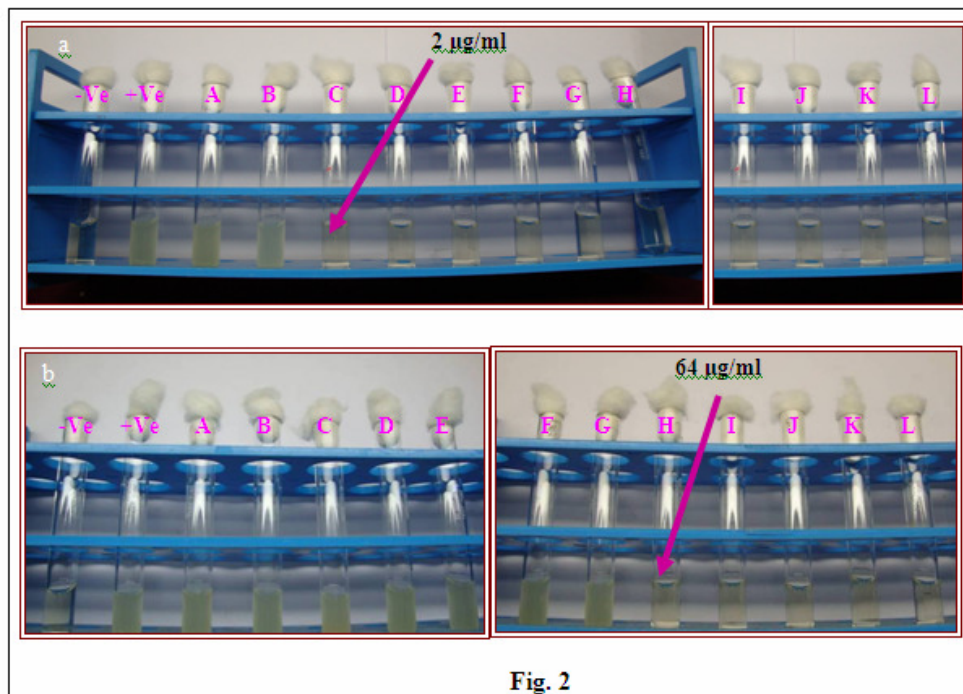


Fig. 1

Antibiotic susceptibility testing:

MIC of antibiotics: The MIC values of penicillin G, ampicillin, cephotaxime, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin for isolates were determined. In each set of experiment, bacterial control tubes showed no growth inhibitory effect of antibiotics. These MIC values were compared with the NCCLS breakpoints Minimum Inhibitory concentration for *Staphylococcus aureus*. It was observed that of MIC values of penicillin G, ampicillin and erythromycin for 30% of isolated strains; cephotaxime, gentamycin, streptomycin, tetracycline, chloramphenicol, norfloxacin, methicillin and vancomycin for 26.67% of isolated strains are beyond the sensitive range (Fig. 2).

Figure-2: Determination of Minimum Inhibitory Concentration value of antibiotics for clinical isolates. MIC of vancomycin for MMC 1 is 2 µg/ ml (2 a) and MMC 4 is 64 µg/ ml (2 b).



MBC of antibiotics: The MBC values of penicillin G, ampicillin, cephotaxime, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin for isolates were determined. It was observed that MBC values of penicillin G, ampicillin and erythromycin for 30% of isolated strains; cephotaxime, gentamycin, streptomycin, tetracycline, chloramphenicol, norfloxacin, methicillin and vancomycin for 26.67% of isolated strains are beyond next two concentrations of MIC values (Fig. 3).

Figure-3: Determination Minimum Bactericidal Concentration of antibiotics for clinical isolates. MBC of vancomycin for MMC 1 is 4 µg/ ml (3 a) and MMC 4 is 512 µg/ ml (3 b).

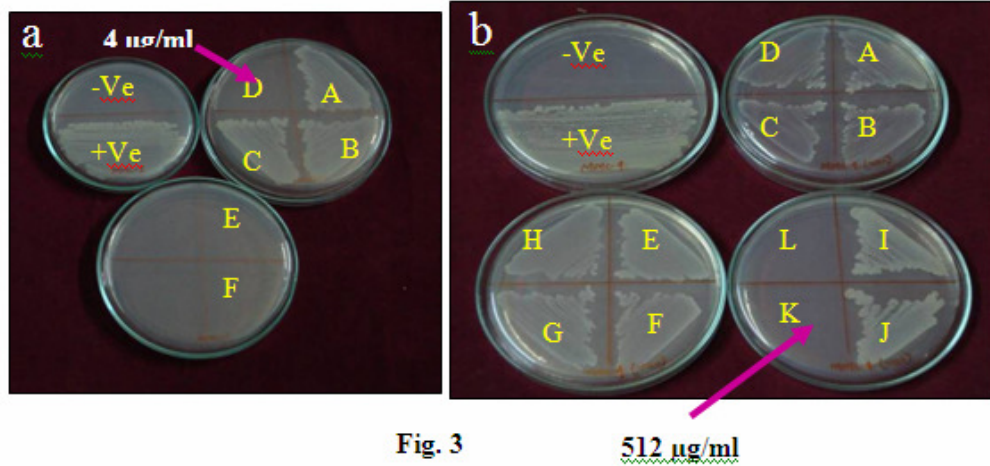


Fig. 3

512 µg/ml

DAD test: The antibiotic-resistance profile, as determined by DAD test, revealed that out of 30 Gram positive isolates, 30% strains were resistant to penicillin G, ampicillin and erythromycin and 26.67% strains were resistant to cephotaxime, gentamycin, streptomycin, tetracycline, chloramphenicol, norfloxacin, methicillin and vancomycin (Fig. 4).

Figure-4: Disc agar diffusion test of MMC 1 and MMC 4 against 11 antibiotic discs.

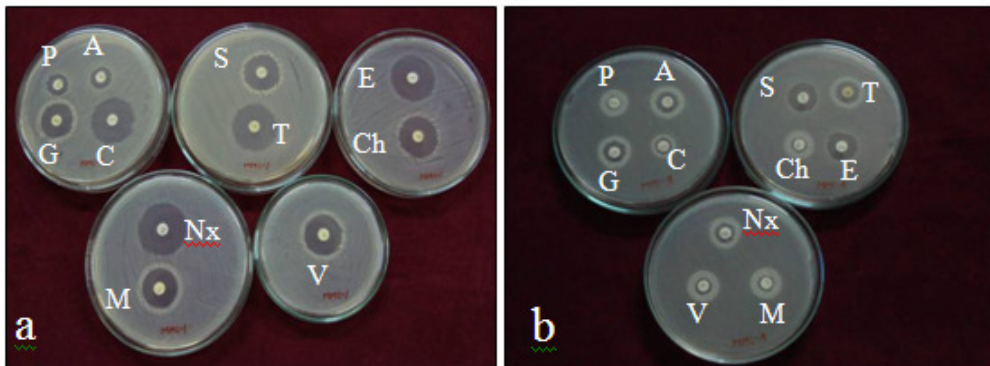


Fig. 4

BHI vancomycin screen agar: Out of 30 Gram positive clinical isolates eight (26.67%) strains were grown in Brain Heart Infusion vancomycin screening agar (Fig. 5).

Figure-5: Growth of all clinical isolates on BHI vancomycin screen agar.

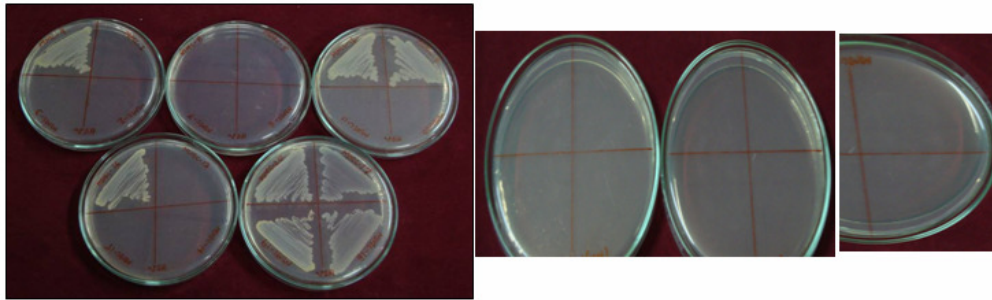


Fig. 5

Confirmation of VRSA by detection of vanA and vanB gene by PCR: PCR amplification of the *vanA* and *vanB* gene using the gene-specific primers and the plasmid DNA preparation of clinical isolates that was suspected to be VRSA yielded 474 bp and 800 bp amplicon respectively (Fig. 6 & 7).

Figure-6: Agarose gel electrophoresis of PCR-amplified *vanA* genes of suspected clinically VRSA strain (Lane1:100 bp ladder, 2: negative control, 3-10: suspected as VRSA by standard antibiotic assay).

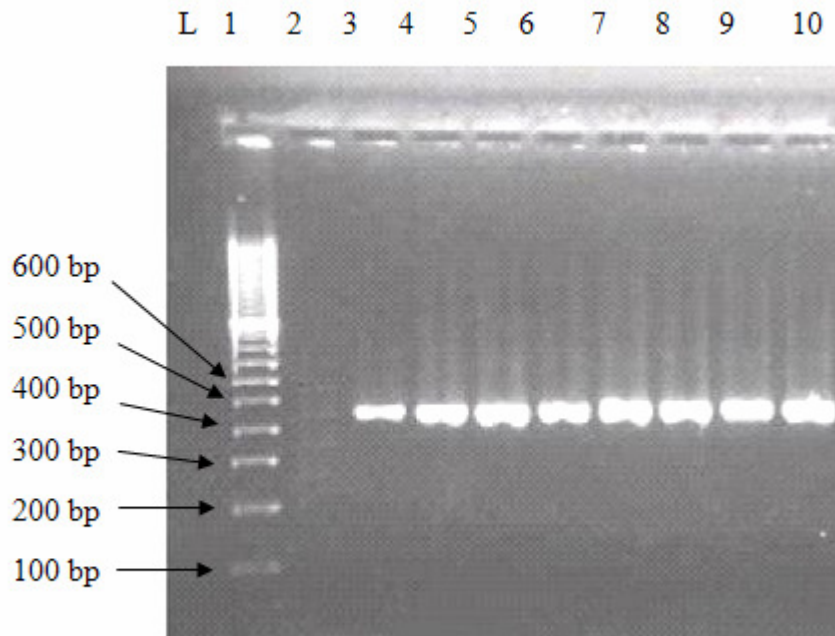


Fig. 6

Figure-7: Agarose gel electrophoresis of PCR-amplified *vanB* genes of suspected clinically VRSA strain (Lane1:100 bp ladder, 2: negative control, 3-10: suspected as VRSA by standard antibiotic assay).

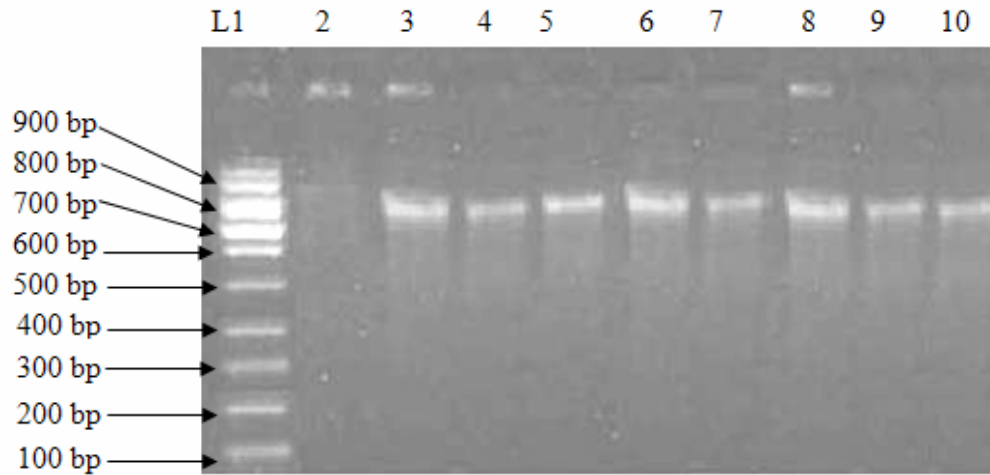


Fig. 7

Discussion

The development and spread of bacterial strains that are resistant to antibacterial drugs has emerged as a global problem [49]. The appearance of antibiotic resistant bacteria over the past decades has been regarded as an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a crucial role in the evolution of antibiotic resistant bacteria. These bacteria then pass the antibiotic resistance plasmid among other bacterial cells and species [50].

Throughout the study, thirty post operative pus samples were collected from nearby Medical College and Hospital, samples were transported to the laboratory within 30 minutes of collection and species identification was carried out by Gram staining and standard biochemical tests. In this study, 73.17% of clinical isolates were Gram positive and 26.83% isolates were Gram negative. Gram negative clinical isolates were not involved in this study as it is commonly known that *Staphylococcus aureus* is gram positive. Clinical isolates were gram positive, which may be due to thicker and denser peptidoglycan layers of their cell walls; iodine penetrates the cell wall of these isolates and alters the blue dye to inhibit its diffusion through the cell wall during decolourisation [32]. Our results showed that 100% of gram positive isolates were oxidase positive, catalase positive and coagulase positive. Isolates were oxidase positive that may due to the presence of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride as artificial electron acceptor which takes the electron from cytochrome oxidase in the electron transport chain and changes colour to a dark blue. Oxidase positivity of clinical isolates distinguishes them from *Micrococcus* spp. [33].

Clinical isolates were catalase positive which may be due to the production of catalase enzyme by isolates which catalyzes H_2O_2 , a potent oxidizing agent into water and oxygen. Catalase positivity of clinical isolates distinguishes them from *Streptococcus* spp. [34]. Isolates were coagulase positive which may be due to the production of coagulase enzyme by isolates, that reacts with prothrombin and form staphylothrombin which causes blood to clot by converting fibrinogen to fibrin. Coagulase positivity of isolates distinguishes them from other *Staphylococcus* spp. except *Staphylococcus aureus* and represents them as possible *Staphylococcus aureus* [35]. It is evident from our study that all isolates were non-motile and gave positivity in latex agglutination test. Non-motility of clinical isolates may be due to absence of flagellum [37]. Latex agglutination of isolates may be due to interaction of human antibody attached to the latex particles with protein A bound to the bacterial cell surface or interaction between cell-associated clumping factor and plasma constituents adsorbed to the latex particles [31]. Our results also demonstrated that, 100% of gram positive clinical isolates have haemolytic activity (α haemolysis-45% and β haemolysis-55%), thermonuclease activity, mannitol fermentation activity and lysostaphin susceptibility. Isolates have haemolytic activity that may be due to the production of haemolysin by isolates, which binds with the haemolysin receptor present on the surface of RBC, that favor haemolysis and makes the clear zone surrounding the isolates (clear zone - α haemolysis and greenish surround the colony - β haemolysis) [1]. Nuclease production was suggested as an indicator of potentially pathogenic staphylococci over a decade ago [51]. Thermonuclease activity of clinical isolates may be due to the breakdown of DNA present in the media by production of nuclease enzyme; suggests that these isolates have the ability to break down the DNA [38]. Mannitol salt agar is a selective media for *Staphylococcus aureus*. The clinical isolates ferment mannitol and produced yellow colour. Lysostaphin susceptibility of isolates may be due to the inactivation of the lysostaphin due to structural modification by enzymatic action [40]. Non-motility, latex agglutination activity, haemolytic activity, thermonuclease activity, mannitol fermentation activity and lysostaphin susceptibility of clinical isolates suggests that these may be *Staphylococcus aureus*. All isolates were coagulase positive and have haemolytic activity that suggests that all these isolates were pathogenic.

The thermostable nuclease-encoding *nuc* gene is highly specific for *S. aureus*. PCR amplification of the *nuc* gene of clinically isolated strains using gene specific primer and genomic DNA preparation yielded a 230 bp amplicon (Fig. 1). This result confirmed, isolated strains are *Staphylococcus aureus*. The clinically isolated *S. aureus* strains were newly named as MMC (Midnapore Medical College) from MMC 1 to MMC 30. In recent years, *Staphylococcus aureus* become resistance to both synthetic and traditional antibiotics. Treatment of antibiotic resistant bacteria is a therapeutic problem. Susceptibility pattern is useful to determine the future challenges of effective therapy. In this study, the result of MIC (Fig. 2), MBC (Fig. 3) and DAD (Fig.4) suggests that 30% of isolated *Staphylococcus aureus* strains (MMC 1, MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) were resistant to penicillin and ampicillin; 30% of isolated *Staphylococcus*

aureus strains (MMC 4, MMC 5, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) were resistant to erythromycin; 26.67% of isolated *Staphylococcus aureus* strains (MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) were resistant to cephalexin, gentamicin, streptomycin, tetracycline, chloramphenicol, norfloxacin, methicillin and vancomycin (Fig. 8). From our study, it was observed that MMC-1 is resistant to penicillin G and ampicillin, MMC-5 is resistant to erythromycin and MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20 are resistant to penicillin G, ampicillin, cephalexin, gentamicin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin. Henceforth 26.67% isolated *S. aureus* strains (MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) are multi drug resistant (MDR) (Fig. 8). In our study, the result of screening of isolated *Staphylococcus aureus* strain on vancomycin agar (Fig. 5) indicated that 26.67% of isolated strains (MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) were resistant to vancomycin.

Figure-8: Antibiotic susceptibility profile of thirty *Staphylococcus aureus* strains. Here Pen: Penicillin G, Amp: Ampicillin, Cep: Cephalexin, Gen: Gentamicin, Str: Streptomycin, Tet: Tetracycline, Ery: Erythromycin, Chl: Chloramphenicol, Nx: Norfloxacin, Met: Methicillin and Van: Vancomycin.

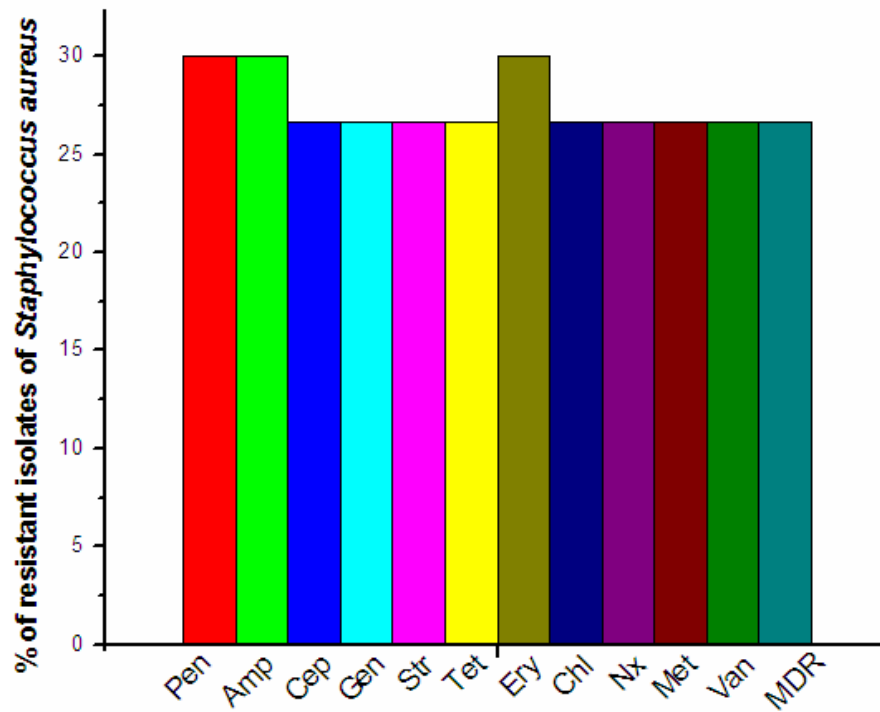


Fig. 8

vanA and *vanB* genes are highly specific for Vancomycin Resistant *S. aureus*. PCR amplification of *vanA* and *vanB* gene of suspected clinically isolated VRSA strains (MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) using gene specific primer and plasmid DNA preparation yielded 474 bp (Fig. 6) and 800 bp (Fig. 7) amplicon respectively. These results confirmed that these suspected clinically isolated VRSA strains (MMC 4, MMC 9, MMC 12, MMC 16, MMC 17, MMC 18, MMC 19 and MMC 20) were truly VRSA.

Clinically isolated *S. aureus* strains from pus sample are resistant to β -lactam antibiotics, aminoglycosides, macrolides, quinolones, tetracycline, chloramphenicol and vancomycin that may be due to (i) inactivation of the antibiotic due to structural modification by enzymatic action, (ii) prevention of access to target by altering the outer membrane permeability, (iii) alteration of the antibiotic target site, (iv) efflux pump which pumps out the antibiotic, (v) target enzyme bypass or over production. In brief, from this study, twenty two (22) pathogenic vancomycin sensitive *Staphylococcus aureus* strains and eight (8) pathogenic vancomycin resistant *Staphylococcus aureus* strains were isolated from post operative pus sample.

Conflict of interest: There is no conflict of interest.

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Abbreviations

ATP	: Adenosine triphosphate	NA	: Nutrient agar
BHI	: Brain heart infusion	NaOH	: Sodium hydroxide
CFU	: Colony formation unit	NCCLS	: National Committee for Clinical Laboratory Standards
DAD	: Disc agar diffusion	<i>nuc</i>	: <i>nuclease</i>
DNA	: Deoxyribonucleic acid	PCR	: Polymerase chain reaction
<i>E. coli</i>	: <i>Escherichia coli</i>	PBP	: Penicillin Binding Protein
EDTA	: Ethylene diamine tetra acetate	PG	: Peptidoglycan
H ₂ O ₂	: Hydrogen peroxide	<i>S. aureus</i>	: <i>Staphylococcus aureus</i>
LB	: Luria broth	SDS	: Sodium dodecyl sulfate
MBC	: Minimum bactericidal concentration	<i>S. epidermidis</i>	: <i>Staphylococcus epidermidis</i>
MgCl ₂	: Magnesium chloride	TSB	: Tryptic soy broth
MHB	: Mueller-Hinton broth	VRSA	: Vancomycin resistant <i>Staphylococcus aureus</i>
MIC	: Minimum inhibitory concentration		
MRSA	: Methicillin resistant <i>Staphylococcus aureus</i>		

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