Clonal dissemination of vanA positive Enterococcus species in tertiary care hospitals in Karachi, Pakistan

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Introduction

Emergence and global spread of vancomycin resistant Enterococcus spp. (VRE) is of great concern due to limited therapeutic options. Infections with VRE have high mortality rate, prolonged hospital stay and increased costs. Epidemiology of VRE is complex. This is due to clonal spread of strains because of breach in infection control measures as well as evolution of new strains due to antibiotic pressure. Moreover resistant genes in Enterococcus spp. mainly vanA and vanB genes are plasmid mediated and have the potential to disseminate to more virulent pathogens such as Staphylococcus aureus (S. aureus). Thus large scale emergence of vancomycin resistant S. aureus may be the next stage in the global crisis of antimicrobial resistance. Major strategies to control nosocomial transmission of VRE include "passive surveillance", with isolation of all patients with known previous or current VRE colonization, and "active surveillance" which uses admission cultures, with subsequent isolation of patients who are found to be colonized with VRE.

Increase in the isolation rates of VRE has been reported from hospitals globally including various countries in Asia. VRE was first reported from Pakistan in year 2002 from Aga Khan University Hospital (AKUH). The results of that study revealed monoclonal spread of vanA gene containing strain in adult and neonatal intensive care unit settings. Cross transmission was identified as probable mode of spread. Analysis of AKUH clinical laboratory data over a period of 6 years (from 2002 to date) has revealed increased VRE isolation in clinical samples from other centers in the city (unpublished data). This increase in frequency of VRE is alarming.

In order to curtail this emerging burden of VRE, it is imperative to conduct studies that will give better insight of molecular epidemiology of local VRE strains. Therefore in this study we determined the molecular epidemiology of nosocomial strains of VRE from 6 tertiary care centers of Karachi, Pakistan.

Material and Methods

This cross sectional study was conducted in the clinical and research laboratory of AKUH, Karachi, Pakistan from October 2007 to September 2008. We studied 86 VRE isolates (65 from AKUH and 21 from five other tertiary care hospitals of Karachi) from clinical and surveillance samples. Conventional sampling technique was utilized to collect the isolates. Sources were pus, tissue, urine, blood and rectal swab. Enterococcus spp. was identified using standard methodology. Antimicrobial testing were performed by...
Kirby-Bauer disk diffusion method according to Clinical Laboratories Standards Institute (CLSI).\textsuperscript{14} Vancomycin (30µg) disc was used for screening, followed by agar plate containing 6mg/L vancomycin to confirm vancomycin resistance. Ampicillin (10µg), amoxicillin/clavulanic acid (20/10µg), chloramphenicol (30µg), gentamicin (120µg), erythromycin (15µg), tetracycline (30µg), teicoplanin (30µg) and linezolid (30µg) were used to detect susceptibility. VRE were stored at -80°C in glycerol phosphate buffer.

**DNA Extraction:**

DNA was extracted using the Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer’s instruction with lysozyme (10mg/ml) and mutanolysin (50U). The DNA concentration was determined by Nanodrop (NDU1000; NanoDrop Technologies Inc., USA).

**Polymerase Chain Reaction (PCR):**

The vanA and vanB genes were detected by performing conventional PCR. The primers used to amplify vanA and vanB genes were VanABF; GTAGGCTGGATATTCAAAAGC, Van AR, CGATTCAATTGCGTAGTCCAA and VanBR, GCCGACAATCAATCATCCTC. PCR amplifications were performed in a total reaction mixture of 25µl with PCR master mix (Promega, USA) containing 1µl of 30ng template DNA and 0.4µM of each primer. The reactions were performed on a Perkin-Elmer Gene Amp® 9700 thermal cycler for 30 cycles with the following cycling parameters: initial denaturation at 94°C for 3min, denaturation at 94% for 30s, annealing at 55°C for 30s, extension at 72°C for 1min, and final extension at 72°C for 5 min. E. faecalis ATCC 51299 and E. faecalis ATCC 19433 were positive control for vanA and vanB respectively. E. faecium ATCC 19433 was included in each experiment as negative control in vanA and vanB detection respectively along with a reagent control blank. PCR products were then analyzed by loading 10µl of the amplicon on a 1% agarose gel prepared in 1X Tris Borate EDTA (TBE) buffer run at 100V. The gels were stained with ethidium bromide and photographed using Quantity One Software gel documentation system (Bio-Rad, USA).

**Pulsed Field Gel Electrophoresis (PFGE):**

VRE isolates were subcultured on blood agar for 4 hours at 37°C and isolated colonies were grown overnight in brain heart infusion broth for 16-18 hours. The cells were pelleted by centrifuging at 12,000xg twice for 5 minutes and washed with 2ml Pett IV (PIV) solution (10Mm Tris-Cl [pH 7.4], 1M NaCl) and then pellets resuspended in 2ml PIV solution. 500µl of resuspended solutions was mixed with 125U Mutanolysin and equal amount of 1.6% low melting point agarose (Sigma-Aldrich, USA) and cast into plugs. The plugs were allowed to solidify for 15 minutes at room temperature and placed in lysis solution (6mM Tris-Cl [pH 7.4], 1M NaCl, 0.1M EDTA, 0.5% Brij58, 0.2% deoxyrate, 0.5% Sodium lauroysarcosine, lysozyme [1mg/ml], RNase [0.028mg/ml]) to incubate overnight at 370°C. The lysis solution was replaced by 10ml proteolysis solution (0.4 M EDTA, 1% Sodium lauroysarcosine, Proteinase K [1mg/ml; Promega, USA]) and the tubes were incubated overnight at 50°C. The plugs were then washed thrice with 15 ml 1X TE buffer (10 mM Tris-Cl, 1mM EDTA [pH 8.0]) for 45 min and stored at 40°C. A thin slice (2 mm) of the plug was equilibrated with 100µl restriction buffer (1X Buffer J, 0.1 mg/ml BSA, Nuclease free water) and the tubes were placed on ice for 30 minutes, which was replaced by restriction enzyme buffer to digest the DNA with 20U Sma-I (Promega, USA). The tubes were again kept on ice for 15 minutes and the reaction mixture was incubated overnight at 25°C. Plugs were loaded into the wells of 1.2% agarose (Bio-Rad, USA) gel and run in 0.5X TBE using a contour-clamped homogenous electric-field electrophoresis (Bio-Rad, USA). The following parameters were set for electrophoresis: 200V (6v/CM), temperature: 14°C, initial switch time: 5s; final switch time: 35s, inclined angle:120° and a total run time of 24 hrs. The gel was stained with ethidium bromide for 30 minutes and visualized under a UV light.

**PFGE Fingerprint Analysis:**

The visual analysis of PFGE fingerprints was performed according to the criteria of Tenover et al.\textsuperscript{15} Isolates with PFGE profiles differed by three or fewer bands were grouped in same PFGE type and those differed by four to six bands were assigned to subtypes of the same type. Isolates with difference of more than six bands were considered unrelated and assigned to different group. Major PFGE patterns were named using capital letters (e.g. A, B, C) and similar PFGE patterns were classified as subtypes of a major pattern using an Arabic number following the capital letters. (e.g. A1, A2). A computer assisted dendogram of fragment patterns was constructed by using BioNumerics software (version 4.5; Applied Maths). Clustering was obtained by the unweighted pair group method using average linkages (UPGMA) with the Dice similarity coefficient.

**Data Management and statistical analysis:**

The data was coded and entered into SPSS 16.0 software for statistical analysis. Frequencies of VRE genotype A and B were calculated and the percentage similarity of VRE pulsotypes from within AKUH and other Karachi hospitals was determined.

**Result**

A total of 86 strains were used in this study, of which
were from AKUH and 21 from other 5 tertiary care hospitals of Karachi.

**PFGE Profiles:**

Analysis of PFGE data (Figure-U1) by Tenover scheme showed 1 major pulsotype A with 3 subtypes A1, A2 and A3 among 65 AKUH isolates. The major pulsotype A was 62% (n=40) and 3 subtypes, were A1 25% (n=16), A2 9% (n=6) and A3 4% (n=3) respectively. Among 21 non-AKUH strains same major pulsotype A with its subtypes A1, A2 and A3 were present. Pulsotype A was 38% (n=8) and subtypes, A1,
A2 and A3 were 19% (n=4), 24% (n=5) and 19% (n=4) respectively. Among total 86 isolates (AKUH and non-AKUH), the major pulsotype A was 56% (n=48) and 3 subtypes, were A1 23% (n=20), A2 13% (n=11) and A3 8% (n=7) respectively (Table).

The dice coefficient of similarity among AKUH, non-AKUH and total 86 had a value of 90%, 88% and 89% reflecting their clonal relatedness (Figure-2).

**Genotype Profile:**

60/65 (92%) isolates from AKUH and 19/21 (90%) isolates from non-AKUH had vanA gene (Figure-3). None of isolates showed PCR signals for vanB gene.

**Antimicrobial Susceptibility Profile:**

Analysis of susceptibility profile revealed that all isolates from AKUH and non-AKUH were sensitive to chloramphenicol and linezolid and resistant to ampicillin, amoxicillin/clavulanic acid, erythromycin, teicoplanin, tetracycline and gentamicin, suggesting clonal relatedness.

**Discussion**

Analysis of our results indicate vanA gene as common determinant for glycopeptide resistance in Enterococcus spp and clonal dissemination of VRE in different hospitals in our population.

As epidemiology of VRE is composite, involving clonal spread, transfer of genetic elements and the introduction of new strains. Increase in incidence has been associated with different factors such as antibiotic selective pressure (uncontrolled use of vancomycin), lack of surveillance and hospital infection control policies.

Predominance of one clone suggests frequent transfer of patients from one ward to another ward as well as from one hospital to another. These findings highlight lack of infection control policies and surveillance of VRE colonized/infected patients during inter/ intra-hospital transfer in the city.

Clonal dissemination of vanA gene encoded VRE have been reported from other parts of world. A study from UK reported cross transmission resulting in spread of VRE in their hospital, with predominance of single pulsotype with 92% containing vanA gene. Similarly molecular typing of VRE strains from Argentina have shown predominance of one epidemic clone carrying vanA element in different hospitals, reflecting increase in incidence of clonal spread of VRE. A study from India showed presence of only vanA gene in their isolates. Similarly molecular typing of VRE strains from Brazil showed 7 pulsotypes, with predominance of one type in different hospitals, reflecting spread of single strain in various hospitals. But they also discussed heavy antibiotic selective pressure due to frequent use of vancomycin was the reason of presence of different pulsotypes. In contrast to our findings Bell et al from Australia showed considerable diversity in VRE phenotypes and genotypes and related this finding with higher of usage of vancomycin in both human and animals.

Improper surveillance and infection control measures remain the major factor responsible for dissemination of VRE. To control its spread, the society for Healthcare Epidemiology of America (SHEA) made a guideline implicating the search and destroy policy. According to that active surveillance cultures are essential to identify the reservoir and make control possible using the CDCs recommended contact precautions. Calfee et al from Virginia was able to reduce their VRE incidence rate from 2.07 to 1.25% and maintained it after application of active surveillance and contact isolation. Price et al from Chicago found lower VRE bacteraemia and a more polyclonal population resulted from active surveillance and then contact isolation of high risk population.

In Pakistan, uncontrolled use of vancomycin sets the stage for selection of VRE while lack of active surveillance to detect VRE has lead to its clonal spread to major district health hospitals of Karachi. This finding is a warning for health agencies in Pakistan as majority of strains have vanA genotype which has the potential to transmit resistant determinant to more virulent organism like S. aureus, mostly endemic in our tertiary care hospitals. Prompt attention for the detection of new cases of VRE colonization and employment of infection control policies are strongly emphasized.

Although advance molecular techniques are important in identifying breakdown in infection control measures, however in resource limited country like Pakistan, role of a simple measure like careful interpretation of antibiogram should not be underestimated and can be a useful screening tool for early recognition of clonal spread of resistant isolates in our setting.

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**References**


