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In vivo transfer of vancomycin resistance gene (*van*a) in *staphylococcus aureus*

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ABSTRACT

Studies show that different strains of *Staphylococcus aureus* are resistant to a wide variety of antibiotics including vancomycin. The present study was performed to isolate vancomycin resistant strain of *S. aureus* from clinical specimens and to understand how they acquire the resistance gene in vivo. A total of 38 *S. aureus* isolates from clinical specimens were tested against vancomycin for antibiotic sensitivity by disk diffusion and tube dilution method. The MIC of vancomycin for these isolates ranged between 16 µg/ml and 1024 µg/ml. The MBC value ranged between 128 µg/ml and 1024 µg/ml. One of the 38 identified isolates showed resistance against vancomycin (MIC of 64 µg/ml and MBC 128 µg/ml). The vancomycin resistant operon *vanA* was characterized in the Vancomycin resistant *S. aureus* strain. The resistant isolate was further inoculated into hartley's guinea pigs along with a susceptible strain to facilitate conjugal transfer. Samples drawn from the animals at regular intervals were further analyzed to determine antibiotic susceptibility and detection of resistant gene. The molecular examination of the genomic DNA of the *S. aureus* isolated recombinant strain revealed that all the new strains have acquired *vanA* operon in vivo essential for antibiotic resistance to *S. aureus*.

KEYWORDS

VRSA;
MIC;
MBC;
Antibiotic susceptibility;
LGT.

INTRODUCTION

The resistance towards vancomycin by *Staphylococcus aureus* strains has become a major health concern. Vancomycin has been highly prescribed due to growing resistance of *S. aureus* against β-lactam antibiotics. The first case of vancomycin insensitivity was reported in Japan in 1996 and the following year experienced the first case of vancomycin resistant S. aureus strain isolated in the USA[1]. Recently many other countries including neighboring India reported several cases of vancomycin resistance in hospital patients[2]. The *blaZ* gene isolated from several MRSA strains encodes β-lactamase, an enzyme that degrades β-lactam thereby rendering the *S. aureus* isolates to be resistant against pencillin. As a result of this, vancomycin has remained the only drug of choice to treat *S. aureus* infection[3]. However, the emergence of vancomycin resistant strains though rare but not improbable. The resistance to

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vancomycin by *S. aureus* is believed to be acquired through conjugal transfer of *vanA* operon from vancomycin resistant *Enterococcus faecalis*. The *vanA* operon harbors cluster of several genes vanH, *vanA* and vanX. Nucleotide sequencing suggests partial homology with *van* genes of an enterococcal transposon *Tn1546*-like element[4]. Vancomycin acts by binding with high affinity to the D-Ala-D-Ala C-terminus of the pentapeptide, thus blocking the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation. Vancomycin does not interact with cell wall biosynthetic enzymes but forms complexes with peptidoglycan precursors. Resistance to vancomycin is due to the presence of operons that encode enzymes[5]. The present study was designed to investigate whether the vancomycin resistant strain of *S. aureus* could transfer the resistant *vanA* operon to vancomycin susceptible strains in vivo. Thus a resistant strain of *S. aureus* isolated from clinical specimens of hospital patients at Chittagong in Bangladesh was subjected to isolation of DNA and characterization of the resistant *vanA* operon. The strain was then co-inoculated with a susceptible strain in a laboratory animal. The resultant strain was further isolated and the genomic DNA of that strain was characterized to investigate if a recombinant strain with vancomycin resistance has developed through conjugal transfer.

EXPERIMENTAL

Isolation and Identification of S. aureus

The clinical samples (blood, pus and cerebrospinal fluid) received at Chittagong Medical College Hospital were tested to isolate *S. aureus* as described elsewhere[3]. The isolates were confirmed by morphological and biochemical properties as outlined in the Bergey's Manual of Determinative Bacteriology (9th edition). The genomic DNA was investigated through PCR amplification using oligonucleotide primers as described previously[6]. The identified strains were sub-cultured and preserved for further investigation.

Antibiotic Susceptibility tests

The antibiotic sensitivity test was performed by disk diffusion method[7]. The standard powder of vancomycin was used to prepare a serial two-fold concentration dilution. The prepared dilutions were then tested against the resistant isolate of *S. aureus* to determine MIC and MBC using agar dilution method.

Molecular Detection of Vancomycin resistant gene

Total DNA was isolated using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according manufacturer's instruction. The genomic DNA was prepared as described elsewhere[8]. The primers and conditions for PCR amplification of *vanA* gene were also described previously[2]. The thermal cycler used for PCR amplification was ABI 9700 (Applied Biosystem, USA).

DNA sequencing

The resulting PCR products were purified using QIAquick PCR purification kit (QIAGEN). The eluted PCR products were then subjected to nucleotide sequencing using ABI Prism automated DNA sequencer (Applied Biosystems) with the single primer. Saha *et al.* (2008) described the sequencing protocol and the sequences obtained were subjected to BLAST search in the GenBank database for identification.

In Vivo conjugal transfer

Single colony of the identified vancomycin resistant and vancomycin susceptible strains of *S. aureus* were allowed to grow in Luria Bertani (LB) broth at 37^0C for overnight with shaking. To facilitate *in vivo* conjugal transfer, chambers made of sterile stainless steel were implanted subcutaneously on the back of Hartley guinea pigs[9]. Three healthy laboratory animals (535g, 610g and 685g) were used for the purpose. The guinea pigs were inoculated with 10^8 cells through the stainless steel chambers with both vancomycin resistant and susceptible strains of *S. aureus*. The fluid was removed with sterile syringe and was plated directly on blood agar. The samples were drawn from each chamber at 24, 48 and 72h of incubation. The blood agar plates were then allowed to grow at 37^0C for 24h. The confirmatory tests for *S. aureus* were performed according to Bergey's manual (9^{th} ed.). The *S. aureus* con-

jugates were then tested for antibiotic sensitivity by both disk diffusion and broth dilution method.

DNA extraction and identification of *vanA* gene in *S. aureus* conjugates

The isolation, purification and sequencing of DNA was performed using the same procedure described earlier in this paper.

RESULTS AND DISCUSSION

Isolation of bacterial strain

The samples were collected from 278 outdoor patients of Chittagong Medical College Hospital who were prescribed vancomycin. The samples were principally blood (44%) and pus (38%) with few swab and body fluid samples (18%). Only 29 samples yielded positive for *S. aureus*. The isolates were further confirmed using molecular technique, which involved PCR amplification of 16s rDNA using specific primers for *S. aureus* 16s rRNA. The primer was 1.5 kb amplicon identical to 16s rDNA in both resistant and susceptible strains[10].

Antibiotic Sensitivity

The isolates were tested for sensitivity against vancomycin using disk diffusion method. One of the 38 isolates was found to be completely resistant to vancomycin while 3 others showed intermediate sensitivity. Majority of the isolates were sensitive to vancomycin treatment. The MIC and MBC of the isolates were determined using broth dilution method. The resistant strain of *S. aureus* was isolated from the pus sample of an outdoor patient. VRSA and VISA were resistant against a wide range of antibiotics. The MIC value of vancomycin for isolated VRSA was found to be 64 µg/ml. However, the MIC value increased with subsequent transfer of the culture on agar plates. The gene responsible for conferring vancomycin resistance to *S. aureus* is *vanA* gene cluster acquired by *S. aureus* strains through transposon Tn1546 from co-infected *Enterococcus faecium* BM4147 and *E. faecalis*[4]. This study is in conformity to the finding that the inducible nature of vancomycin resistance is due to the presence of *vanA* gene cluster[2] [5].

Transconujgal transfer *vanA* operon in vivo

The VRSA culture obtained from the removal of body fluids of laboratory animals inoculated with resistant and susceptible strains of VRSA were all resistant to vacomycin. The MIC value of the recombinant *S. aureus* strain was reduced to 64 µg/ml. The susceptibility of this strain was analyzed using DAD and Tube dilution methods. This strain was then subjected to DNA profiling.

Detection of *vanA* gene cluster

The PCR amplification of the only VRSA strain showed that it possessed *vanA* operon. The DNA profiling of this recombinant strain showed that it contained both *vanA* and mecA genes in its genome. The *vanA* gene identified using the primers described previously[2]. The recombinant strains were allowed to grow on LB Agar containing vancomycin and teicoplanin as well as LB agar without antibiotic. Both culture yielded vancomycin resistant strains. This finding suggests that *vanA* operon was successfully transferred in vivo from the VRSA strain to VSSA strain. Finally, the plasmids specific for containing *vanA* operon was identified. The 55.6 kb plasmid was obtained from the wild strain of VRSA. The recombinant strain produced another 57.5 kb plasmid. The *vanA* gene cluster was approximately 2.3 kb. The vanHAX gene isolated by Saha et al. (2008) was ~2.6 kb. The only gene cluster that has been identified solely in *S. aureus* strains is *vanA*[5]. This gene cluster encodes for enzyme that reduces pyruvate to D-Lac, and the *VanA* ligase, which catalyzes the formation of an ester bond between D-Ala and D-Lac. The enzyme responsible for this conversion is VanH dehydrogenase encoded by vanH gene, constituent of the *vanA* operon. VanH dehydrogenase mimics the action of lactate dehydrogenase, an essential element of Tricarboxylic Acid cycle and glycolysis. The study suggests that the *vanA* genes cluster is responsible for the vancomycin resistance in *S. aureus*. The van gene cassette was effectively transferred in vivo. Roberts & Falkow (1979) previously recorded the intra species transfer of R- plasmid in an animal model. The transfer was detected in absence of any antibiotic pressure. The PCR product showed 45% homology with the nucleotide sequence of VanH dehydrogenase of the Tn-1546- like element[12].

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