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Original Article

Disodium edetate as a conjugation inhibitor: A novel approach to curb growing conjugal transfer of vanA in gram-positive bacteria

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ARTICLE INFO

Article history:
Received 12 December 2012
Accepted 22 January 2013
Available online 19 February 2013

Keywords:
Antimicrobial resistance
Conjugation
Disodium edetate
Methicillin-resistant *Staphylococcus aureus*
Van A

ABSTRACT

Objective: The aim of the present study was to identify the *vanA* gene among clinical isolates of vancomycin-resistant *Staphylococcus aureus* (VRSA). Thereafter, transfer of *vanA* gene through conjugation from *vanA* positive VRSA to a vancomycin-sensitive *S. aureus* was evaluated. Next, we examined the effect of various concentrations of chemicals including ethylenediaminetetraacetic acid (disodium edetate), ethylene glycol tetraacetic acid (EGTA) and boric acid on conjugation.

Methods: A total of fourteen clinical isolates of VRSA were analyzed for the presence of *vanA* gene using previously reported primer by polymerase chain reaction (PCR). The *vanA* positive isolate of VRSA served as donor and vancomycin-sensitive *S. aureus* (*vanA* negative) served as recipient. Conjugation was carried out according to the broth mating method in the absence and presence of various concentrations of chemicals.

Results: The *vanA* gene was detected in eight of the clinical isolates of VRSA. Findings of our study revealed that *vanA* gene was successfully transferred *in-vitro* from VRSA donor to vancomycin-sensitive recipient *S. aureus* by a broth making procedure suggesting possibility of horizontal gene transfer (HGT). Of the evaluated chemicals on conjugation, disodium edetate and EGTA found to be inhibiting the conjugal transfer of *vanA* gene from donor to recipient. Interestingly, it was observed that disodium edetate at a concentration of 10 mM and above strongly inhibited conjugal transfer of *vanA* gene from donor to recipient while EGTA inhibited the same at 120 mM. However, boric acid failed to prevent conjugal transfer of *vanA* gene from donor to recipient.

Conclusion: Bacteria transfer antibiotic resistance from one gram-positive species of bacteria to other bacterial species and thus generating multi-drug resistant bacterial strains. From above study, it can be conclude that disodium edetate at 10 mM and above exhibited a potential effect on the inhibition of transfer of vancomycin resistant gene *vanA* from vancomycin-resistant *S. aureus* to vancomycin-sensitive *S. aureus*. Therefore, the inhibition of conjugation process by 10 mM EDTA can be potentially a novel approach to combat spreading of antibiotic resistant gene.

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0974-6943/$ – see front matter
http://dx.doi.org/10.1016/j.jopr.2013.01.013

1. Introduction

Staphylococcus aureus is one of the most common causes of community and hospital-acquired infections.[1] Vancomycin has been considered the drug of choice for the treatment of methicillin-resistant *S. aureus* (MRSA) infections, but in the last decade, MRSA strains with reduced susceptibility to vancomycin have been reported owing to increase use of vancomycin.[2] Vancomycin resistance is mediated by three classes of gene clusters that confer inducible resistance to high levels of vancomycin and teicoplanin (*vanA*) inducible resistance to various levels of vancomycin (*vanB*), or resistance to vancomycin and low levels of teicoplanin (*vanD*).[3,4] The most common mechanism of vancomycin resistance in MRSA is plasmid-mediated conjugal transfer of the *vanA* gene. The *vanA* gene which codes for an altered target such that the binding of vancomycin to the target is significantly reduced and thus it cannot carry out its normal function of inhibiting bacterial cell wall synthesis.[5] However, the first reported case of reduced vancomycin susceptibility in a clinical isolate of *S. aureus* has not been mediated via acquisition of *vanA*, but by an unusually thickened cell wall containing di-peptides capable of binding vancomycin, thereby reducing availability of the drug for intracellular target molecules.[6,7]

Conjugation is one of the main mechanism of horizontal gene transfer,[8,9] and to be considered one of the major reasons for the development of the multiple-antibiotic resistance. Thus, conjugative transfer of bacterial plasmids carrying resistant genes and spreading of these genes represents a severe problem in antibiotic treatment.[10]

Conjugative transfer of vancomycin resistance from *Enterococcus faecalis* to *S. aureus*,[11,12] from vancomycin-resistant *S. aureus* to vancomycin-sensitive *S. aureus*[13] and transfer of glycopeptide and macrolide-resistance genes by transconjugation among enterococci and from *E. faecalis* to *S. aureus*[14] have been reported. Vancomycin-resistance gene acquisition by *S. aureus* from *Enterococcus faecium* in the clinical environment has also been reported earlier.[15]

In view of the increased spreading vancomycin-resistant *vanA* gene through conjugation, compelled us to explore chemicals that could be used as non-antibiotic adjuvants to control the spreading of resistance gene via conjugation from one gram-positive bacterial species to another species of bacteria. There are no recent study regarding controlling of the spreading of *vanA* gene among the clinical isolates. The aim of the present study was to identify the *vanA* gene among clinical isolates of vancomycin-resistant *S. aureus* (VRSA). Thereafter, transfer of *vanA* gene through conjugation from *vanA* positive VRSA to a vancomycin-sensitive *S. aureus* (VSSA) was evaluated. Next, we examined the effect of various concentrations of chemicals including ethylenediaminetetraacetic acid (disodium edetate), acid (EGTA) and boric acid on conjugation.

2. Materials and methods

2.1. Chemicals

All of the chemicals, such as ethylenediaminetetraacetic acid (disodium edetate), ethylene glycol tetraacetic acid (EGTA) and boric acid were procured from Himedia (Mumbai, India) and were reconstituted with water for injection. Working solutions were prepared using MH (Mueller Hinton, Himedia, Bombay, India) broth.

2.2. Bacterial strains and growth conditions

A total of fourteen clinical isolates of VRSA were used in the present study of which four from patients suffering from surgical wounds and three from bacteremia and seven from patients suffering with burns were recovered. All of the isolates were obtained from Vijayanagar Institute of Medical Sciences (VIMS), Bellary, India. Re-identification of these clinical isolates was done using standard microbiological and biochemical tests.[16,17] The *vanA* positive isolate of VRSA served as donor and was grown overnight at 37 °C in Mueller-Hinton broth (MHB, Himedia, Mumbai, India) and *S. aureus* (MTCC 737) obtained from Institute of Microbial Technology, Chandigarh, India, served as recipient as well as negative control was also grown overnight in MHB. These bacterial suspensions were used as the inoculum at a concentration of 10^6 colony-forming units/milliliter (cfu/ml). *E. faecium* ATCC 51559, which contains *vanA* gene served as a positive control.

2.3. Screening for vanA gene

All of the clinical isolates were processed for screening of *vanA* gene.

2.4. Deoxyribonucleic acid (DNA) isolation

DNA from all of the clinical isolates, recipient, transconjugants, positive and negative controls was isolated using following methods: five ml of each at concentration of 10^{10} cfu/ml was centrifuged at 5000 revolutions per minute (rpm) for 4 min at 25 degree celsius (°C) and pellet was washed once in phosphate buffer saline (0.05 Molar (M) pH 7.2). After addition of 0.2 ml ice-cold solution 1 [25 millimolar (mM) Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 and 50 mM glucose] and 0.4 ml solution 2 [1% sodium dodecyl sulfate (SDS) and 0.2 N sodium hydroxide (NaOH)] Eppendorf tubes were inverted five times gently, and allowed to stand at room temperature for 5 min. Subsequently, incorporated 0.3 ml ice-cold solution 3 (3 M Potassium acetate and 5 M glacial acetic acid) into each tube and inverted five times gently, and allowed to stand on ice for 10 min. After centrifugation (14,000 rpm, 2 min) pellet was dissolved in 0.5 ml of TE (Tris–EDTA, 0.05 M, pH 8.0) and incubated for 5 min at 65 °C, added 0.5 ml of Phenol–Chloroform–Isoamyl alcohol (25:24:1) and shaken thoroughly for 10 min and then solution was centrifuged at 14,000 rpm for 3 min at 4 °C. Supernatant was transferred to another tube and added 1 ml of ice-cold 70% ethanol and centrifuged at 4 °C for 7 min at 7500 rpm. The pellet was air dried and suspended in an appropriate volume of Tris–EDTA buffer. DNA purity and concentration were assayed in a spectrophotometer (260/280).

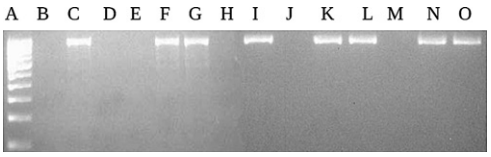

Fig. 1 – Screening of *vanA* genes in clinical isolates. A = ladder (100 bp); B–E clinical isolates recovered from surgical wounds; F–H clinical isolated recovered from bacteremia; I–O clinical isolated recovered from burns. Clinical isolates (I) used as donor in conjugation study.

2.5. *Polymerase chain reaction (PCR)*

The *vanA* gene was detected using previously reported primers.[18] Primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd., Banglore, India. Primer used for *vanA*-F-5′-CATGAATAGAAT AAAAGTTGCAATA-3′ and *vanA*-R-5′-CCCCTTTAACGCTAAT ACGACGATCAA-3′ that amplify a fragment of about 1030 bp. PCR assay was performed in a total volume of 20 microliter (μl) containing 200 picogram (pg) of DNA, 0.5 mM of deoxynucleotide triphosphates (dNTPs), 1.25 micromolar (μM) of each primer and 1.5 U of *Taq* polymerase (Banglore Genei). PCR amplification was carried out on an Eppendorf thermocycler (Germany) with cycling conditions: initial denaturation at 94 °C for 10 min followed by 30 cycles each of denaturation (94 °C for 30 s), annealing (50 °C for 45 s), extension (72 °C for 30 s) and final extension (72 °C for 10 min), for the amplification of *vanA* gene.

2.6. *Gel electrophoresis*

The PCR products were analyzed in 1% (w/v) agarose gel containing 25 μg of ethidium bromide in Tris–EDTA buffer and the gel was photographed under ultraviolet illumination using gel documentation system (Bio-Rad, USA). After electrophoresis, density of PCR product bands were measured by Image J software.

2.7. *Conjugation*

Conjugation study was done by a broth mating method as described elsewhere.[13] Briefly, donor (*vanA* positive VRSA) and recipient (*vanA* negative *S. aureus*) cells at a concentration of 10^6 cfu/ml cells were mixed in one to nine ratio (0.1 ml donor cells and 0.9 ml recipient cells), and was swirled for a few minutes and then incubated at 37 °C for 6 h in M-H broth (without shaking). Transconjugants were selected by plating 0.2 ml on MH agar plate containing 16 μg/ml vancomycin and 2.5 μg/ml ciprofloxacin. Colonies were counted after 48 h of incubation. Donor and recipient cells were also plated separately to check their disability to grow on the vancomycin plus ciprofloxacin plate, because the donor was ciprofloxacin-sensitive and the recipient was susceptible to vancomycin. The transfer of *vanA* was also confirmed by *vanA* gene amplification in transconjugants. To assess the effect of chemicals on conjugation, various concentrations of chemicals ranging from 1 to 150 mM were used. The relative gene transfer was calculated by dividing the % value of each treatment by the % value for the standard. Here transconjugants serve as a standard.

2.8. *Statistical analysis*

Data were analyzed using Graph Pad InStat-3 and expressed as mean ± standard deviation (SD) of three independent experiment. The continuous variables were tested with one-way analysis of variance (ANOVA) and Dunnett's test. Values <0.05 were considered statistically significant.

3. Results and discussion

Re-identification of all of the clinical isolates were done and found to be of VRSA. Among the clinical isolates, only 8 clinical isolates (1 surgical wounds, 2 bacteremia and 5 burns)

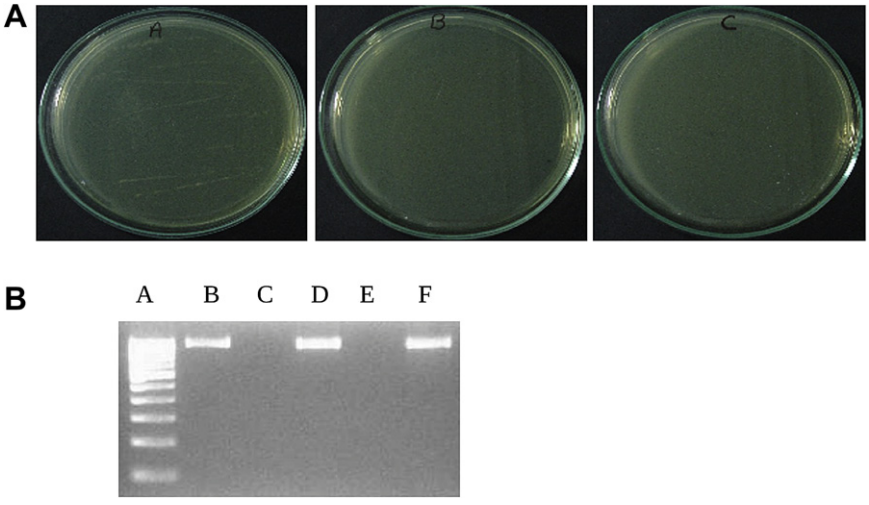

Fig. 2 – A. Proof of concept for formation of Transconjugants. A = transconjugants with vancomycin (16 μg/ml) and ciprofloxacin (2.5 μg/ml); B = donor with vancomycin (16 μg/ml) and ciprofloxacin (2.5 μg/ml); C = recipient with vancomycin (16 μg/ml) and ciprofloxacin (2.5 μg/ml). B. PCR amplification of *vanA* gene in donors, recipients, transconjugants positive and negative controls. A = Ladder (100 bp); B = donor; C = recipient; D = transconjugant; E = negative control; F = positive control

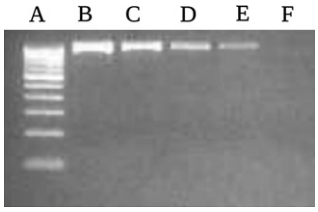

Fig. 3 – Effect of different concentrations of EDTA on conjugation. Detection of *vanA* gene amplification. A = Ladder (100 bp); B = 1.0 mM EDTA; C = 3.0 mM EDTA; D = 5.0 mM EDTA; E = 7.0 mM EDTA; F = 10.0 mM EDTA.

were found to be positive for *vanA* (Fig. 1) and one of the *vanA* positive isolates (from burns sample) used as a donor for conjugation study. Transconjugants were selected by using 16 μg/ml of vancomycin and 2.5 μg/ml ciprofloxacin because these were able to grow in the presence of both of the drugs. Further analysis of transconjugants through PCR confirmed that transconjugants carrying the same gene as donor suggesting that gene transfer had taken place from donor to recipient (Fig. 2A and B). Conjugative transfer of resistant gene has been demonstrated *in-vitro*,[13,14,19] suggesting that genetic exchange of resistance may occur naturally. Moreover, results of conjugation study revealed that when conjugative system was provided with disodium edetate caused a concentration dependent inhibition of conjugation. Treatment with disodium edetate showed a significant conjugation inhibition which started from 4.0 mM (77.5 ± 4.9; $p > 0.05$) and continued up to 10 mM of disodium edetate (Fig. 3 & Table 1). The author hypothesized that 10 mM disodium edetate in combination of antibiotic can be a novel approach to control and spreading of antibiotic resistance. Our lab has already established that disodium edetate to be safe upto 40 mg/kg/body weight when administered intravenously to Swiss albino mice (communicated for publication). Additionally, disodium edetate has been using intravenously in combination with vitamins and minerals in the treatment of various diseases including atherosclerotic vascular disease and renal ischemia.[20,21]

Similarly, when conjugation was studied with various concentration of EGTA and boric acid, EGTA was found to inhibit conjugal transfer for *vanA* gene from donor to recipient at very high concentration that is 120 mM whereas boric acid failed to produces conjugation inhibition upto 150 mM (data not shown). The inhibition of conjugation by disodium edetate could be due to the inhibition of relaxases enzyme. DNA conjugative relaxases and rolling-circle replicating (RCR) initiator proteins, have been known to participate in the binding and coordination of the metal cation (Mg^{2+} or Mn^{2+}) needed for cleavage of the DNA substrate.[22] The relaxases of conjugative and mobilizable plasmids from gram-positive bacteria mainly belong to two families, the IncQ-type family and the pMV158-type family.[23,24] The relaxases encoded by pIP501, pRE25, pSK41, pMRC01, and pGO1 belong to the IncQ-type family.[25]

Table 1 – Effect of EDTA on *vanA* gene transfer determined by Image J software.

Concentration of EDTA (mM)	Relative gene transfer (%)	*p*-value
0	100 ± 5.7	
2.0	94.6 ± 5.2	ns
4.0	77.5 ± 4.9	*
6.0	43.6 ± 4.8	**
8.0	22.5 ± 3.5	***
10.0	0.0	Highly significant

The results were quantified by PCR products band density (mean ± SD). Where ***p-value<0.001, **p-value<0.01, *$p < 0.05$, ^{ns}p>0.05 (non-significant).

4. Conclusion

Bacteria transfer antibiotic resistance from one gram-positive species of bacteria to other bacterial species and thus generating multi-drug resistant bacterial strains. From above study, it can be conclude that disodium edetate at 10 mM and above exhibited a potential effect on the inhibition of transfer of vancomycin resistant gene *vanA* from vancomycin-resistant *S. aureus* to vancomycin-sensitive *S. aureus*. Therefore, the inhibition of conjugation process by 10 mM disodium edetate can be potentially a novel approach to combat spreading of antibiotic resistant gene.

Conflicts of interest

All authors have none to declare.

Acknowledgments

Authors also thankful to sponsor, Venus Pharma GmbH, AM Bahnhof 1-3, D-59368, Werne, Germany, for providing assistance to carry out this study. Dr. J. Mariraj, Vijaynagar Institute of Medical Sciences (VIMS), Bellari, India for providing clinical isolates.

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