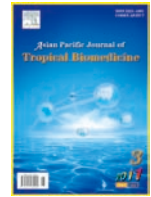




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Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates

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ABSTRACT

Objective: To observe the biochemical characters and antibiotic susceptibility of isolated *Staphylococcus aureus* (*S. aureus*) strains against some conventional and traditional antibiotics. **Methods:** Thirty post operative pathogenic isolated *S. aureus* strains were used in this study. Bacterial culture was done in Mueller–Hinton broth at 37 °C. Characters of these strains were determined by traditional biochemical tests such as hydrolysis test of gelatin, urea, galactose, starch and protein, and fermentation of lactose and sucrose. Antibiotic susceptibility were carried out by minimum inhibitory concentration test, minimum bactericidal concentration test, disc agar diffusion test and brain heart infusion oxacillin screening agar. **Results:** From this study, it was observed that 100% *S. aureus* isolates showed positive results in gelatin, urea and galactose hydrolysis test, 50% isolates were positive in starch hydrolysis test, 35% in protein hydrolysis test, 100% isolates in lactose fermenting test, but no isolate was positive in sucrose fermenting test. Antibiotic susceptibility testing suggested that 20% of isolates were resistant to kanamycin and 46.67% were resistant to oxacillin. **Conclusions:** These findings show that all these isolates have gelatin, urea, galactose hydrolysis and lactose fermenting activity. 20% of these isolates were resistant to kanamycin and 46.67% were resistant to oxacillin.

1. Introduction

Staphylococcus aureus (*S. aureus*), a gram positive cocci, is a 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 and life-threatening diseases such as endocarditis, sepsis and soft tissue, urinary tract, respiratory tract, intestinal tract, bloodstream infections[6,7].

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[8]. More than 90% *S. aureus* strains are resistant to penicillin[9]. Methicillin, a semi synthetic penicillin was

used to treat penicillin resistant *S. aureus* but resistance finally emerged in 1962[10,11]. Methicillin resistant *S. aureus* (MRSA) is mediated by the presence of PBP-2a which is expressed by an exogenous gene, *mecA*[12]. High prevalence of MRSA in hospitals has been reported from many states of India[13]. In India, it is reported that 70% of the strains are resistant to methicillin in some cities[14]. Recently, we have isolated thirty pathogenic *S. aureus* from post operative pus sample by standard biochemical test and detected specific nuc gene of *S. aureus*. Out of them twenty two were vancomycin-sensitive and the rests were vancomycin-resistant[15].

The present study aimed to observe the biochemical characters of isolated *S. aureus* strains, and antibiotic susceptibility against some conventional and traditional antibiotics.

2. Materials and methods

2.1. Culture media and chemicals

Luria broth, nutrient broth, nutrient agar, tryptic soy

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broth, agar powder, beef extract, pancreatic digest of casein, Mac Conkey agar, Tris Citrate Bile Salts Sucrose agar, Eosin–Methylene–Blue agar, peptone, Mueller–Hinton broth, antibiotic discs, glucose, phenol red, imipenem, amikacin, kanamycin and oxacillin were purchased from Himedia, India. Sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), gelatine, urea, starch, were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. The other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2. Bacterial strain

Thirty *S. aureus* strains were clinically isolated from post operative pus samples of patients admitted to Burn and Wound section of Midnapore Medical College and Hospital, Midnapore, West Bengal, India from December 15, 2008 to June 15, 2009^[15]. These samples were tested for their characters and antibiotic susceptibility. Bacterial culture was done in Mueller–Hinton broth at 37 °C.

2.3. Characters of isolated *S. aureus* strains

2.3.1. Gelatin hydrolysis test

Gelatin is solid at room temperature. When the bacterium produces the enzyme gelatinase, the gelatin is hydrolyzed and becomes liquid. There is no indicator or reagent to test solidification or liquefaction. At first the gelatin was stabbed deep with a needle to the bottom and incubated at 25 °C for a couple days. Then the tube was placed on ice for about 15 min or in the fridge for about 30 min, to determine liquefaction. The liquefaction can be complete or perhaps partial throughout the tube^[16].

2.3.2. Urea hydrolysis test

Three test tubes were prepared with urea (20.0 gm/L), agar (15.0 gm/L), NaCl (5.0 gm/L), KH_2PO_4 (2.0 gm/L) and phenol red (0.012 gm/L) in slant position and were divided into sample, urea control and blank group. Another one test tube was prepared with the same reagents but without urea as bacteria control group. Isolates were streaked on the slant in sample and bacteria tubes. All these four test tubes were kept in 37 °C for 20–22 h. Reddish pink or red colour of test tube was regarded as positive^[16].

2.3.3. Lactose fermenting test

5.15 mg% Mac Conkey media in sterile distilled water was suspended, then sterilized in autoclaving at 15lb (121 °C) pressure for 15 minutes, cooled to (45–50) °C, poured into sterile petridish and checked overnight at 37 °C. On the next day, overnight growing isolates in Mueller–Hinton broth (MHB) were streaked on overnight checked Mac Conkey agar plates and plates were kept at 37 °C overnight. The appearance of pinkish or whitish or reddish colony was regarded as positive result^[16].

2.3.4. Sucrose fermenting test

8.9 mg% tris citrate bile salts sucrose (TCBS) media was suspended in sterile distilled water, then dissolved completely by heating, cooled to 50 °C, poured into sterile

petridishes and checked overnight at 37 °C. On the next day, overnight growing isolates in MHB were streaked on overnight checked tris citrate bile salts sucrose plates and plates were kept at 37 °C overnight. A positive result indicates the appearance of yellow colony^[16].

2.3.5. Galactose hydrolysis test

3.7 mg% eosin–methylene–blue (EMB) media was suspended in sterile distilled water, sterilized in autoclaving at 15lb (121 °C) pressure for 15 min. The autoclave mixture was cooled to (45–50) °C, poured in sterile petridishes and checked overnight at 37 °C. On the next day, overnight growing isolates in MHB were streaked on overnight checked EMB plates and plates were kept at 37 °C overnight. The appearance of bacteria as green metallic sheen to brown coloured colony was regarded as positive^[16].

2.3.6. Starch hydrolysis test

The enzyme amylase was excreted out of the cells (an exoenzyme) into the surrounding media, catalyzing the breakdown of starch into sugars. At first, one starch agar plate was picked up and divided in half. Then the isolates were incubated on the one plate in either a straight line or a zig–zag at 37 °C overnight. After incubation and growth the plate was flooded with iodine. The appearance of yellow or gold zone around the growth indicated positive result^[16].

2.3.7. Protease hydrolysis test

2.0 mg% Luria broth and 1.5 mg% nutrient agar were dissolved in a same conical flask, while 1 mg% casein was dissolved in another conical flask and sterilized in autoclave at 15lb (121 °C) pressure for 15 minutes. Both autoclave mixtures were cooled to (45–50) °C, mixed in a same conical flask properly, poured in sterile petridishes and checked overnight at 37 °C. On the next day, overnight growing isolates in MHB were streaked on overnight checked caesine plates and plates were kept at 37 °C overnight. The appearance of clear zone around the bacterial growth indicated the positive result^[16].

2.4. Antibiotic susceptibility testing of isolated *S. aureus* strains

2.4.1. Determination of minimum inhibitory concentration (MIC)

The MIC values of imipenem, amikacin, kanamycin and oxacillin were determined by a broth dilution method using MHB, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS)^[17]. About 5×10^4 cells in MHB were treated with different concentrations of antibiotics and shaken for 16 h at 37 °C. The minimum concentration at which there was no visible turbidity was taken as the MIC of that antibiotic.

2.4.2. Determination of minimum bactericidal concentration (MBC)

The MBC value of antibiotics was determined according to Okore 2005^[18] with some modification. This is an extension of the MIC procedure. Antibiotics which were used in bacterial culture showing growth or no growth in the MIC tests were selected for this test. Bacterial culture used for the MIC test

were inoculated onto the Mueller–Hinton agar at 37 °C for 24 h. 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.

2.4.3. Disc agar diffusion (DAD) test

Susceptibility of isolates to imipenem, amikacin, kanamycin and oxacillin, were determined by DAD technique according to Acar and Bauer *et al*[19,20]. The tested bacterium was from an overnight culture (inoculated from a single colony) and freshly grown for 4 hours at 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 (μ g); Imipenem (10), amikacin (30), kanamycin (30) and oxacillin (1)]. 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 the standard for each drug. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance[20]. *S. aureus* ATCC 25923, an all-sensitive reference strain, was used as a quality control strain for the DAD test.

2.4.4. Inoculation on brain heart infusion (BHI) oxacillin screen agar

Isolates were inoculated on BHI screen agar according to Tiwari and Sen[21]. BHI agar screen plates with oxacillin at 6

μ g/mL were prepared. Colonies were selected from overnight growth on nutrient agar plates for inoculum suspension. The colonies were transferred to sterile saline and suspended at the turbidity of a 0.5 McFarland standard. The final inoculum concentration of 10^5 to 10^6 CFU per spot was obtained by adding the sterile saline to the bacterial suspension. These suspensions were inoculated onto BHI screen agar plates for 24 h at 35 °C in ambient air. Any visible growth indicated the oxacillin resistance. *Enterococcus faecalis* ATCC 51299 and *S. aureus* ATCC 29213 were used as oxacillin susceptible control strains and oxacillin resistant control strain, respectively.

3. Results

3.1. Characterization of isolated *Staphylococcus aureus* strains

The clinical *S. aureus* isolates were characterized by standard biochemical tests. It showed that 100% isolates had gelatin hydrolysis activity, urea hydrolysis activity and galactose hydrolysis activity; 50% isolates had starch hydrolysis activity; 35% isolates had protease hydrolysis activity; 100% isolates had lactose fermentation activity and none of these isolates had sucrose fermentation activity.

3.2. Antibiotic susceptibility testing

3.2.1. MIC of antibiotics

The MIC values of imipenem, amikacin, kanamycin and

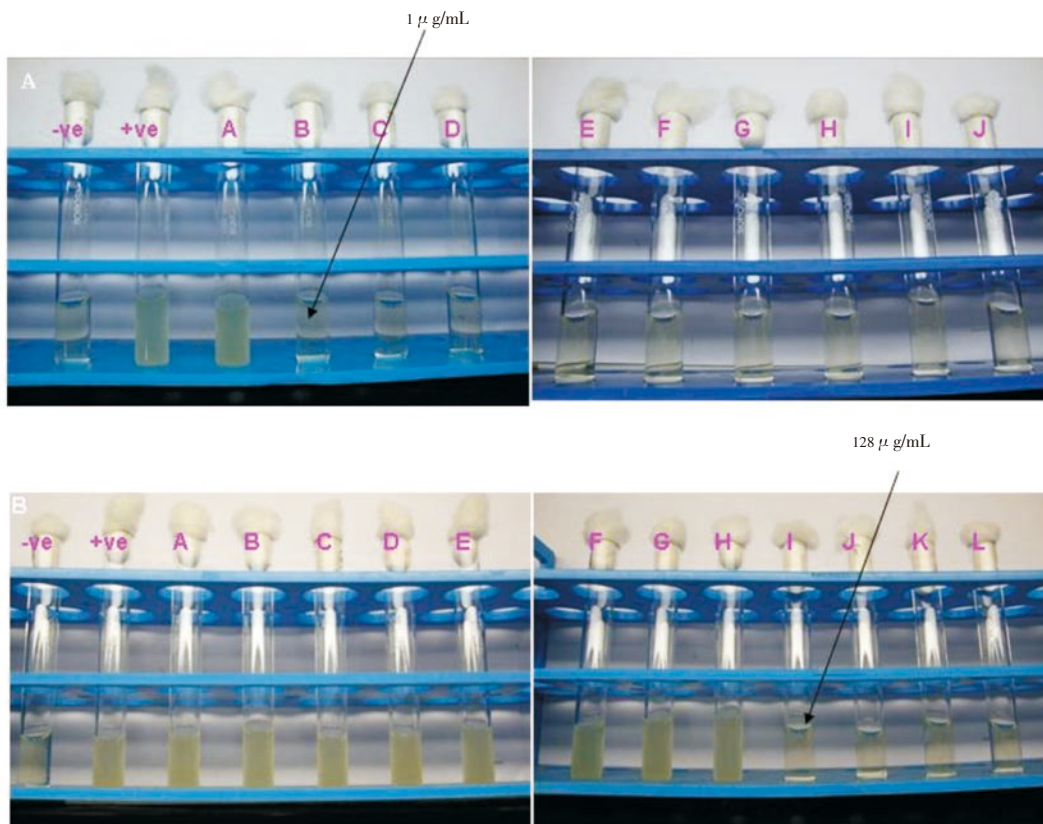


Figure 1. Determination of MIC value of antibiotics for *S. aureus* isolates.

MIC of oxacillin for MMC 7 is $1 \mu\text{g/mL}$ (A) and MMC 12 is $128 \mu\text{g/mL}$ (B). Here, -ve: negative control, +ve: positive control, A= $0.5 \mu\text{g/mL}$, B= $1 \mu\text{g/mL}$, C= $2 \mu\text{g/mL}$, D= $4 \mu\text{g/mL}$, E= $8 \mu\text{g/mL}$, F= $16 \mu\text{g/mL}$, G= $32 \mu\text{g/mL}$, H= $64 \mu\text{g/mL}$, I= $128 \mu\text{g/mL}$, J= $256 \mu\text{g/mL}$, K= $512 \mu\text{g/mL}$, L= $1024 \mu\text{g/mL}$.

oxacillin for *S. aureus* 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 of MIC for *S. aureus*. It was observed that MIC values of kanamycin for 20% of isolated strains and oxacillin for 46.67% of isolated strains were beyond the sensitive range (Figure 1).

3.2.2. MBC of antibiotics

The MBC values of imipenem, amikacin, kanamycin and oxacillin for *S. aureus* isolates were determined. In each set of experiment, bacterial control plates showed no growth. It was observed that MBC values of kanamycin for 20% of isolated strains and oxacillin for 46.67% of isolated strains were beyond next two concentrations of MIC values (Figure 2).

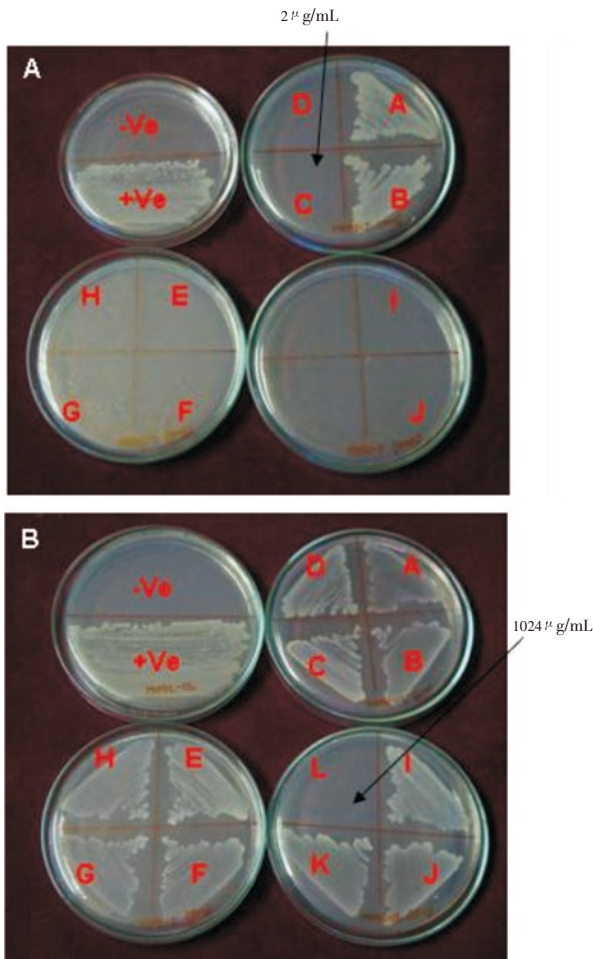


Figure 2. Determination MBC value of antibiotics for *S. aureus* isolates. MBC of oxacillin for MMC 7 is 2 µg/ mL (A) and MMC 12 is 1 024 µg/ mL (B) Here, -ve: negative control, +ve: positive control, A=0.5 µg/mL, B=1 µg/mL, C=2 µg/mL, D=4 µg/mL, E=8 µg/mL, F=16 µg/mL, G=32 µg/mL, H=64 µg/mL, I=128 µg/mL, J=256 µg/mL, K= 512 µg/mL, L=1 024 µg/mL.

3.2.3. DAD test

The antibiotic–resistance profile, as determined by DAD test, revealed that 20% isolates were resistant to kanamycin and 46.67% were resistant to oxacillin (Figure 3).

3.2.4. BHI oxacillin screen agar

Out of 30 gram positive clinical isolates thirteen (46.67%) isolated strains were grown in BHI oxacillin screening agar (Figure 4).

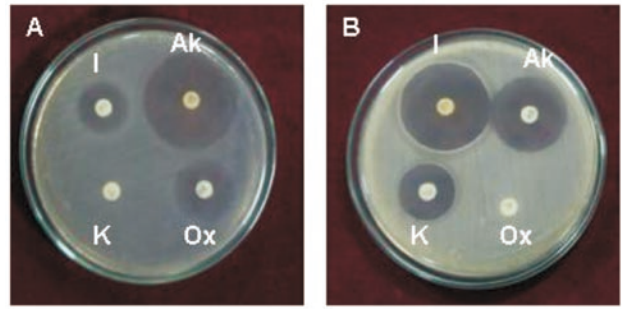


Figure 3. Disc agar diffusion test of MMC 7 (A) and MMC 12 (B) against 4 antibiotic discs.

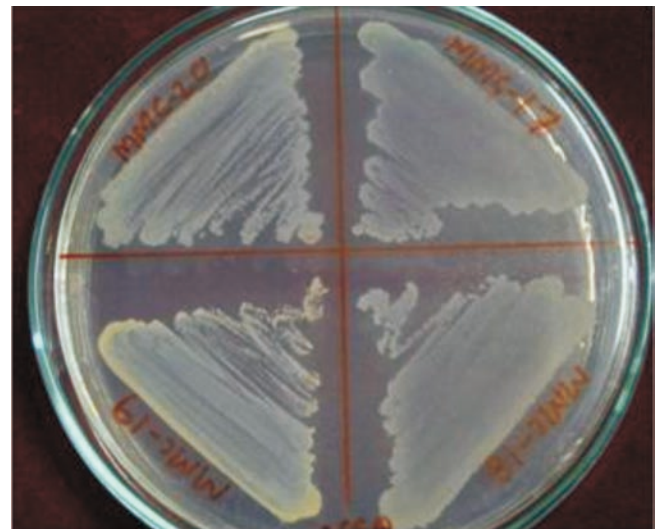


Figure 4. Growth of some clinical *S. aureus* isolates on BHI oxacillin screen agar.

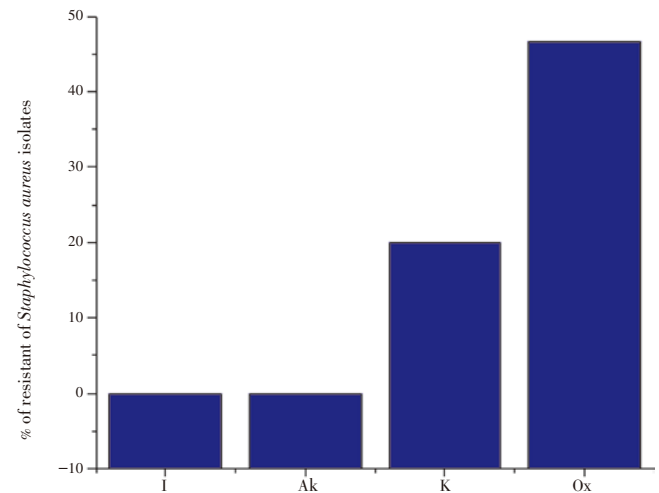


Figure 5. Antibiotic susceptibility profile of thirty *S. aureus* isolates. Here I: Imipenem, Ak: Amikacin, K: Kanamycin and Ox: Oxacillin.

4. Discussion

The development and spread of bacterial strains that are resistant to antibacterial drugs has emerged as a global problem[22]. It was observed from our study that all isolated

S. aureus strains liquefy the gelatin. The enzyme gelatinase was secreted from bacteria, hydrolysis the gelatin into soluble carbohydrates. Thus isolated *S. aureus* liquefy gelatin and showed positive results^[16]. Our study reveals that all isolated *S. aureus* strains changes the colour of media from yellow to red, indicating positive reactivity in urea hydrolysis. That may be due to the liberation of end product of urea hydrolysis which is ammonia^[16]. In this study, all isolated strains showed a positive result in lactose fermentation test. Lactose fermenting bacteria usually developed into red colours surrounded by a violet–pink zone in the Mac Conkey agar screening plates as it contains crystal violet and bile salt^[16]. It was observed in this study that all isolated strains showed a negative result in sucrose fermentation test indicating that these isolates have no sucrose fermenting activity^[16]. It was observed from our study that all isolated strains have galactose activity and it may be due to the enzyme, galactase, which was secreted from bacteria, hydrolysis the galactose into soluble carbohydrates^[16]. It was evident from this study, 50% strains showed a positive result in starch hydrolysis test. It may be due to the reaction of starch and lugal's iodine that turns some part of the plate colourless^[16]. Protein hydrolysis test (casein plate) was performed to identify protease activity strains. In this study, 35% strains showed the positive reactivity with a clear zone around the bacterial growth. That may be due to the activity of protease enzyme in bacteria hydrolysis the casein^[16].

In recent years, *S. 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 suggests that 20% (MMC 6, MMC 7, MMC 10, MMC 13, MMC 14 and MMC 15) were resistant to kanamycin, 46.67% (MMC 4, MMC 5, MMC 8, MMC 9, MMC 12, MMC 17, MMC 18, MMC 19, MMC 20, MMC 22, MMC 26, MMC 27, MMC 29 and MMC 30) were resistant to oxacillin, and 46.67% of isolated strains (MMC 4, MMC 5, MMC 8, MMC 9, MMC 12, MMC 17, MMC 18, MMC 19, MMC 20, MMC 22, MMC 26, MMC 27, MMC 29 and MMC 30) were resistant to oxacillin.

Isolated *S. aureus* strains are resistant to kanamycin and oxacillin. It may be due to inactivation of the antibiotic as a result of structural modification by enzymatic action, prevention of access to target by altering the outer membrane permeability, alteration of the antibiotic target site, efflux pump which pumps out the antibiotic, and target enzyme bypass or over production. In brief, from this study, six kanamycin resistant *S. aureus* strains and fourteen oxacillin resistant *S. aureus* strains were identified.

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Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Bannerman TL. Staphylococcus, micrococcus, and other catalasepositive cocci that grow aerobically. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Tenover FC, Tenover FC. (eds.) *Manual of clinical microbiology*. Washington DC: ASM Press; 2003, p. 384–404.
- [2] Giacometti A, Cirion O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM, et al. Epidemiology and microbiology of surgical wound infections. *J Clin Microbiol* 2000; **38**(2): 918–922.
- [3] Perichon B, Courvalin P. Synergism between beta-lactams and glycopeptides against *vanA*-type methicillin-resistant *Staphylococcus aureus* and heterologous expression of the *vanA* operon. *Antimicrob Agents Chemother* 2006; **50**: 3622–3630.
- [4] Tyagi A, Kapil A, Singh P. Incidence of methicillin resistant *Staphylococcus aureus* (MRSA) in pus samples at a tertiary care hospital, AIIMS, New Delhi. *J Indian Acad Clin Med* 2008; **9**(1): 33–35.
- [5] Kac G, Buu-Hoi A, Herisson E, Biancardini P, Debure C. Methicillin-resistant *Staphylococcus aureus* nosocomial acquisition and carrier state in a wound care center. *Arch Dermatol* 2000; **136**(6): 735–739.
- [6] Jarvis WR. Infection control and changing health care delivery systems. *Emerg Infect Dis* 2001; **7**: 170–173.
- [7] Rallapalli S, Verghese S, Verma RS. Validation of multiplex PCR strategy for simultaneous detection and identification of methicillin resistant *Staphylococcus aureus*. *Indian J Med Microbiol* 2008; **26**(4): 361–364.
- [8] Saha B, Singh AK, Ghosh A, Bal M. Identification and characterization of a vancomycin resistant *Staphylococcus aureus* isolated from Kolkata (South Asia). *J Med Microbiol* 2008; **57**: 72–79.
- [9] Livermore DM. The need for new antibiotics. *Clin Microbiol Infect* 2004; **10**: 1–9.
- [10] Livermore DM. Antibiotic resistance in staphylococci. *Int J Antimicrob Agents* 2001; **16**: 3–10.
- [11] Cui L, Murakami H, Kuwahara-Arai K, Hanaki H, Hiramatsu K. Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *J Antimicrob Chemother* 2000; **44**: 2276–2285.
- [12] Hiramatsu K. Vancomycin-resistance *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infect Dis* 2001; **1**: 147–155.
- [13] Rajadurai K, Mani KR, Panneerselvam K, Mani M, Bhaskar M, Manikandan P. Prevalence and antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus aureus*: a multicentre study. *Indian J Med Microbiol* 2006; **24**(1): 34–38.
- [14] Anupurba S, Sen MR, Nath G, Sharma BM, Gulati AK, Mohapatra TM. Prevalence of methicillin resistant *Staphylococcus aureus* in a Tertiary Care Referral Hospital in Eastern Uttar Pradesh. *Indian J Med Microbiol* 2003; **21**: 49–51.
- [15] Chakraborty SP, KarMahapatra S, Bal M, Roy S. Isolation and identification of vancomycin resistant *Staphylococcus aureus* from post operative pus sample. *Al Ameen J Med Sci* 2010; **4**(2): 152–168.
- [16] Hass D, Defago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 2005; **3**(4): 369–372.
- [17] NCCLS. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. 5th ed. 17, No 2. Approved standard M7–A5. Wayne, PA: NCCLS; 2000.
- [18] Okore VC. Evaluation of chemical antimicrobial agents. Bacterial resistance to antimicrobial agents. *Pharm Microbiol* 2005; **55**–120.
- [19] The disc susceptibility test. In: Acar JF, Lorian V. (eds.) *Antibiotics in laboratory medicine*. Baltimore: Williams & Wilkins; 1980, p. 24–25.
- [20] Bauer AW, Kirby WMM, Sherris JC, Tenover FC. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; **45**: 493–496.
- [21] Tiwari HK, Sen MR. Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. *BMC Infect Dis* 2006; **6**: 156–161.
- [22] Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science* 1992; **257**: 1050–1055.