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Original Research

Molecular and phenotypic characterization of the vancomycin-resistant gene in bacterial isolates acquired from catheter tips

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Abstract: Gram-positive bacteria, particularly *Staphylococcus aureus* is a significant pathogen, not only in the hospital setting but the community also. *S. aureus* is a major cause of serious hospital and community-acquired infections, particularly in the colonized individuals. The emergence of vancomycin-resistant *S. aureus* (VRSA) strains has led to global concerns about treatments for staphylococcal infections. Until now, few strains of VRSA have been reported worldwide. The conventional disk diffusion method for determination of vancomycin sensitivity often misclassifies intermediately susceptible isolates to fully sensitive. However, non-automated minimum inhibitory concentration (MIC) detection methods are the gold standards. Hence there is a dire need of some advanced methods for rapid detection of VRSA strains. In the present study, Gram-positive clinical isolates were collected from different wards of K.G.M.U. Hospital, among them, 12 bacterial isolates were identified as *Staphylococcus aureus* and 18 isolates as *Klebsiella spp*. Genomic DNA of *S. aureus* was isolated and used as template in PCR for detection of the presence of *van A* and *van X* gene based on a given protocol. Nosocomial infections have an impact on morbidity and probably on mortality as well, and pose a significant economic burden. Rapid molecular identification of antibiotic-resistant strains undoubtedly helps to prevent the hospital-induced infections.

Key words: Vancomycin; Resistance; Minimum inhibitory concentration; Nosocomial infections.

Introduction

In general, infection occurs when a microorganism invades a susceptible host and subsequently causes disease. Nosocomial infections cause substantial morbidity and mortality, prolong hospital stay off affected patients, and increase direct patient-care costs (1-4). *Staphylococcus aureus (S. aureus), Klebsiella sp. Escherichia coli (E. coli)* are the most frequently encountered bacteria in a hospital environment (5). Most hospitals acquired infections result from bacterial infections, and the infectious agents may be transmitted by either exogenous or endogenous means. The vast majority (95%) of surgical infections are exogenous in origin. Hospital-acquired infection is defined as – One that was neither present nor incubating at the time of admission to a hospital. It is a major public health problem and is estimated that more than 1.4 million people are suffering from the nosocomial infection in the world. Microorganisms can adhere to catheter surface and embed themselves in a layer of biofilms, a product of microorganism and the human body itself (6). A type of vessel can designate a catheter, it occupies peripheral venous, central venous or arterial its intended lifespan (e.g. temporary or short-term versus permanent or long-term), its site of insertion (e.g. subclavian, femoral internal); jugular, peripheral and peripherally inserted central catheter (PICC); its pathway from skin to vessel (e.g. tunneled or on tunneled); its physical length (long or short) or some unique characteristics of catheter (e.g. presence or absence of or cuff, impregnation with heparin, antibiotics, antiseptics and the number of lumens).

Moreover, nosocomial infections are transmitted by air containing infected droplets, vectors, and by direct and indirect contact (7). Pathogens responsible for the nosocomial infections are bacteria, virus, parasite, fungi or other agents. Among these bacteria and more specifically *E. coli, klebsiella* sp. *Pseudomonas aeruginosa, S. aureus, and Salmonella* sp. are the commonly occurring Pathogenic Microorganisms. The most common reservoirs for nosocomial colonizers are oropharynx, gastrointestinal tract, and urinary tract. *S. aureus and Klebsiella* sp. are the common cause of community-acquired and nosocomial bacteremia. *S. aureus* and *Klebsiella* sp. can develop resistance to many antibiotics which can enhance their importance as human pathogens, especially in the hospital environment. Infections with *Klebsiella* sp. have increased among hospital patients, and

it has been grown in prevalence as the cause of severe nosocomial infections. The appearance of multiple antibiotic resistant agents has created severe problems in the treatment of such diseases (8). In addition to this, it has been demonstrated that multiple drug resistance in these organisms is associated with the widespread use of antimicrobial agents.

Additionally, neonatal nosocomial infections are the major cause of neonatal morbidity and mortality. However, such cases in India has been non-uniform (9). The reported incidence of nosocomial sepsis in India ranges from 1.5-3.7%. In many studies, the impact of antibiotics on acquiring ESBL, *Klebsiella pneumonia* was questioned by historical or indirect arguments. Etiologic investigations that included antibiotic administration as a risk factor gave conflicting results. *S. aureus* is a common cause of community-acquired and nosocomial bacteremia. The pathogenesis usually involves the colonization of site by *S. aureus* followed by its invasion of the bloodstream. Most infections are thought to be monoclonal.

Mechanism of antibiotic resistance varies between pathogen *S. aureus* has been a threat in the hospital and particularly ICU for many decades (10, 11). A particular alarming observation in the treatment of *S. aureus* infections is the doubling in the incidence of methicillin resistance 15% in 1990 to 20-25% in 1997 and consistently increasing. The first outbreak of methicillin-resistant *S. aureus* infections was documented in 1968, and since then its rate in the hospital setting has risen immensely. It is evident from the fact that patients who are at risk of acquiring a nosocomial ventilated – typically experienced a cardiopulmonary arrest and early onset pneumonia after trauma (who has undergone neurosurgery). In this study, we characterized the microorganism those are responsible for nosocomial infection in the hospital system and also established the antibiotic resistance pattern as well as characterized the genes which are responsible for antibiotic resistance.

Materials and Methods

Sample collection

The samples were collected from different wards (NICU, CHDS, NSW, OPD) of King George Medical University (KGMU). These samples were dipped into the Robertson cooked meat medium (RCM) and incubated overnight at 37ºC and further grown on Mac-Conkey agar. Positive cultures were processed in a usual manner for identification of *S. aureus* and *Klebsiella spp*. Susceptibility test was performed against the appropriate antibiotics on Mueller-Hinton agar plate.

Morphological and biochemical identification

Gram staining was done for identification of selected bacterial isolates. Bacteria that appeared purple were referred to as gram-positive (+ve) while those seemed to be pink were described as gram-negative (-ve). Most bacteria were identified and classified primarily by their results in a sequence of biochemical analyses. Some examinations are routinely used for the identification of many groups of bacteria (oxidase, nitrate reduction); others are limited to a particular family, genus, or species (coagulase test for staphylococci). Hence, selected bacterial isolates were subjected to biochemical tests such as IMVIC, Indole Production, Methyl red test, Voges-Proskauer test, citrate utilization, catalase, coagulase, oxidative activity, urease, nitrate reduction, and fermentation of carbohydrates (i.e. lactose, dextrose, sucrose).

Antibiotic Susceptibility Test

Antibiotic susceptibility test (AST) of *S. aureus and Klebsiella sp.* was done on Mueller-Hinton agar by disc diffusion method as described by Matuschek *et al* (12). Various antibiotics such as amikacin, cefoxitin, gentamicin, ciprofloxacin, levofloxacin, oxacillin, erythromycin, vancomycin, and penicillin were used for AST. Plates were incubated at 37ºC for 24 hours. Zone diameter was measured in millimetres, the size of inhibition was interrupted by referring to the CLSI (Clinical Laboratory Standard Institute) 2005 guidelines and organism was labelled as susceptible, intermediate or resistant accordingly.

Isolation and quantification of bacterial DNA

The genomic DNA of *S. aureus* was isolated using commercially available DNA isolation kits (Bangalore Genei). All the DNA samples were kept at -20º C until used (13). The isolated bacterial DNA from *S. aureus* was estimated spectrophotometrically. The isolated DNA was analyzed qualitatively by running 10 µg of DNA on 0.8% agarose gel with 1X TAE (pH 8.3) running buffer on the horizontal electrophoretic unit (Bangalore Genei). A molecular weight marker was included on each gel for the identification of the molecular weight of the DNA band appeared on the gel. The gels were stained with ethidium bromide (EtBr) and visualized under UV trans illuminator at 265 nm.

PCR-amplification of the *VANA* and *VANX* gene from *S aureus*

A partial *vanh* and *vanx* gene were amplified using gene primers, which were selected by the published nucleotide sequence gene derived from the *S. aureus* (14). The PCR kit (Fermentas Pvt. Ltd) was used, and the cycling parameters consisted of 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 50ºC for 1 min, and extension at 72ºC for 1 min 30s.

Results and discussion

A total of 100 catheter tip samples were collected and processed for analysis. Out of the 100 samples analysed, 12 bacterial isolates were found to be *S. aureus* positive and 18 samples confirmed as *Klebsiella sp.* by Gram's staining and biochemical tests analysis. Remaining of the above samples showed growth of various microorganism such as *Escherichia coli, Enterobacter, Acinetobacter* and *Pseudomonas species* etc. as enumerated in the Table-1.

Biochemical test analysis for evaluating the bacterial isolates present in the catheter tip specimens were comprising of gram positive as well as gram negative bacteria. We presented the data in the form of bar of various bacterial isolates and its percentage in the specimens is represented in the Figure-1.

The Gram's staining data analysis also revealed that

Table 1. Isolation rate of prevalent bacterial pathogen in (48+52) 100 Samples (catheter Tips).

Bacteria	No. of isolates	Percentage of bacterial isolates
Staphylococcus aureus	+12	12 %
Klebsiella species	-18	18 %
Pseudomonas species	-15	15 %
Escherichia coli	-15	15 %
Acinetobacter species	-18	18 %
Enterobacter	-02	02 %
Contaminated	08	08 %
Sterile	12	12 %

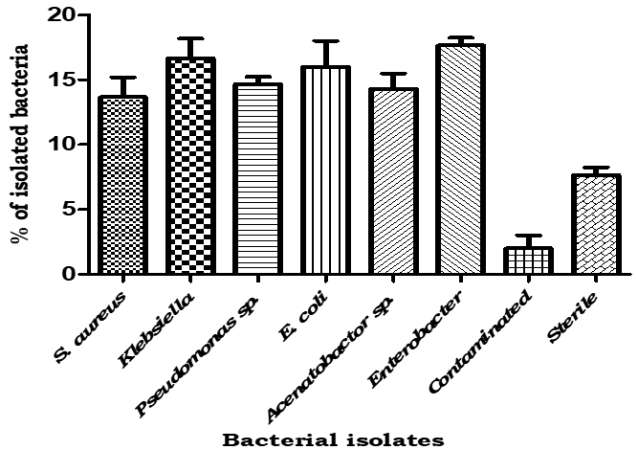

Figure 1. Respective % of bacteria isolated from the catheter tips as compared to the contaminated and sterile.

most of the catheter tip samples were Gram's negative 68 % while Gram's positive bacterial strains were only 12 % as compared to sterile 12% and contaminated was 8%. The data was enumerated in the Table-2.

Next, we analyzed the antibiotic sensitivity pattern of the *S. aureus* bacteria isolated from the catheter tip specimen. Our data revealed that Vancomycin is a most sensitive antibiotic *for S. aureus* isolates while Cefoxitin, Oxacillin and Erythromycin are the antibiotics which were found non-sensitive against the *S. aureus* isolates. In antibiotic sensitivity pattern, Amikacin and Ciprofloxacin were found sensitive for 50% and 33% bacterial isolates, while Oxacillin and Erythromycin were 100% and 83% bacterial isolates resistant respectively. Gentamycin, Levofloxacin and Penicillin showed almost similar sensitivity of 16.67 % among the various isolates of *S. aureus.* However, they showed different moderate as well as resistant profile percentage. The sensitivity profile percentage of various antibiotics against the *S. aureus* isolates was enumerated in the Table-3.

To better understand the relative sensitivity, moderate sensitivity and resistant percentage of *S. aureus* isolates obtained from the catheter tip specimen. The

Table 2. Isolation rate of Gram positive and negative pathogenic bacteria in the samples.

Gram Positive	12 %
Gram Negative	68 %
Sterile	12 %
Contaminated	08 %
Total	100 %

Table 3. Antibiotic sensitivity pattern of *Staphylococcus aureus* isolates from catheter tip specimen.

Antibiotic Used	Sensitive (%)	Moderate sensitive (%)	Resistant (%)
Amikacin	50%	0	50%
Cefoxitin	0	33.33%	66.67%
Gentamycin	16.67%	50%	33.33%
Ciprofloxacin	33.33%	33.33%	33.33%
Levofloxacin	16.67%	33.33%	50%
Oxacillin	0	0	100%
Erythromycin	0	16.67%	83.33%
Vancomycin	66.67%	16.67%	16.67%
Penicillin	16.67%	0	83.33%

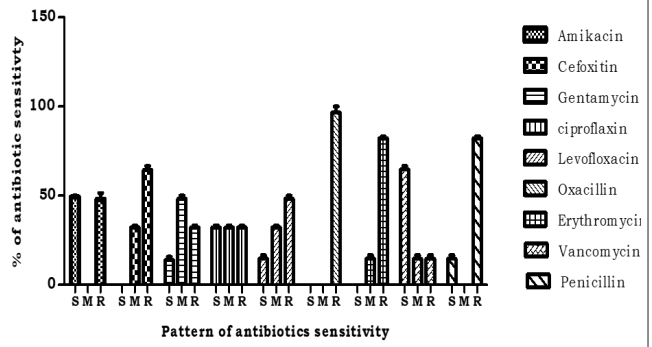

Figure 2. Percentage of antibiotic sensitivity against *S. aureus* various strains. S refers to the sensitive, M refers to the moderate sensitive and R refers to the resistant strains.

above data obtained after performing the antibiotic sensitivity test was then represented in the form of bar chart in the Figure-2.

Similar to the *S. aureus*, we then analyzed the sensitivity pattern percentage of various antibiotics against the *Klebsiella* sp. isolated from the catheter tip specimen. Our results showed that Cefoperazone/Sulbactam was the most sensitive (66.67%) antibiotic for *Klebsiella sp.* Ciprofloxacin was sensitive for 44% bacterial isolates and moderately sensitive for 22%. Whereas Levofloxacin was found to be sensitive for 33.33 % while resistant for 55% bacterial isolates. Amikacin, Gentamicin and Norfloxacin were found to be almost equally sensitive for 11 % bacterial isolates and the trio antibiotic combination showed the maximum resistant percentage among the bacterial isolates. The antibiotic sensitivity pattern of various antibiotics used against the *Klebsiella* species was enumerated in the Table-4.

Table 4. Antibiotic Sensitivity Pattern of *Klebsiella* species isolates from catheter tip specimen.

Antibiotic Used	Sensitive (%)	Moderate sensitive (%)	Resistant (%)
Ampicillin	22.22%	11.11%	66.67%
Ciprofloxacin	44.45%	22.22%	33.33%
Amikacin	11.11%	11.11%	77.78%
Levofloxacin	33.33%	11.11%	55.55%
Cefoperazone/ sulbactum	66.67%	0	33.33%
Gentamycin	11.11%	0	88.89%
Norfloxacin	11.11%	11.11%	77.78%

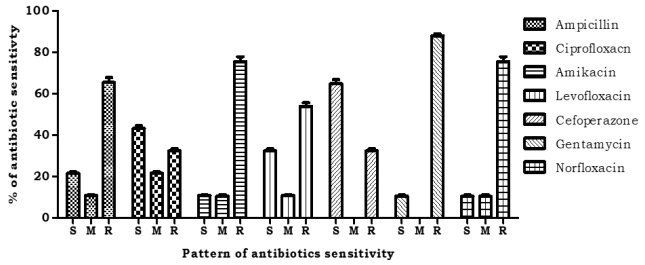

Figure 3. Percentage of antibiotic sensitivity in *klebsiella* sp. S refers to the sensitive, M refers to the moderate sensitive and R refers to the resistant strains.

To further enhance the broader view of the antibiotic sensitivity pattern data obtained against *Klebsiella* species isolated from the catheter tip specimen are illustrated in the Figure-3.

Currently available phenotypic methods for detection of antibiotic-resistant *S. aureus* and *Klebsiella sp.* are problematic. To overcome this difficulty, many phenotypic and genotypic characterizations have been done. But still, phenotypic characterization is an inevitable part of the detection of a pathogenic microorganism.

Keeping the above facts into consideration, we evaluated different phenotypic tests for the detection of *S. aureus* and *Klebsiella sps*. On the Mac Conkey and Blood agar plates, the colonies were grown and found that some isolates were golden yellow and some were red coloured big, mucoid and non-motile. By Gram's staining, we observed that the golden-yellow colonies were gram (ve+) because these taken blue stain and looked like bunches under the microscope, i.e. *S. aureus*. On the other hand, mucoid colonies were gram (-ve) because these not taken blue stain and remained pink coloured, i.e. safranin (counterstain) and seem to be non-motile under the microscope, i.e. *Klebsiella* sp. All the species of *S. aureus* had given a positive test for catalase, coagulase, methyl–red and Voges-Proskauer test, but in the case of *Klebsiella sp.*, common species were detected using biochemical assays. Out of 18 isolates of *Klebsiella* sp. some were *Klebsiella pneumonia*, while the other identified as *Klebsiella oxytocin* as *Klebsiella oxytocin* had given the ve+ for Voges-Proskauer, Lactose and urease test. *Klebsiella pneumonia* had given the +ve test for citrate utilization test, and *Klebsiella oxytoca* had also given the +ve test for indole test.

Cut off zone diameters for vancomycin was followed as recommended by the CLSI. Initially, genomic DNA of *S. aureus* was used as the template for PCR amplification of vancomycin-resistant gene, specifically *van A* and *van X* using specific primers for *van A* (969 bp) and *van X* (609 bp) as previously used by Donabedian et al. (2000) (18). PCR products of 1 kb for *van A*, and 600 bp for *van X* were obtained. In view of this antibiotic resistance, vancomycin has been the drug of last resort. Vancomycin-resistant *Enterococcus faecium harbors* the *van A* operon, which contains five genes, *van S, van R, van H, van A* and *van X*. Among them, the *van S* and *van R* are the regulator genes. The *van H* is a D-hydroxy acid dehydrogenase that reduces pyruvate to D-lactate which could be used by *van A* ligase in conjunction with ATP and D-Ala to make a D-Ala D-lactate depsipeptide, which is incorporated into the PG layer. Conjugative transfer of high-level vancomycin resistance from *Enterococcus faecalis* to *S. aureus* (15,16) and transfer of glyco-peptide and macrolide-resistance genes by transconjugation among enterococci and from *Enterococcus faecalis* to *S.* aureus (17), have been reported. Vancomycin-resistance gene acquisition by *S. aureus* from *Enterococcus faecium* in the clinical environment has also been reported by (18). Infections caused by antibiotic-resistant pathogens are known to affect both patient and economic outcomes (19). The acquirement of high-level vancomycin resistance genes (*nuc, van A*, and *van X*) is responsible for the multidrug resistance ability of *S. aureus* and *Klebsiella sp*. strains to antibiotics which are a major public health problem.

A significant proportion of patients find their hospital stay complicated by an infection. Patients in intensive care units are even more susceptible to nosocomial infections. Also, they are frequently resistant to many commonly used antibiotics, partly as a result of antibiotic selective pressure. The pathogens isolated in this environment are not usually encountered elsewhere. By the isolation, identification and phenotypic as well as molecular characterization of *S. aureus* and *Klebsiella* species, we concluded that both of these strains are responsible for hospital-acquired infections. The result of the present study revealed the high contamination rate by the Gram positive as well as Gram negative strains of the catheter tips. Not only this, the results also confirmed the vancomycin resistant strains of *S. aureus* in the catheter tips. In conclusion, caution should be taken when dealing with the catheter tip to minimize bacterial infections.

Author Contributions

Conceptualized and planned the study and experiments S.S., M.S.,A., K.M.S., S.M.F., M.K. and I.A. Performed the experiments: S.S., M.S., A.A, K.M.S., S.M.F., M.K. and I.A. Analyzed the data: S.S., M.S, S.M.F. and I.A. Contributed reagents/materials/analysis tools: experiments S.S., M.S., K.M.S., S.M.F. and I.A. Wrote the paper: S. S., M.S., S.M.F., A.A, K.M.S., M.K. and I.A. All authors reviewed the manuscript.

Conflict of interest

The authors state that there is no conflict of interest.

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