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Surveillance of vancomycin-resistant *Staphylococcus aureus* (VRSA) from a teaching hospital, Bhubaneswar, India

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Abstract

Background: Glycopeptides such as vancomycin are frequently the antibiotics of choice for the treatment of infections caused by methicillin resistant *Staphylococcus aureus* (MRSA). For the last 7 years incidence of vancomycin intermediate *S. aureus* and vancomycin resistant *S. aureus* (VISA and VRSA respectively) has been increasing in various parts of the world. The present study was carried out to find out the presence of VISA and VRSA in the Department of Medicine, IMS and SUM Hospital Bhubaneswar.
Methods: A total 1681 staphylococcal isolates consisting of 783 *S. aureus* and 898 coagulase negative staphylococci (CoNS) were isolated from different clinical specimens from various outpatient departments and wards. All *S. aureus* and 93 CoNS were subjected to MIC testing (against vancomycin, teicolplanin and oxacillin); Brain Heart Infusion (BHI) vancomycin screen agar test, and disc diffusion testing.
Results: Out of 783 *S. aureus* two *S. aureus* strains were found to be vancomycin and teicoplanin resistant (one strain with MIC 32 g/ml and the other strain with MIC 64 g/ml); six strains of *S. aureus* have shown to be vancomycin intermediate (two strains with MIC 16 g/ml and four strains with MIC 8 g/ml); and two strains with teicoplanin intermediate (MIC 16 g/ml). One CoNS strain was resistant to vancomycin and teicoplanin (MIC 32 g/ml), and two CoNS strains were intermediate to vancomycin and teicoplanin (MIC 16 g/ml). All VRSA, VISA and vancomycin resistant CoNS had shown growth on BHI vancomycin screen agar (vancomycin 6 g/ml).
Conclusion: The present study reveals for the first time emergence of VISA/VRSA from this part of world and indicates the magnitude of antibiotic resistance in and around the study area. The major cause of this may be unawareness and indiscriminate use of broad-spectrum antibiotics.

Keywords: Vancomycin-resistant *Staphylococcus aureus, methicillin resistant Staphylococcus aureus*, VISA, Brain Heart Infusion

Introduction

Background

Staphylococcus aureus has long been recognized as a major pathogen of hospital acquired

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infections. Over the last decade, methicillin resistant *S. aureus* (MRSA) strains have become endemic in hospitals worldwide. In addition, it is now incipient community pathogen in many geographical regions [1]. MRSA is important because, in addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotic, with the exception of glycopeptide antibiotics [2, 3]. In 1980s, because of widespread occurrence of MRSA, empiric therapy for staphylococcal infections (particularly nosocomial sepsis) was changed to vancomycin in many health care institutions. Vancomycin use in United States also increased during this period because of the growing numbers of infections with *Clostridium difficile* and coagulase negative staphylococci (CoNS) in health care institutions [4, 5]. Thus, the early 1990s have shown a discernible increase in vancomycin use. As a consequence, selective pressure was established that eventually lead to the emergence of strains of *S. aureus* and other species of staphylococci with decreased susceptibility to vancomycin and other glycopeptides [6]. In 1997, the first strain of *S. aureus* with reduced susceptibility to vancomycin and teicoplanin was reported from Japan [7]. Shortly after, two additional cases were reported from United States [8]. However, first clinical isolate of vancomycin resistant *S. aureus* (VRSA) was reported from United States in 2002 [9]. More recently some workers have reported vancomycin resistant staphylococcal stains from Brazil [10] and Jordan [11].
Strains of Vancomycin Intermediate *S. aureus* (VISA) with vancomycin MIC of 8 g/ml have been reported from Japan [7], United States [12-14], France [15], United Kingdom [16] and Germany [17]. Most of these isolates appear to have developed from preexisting MRSA infections. Assadullah *et al*. from India have reported reduced susceptibility of vancomycin against MRSA and CoNS [18]. Keeping this in view we performed extensive longitudinal study of total 1681 staphylococcal strains for the assessment of current situation of vancomycin resistance in IMS and SUM Hospital, Bhubaneswar.

Methods

Staphylococcal isolates

A total 1681 staphylococcal isolates consisting of 783 *S. aureus* and 898 CoNS were investigated for the period of three years from August 2002 to July 2005. The strains were collected from various clinical specimens including pus, urine, wound swabs, catheters, blood, sputum and CSF from the patients of different inpatient and outpatient departments of IMS and SUM Hospital, Bhubaneswar. This is a tertiary care teaching hospital, which serves the population of eastern region of Odisha.

Media and Culture conditions

All clinical samples except urine were first inoculated onto blood agar (Hi-Media, India) and MacConkey agar (Hi- Media, India) plates whereas the urine samples were inoculated only on CLED agar (Hi-Media, India) plates. The plates were incubated at 37 °C for 24-48 h. The identification of isolates was done according to standard method described elsewhere [19].

Growth on mannitol salt agar

All staphylococcal isolates were again inoculated onto mannitol salt agar (Hi-Media, India) and plates were incubated at 37 °C for 24-48 h. Mannitol fermentation was observed and recorded.

Coagulase test

Slide coagulase tests of all 1681 isolates were performed by emulsifying few pure colonies of

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staphylococci from blood agar on undiluted rabbit plasma. Tube coagulase tests were performed by diluting the plasma in freshly pre-pared normal saline (1:6). Three to four pure colonies were emulsified in 1 ml of diluted plasma and the tubes were incubated at 37°C. Readings were taken at 1 h, 2 h, 3 h, and 4 h and further incubated overnight at room temperature if no clot formation was observed. *S. aureus* ATCC 29213 was used as control strain [19].

Latex agglutination test (slidex staph plus)

Latex agglutination test on all clinical isolates was performed according to the protocols supplied by the manufacturer (Biomerurix India Ltd, New Delhi, India).

Determination of minimum inhibitory concentration (MIC)

MIC of oxacillin (Hi-Media, India), vancomycin (Lilly Pharma, Giessen, Germany) and teicoplanin (Gruppo Lepetit, Angani, Italy) were determined by agar dilution method as described elsewhere [20]. Briefly, gradient plates of Mueller-Hinton agar (Hi-Media, India) were prepared with oxacillin (0.25-256 g/ml) (with 2% NaCl), vancomycin (0.5-128 g/ml) and teicoplanin (0.5-128g/ml). By direct colony suspension method 0.5 McFarland equivalent inouculum were prepared in normal saline from 18-24 h agar plate culture. The suspension was further diluted to achieve desired inoculum concentration of 105 CFU/ml. All strains were spotted onto gradient plates. Plates were incubated overnight at 35 °C for any visible growth. Readings were taken according to NCCLS guidelines [20]. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains were used as vancomycin susceptible controls and *E. faecalis* ATCC 51299 as vancomycin resistant control.

Inoculation on BHI vancomycin screen agar

In-house prepared BHI agar (Hi-Media, India) screen plates containing 6 g/ml vancomycin (Lilly Pharma, Giessen, Germany) 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 105 to 106 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 h at 35 °C in ambient air. Any visible growth indicated the vancomycin resistance. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains were used as a vancomycin susceptible control strains and *E. faecalis* ATCC 51299 as vancomycin resistant control strain.

Disc diffusion

Disc diffusion test of Penicillin (10 U), Oxacillin (1g), Gentamicin (10g), Tobramycin (30g), Amikacin (30g), Netilmicin (30g), Norfloxacin (10g), Ciprofloxacin (5g), Chloramphenicol (30g), Erythromycin (15g), Tetracycline (15g), Trimethoprim/Sulfamethaxole (1.25/23.75g), Nitrofurantoin (300 g), Vancomycin (30g), Cefoperazoneoxacillin/Sulbactam (75/30g) was carried out using Kirby-Bauer Method as described elsewhere [20]. Mueller-Hinton agar plates were overlaid with the inoculum (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical strains. Zone diameters were measured at 24 and 48 h following NCCLS criteria [20]. *S. aureus* ATCC 29213 was used as reference strain.

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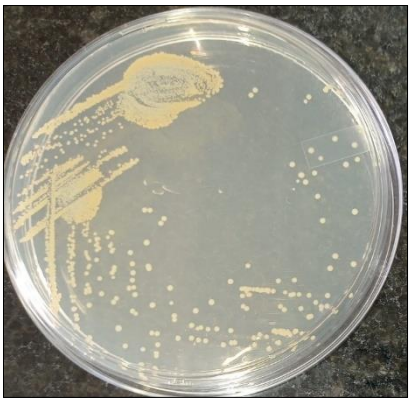

Fig 1: Staphylococcus aureus on nutrient agar

Results

Out of 1681 staphylococcal isolates, 761 isolates were slide coagulase positive, 772 were slidex staph plus positive and 783 were tube coagulase positive. Thus, 783 strains were confirmed as *S. aureus* and remaining 898 strains as CoNS. MIC of 783 *S. aureus* strains and 93 CoNS against oxacillin had shown that 318 *S. aureus* stains and 51 CoNS strains were resistant to oxacillin (MIC µ 4 g/ml for *S. aureus* and MIC µ 0.5 g/ml for CoNS). Out of 318 oxacillin resistant *S. aureus* strains, total two strains were found to be VRSA strains. One strain had shown vancomycin and teicoplanin MIC 32 g/ml and the other strain with vancomycin and teicoplanin MIC 64 g/ml. Out of 51 oxacillin resistant CoNS, one strain had shown vancomycin and teicoplanin MIC 32 µg/ml. Six strains of *S. aureus* were to be found vancomycin intermediate *S. aureus* (two strains with MIC of 16 g/ml and four strains with MIC of 8 g/ ml). Two *S. aureus* strains were to be teicoplanin intermediate (MIC 16 g/ml). The detailed description of all vancomycin intermediate/resistant *S. aureus* strains (Fig 1).

Discussion

In the routine microbiology laboratory prompt identification of the staphylococcal strains up to species level were done by catalase, coagulase and other standard biochemical test. However during routine screening by slide coagulase test many strains of *S. aureus* are missed due to their poor sensitivity and falsely reported as coagulase negative staphylococci during routine screening process. In our study we performed tube coagulase tests of all 1681 staphylococcal strains. Therefore the main criterion used for the *S. aureus* identification was tube coagulase test. However, the slidex staph plus test was also found to be good to differentiate *S. aureus* and CoNS. MICs of oxacillin, vancomycin and teicoplanin against *S. aureus* and CoNS revealed that there is a rise in the resistance. *S. aureus* and CoNS have shown 40.61% and 54.83% oxacillin resistance, respectively. We have found two MRSA strains which were showing vancomycin resistance (VRSA). These VRSA strains have been isolated from the pus of two different patients who were admitted in the different wards. One VRSA (vancomycin MIC 64 g/ml) strain was isolated from pus of a 56 years old patient who was admitted in Medicine ward and; the other VRSA (vancomycin MIC 32 g/ml) strain was isolated from pus of a 49 years old patient who was admitted in Skin and VD ward of our hospital. Both the isolates were also found to be resisistant to several other antimicrobials such as gentamicin, tobramycin, amikacin, norfloxacin, ciprofloxacin, erythromycin, tetracycline, trimethoprim/sulfamethoxazole and cefoperazone/sulbactam. While going through the detailed history of these two patients it was found that they were already treated with glycopeptides for more than 20 days. These patients were not responding to further vancomycin therapy and later died due to complication of the disease.

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The present study also encountered one vancomycin resistant CoNS strain with vancomycin and teicoplanin MIC 32 g/ml. The strain was later identified as *Staphylococcus epidermidis*. This strain was isolated from culture of tip of endotrachial tube of a patient who was admitted in the intensive care unit of teaching hospital. Of total six VISA strains, four had their MIC 8 g/ml and two strains showed vancomycin and teicoplanin MIC of 16 g/ml. Out of six VISA strains, two were isolated from the pus specimens of the patient visiting outpatient department and remaining four were from the pus specimens of patients admitted in post-operative ward. Out of two strains of CoNS (*Staphylococcus epidermidis*) with vancomycin and teicoplanin MIC 16 g/ml, one was isolated from the blood of a patient who was admitted in orthopedic ward, and the other was isolated from the urine specimen of a patient visiting the endocrinology outpatient department. In this study we have found almost similar pattern of MIC of vancomycin and teicoplanin. The present study shows that there is a significant rise of reduced susceptibility of oxacillin, vancomycin and teicoplanin. The emergence of the glycopeptide resistance is of great concern. Though first case of VRSA was reported in 2002 in USA [9] but few other countries have reported the reduced susceptibility of *S. aureus* and CoNS against glycopeptides [6-8, 10, 11, 13-18]. Recently Pallazo *et al*. [10] have also reported some vancomycin resistant strains of CoNS in Brazil. More recently Bathaineh has reported VRSA strains from Jordan [11]. Ashdulla *et al*. [18] have reported some strains of vancomycin intermediate *S. aureus* (VISA) from India. Song *et al*., 2004 have also been reported the emergence of heterogeneous vancomycin resistant *S. aureus* strains from India and its neighboring countries [24]. The current van-comycin resistant staphylococci in hospital as well as in community are alarming situation to the clinicians. The development of antibiotic resistance in developing countries like ours seems to be very much related to the irrational antibiotic usage due to its easy availability at the drug store without prescription, injudicious use in hospitals and uncontrolled use in agriculture, animal husbandry and fisheries [25]. This emergence of VRSA/VISA may be due to building of selective pressure of vancomycin. Vancomycin, a glycopeptide is currently the main antimicrobial agent available to treat life-threatening infections with MRSA. Until recently vancomycin resistance among gram-positive bacteria had been thought to be uncommon but the confirmed reports of vancomycin resistance in *Enterococcus* spp; *S. aureus* and CoNS have been reported from various part of world. Widespread use of vancomycin to treat infections caused by MRSA and other gram-positive cocci has led to the emergence of vancomycin resistance. The large scale of development and subsequent spread of resistance to vancomycin has been perceived as a fearsome threat to the already challenging therapy of MRSA.

The true mechanism of vancomycin resistance in *S. aureus* is not known. It was initially feared that *S. aureus* would acquire the *van* gene that code for vancomycin resistance in *Enterococcus s*pp; this phenomenon was successfully accomplished in the laboratory [26]. Further, Showsh *et al*., 2001 [27] have demonstrated the presence of sex pheromone in *S. aureus* that promotes plasmid transfer in *Enterococcus* spp. Release of these pheromones by *S. aureus* with proximity to vancomycin-resistant enterococci causes the transfer of plasmids encoding *van* gene to the *S. aureus*.

Moreover, a higher concentration of vancomycin would be required to saturate all the murein monomers that are supplied at an increased rate in Mu50. Besides the vancomycin-trapping mechanism, designated "affinity trapping" [31-33], Hiramatsu has suggested that dense accumulation of vancomycin molecules within the thickened cell-wall significantly delays the timing of com- plete inhibition of cell-wall synthesis by not allowing efficient penetration of vancomycin molecules through the thickened cell-wall layers [30].

The thickened cell wall of VRSA strains become thinner with the loss of vancomycin resistance during the drug free passage and again become thick in resistant mutants. Pallazo *et al*. [10] have also demonstrated the thickening of cell wall in vancomycin resistant staphylococci. This could be the possible mechanism behind the vancomycin resistant staphylococcal isolates that we have reported though we could not perform the test for the

demonstration of cell wall thickening in these isolates.

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