*van*A HARBORING ENTEROCOCCAL AND NON-ENTEROCOCCAL SURFACE WATER ISOLATES MONITORED BY AN OLIGONUCLEOTIDE DNA PROBE

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_van_A HARBORING ENTEROCOCCAL AND NON-ENTEROCOCCAL SURFACE WATER ISOLATES MONITORED BY AN OLIGONUCLEOTIDE DNA PROBE

submitted by **MUSTAFA NAKİPOĞLU** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Mevlüde Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Head of Department, **Biotechnology** _____

Assoc. Prof. Dr. Bülent İçgen
Supervisor, **Environmental Engineering Dept., METU** _____

Prof. Dr. Sema Çetin
Co-advisor, **Biology Dept., Kırıkkale University** _____

Examining Committee Members:

Prof. Dr. Aysun Ergene
Biology Dept., Kırıkkale Üniversitesi _____

Assoc. Prof. Dr. Bülent İçgen
Environmental Engineering Dept., METU _____

Prof. Dr. Sema Çetin
Biology Dept., Kırıkkale Üniversitesi _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Biology Dept., METU _____

Asist. Prof. Dr. Robert Waller Murdoch
Environmental Engineering Dept., METU _____

Date: 03.02.2016

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Mustafa Nakipoğlu

Signature :

ABSTRACT

*van*A HARBORING ENTEROCOCCAL AND NON-ENTEROCOCCAL SURFACE WATER ISOLATES MONITORED BY AN OLIGONUCLEOTIDE DNA PROBE

Nakipoğlu, Mustafa
M.S., Department of Biotechnology
Supervisor: Assoc. Prof. Dr. Bülent İçgen
Co-supervisor: Prof. Dr. Sema Çetin

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Untreated wastewaters and treated effluents even after final disinfection inhabitate antibiotic resistant bacteria and resistance genes before they are released into surface waters. A correlation between resistant bacteria with antibiotic resistance genes in surface waters has been found. Of particular interest are vancomycin-resistant enterococci (VRE) harboring *van*A gene that confers their high-level resistance to glycopeptide antibiotics including teicoplanin. Therefore, in this study, river water samples were analyzed to investigate vancomycin- and teicoplanin-resistant bacterial isolates harboring *van*A gene. Out of 290, 18 surface water isolates displayed resistance to both antibiotics. These glycopeptide resistant enterococcal and non-enterococcal isolates, identified by 16S rRNA sequencing, were found to harbor *van*A gene with sequence similarities of 58 to 100%. The presence of D-alanine-D-lactate ligase encoded by *van*A gene was also shown for all vancomycin- and teicoplanin-resistant isolates through western blotting. The fate of *van*A gene in surface waters provides information on the exposure and potential threats of those bacteria for the environment and human health. For this purpose, a 25-mer-oligonucleotide DNA probe based on the 909 bp *BamH*I-*Cla*I fragment from

Enterococcus faecium plasmids pVEF1 and pVEF2 was also prepared by using Vector NTI Express software. Under the hybridization stringency conditions of 46 °C, 55 % formamide and 0.020 M NaCl, designed *van*A probe appeared to be highly specific to *van*A-positive *Enterococcus faecalis* tested. *In situ* fluorescent hybridizations under the same stringency conditions were also used to monitor the river water samples by using fluorescent microscopy. The results indicated that newly designed *van*A-targeted oligonucleotide DNA probe was highly specific and quantitative tool for monitoring vancomycin- and teicoplanin-resistant bacteria in surface waters. Due to reuse of treated wastewater, antibiotic resistant bacteria and resistance genes are being introduced into surface waters and possess human health risks. Therefore, surface waters are not only hot spots for *van*A harboring enterococcal isolates but also non-enterococcal ones due to gene dissemination and require special scientific consideration.

Key Words: Vancomycin, teicoplanin, *van*A, *van*A DNA probe, D-alanine-D-lactate ligase, glycopeptide resistance, surface waters

ÖZ

*van*A GENİ TAŞIYAN ENTEROKOK VE ENTEROKOK OLMAYAN YÜZEY SUYU İZOLATLARININ OLİGONÜKLEOTİT DNA PROBU KULLANILARAK İZLENMESİ

Nakipoğlu, Mustafa
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Doç. Dr. Bülent İçgen
Ortak Tez Yöneticisi: Prof. Dr. Sema Çetin

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Atık sular doğaya salınımlarından önce arıtım tesislerinin tüm proseslerinden geçmiş olsalar bile antibiyotik direnç genlerini ve antibiyotiklere dirençli bakterileri barındırabilmektedirler. Bu bağlamda *van*A geni taşıyan ve teikoplanin de dahil olmak üzere vankomisin gibi glikopeptit antibiyotiklere yüksek seviyede direnç gösteren enterokoklar (VDE) oldukça önem arzetmektedir. Bu nedenle, bu çalışmada, ırmak suyunda *van*A geni taşıyan vankomisin ve teikoplanin dirençli bakteriler araştırılmıştır. Yapılan ön çalışmalar sonucu 290 yüzey suyu izolatı arasından 18 izolatın her iki glikopeptit antibiyotiğe karşı direnç gösterdiği tespit edilmiştir. 16S rRNA sekans analizi ile tanımlanan glikopeptit dirençli bu enterokok ve enterokok olmayan izolatlar içerdikleri *van*A geni bakımından %58-100 oranında homoloji gösterdikleri bulunmuştur. Ayrıca tüm dirençli izolatlarda *van*A geni tarafından kodlanan D-alanin-D-laktat ligaz enziminin varlığı western blot yöntemi kullanılarak gösterilmiştir. *van*A geninin yüzey sularındaki yayılımı ve bunun izlenmesi, dirençli bakterilerin çevreye ve insanlara karşı olası potansiyel zararı hakkında bilgiler sağlayabilmektedir. Bu amaçla Vector NTI Express yazılımı

kullanılarak *Enterococcus faecium* pVEF1 ve pVEF2 plazmidlerinin 909 bp'lik *BamH*I-*Cla*I enzimleriyle kesilmiş kısmından 25-mer'lik bir oligonükleotit DNA probu tasarlanmıştır. Dizayn edilen probun 46 °C, 55 % formamit ve 0.020 M NaCl hibridizasyon koşullarında *van*A-pozitif *Enterococcus faecalis* için yüksek oranda seçici olduğu tespit edilmiştir. Aynı hibridizasyon koşulları kullanılarak ırmak suyundan alınan örnekler FISH yöntemi kullanılarak floresan mikroskop ile analiz edilmiştir. Sonuçlar, yeni dizayn edilen *van*A spesifik oligonükleotit DNA probunun yüzey sularındaki vankomisin ve teikoplanin dirençli bakterilerin izlenmesinde yüksek oranda ayırt edici ve kantitatif bir araç olduğunu göstermiştir. Arıtılmış atık suların tekrar kullanımından dolayı antibiyotik direnç genleri ve antibiyotik dirençli bakteriler yüzey sularına karışmakta ve insan sağlığı için risk oluşturmaktadır. Bu nedenle yüzey suları, direnç genlerinin yayılımı nedeniyle sadece *van*A taşıyan enterokoklar için değil, aynı zamanda enterokok olmayan bakteriler için de sıcak bölgeler oluşturmakta ve özel bilimsel yaklaşımlar gerektirmektedir.

Anahtar Kelimeler: Vankomisin, teikoplanin, *van*A, *van*A DNA probu, yüzey suları, D-alanin-D-laktat ligaz, glikopeptit dirençliliği, VDE

I heartedly present my deepest thankfulness to The Almighty. It is He Who brought me forth from the womb of my mother when I knew nothing; and He gave me hearing and sight and intelligence and affections; that I may give thanks to Him.

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ABBREVIATIONS

ANOVA	Analysis of variance
ARG	Antibiotic resistance genes
BLAST	Basic local alignment search tool
DAPI	4',6-diamidino-2-phenylindole
DD	Disc diffusion
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
FDA	U.S. Food and Drug Administration
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
HGT	Multiple drug resistant
LB	Luria broth
MDR	Horizontal gene transfer
MIC	Minimum inhibitory concentration
NA	Nutrient agar
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NB	Nutrient broth
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDR	Pandrug resistant
PG	Peptidoglycan
PI	Pathogenicity island
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris-EDTA-boric acid
TE	Tris-EDTA

TEC	Teicoplanin
UDP	Uridine diphosphate
VAN	Vancomycin
VISA	Vancomycin intermediate resistant *S. aureus*
VRE	Vancomycin resistant Enterococci
VRSA	Vancomycin resistant *S. aureus*
WWTP	Wastewater treatment plants
XDR	Extensively drug resistant

CHAPTER 1

INTRODUCTION

1.1 Antibiotics and Resistance

Antibiotic resistance is the presence of a genetically determined resistance mechanism categorizing the pathogen as resistant or susceptible based on application of a set procedures in a phenotypic laboratory test (MacGowan and Macnaughton, 2013). Since the first utilization of antibiotics, such as penicillin and streptomycin in 1940's, a dramatic decrease was achieved in mortality caused by certain microbial infections. Most of the drugs still being used today were introduced during the 'golden age' of antibiotics between 1940-1960's, including vancomycin. The origion first antibiotics were natural, while currently they are synthesized chemically. They are several classes of antibiotics; aminoglycosides, quinolones, tetracyclines, aminoglycosides, carbapenems, sulfonamides, β-lactams, macrolides and cephalosporins (Figure 1.1) (Kümmerer, 2009). These antimicrobials may be evaluated on the basis of their principal mechanism of action. There are four major modes of action: (1) inhibition of peptidoglycan (PG) synthesis, (2) inhibition of protein synthesis, (3) inhibition of nucleic acid synthesis, and (4) interference with a metabolic pathway (Lin *et al.*, 2015).

β-lactams such as penicillins, cephalosporins, carbapenems and mono-bactams, and the glycopeptides, including vancomycin and teicoplanin interfere with bacterial peptidoglycan synthesis (MacGowan and Macnaughton, 2013). β-lactams inhibit cell wall synthesis by distrupting the enzymes responsible for peptidoglycan (PG) synthesis (Lin *et al.,* 2015). Vancomycin and teicoplanin prevent peptidoglycan synthesis by binding to the D-alanine precursors of the growing PG chain, thereby interfering with the cross-linkage of the PG subunits (Courvalin, 2006).

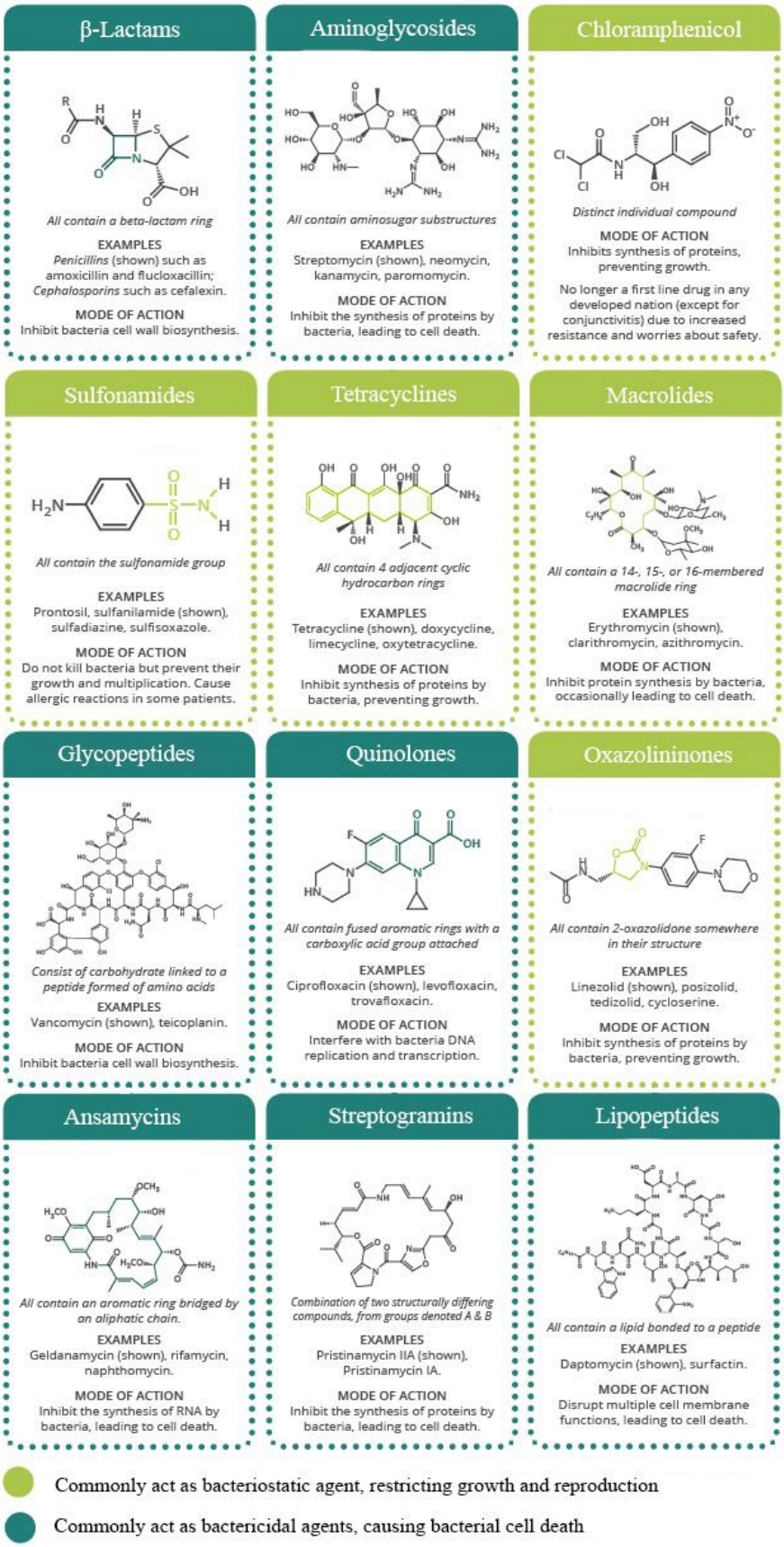

Figure 1.1 Classes of antibiotics and their mode of action (Compound Interest, 2014)

Chloramphenicol, aminoglycosides, streptogramins, tetracyclines, macrolides and oxazolidinones exert their bactericidal effect by blocking protein synthesis (McManus, 1997). Bacterial ribosomes have differences in terms of structure when compared to eukaryotic ribosomes. Therefore, antibacterial agents are able to selectively inhibit bacterial growth by specifically targeting prokaryotic ribosomes. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit (Tenover, 2006)

Fluoroquinolones act on DNA synthesis bringing about lethal double-strand DNA breaks in DNA replication process. Sulfonamides and trimethoprim inhibit the pathway for folate synthesis, which ultimately inhibits DNA synthesis (Petri, 2006)

Disruption of bacterial membrane structure is another mechanism of antibacterial action. It is argued that polymyxins increase cell membrane permeability which in turn cause cell lysis (Falagas *et al.,* 2005) The cyclic lipopeptide daptomycin apparently inserts its lipid tail into the bacterial cell membrane, causing membrane depolarization and eventual death of the bacterium (Carpenter and Chambers, 2004).

Due to the unguided overuse of this antibacterial weaponry, an unexpected scenario has been faced. In addition to the therapeutic use of antibiotics by humans, it has also been used for non-therapeutically purposes such as to boost animal growth (Davies, 2006). Eventually, this has led to the acquisition of resistance against the antibiotics such as penicillin and cephalosporin. By the decline of these antibiotics and the lack of discovery in finding newer drugs, there has been a hectic pursuit of new ways to tackle the ever-expanding microbial resistance threat after 1990’s (Figure 1.2).

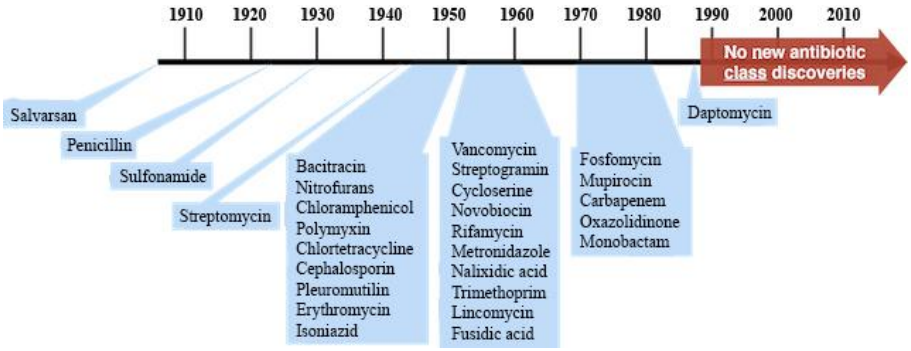

Figure 1.2 Chronograph of the antibiotic discoveries (Silver, 2011)

According to the First Global Report on Antimicrobial Resistance, very high rates of antibiotic resistance have been monitored in bacteria associated with nosocomial and community-acquired infections in all World Health Organization (WHO) regions (Figure 1.3) (WHO, 2014).

It is important to note that resistance mechanisms originate pre-therapeutic use of antibiotics, in non-pathogenic bacteria. There are strong evidences support the notion that many types of resistance mechanisms and resistant bacteria have existed long before production, utilization and spread of antibiotics (Aminov *et al.,* 2007; Mindlin *et al.,* 2008; Allen *et al.,* 2009; D'Costa *et al.,* 2011).

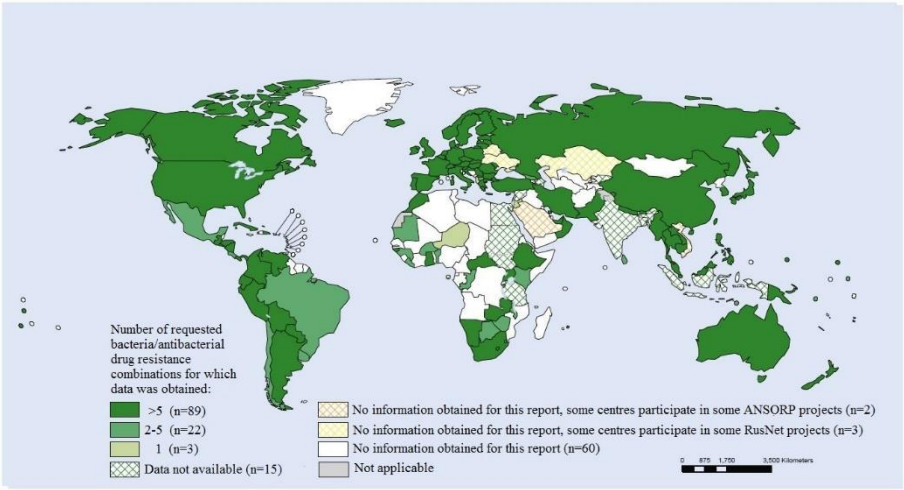

Figure 1.3 Availability of data on resistance for selected bacteria–antibacterial drug combinations (WHO, 2014)

Ancient gene libraries of antibiotics and resistance mechanisms have been discovered among certain bacteria that shed light onto millions of years of evolution. Super integrons, large chromosomal DNA elements as shown in *Proteobacteria*, appear to be such a gene library. *Vibrio vulnificus* harbors such an integron that contains hundreds of gene cassettes with various resistance factors, whose individual functions have not been discovered completely yet (Bradley, 2013).

Bacteria respond to the previously mentioned effects of antibiotics by several ways which can be categorized in four types:

Modifying or eliminating the target protein to which the antibacterial antibody binds by a spontaneous mutation or a gene transfer. Change in penicillin-binding protein 2b in Pneumococci that results in penicillin resistance, alteration of PG precursors to D-alanine-D-lactate from D-alanine-D-alanine in Enteroccocci that results in vancomycin resistance. *mec*A gene encoding methicillin resistance in *S. aureus* can be given as example to these mechanisms (Lambert, 2005).

Production of enzymes that distrupt the antimicrobial agent is another way of resistance against antimicrobials. These include hydrolysis, group transfer, and redox mechanisms. Although hydrolysis is clinically important, particularly as applied to β-lactam and macrolide antibiotics, the group transfer approaches are the most diverse and include the modification by acyltransfer (aminoglycoside, chloramphenicol, type A streptogramin), phosphorylation (aminoglycoside, macrolide, rifamycin), glycosylation (macrolide, rifamycin), nucleotidylation (aminoglycoside, lincosamide), ribosylation (rifamycin), and thiol transfer (fosfomycin) (Wright, 2005).

Downregulation of an outer membrane protein that the antibacterial agent requires for cell infiltration. Downregulation of OmpC porins in *Escherichia coli* against β-lactams, OprD porins in *Pseudomonas aeroginosa* against carbapenems, OmpU

porins in *Vibrio cholera* against cephalosporins are examples of such resistance mechanism (Fernández and Hancock, 2012).

Upregulating efflux pumps that expel the drug from the cell is another well studied mechanism of antibiotic resistance. Efflux of fluoroquinolones in *S aureus*, aminoglycosides in *Acinetobacter baumannii*, macrolides in *Enterococcus faecium* are examples to such resistance mechanisms (Figure 1.4) (Tenover, 2006).

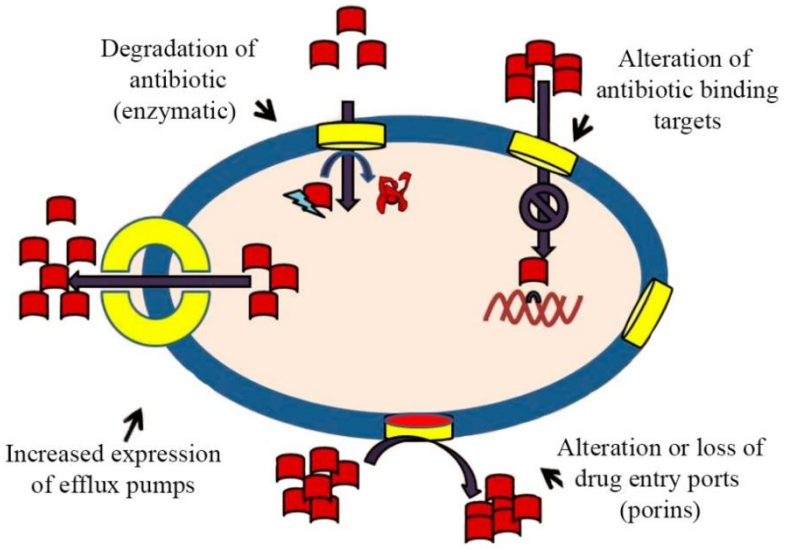

Figure 1.4 Bacterial antibiotic resistance mechanisms (Andersen *et al.*, 2015). Red blocks indicate antibiotics. Yellow channels indicate drug entry ports/porins

Discovery and availability of new antimicrobials, increasing or decreasing public health importance are all relevant in scientometric analyses, which in turn give ideas on the medical need for the invention or discovery of novel antimicrobials. In total, 49690 publications released for the years 1940–2013 related to 'fluoroquinolones resistance' (2901), 'tetracycline resistance' (2933), 'methicillin resistance' (18706), 'β-lactamases' (22275) and 'vancomycin resistance' (2875) (Figure 1.5a). For estimation of diseases by individual species, 54381 publications released between 1940 and 2012. In these publications, 21737 referred to *Staphylococcus aureus*, 10241 to *Pseudomonas aeruginosa*, 17447 to *Escherichia coli*, 3512 to *Klebsiella*

pneumoniae and 1444 to *Acinetobacter baumannii* (Figure 1.5b). The relative research interest is contrasted with the data from EARS-Net (ECDC, 2011) to assess the accuracy of the search results regarding the development of resistance (Figure 1.5c) (Brandt *et al.*, 2014).

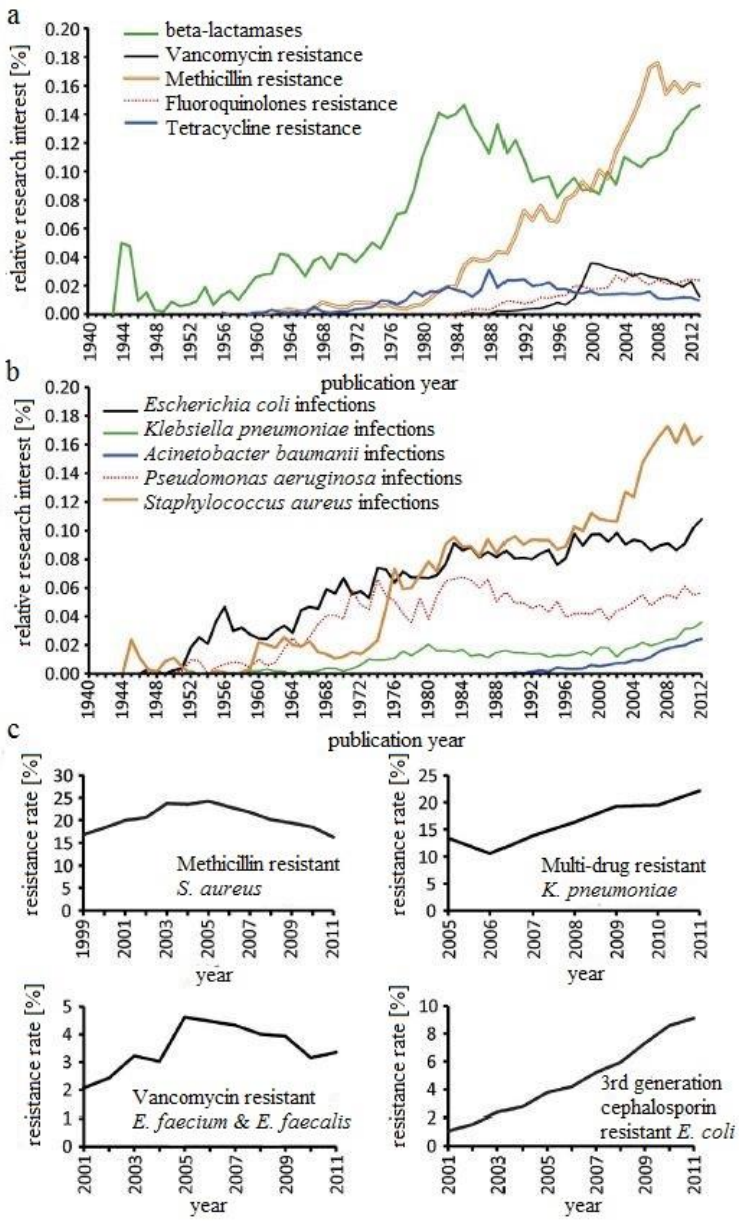

Figure 1.5 Scientometric analyses of antibiotic resistances. (a) Global resistance trends calculated by scientometric analysis, (b) global infection trends calculated by scientometric analysis and (c) resistance rate for each year calculated out of the European Antimicrobial Resistance Surveillance Network (EARS-Net) database. The x-axes in (c) begin with meaningful ascertainment (>5 contributing countries/year) of EARS-Net (Brandt *et al.*, 2014)

1.2 Reservoirs of Resistance

The increase in antibiotic resistance can be examined in various major areas suc as; antimicrobial resistance in livestock and poultry animals, industrial food chain, environment, and within the healthcare setting (Goñi-Urriza *et al.,* 2000). As enormous amounts of antibiotics are still being used as growth boosters as well as cures for many different infections among farm and aqua cultures, the selective pressure on commensal and pathogenic bacteria increase. Although it remains controversial as to state clearly that such manner of usage poses a threat on human health, it obviously turns food animals into antimicrobial resistance pools (Figure 1.6) (Marshall *et al.,* 2011; Liebana *et al.,* 2013).

Figure 1.6 Antibiotics are over-used in animal farms such as chicken poultries (Wendy *et al.,* 2015)

Antimicrobial resistance has also been increasing in the community in the last decades (Martinez, 2014). One of the most important reasons lying beneath this fact is that antibiotics are used inappropriately due to either obsolete guidelines or pharmaceutical companies' pressure on prescribers. Moreover, since the community's awareness in this field is inadequate, antibiotic resistance continued to rise worldwide, posing a threat to our society (Spellberg *et al.,* 2008; Coenen *et al.,* 2013). The overuse of antibiotics by humans and especially animals (Figure 1.7) has also a great effect on the accumulation of these compounds (Wellington *et al.,* 2013).

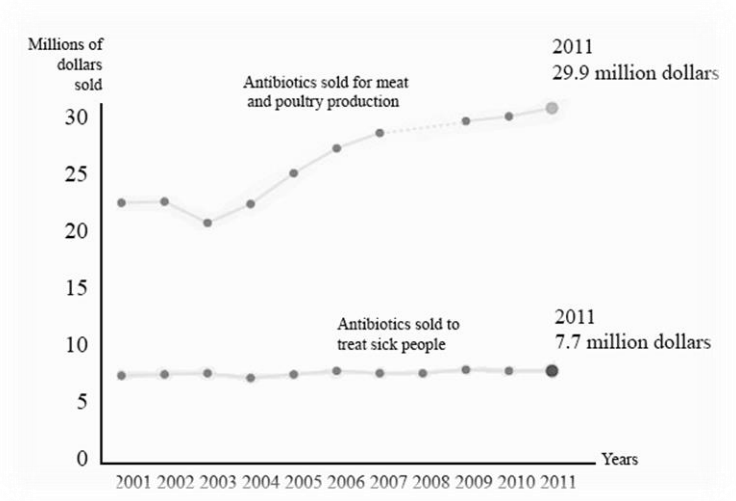

Figure 1.7 Antibiotics used for meat production in the United States nearly quadruples the amount used for humans (The Pew Charitable Trusts, 2013)

Antimicrobials and their metabolites have various ways of infiltration to the environment, including sewage from domestic areas or hospitals through manure and water bodies (Finley *et al.*, 2013; Heuer *et al.*, 2009). Wastewater treatment plants contain a higher proportion of resistant bacterial populations compared to surface waters (Figure 1.8) (Huang *et al.*, 2012). Suggesting that WWTP's have become hot zones for horizontal gene transfer (HGT).

Figure 1.8 Wastewater treatment facilities can be hotspots for horizontal transfer of antibiotic resistance. The photo shows a clarifier in the United States (EPA, 2015)

This results in co-selection of genes providing resistance to antimicrobials, biocides, heavy metals, pollutants, disinfectants or detergents (Baquero *et al.*, 2008). Schwartz *et al.* (2003) examined the existence of antibiotic resistant bacteria in biofilms collected from nosocomial and domestic wastewater, river and tap water in Germany. Vancomycin-resistant enterococci (VRE) and β-lactam-hydrolyzing *Enterobacteriaceae* have been detected more frequently in wastewater biofilms when compared to river or tap water biofilms. Also, antibiotic resistance genes such as *van*A, *mec*A, and *amp*C were observed at escalated levels in nosocomial wastewater. Willems *et al*. (2005) determined the population structure of 411 VRE and vancomycin-susceptible *Enterococcus faecium* isolates by using multilocus sequence typing. They assessed the evolutionary homology of bacteria associated with documented nosocomial outbreaks and other isolates. In addition, the association with ampicillin resistance and the presence of a recently discovered putative pathogenicity island (PI) in *E. faecium* was assessed. (Bouki *et al.*, 2013). Gaddad *et al.* (2009) investigated the presence of vancomycin resistance among methicillin resistant *S. aureus* isolates from intensive care units of hospitals in India by using conventional methods and found that all methicillin resistant *S. aureus* (MRSA) isolates were also VRSA with vancomycin MIC range of 16-64 mg L^{-1}.

Microorganisms are in a tight interaction with in the environment. In particular, bacteria effect and get affected by changes in interaction network. As a part of this interaction, vast amounts of gene flux takes place among different bacterial populations, to such an extent that they demonstrate similar survival characteristics, typically by antibiotic resistance genes. Through this way, resistance genes may rapidly spread from on region to another, from one species to the other (D'Costa *et al.*, 2006). The widespread dissemination and distribution of antimicrobials in hospitals and agricultural environments lead to constant selective pressure that favors pathogenic bacteria over non-pathogenic (Figure 1.9).

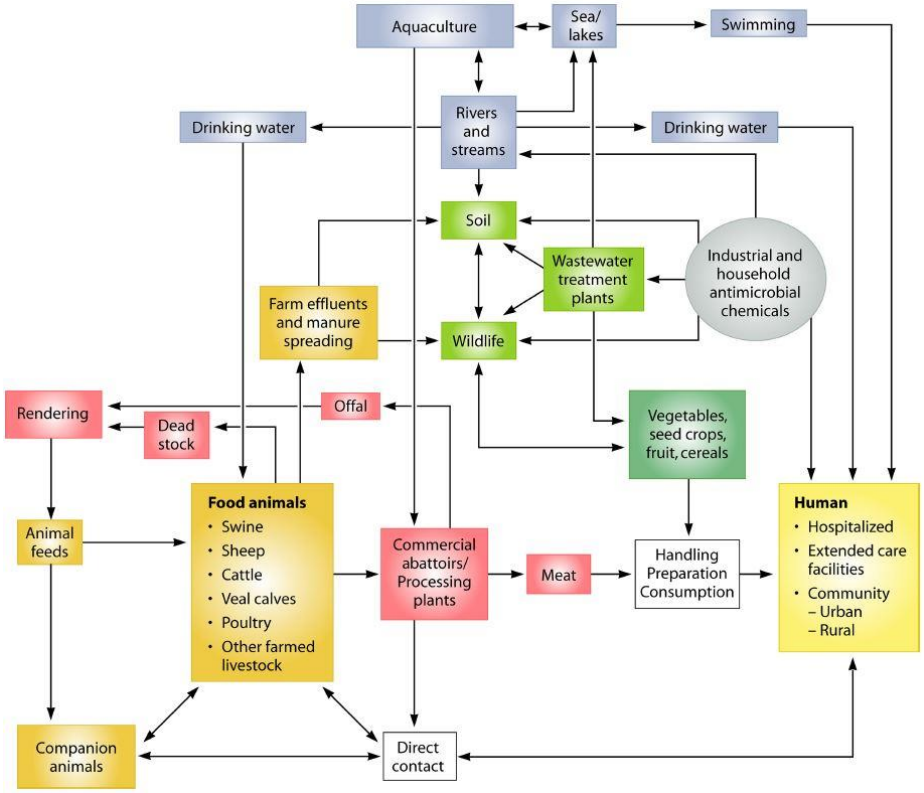

Figure 1.9 Spread of antibiotics and antibiotic resistance within community, WWTP, agricultural, hospital and associated environmental settings (Doyle *et al.*, 2006)

In the aquatic environments, some of the microbiotas are killed by the antibiotic residues thus affecting the microenvironment as well as selection of resistant bacterial organisms. Some members of the microbiota have genes for drug resistance as a part of their genomes. When the population of bacterial cells is treated with an antibiotic, the sensitive members gradually fade away and resistant strains become dominant. The pathogenic bacteria then multiply and disseminate in the environment with larger numbers (Figure 1.10) (Bbosa *et al.*, 2014).

As antimicrobial compounds accumulate in the environment, it selects the resistant microorganisms over non-resistant ones and resistance ones become dominant (Andersson *et al.*, 2012). The more dominant they become the more exposed humans and animals to them.

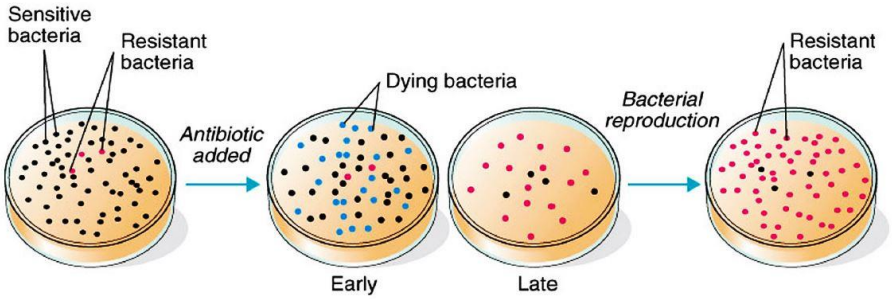

Figure 1.10 Selection of microbial resistance (Bbosa et al., 2014)

Antibiotics in the environment, as emerging pollutants, require urgent remediation with particular focus on urban and heavily populated demographic sites. Current studies indicate that metabolites of antibiotics can be persistent, and accumulate in foods and water sources, including ground-waters (Manzetti and Ghisi, 2014). The major source of these contaminations is wastewaters, which are released to surface waters later. Modern decontamination approaches in WWTP's do not remove the antibiotic compounds fully, resulting in a low but constant concentration remaining present in the environment (Michael *et al.*, 2013). The improvement of wastewater decontamination methods, and prolonged incubation in biological reactors can significantly reduce the diffusion of antibiotics in the environment (Manzetti and Ghisi, 2014).

Agricultural sector also has a role in the dissemination of antibiotic resistance. Antibiotics and their metabolites are transferred to agricultural fields via manure and sludge, resulting in local cycles of antibiotic transfer from animals to soil, and from soil back to animals and ultimately to humans (Figure 1.11) (Heuer *et al.*, 2011). Further studies are mandatory for the antibiotics in the environment. New and different compounds with different half-lives and different environmental fates are continuously emerging which may thus require new and different decontamination methods to halt further accumulation in the environment.

Figure 1.11 Antibiotics and their metabolites are transferred to agricultural fields via manure and sludge. The photo shows a large farm growing a variety of foods in Vietnam (Jarvis, 2009)

The current legislations on water quality mainly take indicator microorganisms as the sole determinant but do not take antibiotic concentrations of sewages and treatment plants into consideration. Mitigation of the risks caused by antibiotic contaminations and accumulations relies on strategies that aim to improve industrial systems for sanitation and decontamination of sewage waters (Pruden *et al.,* 2013). Antibiotic prescription guidelines must be updated with a regulation of active reporting on prescription and consumption. The implementation of such strategies may require significant legislative reforms, which depend on a dedicated commitment by international policy makers (Spellberg *et al.*, 2011).

1.3 Modes of Transmission

As the WHO's report suggests, the resistant microbial biota has not only prevailed during the enforced selective pressure applied by the antimicrobials in the past few decades but also has been able to flourish by a various ways of genetic augmentation (Skalet *et al.*, 2010). After the discovery of transferable resistance in Japan around 1950s, various ways of mutation and horizontal gene transfer (HGT) based defense mechanisms were identified in bacterial cells (Figure 1.12) (Dzidic *et al.*, 2008).

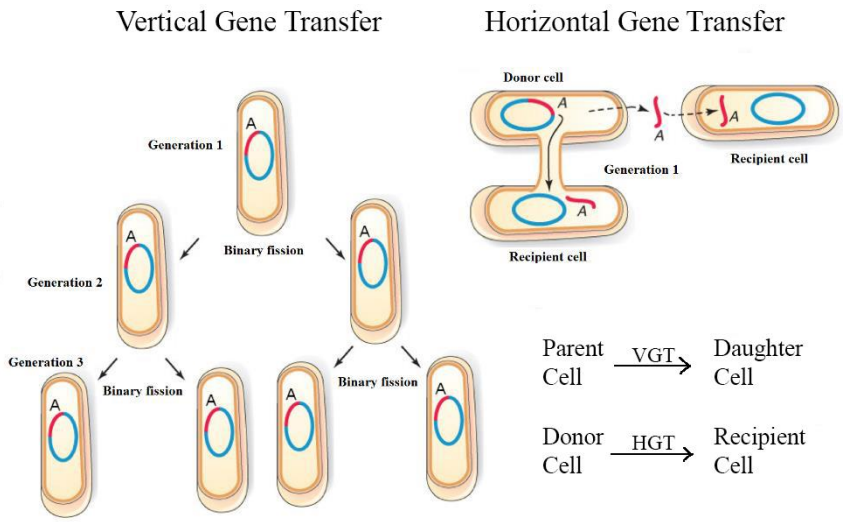

Figure 1.12 Schematic representations of VGT and HGT (Francino, 2012)

Since vertical transfer of genes (VGT) from one generation to the other is not the sole way of genetic transfer, the role of HGT, which occurs by transformation, transduction or conjugation of the encoding genes, in the dissemination of resistance genes in bacteria is of great significance (Figure 1.13) (Schwartz *et al.*, 2003).

Dissemination of antibiotic resistance by HGT has been shown in various settings such as aquatic environments, soil, animals and plants (Miller, 1998). circular independent DNA's called plasmids are the most common mobile genetic elements that carry DNA from a donor to receipient. In contrast to chromosomal DNA, plasmid DNA mainly carries special genes for special circumstances instead of genes required for regular cell growth. Special circumstances may demand antibiotic resistance, heavy metal resistance, unique metabolic functions, or production of antibiotics, toxins and virulence factors (Guardabassi and Dalsgaard, 2002)

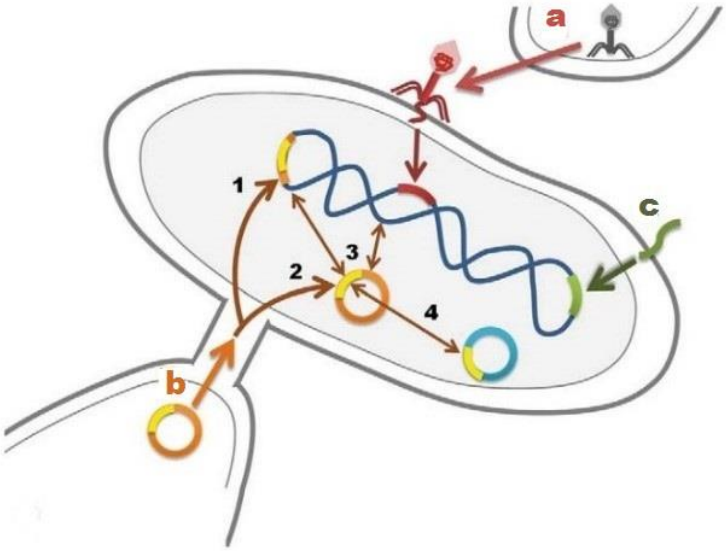

Figure 1.13 Mechanisms of DNA transfer between and within bacteria. (a) Transduction: injection of DNA into a bacterium by a phage. (b) Conjugation: plasmid in a donor bacterium is transferred through a pilus into a recipient bacterium; plasmid may integrate into the chromosome (1) or remain in the cytoplasm (2); plasmid may be transferred between cytoplasmic and chromosomal locations (3); plasmid may exchange insertion sequences or transposons with other plasmids (4) or the chromosome. (c) Transformation: uptake of naked DNA from the environment (Gyles and Boerlin, 2014).

Eventually, multiple resistances against antibiotics have become widespread by the transfer of these pathogenic gene clusters, which is also called as pathogenicity islands (PAI) (Table 1.1) (Figure 1.14) (Bhatt *et al.*, 2015). This phenomenon is common in the some of the Gram-positive bacteria, namely *Enterobacteriaceae* family (Fluit *et al*., 2004). *Acinetobacter baumanii* can be given as prominent example to the appearance of multi-drug resistant bacteria, which is now one of the major nosocomial pathogens. Some of its strains harbor genomic islands composed of 85 genes that encode various proteins against 6 different antimicrobial classes (Fournier *et al*., 2006).

Table 1.1 Mobile genetic elements involved in horizontal gene transfer of virulence determinants (Gyles and Boerlin, 2014)

Element	Definition	Mechanism of transfer
Plasmid	Extrachromosomal self-replicating genetic element	Conjugation
Bacteriophage	Virus that infects and replicates within bacteria	Transduction
Integrative and conjugative element	Self-transmissible but not self-replicating genetic element that encodes the machinery for integration into or excision from the bacterial chromosome and for conjugative transfer.	Conjugation
Pathogenicity island	Part of a genome that shows evidence of past horizontal gene transfer and foreign origin. The pathogenicity island may be part of the chromosome or a plasmid.	Transduction, conjugation, transformation

Another element playing roles in transmission of antibiotic resistance are insertion sequence IS elements. They are the simplest transposable elements that only fulfill the minimum requirement for their own transposition. They encode only the enzyme(s) required for their own transposition.

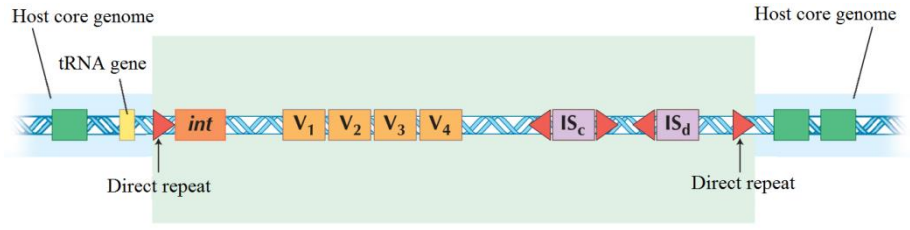

Figure 1.14 Diagram of a PAI (within the large green shaded area). Flanking the islands are core host genes (small blue shaded regions). Immediately flanking the island on the left is a tRNA gene – many islands are found near or within tRNA genes. At the edges of the islands are direct repeats (red triangles). Inside the island are virulence genes (orange boxes) and some insertion sequences (purple boxes) (Schmidt *et al.*, 2004).

Currently, transposons are usually classified into three groups: (i) composite transposons; (ii) Tn*3* family transposons; and (iii) conjugative transposons. Enterococcal transposons from each of these groups is represented in Figure 1.15. Composite transposons have flanking copies of IS of the same family that cooperate to transfer the DNA between them and are associated with *van*B1-related resistance.

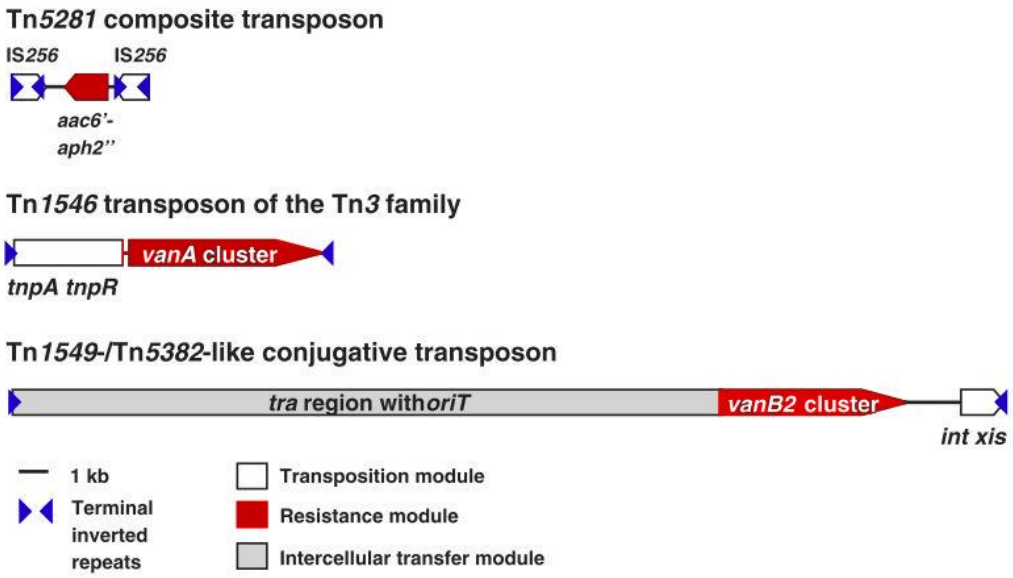

Figure 1.15 Schematic presentation of the three transposons representing the transposon groups transferring resistance genes in Enterococci (Hegstad *et al.*, 2010)

The Tn*3* family of transposons move intracellularly by the help of a transposase and resolvase. Tn*3*-like transposons mediate high-level *van*A-type resistance. Conjugative transposons encode all information necessary for their own excision, conjugation and integration into a new host (Hegstad *et al.*, 2010). There are solid evidences that HGT may have a profound effect on prokaryotic evolution (Rowe-Magnus *et al.*, 2001; Bradley, 2014). In the development and spread of resistance genes, integron structures are also common (Figure 1.16). They are composed of gene cassettes, which may be converted into resistance causative genes by insertion next to active promoters in order to assure increased transcription rates (Shapiro, 2012).

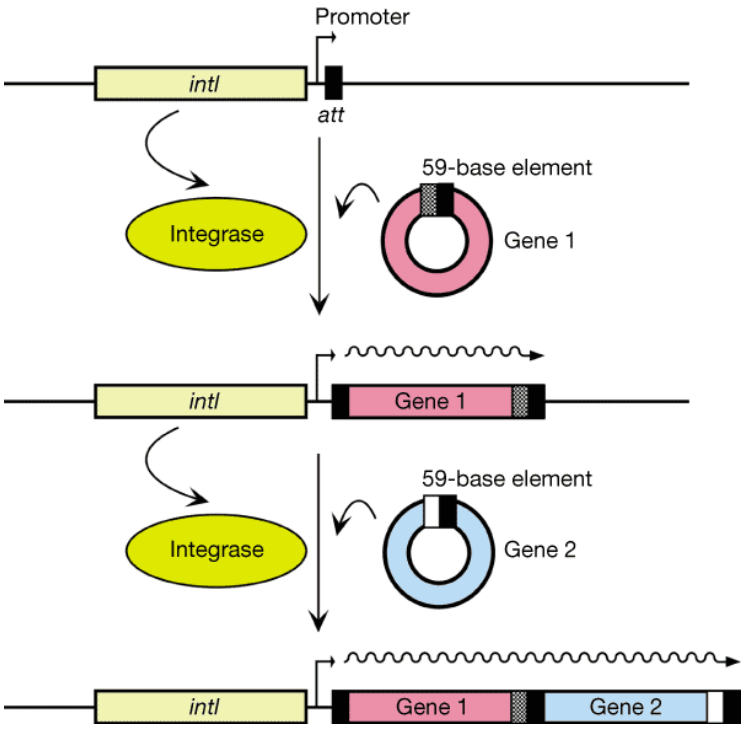

Figure 1.16 Integrons are composed of an attachment site (*att*), a gene encoding a site-specific recombinase (*intI*) and a promoter that regulates expression of the inserted gene cassette. (Ochman *et al.*, 2000).

In addition to these classic HGT types, there are reports of phage-mediated HGT. According to Oliver *et al.*, (2005) a plasmid from *Klebsiella pneumonia* contains a phage-related element, which was detected in *Enterobacteriaceae*. Intermediate stages between transposons and phage genomes can also be found. Wyres *et al.* (2013) reported that antimicrobial resistance-conferring Tn*916*-like elements identified in phages and also found in a PAI. Another example to phage-mediated HGT was given by Mašlaňová *et al.* (2013) by demonstrating a group of bacteriophages that can package concurrently *ccr*A1 and *mec*A resistance genes located at (staphylococcal cassette chromosome *mec*) SCC*mec,* into their capsids. Also, it has been very recently found that phage-like particles have also evolved, that can package random pieces of the producing cell's genome (Penades *et al.*, 2014). These finding indicate that phage-elements also have a significant role in the emergence of HGT.

As Gram-negative bacteria, *Enterobacteriaceae* are also drawing attention as multidrug resistant pathogens that but the health sector in a challenging position (Goñi-Urriza *et al.*, 2000). A significant contributing factor is the acquisition of large plasmids encoding resistance factors for various drug classes. Plasmids with diverse integrons and transposons that encode every class of β-lactamase were found in microorganisms such as *E.coli* and *Klebsiellae* (Bush, 2010). High multi-drug resistance (MDR) rates lead to increasing consumption of second and third level, and finally, last-resort antimicrobials (see Table 1.2 for definitions).

Table 1.2 Definition of different terms of multi-drug resistance (Cantón and Ruiz-Garbajosa, 2011)

Term	Definition
Multidrug resistant (MDR)	Non-susceptibility to at least one agent in three or more antimicrobial categories*
Extensive drug resistant (XDR)	Non-susceptibility to at least one agent in all but two or fewer antimicrobial categories*
Pan-drug resistance (PDR)	Non-susceptibility to all agents in all antimicrobial categories*
Co-resistance	Presence of different resistance mechanisms encoded by mutated or acquired genes
Cross-resistance	Presence of mutated or acquired resistance genes affecting antimicrobials agents from the same class.
Pleiotropic resistance	Presence of a resistance mechanism affecting several antimicrobial classes owing to the same genetic event such as mutation or acquisition of a resistance gene.

*Therapeutic categories (i.e. aminoglycosides, fluoroquinolones, cephalosporins, carbapenems, etc.)

In wealthy countries, a slowly growing number of patients will be untreatable due to pandrug (PDR) resistance. Usually, used ineffective antibiotics in the case of extensively drug resistance (XDR)/PDR infections in critically ill patients will result in high mortality. In poor countries, multidrug resistance will lead to untreatable

resistant infections much earlier in this chain of events as they will not sufficiently have a broad selection of second generation drugs, and novel antibiotics may not be available or affordable for them (Theuretzbacher, 2013).

1.4 Glycopeptide Antibiotics

Ever since the invention of penicillin, many different β-lactam antibiotics have been developed and their molecular mechanism elaborated. Meanwhile, an additional class of antibiotics that attack on the same pathway by a different mechanism than that of penicillin has been described. Glycopeptide antibiotics target the membrane-bound steps of the biosynthesis of cell wall, which eventually cause their death (Schneider and Sahl, 2010). With the help of new methods for elaborating the prokaryotic cellular pathways newer insights will be available for us to exploit in the ongoing pharmaceutical struggle against resilient pathogens.

1.4.1 Importance of Glycopeptide Antibiotics

Vancomycin was first discovered by in 1953 from Actinomycete, later named as *Amycolatopsis orientalis* (McCormick *et al.*,1955) and in 1958, it was approved by U.S. Food and Drug Administration (FDA) for clinical usage. However, methicillin and cephalosporins were rather more utilized due to their less side effects. Nevertheless, the consumption of vancomycin has risen as it has been obtained in a purer way, which reduced its toxicity. Along with teicoplanin (Somma *et al.*, 1984), another glycopeptide antibiotic that was introduced to clinics in 1988, vancomycin is indispensable antimicrobials for the treatment of infections caused by Gram-positive bacteria. These antibiotics inhibit the cell wall synthesis by binding to the precursors in their own unique manner. Vancomycin-type glycopeptide activity is based on dimerization whereas teicoplanin and its derivatives anchor themselves into phospholipid bilayer by fatty acid chains (Figure 1.17) (Jovetic *et al.*, 2010).

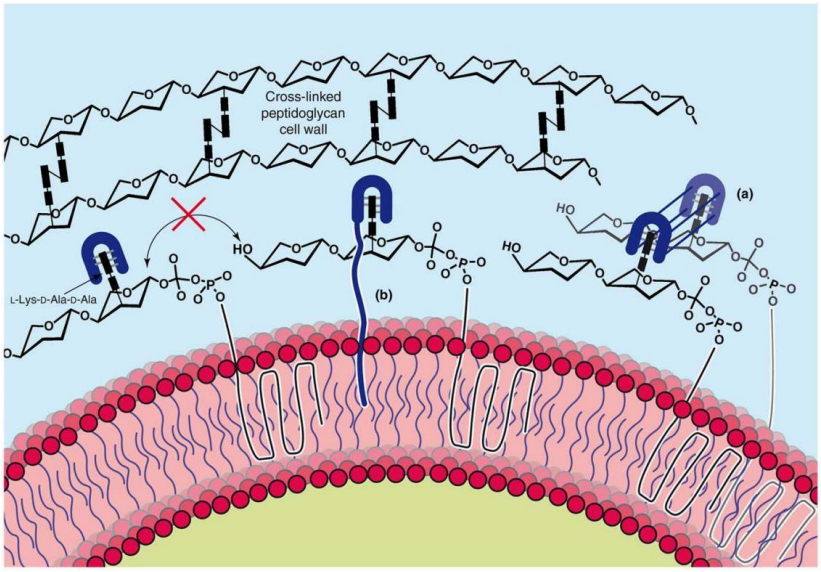

Figure 1.17 PG structure and mechanism of action of glycopeptides antibiotics. Glycopeptides inhibit transglycosylation and transpeptidation by binding to the C-terminal D-alanyl-D-alanine of the late PG precursor. (a) Vancomycin-type glycopeptide activity is based on dimerization, which enhances binding to the target peptide through both cooperative and allosteric effects. (b) Lipoglycopeptides (e.g. teicoplanin and its derivatives) have fatty acyl chains anchored in the phospholipid bilayer that enhance the binding affinity (Jovetic *et al.*, 2010)

Glycopeptide antibiotics that target cell wall biosynthesis are very useful in dealing with Gram-positive pathogens. This is due to the mechanism that is unique to prokaryotes and therefore, less toxic to mammalian eukaryotic cells (Bbosa *et al.*, 2014). In addition, cell wall synthesis is composed of a complex pathway, which can be interfered at many steps, from the biogenesis of the PG monomers to cross-linking of PG subunits. Therefore, glycopeptide antibiotics that target the PG layer are very valuable in struggling Gram-positive pathogens.

To fully comprehend glycopeptide resistance, it is a must to understand the biosynthesis of cell wall. PG, also known as murein, is a biopolymer composed of β-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid residues. The monomers are synthesized in the cytosol as UDP-N-acetylglucosamine and UDP-N-acetylmuramic acid. The latter is later converted to UDP-MurNAc pentapeptide by the addition of five amino acids, usually including the dipeptide D-alanyl-D-alanine.

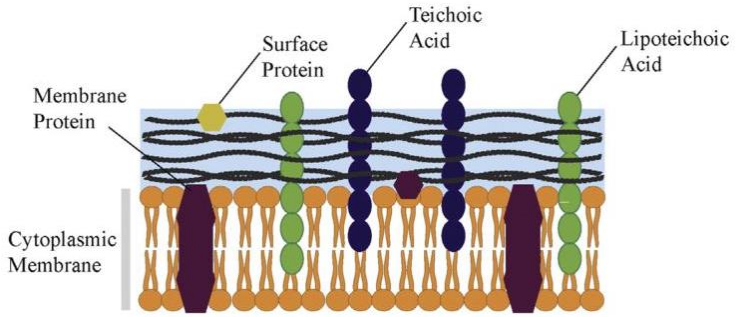

Figure 1.18 Schematic diagram of bacterial cell wall in a Gram-positive bacterium (R.M. Epand *et al.*, 2015)

A transmembrane protein called bactoprenol carries these monomers through the cell membrane and are inserted to the growing glycan chain by crosslinking (White, 2007). PG is an essential component of Gram-positive and Gram-negative bacterial cell wall that increases cell membrane durability against osmotic pressure and lysis (Tortora *et al.*, 2007). Also it has been discovered that actually the MreB protein gives give shape to cells and not PG layer (Popp *et al.*, 2010). PG is also closely involved in binary fission therefore; interruption of its synthesis is bactericidal (Rohrer and Berger-Bachi, 2003). In Gram-positive bacteria, the PG layer is thick (20-80 nanometers) (Figure 1.18), whereas it is thinner (7-8 nanometers) in Gram-negative bacteria (Figure 1.19). PG layer is the main determinant for distinguishing between Gram-negative and -positive bacteria (Hogan, 2010).

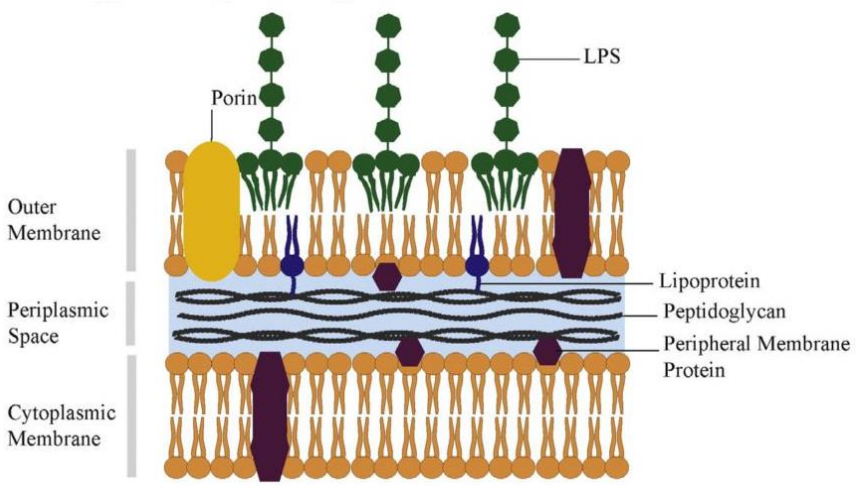

Figure 1.19 Schematic diagram of bacterial cell wall in a Gram-negative bacterium (R.M. Epand *et al.*, 2015)

1.4.2 Glycopeptide Resistance Mechanisms

Vancomycin and teicoplanin (including their derivatives like avoparcin, telavancin, ramoplanin) are glycopeptide antibiotics which are used only in cases when other antimicrobials are proven inefficient. This is due to their nephrotoxicity and therefore, need for meticulous dosage adjustment (Varela *et al.*, 2013). They act on the synthesis of PG layer. In contrast to lysozyme, which breaks the bonds between PG monomers, both antibiotics must be able to reach the PG layer of the cell wall before its biosynthesis to function. Therefore, an outer membrane possessing Gram-negative bacteria are naturally unaffected by vancomycin (Hiramatsu *et al.*, 2014). Vancomycin exerts it effect by binding to D-alanyl-D-alanine residue at the end of the pentapeptide interbridge by five hydrogen bonds. This interaction inhibits the transglycosylation of UDP-N-acetylmuramyl-pentapeptide and transpeptidation of D-alanyl-D-alanine crosslinkage (Figure 1.20) (Wright and Walsh, 1992).

Teicoplanin has the same mode of action except it inhibits more transpeptidation while vancomycin inhibits more transglycosylation (Peng *et al.*, 2013). Also, vancomycin and teicoplanin belong to different side-chain linkage patterns and carbohydrate groups which result in different efficiencies (Loll and Axelsen, 2000).

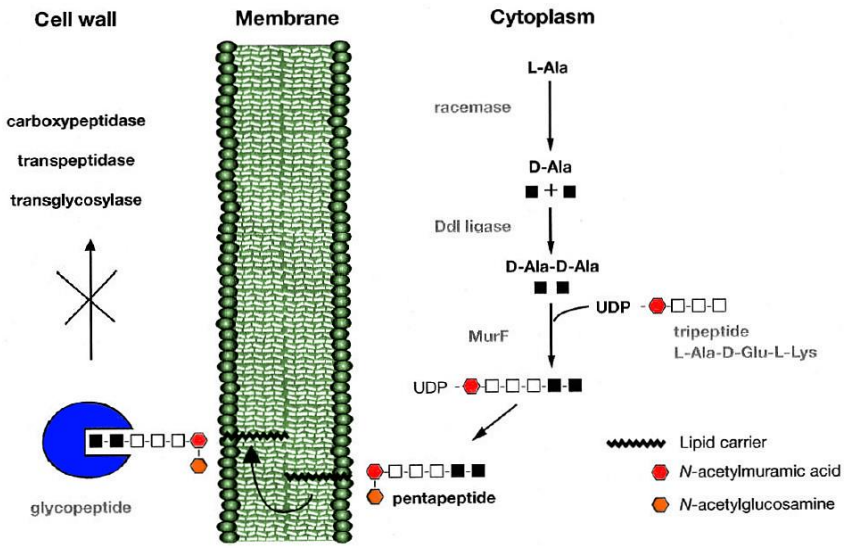

Figure 1.20 PG synthesis and mode of action of glycopeptide antibiotics. Glycopeptides bind to the C-terminal D-alanyl–D-alanine of PG precursors to prevent transglycosylation, transpeptidation, and the D, D-carboxypeptidation. Ddl, D-alanine-D-alanine ligase; MurF, a synthetase protein; UDP, uracil diphosphate (Courvalin, 2006)

Being unable to synthesize the PG layer due to vancomycin inhibition, bacteria either die or react in two ways. In the first, resistance is not based on resistance genes. In this case, murein component of PG has many free D-alanyl-D-alanine residues, towards which vancomycin has a high binding affinity. However, these precursors are actually 'false targets' since binding of vancomycin does not damage the integrity of cell wall significantly (Kawalec *et al.*, 2001). The lipid-murein monomer precursors on the cytoplasmic membrane are the real targets of vancomycin, which takes role in transglycosylation. In other words, cells continue producing PG and provide new PG layers from below the older PG layers. With thickened PG layers, vancomycin cannot completely stop PG synthesis no matter how high a dose of vancomycin is used (Rio-Marques, 2014). Some strains of *S. aureus* show such an intermediate resistance mechanism (Figure 1.21) (Hiramatsu *et al.*, 2014) Investigation of vancomycin and teicoplanin resistances have also led to several corresponding genes called 'the *van* alphabet' (Lebreton *et al.*, 2011). There are several phenotypically specified vancomycin phenotypes, namely; *van*A, *van*B, *van*C, *van*D, *van*E, *van*G, *van*M, *van*N, *van*L (Table 1.3) (Werner, 2012). Phylogenetic analysis of these genes is shown in Figure 1.22.

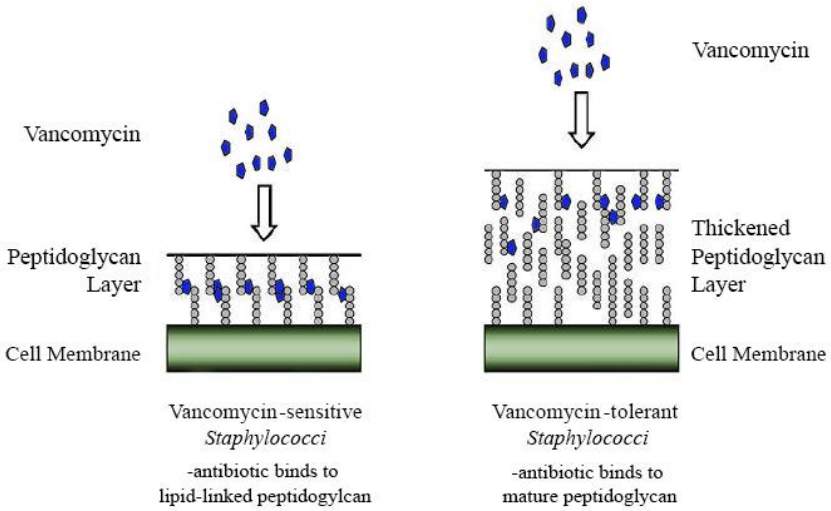

Figure 1.21 A mechanism of vancomycin resistance in Staphylococci. Resistance to vancomycin may not occur in Staphylococci due to the *van* genes. The thickened cell wall of Staphylococci has an abundance of vancomycin targets which binds the antibiotic while layers of PG growing from beneath (Pootoolal *et al.*, 2002)

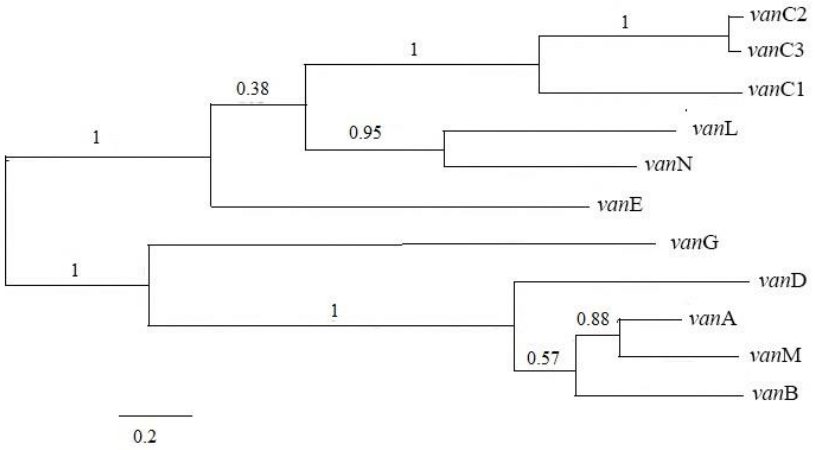

Figure 1.22 Phylogenetic analyses of vancomycin resistance ligases. Branch support values are indicated on the phylogram (Lebreton *et al.*, 2001). The scale bar indicates nucleotide substitutions per site

Table 1.3 Level and type of resistance to vancomycin in Enterococci (Courvalin, 2006; Werner, 2012)

Strain characteristics	Acquired resistance level, type								Intrinsic resistance, low level, type *van*C1/C2/C3
	High	Variable	Moderate	Moderate	Low				
	*van*A	*van*B	*van*M	*van*D	*van*G	*van*E	*van*L	*van*N	
MIC, mg L^{-1}									
Vancomycin	64–1000	4–1000	>256	64–128	16	8–32	8	16	2–32
Teicoplanin	16–512	0.5–1	0.75–96	4–64	0.5	0.5	S	S	0.5–1
Conjugation	+	+	+	-	-	-	-	nd	-
Mobile element	Tn*1546*	Tn*1547* or Tn*1549*	IS*1216*	…	…	…	…	nd	…
Expression	Inducible	Inducible	Inducible	Constitutive	Inducible	Inducible	Inducible	nd	Constitutive Inducible
Location	Plasmid Chromosome	Plasmid Chromosome	Plasmid	Chromosome	Chromosome	Chromosome	Chromosome?	nd	Chromosome
Modified target	D-ala-D-lac	D-ala-D-lac	D-ala-D-lac	D-ala-D-lac	D-ala-D-ser	D-ala-D-ser	D-ala-D-ser	D-ala-D-ser	D-ala-D-ser

MIC, minimum inhibitory concetration;
-, negative; +, positive;
nd, not defined; S, susceptible; ?, not clear;
ala, alanine; lac, lactate; ser, serine

1.5 Glycopeptide Antibiotic Resistance Phenotypes

Although there are nine types of vancomycin resistance (*van*A, *van*B, *van*C, *van*D, *van*E, *van*G, *van*M, *van*N, *van*L) have been characterized by both phenotypic and genotypic basis in Enterococci, the most prevalent types are *van*A and *van*B (Bjørkeng, 2010).

1.5.1 *van*A

The most extensively seen, thoroughly researched phenotype in Enterococci, *van*A, has the highest MIC values among other phenotypes. It also provides resistance not only against vancomycin (64-1000 mg L^{-1}) but also against teicoplanin (16-512 mg L^{-1}) (Howden *et al.,* 2013). The *van*A phenotype is expressed inducibly as a result of the production of PG precursors ending in D-lactate, decreasing the binding affinity of vancomycin a thousand folds (Bugg *et al.,* 1991).

The common resistance mechanism in all *van* phenotypes is first to substitute the D-alanyl-D-alanine which is the affinity binding site for vancomycin and teicoplanin (Courvalin, 2006). Because producing precursors ending with D-alanine and D-lactate or D-serine allow for glycopeptide resistance (Arthur *et al.,* 1996). A two-component regulatory system (*van*R–*van*S) regulates vancomycin resistance in VRE and VRSA strains. *van*S is a membrane-associated sensor of vancomycin that controls the level of phosphorylation of *van*R. *van*R is a transcriptional activator of the operon encoding *van*H, *van*A and *van*X. *van*H is a dehydrogenase that reduces pyruvate to D-lactate, whereas *van*A is a ligase that catalyses the formation of an ester bond between D-alanine and D-lactate. *van*X is a dipeptidase that hydrolyses the normal PG component D-alanyl-D-alanine, preventing it from causing vancomycin sensitivity. *van*Y is a D,D-carboxypeptidase that hydrolyses the terminal D-alanine residue of late PG precursors that are produced if elimination of D-alanyl-D-alanine by *van*X is not complete. Therefore, D-alanine-D-lactate replaces the normal dipeptide D-alanine-D-alanine in PG synthesis resulting in vancomycin resistance. *van*Z confers resistance to teicoplanin by an unknown mechanism (Ranotkar *et al.,* 2014).

The transposon Tn*1546* (10.8 kb), *van*A-type resistance element was originally detected on a plasmid in an *E. faecalis* clinical isolate. Tn*1546* carries nine encoding genes. ORF1 and ORF2 regulate transposition functions; *van*R and *van*S regulate the expression of the resistance gene; *van*H and *van*A synthesize D-alanine-D-lactate depsipeptide; *van*X and *van*Y takes action in the hydrolysis of PG precursors (Figure 1.23 and 1.24) (Ranotkar *et al.*, 2014). *van*Z has an unknown function. The presence of *van* cluster in a transposon is the explanation of its widespread of *van*A mediated glycopeptide resistance (Woodford *et al.*, 1995).

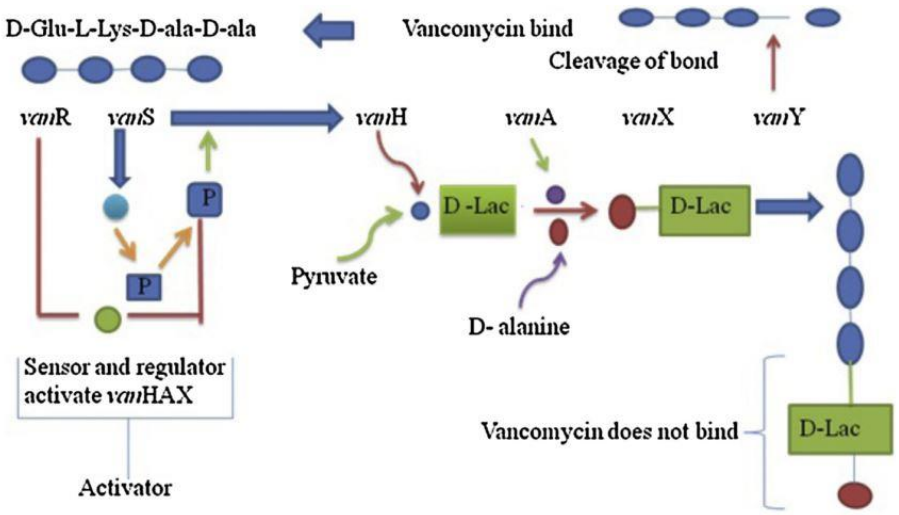

Figure 1.23 Representation of the development of *van*A-mediated vancomycin resistance in enterococci (Ranotkar *et al.*, 2014)

The *van*A gene cluster has been found mainly in *E. faecium* and *E. faecalis* but also in *E. avium*, *E. durans*, *E. raffinosus*, and atypical isolates of *E. gallinarum* and *E. casseliflavus*, which are highly resistant to both vancomycin and teicoplanin (Courvalin, 2006).

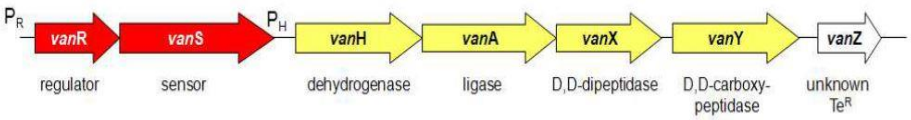

Figure 1.24 The structure and composition of *van*A operon (Werner, 2012)

1.5.2 *van*B

Determinants of *van*B operon reside on large mobile elements of approximately 90 - 250 kb or transposons such as Tn*1547* or Tn*1549*, which can be transferred from one strain of Enterococci to another by either HGT or through plasmid conjugation (Kawalec *et al.*, 2001). The *van*B-mediated glycopeptide resistance is second to *van*A phenotype seen in Enterococci and also Streptococci (Marshall *et al.*, 1997). The mechanism by which the *van*B-mediated vancomycin resistance functions is similar to that of *van*A (Figure 1.25) (Werner, 2012).

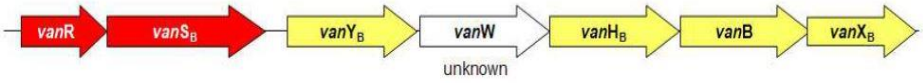

Figure 1.25 The structure and composition of vanB operon (Werner, 2012)

However, *van*B is different in terms of its regulation. Because vancomycin, but not teicoplanin is an inducer of the *van*B cluster (see MIC values in Table 1.3) (Desai, 2005). *van*B operon contains genes encoding a dehydrogenase, a ligase, and a dipeptidase, all of which have a high level of sequence identity (67%–76% identity) with the corresponding deduced proteins of the *van*A operon. *van*R_BS_B regulatory genes however show lower similarity to *van*R_AS_A (34% and 24%, respectively) (Figure 1.26).

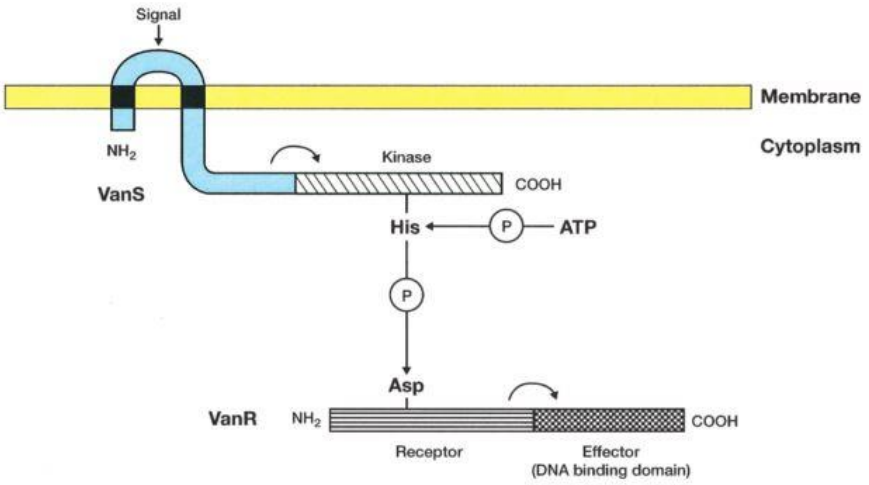

Figure 1.26 *van*RS 2-component regulatory system (Courvalin, 2006).
Asp, aspartate; His, histidine; P, phosphate

A unique gene *van*W can only be seen in *van*B operon and its function is unknown and *van*B operon does not carry *van*Z (Evers *et al.*, 1996). Based on sequence differences, the *van*B gene cluster can be divided into 3 subtypes: *van*B1, *van*B2, and *van*B3 (Dahl *et al.*, 1999). However, there is no correlation between the *van*B subtype and the level of resistance to vancomycin (Courvalin, 2006).

1.5.3 *van*C

The organization of the *van*C operon is different compared to the *van*A, *van*B, and *van*D (Table 1.3) (Figure 1.27) (Werner, 2012). It is characterized by low-level and intrinsic resistance to vancomycin at an MIC of 2 – 32 mg L^{-1} but not to teicoplanin (Reynolds *et al.*, 2005). *E. gallinarum* and E. *casseliflavus*, *E. flavescens* are intrinsically resistant to low levels of vancomycin. There are three subtypes; *van*Cl, *van*C2 and *van*C3 (Dutta and Reynolds, 2003). *van*C favours pentapeptide ending in D-alanyl-D-serine, which weakens binding of vancomycin to the pentapeptide by six-folds. This is thought to be caused of hydroxymethyl side chain of D-Ser which sterically distrupts the binding of vancomycin to the normal D-alanyl-D-alanine termini (Hong *et al.*, 2008).

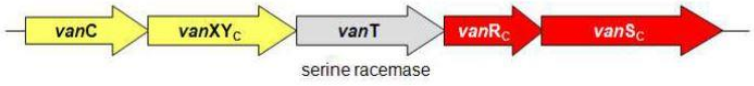

Figure 1.27 The *van*C operon. In the *van*A, *van*B, and *van*D clusters, the genes encoding the 2-component regulatory systems (i.e., *van*RS, *van*R_BS_B, or *van*R_DS_D) are located upstream from the resistance genes, whereas, in the *van*C cluster, these genes are downstream from *van*T (Werner, 2012)

1.5.4 v*an*D

The organizations of the *van*A, *van*B, and *van*D operons are similar (Figure 1.28). In several isolates of *E. faecium* the less common phenotype of acquired glycopeptide resistance includes *van*D (Depardieu *et al.*, 2004).

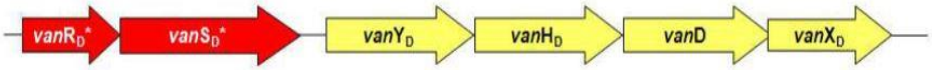

Figure 1.28 The structure and composition of *van*D operon (Werner, 2012)

It has constitutive resistance to moderate levels of glycopeptides. They are located on the chromosome and are not transferable by conjugation to other cocci. Together with the *van*A and *van*B operons, the *van*D operons all have similar organization (Depardieu *et al.,* 2004). One unique aspect of *van*D operon is the diminished susceptibility to teicoplanin (MIC, 4-64 mg L^{-1}) implying a mutation on *van*R_DR_D 2-component regulatory system (Courvalin, 2006).

1.5.5 *van*E

*van*E has an identical operon to that of *van*C (Figure 1.29). This phenotype is characterized by an intrinsic low-level resistance to vancomycin and susceptibility to teicoplanin due to the synthesis of precursors ending with D-alanyl-D-serine (Abadia *et al.,* 2002).

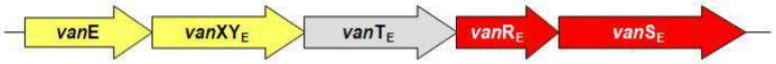

Figure 1.29 The structure and composition of *van*E operon (Werner, 2012)

1.5.6 v*an*G

Acquired *van*G type is characterized by resistance to low levels of vancomycin (MIC, 16 mg L^{-1}) but susceptibility to teicoplanin (MIC, 0.5 mg L^{-1}) and by inducible synthesis of PG precursors ending in D-alanyl-D-serine. The chromosomal *van*G cluster is composed of 7 genes recruited from various *van* operons (Figure 1.30) (Depardieu *et al.*, 2003).

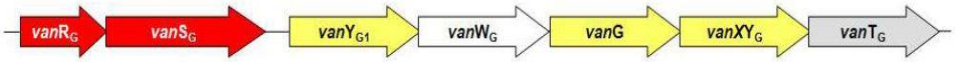

Figure 1.30 The structure and composition of *van*G operon (Werner, 2012)

In contrast to all the other *van* operons, the *van*G cluster encodes three putative gene products with regulatory functions. Besides the known *van*R_G and *van*S_G regulator genes, a *van*U_G gene encoding an additional putative transcriptional activator was identified (Werner, 2012).

1.5.7 *van*L

A single *E. faecalis* isolate from Canada (N06-0364) expressed low level vancomycin resistance by a new mechanism called *van*L (Boyd *et al.*, 2008). The corresponding *van*L gene mediates D-alanyl-D-serine ligation. The *van*L gene cluster was similar in organization to the *van*C operon, but the *van*T serine racemase was encoded by two separate genes, *van*Tm_L (membrane binding) and *van*Tr_L (racemase) resembling the two functional domains of the otherwise combined *van*T type racemase (Figure 1.31) (Boyd *et al.*, 2008)

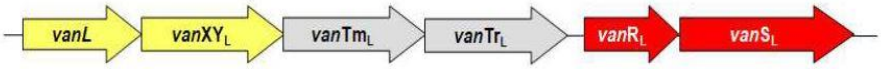

Figure 1.31 The structure and composition of *van*L operon (Werner, 2012)

1.5.8 *van*M

The *van*M genotype was described in seven Chinese VRE isolates originating from a single hospital. A single *van*M VRE has been investigated in details. The translated sequence of *van*M, the corresponding ligase, showed highest similarity to the *van*A, the corresponding *van*M gene product mediates ligation of the D-alanyl-D-lactate peptide. The *van*M gene cluster showed a gene arrangement similar to *van*B and *van*D with the D,D-carboxypeptidase gene *van*YM preceding the ligase gene (Figure 1.32) (Xu *et al.*, 2010).

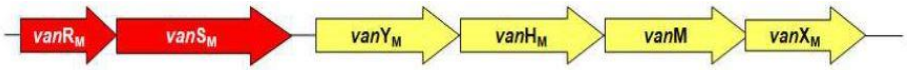

Figure 1.32 The structure and composition of *van*M operon (Werner, 2012)

*van*M type resistance was transferable by conjugation *in vitro* and plasmid-coded. *van*M phenotype showed *in vitro* resistance against vancomycin and teicoplanin in six of seven isolates investigated (Werner, 2012).

1.5.9 *van*N

Lebreton *et al.* (2011) reported that *E. faecium* UCN71, isolated from a blood culture, was resistant to low levels of vancomycin (MIC, 16 μg mL^{-1}) but susceptible to teicoplanin (MIC, 0.5 μg mL^{-1}). The organization of the *van*N gene cluster was similar to that of the *van*C operons. The presence of PG precursors ending in D-serine and D,D-peptidase activities in the absence of vancomycin indicated constitutive expression of the resistance operon. *van*N-type resistance was transferable by conjugation to *E. faecium*. This was the first report of transferable D-alanyl-D-serine-type resistance in *E. faecium*.

1.6 Importance of Surface Waters in the Dissemination of Antibiotic Resistance

Antibiotic resistance is a significant and expanding public health concern, the surveillance for the expansion of this phenomenon in environmental settings is remarkably limited (WHO, 2014). One possible explanation could be the fact that antibiotic concentrations in nonclinical settings are usually very low (Marti *et al.*, 2014). However, recent studies have revealed that selection of resistant bacteria can occur at extremely low antibiotic concentrations (Gullberg *et al.*, 2011) showing that even below MIC's of antibiotics, the resistance may be promoted. Furthermore, the overuse and misuse of antimicrobial agents for human and veterinary medicine, poultry and livestock animals, industrial settings, and their subsequent release in WWTPs have contributed to the emergence and dissemination of resistant bacteria into the environment (Aarestrup, 2005; Cabello, 2006). Given this, aquatic environments including surface waters are ideal settings for the horizontal exchange

of mobile genetic elements encoding antibiotic resistance (Figure 1.33) (Taylor *et al.*, 2011).

Antibiotic resistance is one of the most significant challenges to the health care sector in the 21st century. Due to the emerging problem, large amounts of capital must be invested in the development of new and effective surveillance methods as well as novel antimicrobial drugs. However, there have been ever fewer new antibiotics brought to market, and the pharmaceutical industry increasingly sees antibiotics as a poor investment (Wright, 2012).

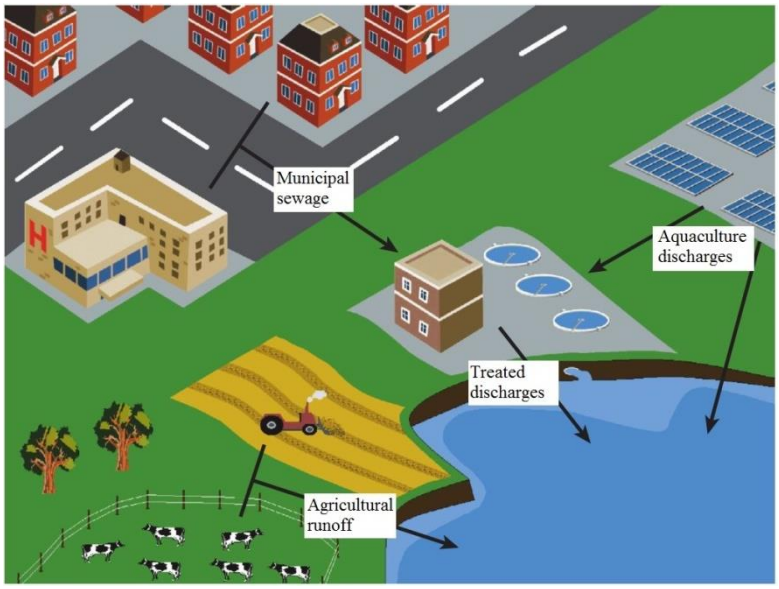

Figure 1.33 Different anthropogenic activities that result in the dissemination of antibiotic resistance genes in aquatic environments (Marti *et al.*, 2011)

The unexpected rapid evolution of antibiotic resistance among pathogenic bacteria left the therapeutic industry in a challenging position. A single nucleotide mutation in resistance causative sequence raises the necessity of development of new antibiotics at a cost of tens of million dollars. Even a brand new compound was to be discovered, there is no guarantee that the new antibiotic will compensate its cost before resistance against it developed. Moreover, a new compound will not take its place in general therapy but rather will stay in the reserve list as a possible cure for difficult cases. This is good to prevent resistance from being developed but at the same time it will limit the profit the developer company will earn (Projan, 2003;

Clardy *et al.*, 2009; Glew, 2010). However, there are companies and researchers working hard to invent novel compounds and surveillance solutions. One example to the brand new antibiotic approaches is External Guide Sequence (EGS) Technology. In this technology, a piece of RNA is put into cell and is complementary to the resistance causative 'pathogenic RNA'. The inserted complementary RNA can withstand 3 mutations in the pathogenic RNA but the fourth one renders it useless. This allows a very flexible dealing of pathogenic strains as when their encoding sequences undergo a mutation it does not causes an immediate by-pass to antibiotics (Wesolowski *et al.*, 2011).

Yarlagadda *et al.* (2015) synthesized vancomycin aglycon dimers to systematically analyze the effect of a linker on biological activity. A dimer having a pendant lipophilic moiety in the linker showed 300-fold more activity than vancomycin against VRE. The high activity of the compound is due to its empowered binding affinity to target peptides, which resulted in improved peptidoglycan (PG) (cell wall) biosynthesis inhibition. Schröder *et al.* (2015) developed a spectroscopy based rapid test method for VRE that reveals the resistance in only 3½ hours. By using a Raman spectroscopic analysis, they demonstrated the characteristic differences in the molecular response of sensitive and resistant *Enterococcus faecalis* and *Enterococcus faecium*. Kim *et al.* (2014) optimized a DNA-based diagnostic technique for the detection of VRE. They utilized the loop-mediated isothermal amplification (LAMP) for a rapid detection of the presence of *van*A gene. They tested 56 clinical isolates and proved that the optimized-LAMP detection is much more sensitive than normal PCR detection. The technique also enables the detection of *van*A gene with the naked eye by observing a white precipitate with just 80 pg DNA template used.

A novel antimicrobial type has been recently found as a result of screening for new compounds. Lantibiotics are a various group of highly modified peptides produced by Gram-positive bacteria (Castigilone *et al.*, 2007). They bind to lipid II but from a different epitope when compared to vancomycin and teicoplanin. Therefore, no cross-resistance has been observed (Hasper *et al.*, 2006). Lipid II consists of a PG

monomer subunit (GlcNAc-MurNAc-pentapeptide, to which vancomycin binds) linked to the lipid transmembrane of a polyisoprenoid anchor 11 subunits long via a pyrophosphate linker. Due to this crucial role in cell-wall biosynthesis and its vulnerability to antibiotics, lipid II has recently been seen as the weakness of antibiotic resistance (Schneider and Sahl, 2010).

Antibiotics are indispensable compounds that will allow us to control and treat infectious diseases. By increasing the public awareness and preventative measurements throughout the world, it will be possible to overcome the menace (Table 1.4). When we consider the fact that there is still a huge fraction of microorganisms yet to be discovered for their new and potent antibiotics, we realize that the ever-developing field of metagenomics will allow us to find new solutions in this regard. Also reducing the rate of resistance emergence might be possible by killing bacteria and removal of antibiotic pollutants in wastewater, sludge, manure (Andersson *et al.,* 2012). Slowing or stopping the initial emergence of the resistance might affect better than trying to control their dissemination later on by restrictive antibiotic usage (Andersson *et al.,* 2012).

Table 1.4 Avoiding and overcoming antibiotic resistance (Davies, 2006; Brown-Jaque *et al.,* 2015)

Various ways of overcoming antibiotic resistance
Optimal use of all antimicrobials through selection, cycling, combination and restriction
Novel antimicrobials and their prudent use
Alternative approaches (immunity, phage, probiotics)
Better understanding of pathogen, commensal and host biology
Increased surveillance and epidemiology of resistance
Improved public and health care specialist education
Improved hygiene
Banning of nontherapeutic uses of antimicrobials
Reduction in bactericide use
Improving our knowledge of HGT strategies with the aim of designing barriers to block the transfer and spread of resistance

Research and development of methods to prevent infection, reducing the need for antibiotics is very important. There is huge scope for advancements in the field of diagnostics. Development of microbiological tests, such as mass spectrometry and

molecular techniques including whole genome sequencing, will improve the speed and accuracy of pathogen identification and susceptibility testing, enabling use of narrower spectrum agents (Pallash, 2003). Better diagnostic tests to distinguish viral infections and non-infectious disease from bacterial infections, to prevent unnecessary antibiotic use, would be of great benefit (Piddock, 2011; Barlett *et al.,* 2011).

In particular, *van*A-mediated vancomycin resistance is on the rise around the world (Figure 1.34). In addition to the previously mentioned evidences, a recent study conducted in Poland shows the important role of transferable genetic elements in the spread of resistance genes (Wardal *et al.*, 2014).

Figure 1.34 Prevalence of VRE in the last two decades. The incidence of VRE is rising due to multidrug resistance and HGT (Ranotkar *et al.*, 2014)

Although *van*A has been disseminated by many different routes, the most common pathogenic carriers of *van*A are Enterococci (VRE) (French, 1998). However, the resistance causative genotype has also been demonstrated in *Staphylococcus* sp. many times in the last decade (VRSA) (Table 1.5) (Courvalin, 2006). The interaction between different bacterial genera in aquatic systems pose a serious threat. Although clinically not considered as vancomycin-resistant (due to their different cell wall structure), *Pseudomonas* spp. may be harboring these genes. As the resistance genes pass to different organisms, they find new reservoirs of accumulation naturally and the resistance disseminates from one region in the world to another.

In order to overcome this emerging issue, not only bold steps must be taken to raise a public, pharmaceutical and clinical awareness on the peril but also robust methods of surveillance and monitoring must be developed. The highly resistant *van*A gene is being disseminated in surface waters through hospital and domestic effluents along with agricultural discharges around the globe. Approaches applied in wastewater treatment plants proven inefficient. Moreover, reports indicate that wastewater treatment plants are hot-spots in the dissemination of these resistance genes (Varela *et al.,* 2013; Rosenberg Goldstein *et al.,* 2014; Roca *et al.,* 2015).

Table 1.5 A summary of vancomycin resistance mechanisms discovered in two common pathogens (Bradley, 2013)

Pathogen	Mechanism of resistance
Staphylococcus aureus	VISA: multiple, poorly defined mutations leading to a thickened, poorly cross-linked cell wall. VRSA: *van*A-or *van*B-mediated replacement of the D-ala-D-ala binding site by D-ala-D-lac.
Enterococcus sp.	Replacement of the D-ala-D-ala binding site by D-ala-D-lac by the *van*A or *van*B operon. Replacement of the D-ala-D-ala binding site by D-ala-D-ser by *van*C, *van*E and *van*G operon.

1.7 Aim of the Study

Transfer of resistant bacteria from environmental compartments to humans may occur through surface water when these waters are used for irrigation or as recreational water. One additional concern is the possible presence of resistant pathogens or resistant bacteria in drinking water. This might occur if surface water is used for drinking water production and treatment is not sufficiently effective. The potential spread of resistance genes in water environments and transfer towards human pathogens is a human health risks. Despite continual increases in the community prevalence of the *van*A gene, there is still speculation on the role of the surface waters as a reservoir of VRE and other potentially pathogenic bacteria that harbor the gene *van*A. Because of these concerns, we investigated the occurrence of VRE and *van*A harboring bacteria in river water.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture Media

Culture mediums used in the study are given below.

2.1.1 Luria Bertani Agar

Luria Bertani (LB) (Merck KGaA, Darmstadt, Germany) agar was prepared according to manufacturer's instructions by weighting 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar suspended in 1 L of distilled water by mixing. The pH of the solution was adjusted to 7.0. The solution was sterilized by autoclaving for 15 min at 121°C (Gerhardt *et al*., 1994).

2.1.2 Nutrient Agar

Nutrient Agar (NA) (Merck KGaA, Darmstadt, Germany) was prepared by weighing 20 g of NA powder in 1 L of distilled water. The solution was boiled to dissolve completely and sterilized by autoclaving for 15 min at 121°C. Then, the solution was mixed well and poured to petri dishes. Finally, the media was stored in a refrigerator at 4°C.

2.1.3 Nutrient Broth

Nutrient Broth (NB) (Merck KGaA, Darmstadt, Germany) was prepared by dissolving 8 g of the medium in 1 L of distilled water. After mixing and dissolving through heating with continued agitation, the solution was poured into appropriate containers and sterilized in autoclave at 121°C for 15 min. The prepared medium was stored at 2-4°C.

2.2 Study Area and Sample Collection

The area of the study was a section of Kızılırmak River in Kırıkkale province of Turkey (Figure 2.1).

Figure 2.1 The main stream of Kızılırmak river (Dogan, 2010)

The strains examined in this study were isolated from water samples collected in 2011 and 2012 from twelve different stations on the river Kızılırmak by Ozer *et al.* (2013). The stations are extending from 39º22’16.39’’N, 33º26’49.26’’E, 890 m to 39º57’22.98’’N, 33º25’04.35’’E, 679 m of the city Kırıkkale.

2.3 Selection of Vancomycin-Resistant Surface Water Isolates

Disc diffusion (DD) test and minimum inhibitory concentrations (MIC) were used for determination of VAN and TEC susceptibilities of the isolates collected.

2.3.1 Disc Diffusion Tests

For the selection of VAN-resistant surface water isolates, antibiotic susceptibility testing was done by using DD test as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). All the isolates were subjected to VAN DD test using a 30 µg disc and TEC DD test by using 30 µg disc on NA with 4% NaCl. The plates were incubated at 30°C for 24 h and zones of inhibition were measured. An inhibition zone of ≥17 mm was considered as susceptible and ≤ 14 mm as resistant for VAN. An inhibition zone of ≥ 14 mm was considered as susceptible and ≤ 10 mm resistant for TEC. (CLSI, 2012).

2.3.2 Minimum Inhibitory Concentration Tests

MIC was carried out for both antibiotics VAN and TEC by agar dilution method (CLSI, 2012). results were interpreted as susceptible if the MIC was ≤ 4 μg mL^{-1} and resistant if the MIC was ≥ 32 μg mL^{-1} for VAN, if the MIC was ≤ 8 μg mL^{-1} the results were interpreted as susceptible and resistant if the MIC was ≥ 32 μg mL^{-1} for TEC. All results were validated by using vancomycin- and teicoplanin-resistant *Enterococcus faecium* E330 with a GenBank number of KU296972 and vancomycin- and teicoplanin-sensitive *Escherichia coli* DH5α.

2.4 Genomic DNA Extraction

Genomic DNA extraction was done in order to obtain genomic DNA samples for chromosomal DNA profiling and PCR analysis by using High Pure PCR Template Preparation Kit (Roche, Germany). The kit was composed of:

- Lysis buffer; 4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.4.
- Binding buffer; 6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4.
- Proteinase K as dissolved in 4.5 mL double-distilled water.
- Inhibitor removal buffer; 20 mL of absolute ethanol was added to 3 mL volume of: 5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6.
- Wash buffer; 80 mL of absolute ethanol was added to 20 mL volume of: 20 mM NaCl, 2 mM Tris-HCl, pH 7.5.
- Elution buffer: 10 mM Tris- HCl, pH 8.5.
- High pure filter tubes and collection tubes.

The pH was adjusted to 7.4 with 1 N HCl. The total volume was completed to 1 L with additional distilled water. Solution was sterilized by autoclaving at 121°C for 20 minutes (CSH Protocols, 2006). Isolated bacteria were grown in 50 mL of NB and incubated while mildly shaking at 30°C for 24 h. 200 μL of bacteria were added to a nuclease-free 1.5 mL microcentrifuge tube and centrifuged for 5 minutes at 3000 g. Supernatants were discarded and cell pellet resuspended in 200 μL of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). 5 μL of lysozyme (10 mg mL^{-1} in 10 mM Tris-HCl, pH 8.0) were added to Gram-positive bacteria and incubated at 37°C for 15 minutes. After that 200 μL of binding buffer and 40 μL of

reconstituted proteinase K were added to the sample and mixed immediately to incubate at 70°C for 10 minutes. Then 100 µL of absolute isopropanol were added and mixed well. High pure filter tubes were assembled into collection tubes and the liquid samples were pipetted into the upper buffer reservoir of the filter tubes. It was then centrifuged for 1 minute at 8000 g. After centrifugation, liquid was discarded. The filter tubes were assembled with new collection tubes and 500 µL of inhibitor removal buffer added to the upper reservoir of the filter tubes and centrifuged for 1 minute at 8000 g. The flow through liquid and the collection tube were discarded and 500 µL of wash buffer added to the filter tubes. The tubes were centrifuged for 1 minute at 8000 g and the flow through was discarded. After repeating the previous step, the tubes were centrifuged for 10 seconds at 12500 g to remove residual wash buffer. Finally, the filter tubes were inserted into clean, sterile 1.5 mL microcentrifuge tubes and 200 µL prewarmed elution buffer added to the filter tubes to centrifuge for 1 minute at 8000 g for eluting DNA. In order to remove RNA from the eluted DNA, 0.5 µL of RNase was added to the eluted DNA and incubated at 37°C for 15 minutes. The amount of DNA was measured by Qubit Fluorometer (Invitrogen, USA) and stored at -20°C (Aljanabi and Martinez, 1997).

2.5 Plasmid DNA Extraction

Plasmid DNA extraction was done in order to obtain plasmid DNA samples for profiling and PCR analyses. Plasmid DNA extraction was accomplished by the alkali lysis method originally developed by Birnboim and Doly (1979). Required reagents for this method are as follows:

- Resuspension Buffer; 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0).
- Lysis Solution; 0.2 N NaOH, 1% (mg mL^{-1}) sodium dodecyl sulfate (SDS).
- Neutralization Solution; 3 M potassium acetate (pH 4.8).
- Phenol (saturated with 10X Tris-EDTA, pH 8.5): chloroform: isoamyl alcohol (25:24:1) solution.
- 70% ethanol.
- Absolute isopropanol.
- Tris – EDTA (TE) (100 mM Tris, 10 mM EDTA, pH 8.0).
- DNAse free RNAse (10 mg mL^{-1}).

The resistant bacteria were grown in 50 mL of LB agar according to each their growth curves. 100 µL VAN or TEC (1000 µg mL^{-1}) was added to the growth medium to enhance plasmid DNA replication. According to growth curves constructed by Göksu, Salık and Ulusoy (2015), cultures were taken at their late exponential or early stationary phase of growth. Then 1.5 mL of grown cultures were poured in a microcentrifuge tube and centrifuged at 4°C for 2 minutes at 12000 g. The supernatant was removed from the tube completely, leaving the bacterial pellet as dry as possible. 100 µL of ice cold resuspension buffer was added and the bacterial pellet was resuspended properly by vortexing or by slow rounds of pipetting with a 100 µL micropipette. The tubes were incubated on ice for 5 minutes. In the next step, 200 µL of freshly prepared lysis solution was added to the bacterial suspension. The tubes were tightly closed and mixed thoroughly by inverting 4-6 times until the solution becomes viscous and slightly clear. After an incubation on ice for 3-5 minutes, 150 µL of chilled neutralization solution was added then mixed immediately by inverting and rolling the tube 4-6 times before incubating on ice for 3-5 minutes. Afterwards, the tubes were centrifuged at 14000 g in a microcentrifuge for 10 minutes at 4°C. Then the supernatant containing plasmid DNA transferred to new microcentrifuge tubes. An equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to the supernatant and mixed by vortexing for 10 seconds. Then the tubes were centrifuged at 14000 g for 5 minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube. An equal volume of isopropanol was added to the supernatant, mixed by inverting the tubes 4-6 times and again centrifuged at 14000 g for 30 minutes at room temperature. Then the supernatant was removed completely. 500 µL of 70% ethanol was added to the pellet. The tube was closed and inverted several times. Then the tubes were centrifuged at 14000 g for 5 minutes at 25°C. The supernatant was removed completely again. After drying the pellets were dissolved in 25 µL of sterile ultra-distilled water. The samples were stored at -20 °C.

2.6 DNA Agarose Gel Electrophoresis

DNA agarose gel electrophoresis was done to obtain chromosomal DNA and plasmid DNA profiles and to confirm whether the extractions were successful. 10X Tris-borate-EDTA (TBE) buffer was prepared by suspending 108 g of Tris base (89 mM),

55 g of boric acid (89 mM) and 7.5 g of EDTA (2 mM) in 1 L of RNAse-free water and stirred before storing at room temperature (pH 8.3). 1X TBE buffer was also prepared by diluting 100 mL of 10X TBE buffer in 900 mL of distilled water (CSH Protocols, 2006). The DNA preparations were electrophoresed in 0.7-1% agarose gels with 1X TBE running buffer at 90 V for 1-5 hours at room temperature in Mini-Sub Cell GT (Bio-Rad, USA) apparatus. The gels were stained with GelRed (Olerup SSP, Sweden). Lambda DNA/*Hind*III Marker (Thermo Scientific, USA) was used as DNA size marker. Visualization of DNA bands were done under UV light. Sizes of the DNA bands obtained on agarose gel electrophoresis were calculated by constructing standard curve. The standard curve was calculated by plotting the distances travelled by marker bands on agarose gel against each bands' corresponding base pairs.

2.7 Screening for *van*A harboring Surface Water Isolates by Polymerase Chain Reaction

VAN- and TEC-resistant surface water isolates were further subjected for the detection of *van*A gene by using polymerase chain reaction (PCR). Extraction of genomic and plasmid DNA from the VAN- and TEC-resistant surface water isolates was performed as described previously. The *van*A gene was amplified by using *van*A specific primers, 1F 5'-ATGAATAGAATAAAAGTTGCAATAC-3' and 1029R 5'-CCCCTTTAACGCTAATACGAT-3' (Miele *et al.,* 1995). Optimization was carried out by changing the amount of DNA template and $MgCl_2$ concentrations at temperatures ranging from 50 to 62 ^{o}C. PCR was performed in 50 µL of a reaction mixture containing DNA (100 ng), 200 µM each of deoxynucleoside triphosphates (dNTP), 3 mM $MgCl_2$, 5X Taq buffer, 25 pmol of each primer and 2.5 units of Taq DNA polymerase (Fermantas, Germany).

DNA extracts from VAN-resistant *E. faecium* E330 was used as positive control and VAN-susceptible *Escherichia coli* DH5α was used as negative control. Furthermore, primers from a ubiquitous and highly evolutionarily conserved part of the core genome that is called *tuf* gene (which encodes the elongation factor EF-Tu) is also utilized to specifically validate the presence of *Enterococci* as described by Ke *et al.* (1999). The primers for *tuf* were 618F 5'- TACTGACAAACCATTCATGATG-3' and 729R 5'- AACTTCGTCACCAACGCGAAC-3'. Amplifications were performed

using a Thermal Cycler (Bio-Rad T-100, USA) with the following optimal conditions: Initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 93°C for 1 minute, annealing at 56 °C for 1 minute, and extension at 72°C for 2 minute, followed by final extension at 72°C for 6 min. The PCR products were electrophoresed on a 1% agarose gel and stained with GelRed (Olerup SSP, Sweden). Quick-Load 100 bp DNA ladder (New England Biolabs) was used as DNA size marker (Miele *et al.*, 1995).

2.9 Identification of *van*A Harboring Surface Water Isolates by 16S rRNA Sequencing

Identification of *van*A harboring two surface water isolates E330 and E07 were done by 16S rRNA sequencing. Genomic DNA was isolated from *van*A-positive surface water isolates and analyzed as indicated previously. Bacterial 16S rRNA was amplified by using the universal bacterial 16S rRNA primers, 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTGTTTGATTGTTACGACTT-3' (Lane *et al.*, 1985). PCR was performed with a 50 µL reaction mixture containing 1 µL (10 ng) of DNA extract as a template, each primer at a concentration of 5 mM, 25 mM MgCl2 and dNTPs at a concentration of 2 mM, as well as 1.5 U of Taq DNA polymerase and buffer used as recommended by the manufacturer (Fermentas, Germany). After the initial denaturation at 94°C for 5 min, the following steps were used; 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR was carried out in a Thermal Cycler (Bio-Rad T-100, USA). The obtained PCR products were purified, using the GeneJET™ PCR Purification Kit (Fermentas, Germany), according to the instructions of the manufacturer, and sequenced. The amplicons were sequenced by using 3730x 1 DNA synthesizer (Applied Biosystems, USA). The two 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI (Basic Local Alignment Search Tool) BLAST program (Benson and Karsch Mizrachi, 2002). The 16S rRNA gene sequences were deposited to GenBank using the BankIt submission tool, and to assign NCBI accession numbers. Phylogenetic trees were constructed by using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura *et al.*, 2013). Ten different strains from GenBank in BLAST program were selected and aligned by Clustal Omega.

2.8 Sequencing of *van*A Amplicons

The *van*A amplicons were purified with the QIAquick PCR Purification Kit (QIAGEN, Toronto, Canada). Then each *van*A amplicons were sequenced with *van*A primers by using 3730x1 DNA synthesizer (Applied Biosystems, USA). Sequence alignment was carried out by using the NCBI Basic Local Alignment Search Tool BLASTn program. A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic tree for *van*A gene from different species was created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) by using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura *et al*., 2013).

2.9 Analyses of D-Alanine-D-Lactate Ligase

In order to validate the presence of D-alanine-D-lactate ligase encoding *van*A, complete protein extraction was performed. Protein extractions were prepared from the *van*A-positive isolates following the method described previously (Hill *et al*., 2010). Strains were grown at 30°C in 100 mL of LB. Cells from exponential-phase cultures were harvested by centrifugation at 8000 g for 10 minutes before being washed with 25 mL of cold PBS with 1 mM ß-mercaptoethanol and Protease Inhibitor Cocktail Tablets (Invitrogen, UK). After centrifugation at 8000 g for 10 minutes, the pellet was resuspended in 2.5 mL of PBS and kept cold on ice. Cells were broken by sonication on ice at 30kHz for 3-5 minutes, the lysate was cleared by centrifugation at 8000 g for 10 minutes at 4°C. The supernatant was stored at -80°C and used for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis (Mahmood and Yang, 2012). The protein concentrations of samples were determined by Quick Start Bovine Serum Albumin (BSA) Standard Kit (Bio-Rad, USA) (Bradford, 1976).

2.9.1 SDS-PAGE Analysis of D-Alanine-D-Lactate Ligase

SDS-PAGE analysis was done to confirm the presence of D-alanine-D-lactate ligase and as a preliminary step for western blot analysis. The D-alanine-D-lactate ligase preparations were first detected with SDS-PAGE according to Laemmli (1970). First, stock solutions were prepared as shown in Table 2.1.

A 20 µL of supernatant protein was mixed with 4 µL of Laemmli sample buffer (125 mM Tris- HCl, 4% SDS, 20% (v/v) glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue, pH 6.8) and boiled for 5 minutes. Prepared samples were stacked in a 4% acrylamide stacking gel and separated in a 12% acrylamide resolving gel (Table 2.2). The running buffer system contained Tris-glycine system of 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS (pH 8.3). Electrophoresis was done in Mini Protean Tetra System (Bio-Rad, USA) apparatus at 150 V for 30 min. After completion of electrophoresis, proteins were visualized by staining with 0.125% Coomassie Brilliant Blue (w/v) in 50% methanol (v/v) and 10% glacial acetic acid (v/v) followed by destaining with 20% methanol (v/v) and 10% glacial acetic acid (v/v) solution, respectively (Laemmli, 1970). The molecular weights of the proteins were estimated from calibration curves prepared by using Color Plus Prestained Protein Marker (New England BioLabs, UK) (Siwach and Singh, 2007). The reference marker possessed 8 proteins with the following sizes; 175, 80, 58, 46, 30, 23, 17 and 7 kDa, respectively.

Table 2.1 Stock solutions of SDS-PAGE

Stock solutions	Preparation
Acrylamide solution (30% acrylamide, 0.8% bisacrylamide, 100 mL	30 g of acrylamide and 0.8 g of N'N'-bismethylene-acrylamide were dissolved in 100 mL of double-distilled water. Solution was stored up to 3 months in the dark at 4°C.
Resolving gel buffer (4X) (1.5 M Tris-HCl, pH 8.8, 100 mL)	18.15 g of Tris-HCl was suspended in 75 mL of double-distilled water and pH adjusted to 8.8 with HCl and diluted to 100 mL of deionized water. Solution was stored at 4°C.
Stacking gel buffer (4X) (0.5 M Tris-HCl, pH 6.8, 100mL)	6 g of Tris-HCl was suspended in 80 mL of double-distilled water and pH adjusted to 6.8 with HCl and diluted to 100 mL of deionized water. Solution was stored at 4°C.
10% SDS	10 g of SDS was dissolved in 100 mL of deionized water. Solution was stored up to 6 months at room temperature.
10% Ammonium persulfate (APS)	0.1 g of APS was dissolved in 1 mL of deionized water. APS was prepared just prior to use.

Table 2.2 Resolving and stacking gel compositions

Reagents	Resolving gel (12%)	Stacking gel (4%)
Acrylamide solution	16 mL	1.33 mL
Resolving gel buffer (4X)	9.6 mL	-
Stacking gel buffer (4X)	-	2.6 mL
10% SDS	0.4 mL	0.1 mL
Deionized water	12.2 mL	6 mL
10% Ammonium persulphate	192 µL	50 µL
TEMED	12.7 µL	5 µL

TEMED, N, N, N', N'-tetramethylethylenediamine;
SDS, sodium dodecyl sulphate

2.9.2 Western Blot Analysis of D-Alanine-D-Lactate Ligase

After SDS-PAGE analysis, the presence of D-alanine-D-lactate ligase was further tested by using western blot analysis.

2.9.2.1 Transferring of D-Alanine-D-Lactate Ligase from SDS-PAGE Gel to Polyvinylidene Difluoride Membrane

For western blot analysis of D-alanine-D-lactate ligase, the separated proteins by SDS-PAGE gel were transferred to polyvinylidene difluoride (PVDF) membrane by using Trans Blot PVDF/Nitrocellulose Starter Kit (Bio-Rad, USA) following manufacturer's instructions. PVDF membrane was immersed in 100% absolute ethanol until membrane became translucent. Then the membrane was transferred to a gel tray containing 30 mL of 1X transfer buffer (200 mL 5X transfer buffer, 600 mL nanopure water and 200 mL ethanol) for 3 min at room temperature. Two transfer stacks were also immersed to a gel tray containing 50 mL of 1X transfer buffer for 3 min at room temperature. Next, one wetted stack was placed on bottom of cassette, then wetted membrane, SDS-PAGE gel containing D-alanine-D-lactate ligase and second wetted transfer stack were placed, respectively. Air bubbles were removed with blot roller. Cassette lid was locked and inserted in the Trans-Blot Turbo System (Bio-Rad, USA) and transferring of D-alanine-D-lactate ligase from SDS-PAGE gel to PVDF membrane was performed at 1.3 mA and 25V for 7 minutes.

2.9.2.2 Blocking of PVDF Membrane

The membrane was removed from the transfer apparatus and placed immediately into Tris-Buffered Saline Tween 20 (TBST) (10 mM Tris-HCl, 100 mM NaCl, 0.1 %

Tween 20) containing 5% non-fat dry milk overnight at 4°C to block the membrane (Mahmood and Yang, 2012).

2.9.2.3 Incubation of PVDF Membrane with Primary and Secondary Antibodies

The previous blocking buffer from the membrane was decanted and the membrane was washed three times with TBST. Afterwards, the PVDF membrane was incubated for 2 h at room temperature with the mouse anti-human *van*A primary antibody (USBiological Life Sciences, USA) diluted 1:1000 in TBST. Then the membrane was washed with TBST and then incubated with the goat anti-mouse immunoglobulin (G/M) horseradish peroxidase (HRP)-conjugated secondary antibody (Millipore, USA) diluted 1:10000 in TBST for 2 h at room temperature (Hill *et al*., 2010).

2.9.2.4 Treatment of PVDF Membrane with Horseradish Peroxidase Conjugate Substrate

After three times washing with TBST, the membrane was treated using HRP-conjugate Substrate Kit solution (Bio-Rad, USA) according to the manufacturer's instructions. The membrane was immersed in the color development solution prepared by adding 600 µL of HRP color reagent B to 100 mL of 1X HRP color development buffer and 20 mL of HRP color reagent A. Then the immersed membrane in the color development solution was incubated at room temperature with gentle agitation until the appearance of the protein band. The incubation was stopped by washing the membrane in distilled water for 10 min with gentle agitation. Finally, the membrane was dried in air. The molecular weights of the proteins were estimated from calibration curves prepared by using Precision Plus Protein WesternC Standards (Bio-Rad, USA). The reference marker possessed 10 proteins with the following sizes; 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa, respectively.

2.10 Monitoring of *van*A-harboring Bacteria in Surface Water through Fluorescent *in situ* Hybridization

Seasonal water samples collected from the study area over the years 2011 and 2012 were monitored in terms of *van*A gene by fluorescent *in situ* hybridization (FISH).

2.10.1 *van*A Probe Design

For the preparation of *van*A-targeted oligonucleotide DNA probe, the *van*A operon from the pVEF1 and pVEF2 plasmids of *E. faecium* was digested with *BamH*I and *Cla*I restriction enzymes by using Vector NTI Express Software 1.2 (IBI, USA). Obtained sequences were subsequently confirmed for their specificity with *van*A gene using BLAST (Benson and Karsch-Mizrachi, 2000). The selected 25 bp DNA fragment was labelled with fluorescein isothiocyanate (FITC) at the 5' end (Alpha DNA, Montreal, Canada). The FITC labelled *van*A probe was evaluated with both paraformaldehyde-fixed *van*A harboring pure cultures of *E. faecalis* E07 and water samples collected from river waters by using FISH.

2.10.2 Sample Preparation and Fixation

For visualization of the *van*A harboring bacteria via FISH, samples were first fixed and permeabilized on glass slides before exposed to hybridization probe. The probe designed for FISH was optimized with pure cultures of *van*A-positive and -negative strains for its optimal stringency for hybridization studies. Microbial samples from culture medium and surface water were processed according to the protocol of Amman *et al*. (1990). VAN-resistant *E. faecalis* E07, VAN-non resistant *E. coli* DH5a strains were grown in 50 µL of NB at 37°C overnight. While the cultures were in the exponential growth phase, 1 mL of the suspension was removed and centrifuged in a 2 mL microcentrifuge tube at 10000 g for 2 minutes. 500 mL of water samples collected over the years of 2011 and 2012 were also centrifuged at 10000 g for 10 minutes. The supernatants were removed and the pellets were resuspended in 1 mL of 1X PBS. After centrifugation, the supernatants were discarded and samples were washed twice. After the second wash, the cells were resuspended in 200 µL of 1X PBS and then fixed with 600 µL of 4% paraformaldehyde prepared in 1X PBS at 4ºC for 24 h. Fixed cells were washed two times in 1X PBS and resuspended in a 1 mL solution of 1:1, PBS: ethanol (Glockner *et al*., 1996; Korzeniewska and Harnisz, 2012). The fixed samples were stored at -20°C for FISH analyses.

2.10.3 Sample Dehydration and Permeabilization

5 µL of the fixed samples were placed on glass slides and distributed by the side of the pipette tip. The samples were dried at 45ºC for 30 minutes. Dry slides were dehydrated by dipping them into glass jars containing 50, 80 and 96% ethanol 3 minutes for each, respectively. Then the slides were dried at room temperature (Nielsen *et al*., 2009). 10 µL of the lysozyme (dissolved to a final concentration of 10 mg mL- 1 in 0.05 M EDTA and 0.1 M Tris-HCl, pH 8.0) was applied to each slide and incubated at 37°C for 15 minutes. The slides were washed 3 times with distilled water, followed by once in absolute ethanol and air-dried (Nielsen *et al*., 2009).

2.10.4 Hybridization of Fixed Samples with Oligonucleotide DNA Probes

The hybridization protocol for samples from pure cultures and water samples was adapted from Amman *et al*. (1990) and Santos *et al*. (2010). First, 2 mL of hybridization buffers were prepared with different formamide concentrations as given in Table 2.3. 50 mL of washing buffer were also prepared by replacing formamide by NaCl as indicated in Table 2.4.

Table 2.3 Composition of the hybridization buffer at various formamide concentrations including 5M NaCl, 360 µL; 1 M Tris-HCl, 40 µL; 10% SDS, 2 µL to a final volume of 2 mL

Formamide (%)	Formamide (µL)	Distilled water (µL)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500

16S rRNA-targeted oligonucleotide probe sequences were selected from those deposited at probeBase (Loy *et al.* 2003). The following 16S rRNA-targeted oligonucleotide probes were used as suggested in the corresponding references: (i) EUB338 (Daims *et al.* 1999), (ii) EUB338 II (Amann *et al.* 2001), (iii) EUB338 III (Daims *et al.* 1999), (iv) NON338 (Daims *et al.* 1999), as a negative control and (v) *van*A probe. The probes were labelled with fluorescein isothiocyanate (FITC) from 5' end (Alpha DNA, Montreal, Canada). DAPI (4′, 6′-diamidino-2 phenylindole) staining was applied to record all living organisms in the samples.

Table 2.4 Composition of the washing buffer corresponding to the formamide concentrations in hybridization buffer, including 1 M Tris-HCl, 1 mL; 10% SDS, 50 μL

Formamide (%)	5 M NaCl (μL)	0.5 M EDTA (μL)
0	9000	0
5	6300	0
10	4500	0
15	3180	0
20	2150	500
25	1490	500
30	1020	500
35	700	500
40	460	500
45	300	500
50	180	500
55	100	500

Reference strains of VAN-resistant *E. faecalis* E07 as a positive control and VAN-non resistant *E. coli* DH5a as a negative control were used for the optimization of hybridization stringency by changing concentration of formamide/ NaCl. Hybridization of probes was processed according to Amman *et al.* (1990). 9 μL of hybridization buffer including 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% SDS and different formamide concentration with 1 μL of specific probe solution (50 ng μL^{-1}) and 1 μL of DAPI (200 ng μL^{-1}) was applied to each slide. Hybridization was performed in humidified incubator at 46°C for 2 h. After hybridization, the slides were washed with a prewarmed washing solution including

20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% SDS and NaCl. After washing, the samples were left to dry at room temperature.

2.10.5 Microscopic Evaluation

*van*A harboring bacteria were observed by a Zeiss Axio Scope A1 fluorescence microscope equipped with DAPI and FITC filter sets: A filter was used for total microorganisms in water samples stained by DAPI and the other filter for *van*A-positive bacteria hybridized with FITC-labelled *van*A probe. Slides were mounted in anti-fading solution AF1 (Citifluor Ltd., London, UK) and viewed under oil immersion at 100X magnification. For each prepared sample, 3 slides were prepared and 10 images were captured by using CCD camera from each slide. The captured digital images were saved in Zeiss Axio Vision software and processed with Adobe Photoshop CC (San Jose, California, USA) to remove the blur areas. Each image was separately counted in terms of the pixel areas of green region conferred by FITC-labelled *van*A probe and blue region conferred by DAPI. The populations of *van*A harboring bacteria were determined depending on the quantification of pixel areas of images by using the below equation (Baker and Irvin 2007);

$$\textit{Biomass of vanA harboring bacteria (\%)} = \frac{Pixel\ area\ of\ FITC\ image}{Pixel\ area\ of\ DAPI\ image}$$

Biomasses (%) of *van*A harboring bacteria were calculated using average values of taken images for pixel areas of FITC probe and DAPI. Before the calculation, the pixel areas were subtracted by the areas of non-binding probe NON338 to remove auto-fluorescence and background interference. The oligonucleotide *van*A probe images were assumed as the total amount of *van*A harboring bacteria, while the images of DAPI stained cells were assumed as the total amount of biomass (Yilmaz and Icgen, 2014). After optimization of hybridization stringency conditions for *van*A probe with positive and negative pure culture controls, the river water samples were screened for the *van*A harboring isolates by using FISH.

2.10.6 Statistical Analyses of FISH Results

All statistical analyses were carried out using Origin Pro 8.5 software (OriginLab Corporation, Northampton, Massachusetts, USA). Shapiro-Wilk test was applied to

identify which data was normally distributed. The significance of all parameters in the regression analyses presented has been verified ($p<0.05$ significance level) by one-way analysis of variance (ANOVA) and Tukey test (Korzeniewska and Harnisz, 2012).

CHAPTER 3

RESULTS & DISCUSSION

3.1 Selection of Vancomycin and Teicoplanin Resistant Surface Water Isolates

A total of 290 surface water isolates collected were tested for their vancomycin and teicoplanin resistance. Glycopeptide resistance was detected by VAN/TEC DD and MIC tests. Out of 290, 18 bacterial isolates inculuding 4 enterococcal (3 *E. faecalis*, 1 *E. faecium*) and 14 non-enterococcal (8 *Pseudomonas*, 3 *Staphylococcus*, 1 *Comamonas*, 1 *Raoultella*, and 1 *Aeromonas*) species displayed resistance to both glycopeptides (Table 3.1). Vancomycin-resistant *E. faecalis* E330, vancomycin-non resistant *E. coli* DH5a isolates were used as positive and negative controls, respectively. The DD zone values of isolates varied in between 6 and 10 mm for VAN (Appendix A) while it differed from 6 to 9 mm for TEC. The MIC of vancomycin-resistant isolates was also determined. The MIC of isolates varied in between 30 and $\geq$512 $\mu g\ mL^{-1}$ for VAN and TEC (Table 3.1). Overuse and misuse of antibiotics are widely regarded as major factors promoting antibiotic resistance (Wright 2010). Antimicrobial agents like avoparcin, one of the glycopeptide widely used as feed additives for growth promotion in animal husbandry, appears to be associated with the emergence of glycopeptide resistance (Bager *et al.* 1997). Horizontal transmission of VRE from poultry to humans through the food chain is important transmission route (Van den Braak *et al.* 1998). Enterococci being part of normal intestinal flora of humans and animals are widespread bacteria and can inhabitate in different ecological sources (Klare *et al.* 1993). VRE have also been found in sewage, from stools of healthy farm animals and animal products, but also in surface water (Harwood *et al.* 2001; Iversen *et al.* 2002). Resistance in farm animals spread mostly to manure and soil, accordingly, resistance in humans mostly impact the water chain. Transfer of resistant bacteria from environmental

compartments to humans may occur through surface waters, manure or food (Schwartz *et al.* 2003). Through surface runoff, leaching and the effluents of wastewater treatment plants resistance genes and resistant bacteria can be transported to surface waters. Therefore, transmission of resistance genes and dissemination of resistant bacteria could be the reason for vancomycin and teicoplanin resistance in these surface waters. Seyedmonir *et al.* (2015) showed the dissemination of methicillin-resistant staphylococcal and non-stapylococcal isolates in river waters. Antibiotic resistance genes in surface waters can increase the chances of human pathogens for acquiring resistance. The contact of human microbiota with other types of microbiota in surface waters increases the possibility of genetic variation and the possible emergence of novel mechanisms of resistance that are re-introduced in the human environment (Baquero *et al.* 2008).

Table 3.1 Enterococcal and non-enterococcal glycopeptide-resistant and *van*A harboring surface water isolates used in the study

Strain designations	Sampling coordinates	VAN DD test[a]	TEC DD test[b]	Vancomycin MIC test[c] (μg mL^{-1})	Teicoplanin MIC test[d] (μg mL^{-1})	Plasmid profiles (kb)	PCR analysis of *van*A	SDS-PAGE analysis of D-alanine-D-lactate ligase	Western blot analysis of D-alanine-D-lactate ligase	16S rRNA sequencing (Homology %)	EMBL access numbers	References
Ag10	39º48'38.97''N, 33º29'14.57''E, 684m	R	R	64	128	47, 50, 55, 205	+	+	+	*Pseudomonas plecoglossicida* (99%)	KJ395363	Koc *et al.*, 2013
Ag11	39º50'28.41''N, 33º28'02.13''E, 686m	R	R	512	≥512	47, 50, 55, 205	+	+	+	*Raoultella planticola* (99%)	KJ395359	Koc *et al.*, 2013
Al11	39º50'28.41''N, 33º28'02.13''E, 686 m	R	R	128	32	55	+	+	+	*Staphylococcus aureus* (99%)	KJ395360	Yilmaz *et al*., 2013
Ba01	39º22'16.39''N, 33º 26'49.26''E, 890m	R	R	128	32	33, 55, 110, 215	+	+	+	*Staphylococcus aureus* (99%)	KJ395371	Yilmaz *et al*., 2013
Cr07	39º37'02.34''N, 33º26'38.26''E, 773m	R	R	64	128	205	+	+	+	*Enterococcus faecalis* (96%)	KJ395365	Icgen and Yilmaz 2014
Co11	39º50'28.41''N, 33º28'02.13''E, 686 m	R	R	128	32	215	+	+	+	*Staphylococcus warneri* (99%)	KJ395373	Yilmaz *et al*., 2013
Cu12	39º57'22.98''N, 33º25'04,35''E, 679m	R	R	512	≥512	205	+	+	+	*Pseudomonas koreensis* (98%)	KJ395364	Icgen and Yilmaz 2014
E07	39º37'02.34''N, 33º26'38.26''E, 773m	R	R	512	512	47, 55, 222	+	+	+	*Enterococcus faecalis* (99%)	KU195302	This study
E330	39º37'02.34''N, 33º26'38.26''E, 773m	R	R	≥512	512	47, 55, 222	+	+	+	*Enterococcus faecium* (99%)	KU296972	This study

[a] ≥ 17 mm interpreted as susceptible for vancomycin and designated with S, ≤ 14 mm interpreted as resistant for vancomycin and designated with R (CLSI 2012);
[b] ≥ 14 mm interpreted as susceptible for teicoplanin and designated with S, ≤ 10 mm interpreted as resistant for teicoplanin and designated with R (CLSI 2012);
[c] ≤ 4 μg mL^{-1} interpreted as susceptible and ≥ 32 μg mL^{-1} as resistant for vancomycin (CLSI 2012);
[d] ≤ 8 μg mL^{-1} interpreted as susceptible and ≥ 32 μg mL^{-1} as resistant for teicoplanin (CLSI 2012);
DD, disc diffusion;
MIC, minimal inhibitory concentration test;
+, positive; -, negative;
PCR, Polymerase Chain Reaction;
SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis;
16S rRNA, 16S ribosomal RNA;
EMBL, European Molecular Biology Laboratory.

Table 3.1 cont'd

Strain designations	Sampling coordinates	VAN DD test[a]	TEC DD test[b]	Vancomycin MIC test[c] (μg mL^{-1})	Teicoplanin MIC test[d] (μg mL^{-1})	Plasmid profiles (kb)	PCR analysis of *van*A	SDS-PAGE analysis of D-alanine-D-lactate ligase	Western blot analysis of D-alanine-D-lactate ligase	16S rRNA sequencing (Homology %)	EMBL access numbers	References
Hg10	39º48'38.97''N, 33º29'14.57''E, 684m	R	R	512	≥512	205	+	+	+	*Pseudomonas koreensis* (96%)	KJ395377	Icgen and Yilmaz 2014
Hg11	39º50'28.41''N, 33º28'02.13''E, 686m	R	R	512	≥512	205	+	+	+	*Pseudomonas koreensis* (98%)	KJ395378	Icgen and Yilmaz 2014
Ni11	39º50'28.41''N, 33º28'02.13''E, 686m	R	R	64	128	205	+	+	+	*Comamonas testosteroni* (99%)	KJ395372	Icgen and Yilmaz 2014
Pb06	39º34'34.39''N, 33º26'11.61''E, 763m	R	R	64	30	55, 69, 205	+	+	+	*Enterococcus faecalis* (98%)	KJ395380	Aktan *et al.*, 2013
SDS3	39º26'03.30''N, 33º24'08.43''E, 781m	R	R	30	≥512	181, 205	+	+	+	*Pseudomonas fluorescens* (99%)	KJ937668	Icgen *et al.*, 2015
SDS7	39º37'02.34''N, 33º26'38.26''E 773m	R	R	≥512	32	89, 140, 181	+	+	+	*Aeromonas veronii* (96%)	KJ937671	Icgen *et al.*, 2015
SDS8	39º39'53.04''N, 33º28'55.46''E, 852m	R	R	≥512	≥512	140, 181	+	+	+	*Pseudomonas baetica* (99%)	KJ937672	Icgen *et al.*, 2015
SDS10-2	39º48'38.97''N, 33º29'14.57''E, 684m	R	R	512	32	181	+	+	+	*Pseudomonas resinovorans* (93%)	KJ937675	Icgen *et al.*, 2015
SDS11	39º50'28.41''N, 33º28'02.13''E, 686m	R	R	128	30	140, 181	+	+	+	*Pseudomonas kilonensis* (99%)	KJ937677	Icgen *et al.*, 2015

[a] ≥ 17 mm interpreted as susceptible for vancomycin and designated with S, ≤ 14 mm interpreted as resistant for vancomycin and designated with R (CLSI 2012);
[b] ≥ 14 mm interpreted as susceptible for teicoplanin and designated with S, ≤ 10 mm interpreted as resistant for teicoplanin and designated with R (CLSI 2012);
[c] ≤ 4 μg mL^{-1} interpreted as susceptible and ≥ 32 μg mL^{-1} as resistant for vancomycin (CLSI 2012);
[d] ≤ 8 μg mL^{-1} interpreted as susceptible and ≥ 32 μg mL^{-1} as resistant for teicoplanin (CLSI 2012);
DD, disc diffusion;
MIC, minimal inhibitory concentration test;
+, positive; -, negative;
PCR, Polymerase Chain Reaction;
SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis;
16S rRNA, 16S ribosomal RNA;
EMBL, European Molecular Biology Laboratory.

3.2 Detection of *van*A Gene among Glycopeptide-Resistant Isolates

The dissemination of resistance genes in surface waters can challenge the population dynamics of indigenous microbial populations. The *van*A gene and other genes involved in the regulation and expression of high level vancomycin resistance are located on a 10581 bp transposon Tn*1546* of *E. faecium*, which often resides on a plasmid (Arthur *et al.*1993). The map of the *van*A gene cluster from several *van*A harbouring isolates revealed some heterogeneity in organization. Tn*1546* exists intact in some strains but has insertion-like sequences in others. These vancomycin resistance gene clusters may be incorporated into even larger mobile elements containing additional insertion-like elements (Handwerger and Skoble 1995; Handwerger *et al.* 1995). Therefore, the *van*A genes may be located either on plasmids or in the chromosome and are transmitted to the next generation through vertical gene transfer or to different taxonomic affiliation through horizontal gene transfer. Therefore, the obtained glycopeptide resistant isolates were analysed in terms of their plasmid profiles (Figure 3.1 and 3.2). According to the plasmid profiling, the sizes of plasmids ranged from 33 to 222 kb and the number of plasmids varied between 1-4 (Table 3.1). Since the whole *van* operon is 10581bp, it may be harbored either by any of the plasmid or the chromosome. The vancomycin- and teicoplanin-resistant isolates found in this study were further characterized by screening the *van*A gene through PCR. Vancomycin- and teicoplanin-resistant *E. faecium* E330 and vancomycin- and teicoplanin-sensitive *Escherichia coli* DH5α isolates were used as *van*A-positive and *van*A-negative controls, respectively. *van*A-positive *E. faecium* E330 was used to optimize PCR conditions by changing concentration of $MgCl_2$ ranging from 1.50 mM to 3 mM and temperature ranging from 50^{o}C to 62^{o}C (Figure 3.3 and 3.4). 56^{o}C and 3 mM $MgCl_2$ yielded the best result and preferred for *van*A PCR analysis conditons.

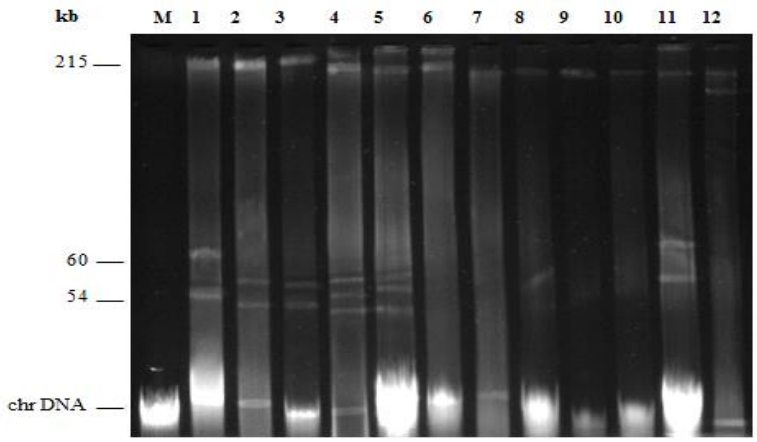

Figure 3.1 Agarose gel electrophoresis of total DNA extracted from surface water; *Agrobacterium tumefaciens* lane 1 (215, 60, 54 kb), *E. faecium* E330 (vancomycin-resistant positive control) lane 2, *E. faecalis* E07 lane 3, *P. plecoglossicida* Ag10 lane 4, *R. planticola* Ag11 lane 5, *E. faecalis* Cr07 lane 6, *P. korensis* Cu12 lane 7, *P. korensis* Hg10 lane 8, *P. korensis* Hg11 lane 9, *C. testosteroni* Ni11 lane 10, *E.faecalis* Pb06 lane 11, *P. fluorescens* SDS3 lane 12. M, Marker Lambda DNA/EcoRI+HindIII (125-21226 bp) (Standard curve of the gel is given in Appendix B)

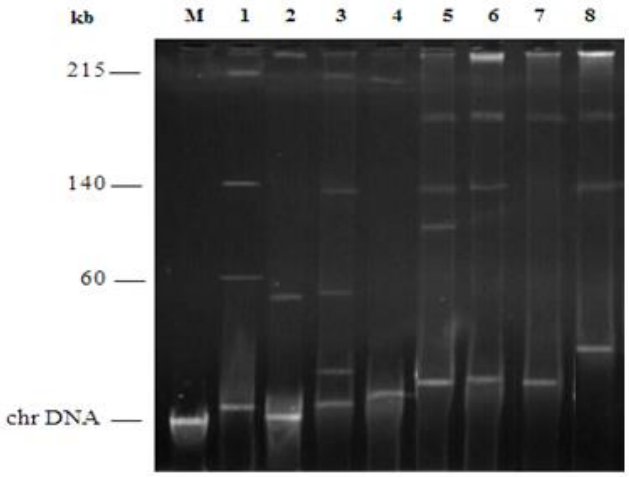

Figure 3.2 Agarose gel electrophoresis of total DNA extracted from surface water; *Agrobacterium tumefaciens* lane 1 (215, 140, 60 kb), *S. aureus* Al11 lane 2, *S. aureus* Ba01 lane 3, *S. warneri* Co11 lane 4, *A. veronii* SDS7 lane 5, *P. baetica* SDS8 lane 6, *P. resinovorans* SDS10-2 lane 7, *P. kilonensis* SDS11 lane 8, M, Marker Lambda DNA/EcoRI+HindIII (125-21226 bp) (Standard curve of the gel is given in Appendix B)

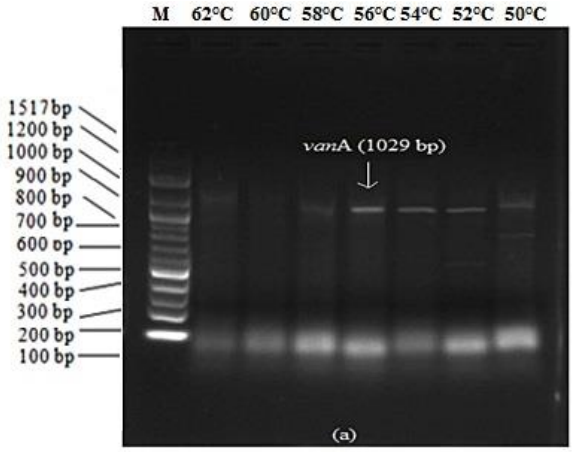

Figure 3.3 Optimization of PCR conditions at various annealing temperatures (°C) for *E. faecium* E330. M, Quick-Load 100 bp DNA Ladder (100–1517 bp)

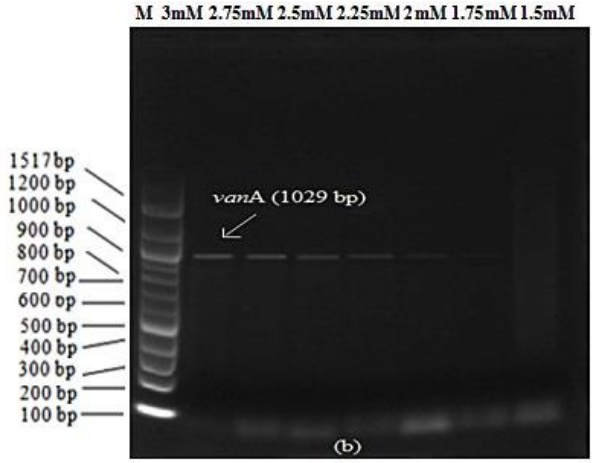

Figure 3.4 Optimization of PCR conditions at various MgCl2 concentrations (mM) for *E. faecium* E330. M, Quick-Load 100 bp DNA Ladder (100–1517 bp)

Presence of *van*A gene was confirmed in all of the vancomycin- and teicoplanin-resistant surface water isolates by the amplification products with the expected sizes of 1029 bp on the gel electrophoresis (Figure 3.5 and 3.6). All of the vancomycin- and teicoplanin-resistant and the *van*A gene harboring isolates were coherently detected. The identities of 4 enterococcal isolates harboring *van*A gene were also

successfully revealed by including partial *tuf* gene primers during the *van*A gene analyses (Figure 3.5 and 3.6). The *tuf* gene encoding the elongation factor EF-Tu has previously been demonstrated as a suitable target to identify enterococcal species (Ke *et al.*, 1999).

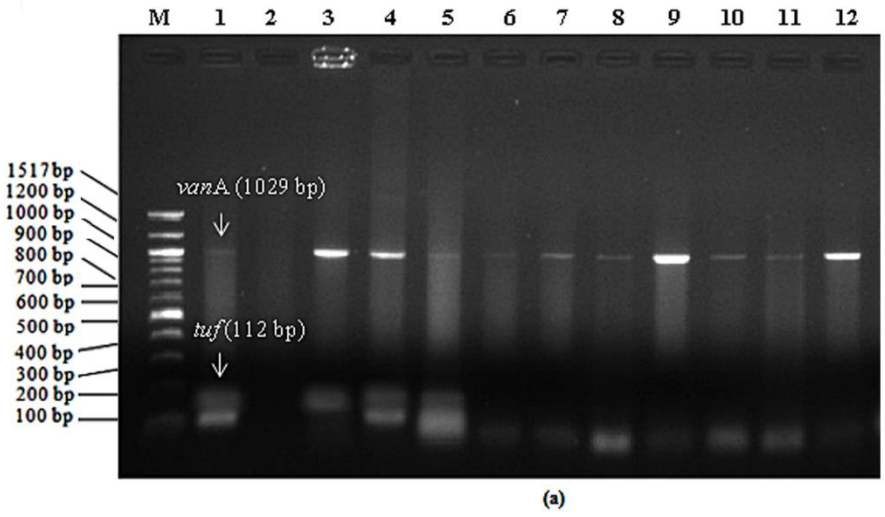

Figure 3.5 The *van*A gene harboring surface water isolates; *E. faecium* 330 (positive control) lane 1, *E. coli* DH5α (negative control) lane 2, *E. faecalis* E07 lane 3, *E. faecalis* Cr07 lane 4, *E. faecalis* Pb06 lane 5, *P. plecoglossicida* Ag10 lane 6, *R. planticola* Ag11 lane 7, *P. koreensis* Cu12 lane 8, *P. koreensis* Hg10 lane 9, *P. koreensis* Hg11 lane 10, *C. testosteroni* Ni11 lane 11, *P. fluorescens* SDS3 lane 12. M, Quick-Load 100 bp DNA Ladder (100–1517 bp) (Standard curve of the gel is given in Appendix C)

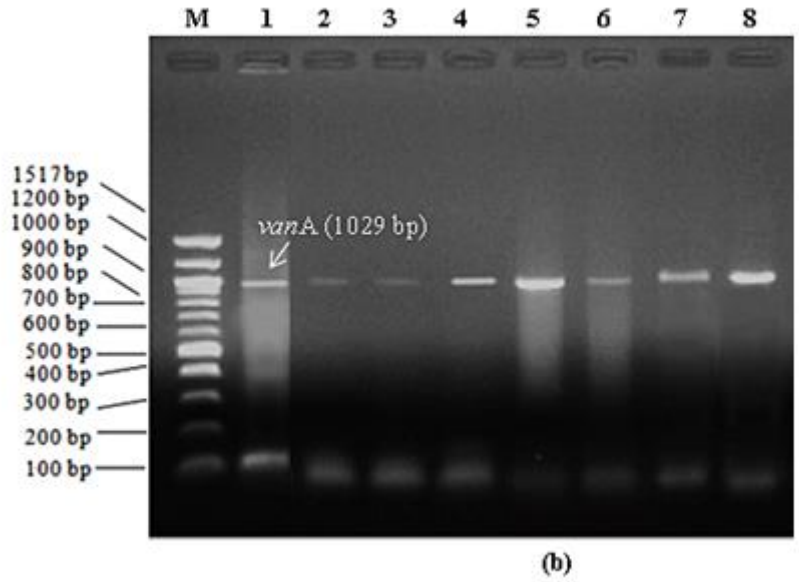

Figure 3.6 The *van*A gene harboring surface water isolates; *E. faecium* 330 (positive control) lane 1, *A. veronii* SDS7 lane 2, *P. baetica* SDS8 lane 3, *P. resinovorans* SDS10-2 lane 4, *P. kilonensis* SDS11 lane 5, *S. warneri* Co11 lane 6, *S. aureus* Ba01 lane 7, *S. aureus* Al11 lane 8. M, Quick-Load 100 bp DNA Ladder (100–1517 bp) (Standard curve of the gel is given in Appendix C)

As in this study, the occurrence of *van*A-mediated vancomycin resistance in *Staphylococcus* has been shown previously although it is still uncommon (Perichon and Courvalin 2009). However, so far, there has been no evidence for the presence of *van*A gene in *Pseudomonas, Comamonas, Raoultella*, and *Aeromonas* species as found in this study. One means by which these species may develop high-level vancomycin and teicoplanin resistance in surface waters is through *van*A gene transfer.

3.3 Identification of *van*A Harboring Isolates by 16S rRNA Sequencing

Out of 23 isolates used in this study, only two of them, namely E07 and E330 were identified. The rest of the isolates had been identified by our group previously (Table 3.1) The phylogenetic trees of the isolates E07 (Figure 3.7) and E330 (Figure 3.8) displayed 99% homologies with *E. faecalis* and *E. faecium* and affiliated with accession numbers of KU195302 and KU296972, respectively (Table 3.1).

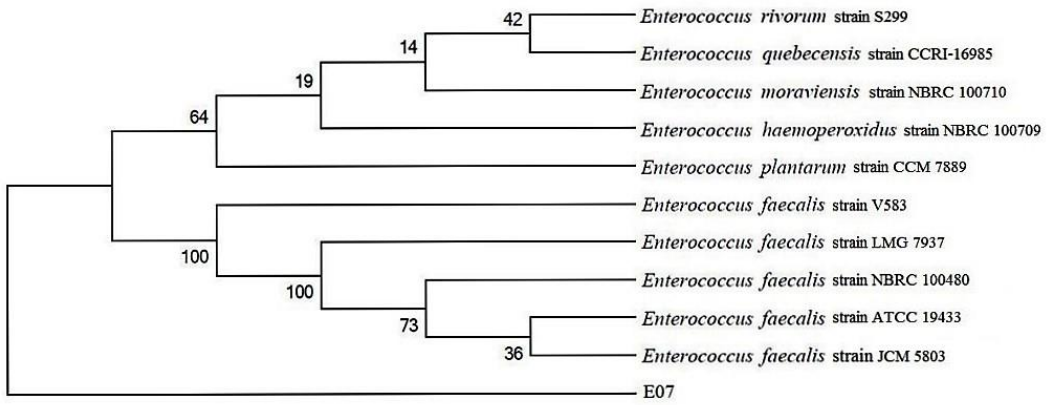

Figure 3.7 Phylogenetic tree of the isolate E07

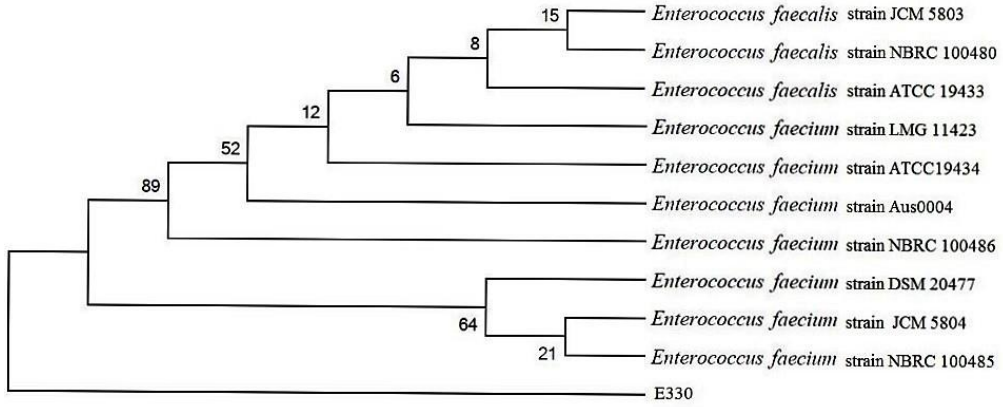

Figure 3.8 Phylogenetic tree of the isolate E330

3.4 Sequencing of *van*A Amplicons

The first step toward building a distance tree is to generate a distance matrix just to count the fraction of identical bases in every pair of sequences in the alignment. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic tree of partial *van*A sequences obtained from enterococcal and non-enterococcal isolates showed sequence similarity values of 58 to 100% (Figure 3.9). The results revealed that the *van*A gene sequence similarity among surface water isolates from enterococcal and non-enterococcal origins were related. Conjugative transfer of high-level vancomycin resistance from *E. faecalis* to *S. aureus* (Noble *et al.* 1992), and transfer of glycopeptide- and macrolide-resistance genes by transconjugation among

enterococci and from *E. faecalis* to *S. aureus* (Młynarczyk *et al*. 2003) have been reported. The *van*A gene acquisition by *S. aureus* from *E. faecium* in the clinical environment has also been reported by Weigel *et al.* (2007). Antibiotic resistance genes, acquired by pathogenic and non-pathogenic bacteria trough horizontal gene transfer have been originated from environmental bacteria (Davies, 1997). This indicates that, once they are integrated in successful gene-transmission elements, antibiotic resistance genes can persist and spread as shown with the presence of *van*A gene in enterococcal and non-enterococcal species found in current study. It is important to understand the heterogeneity of *van*A gene from different origins. The overlapping and non-overlapping sequences of several *van*A genes from different species obtained in this study confirmed that the *van*A gene was not only harbored and conserved among enterococcal species but also non-enterococcal ones.

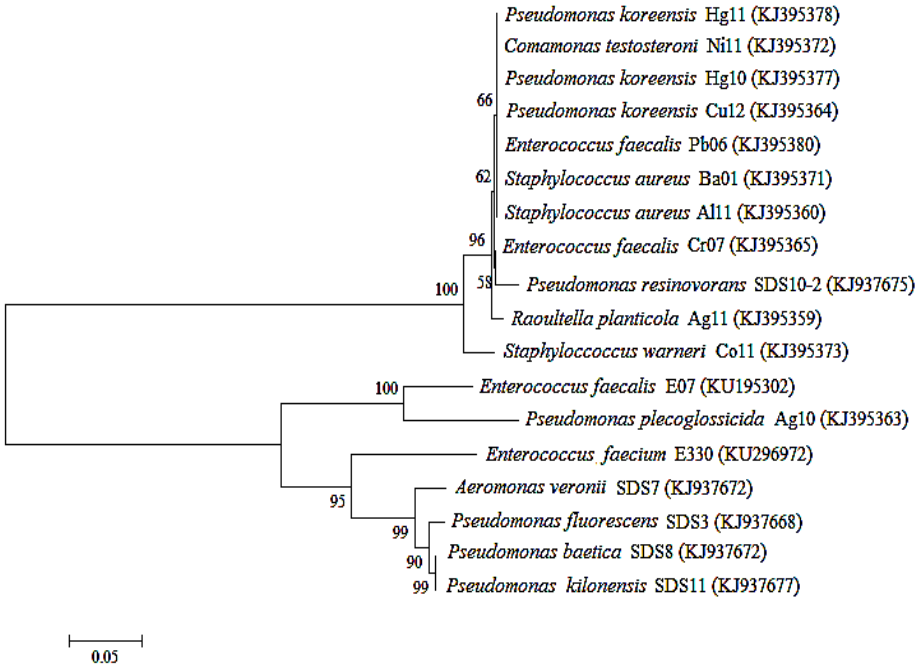

Figure 3.9 Phylogenetic tree of *van*A harboring isolates. The phylogenetic tree was constructed based on common partial sequences by using the Neighbor-Joining Tree method with 1000 bootstrapped data sets. The scale bar indicates substitutions per base pair

```
Hg11         ------------------------------ATGATCGGGGAGTGCATACCTGCAGCGGCCA   31
Ba01         ------------------------------ATGGTTTGGGGAGTGCATACCTGCAGCGGCCA   32
Ni11         ------------------------------AGGTTTTGGGGAGTGCATACCTGCAGCGGCCA   32
Pb06         ------------------------------GGGATTTGGGCAGTGCATACCTGCAGCGGCCA   32
SDS11        ----------------------AGTATTGGTTTCGGGGAGTGCAATACCTGCAGCGGCCA   38
SDS10-2      AACTCGATGCAGTGCTTGCACTTTTATTCTATTCATATAATAAGAGGGATACGACTGTAT   60
Cu12         ------------TTGGTTTTGGGGGAGTGCATACCT--------------GCAGCGGCCA   34
SDS8         ------------GCATTGATTCGGGGGAGTGCAATA-----------CCTGCAGCGGCCA   37
Ag11         -----------GATCCTTCGGGGAAGTGAAATACCT-----------GCAGCGGCCAT--   36
Hg10         -----------ATAGATTCGGGGGAGTGCAATACCT-----------GCAGCGGCCAT--   36
Cr07         -------------CGAGGTTAGGTACTTACATATCT-----------ATCCCTCGACGTC   36
Co11         -----GGGGGTGGGGTGATTGCACTTTTATTCTATT-----------CATATAACGCGGG   44
Al11         ----------------TTGGTTTTGGGAAGTGCATACCTGCAGCGGCCATCATACGGGGA   44
E330         -----------TCTGGCTGCTACGTACATAGTACGAAGTAGTCGTCGACTGGATGGACGC   49
SDS3         -----------------CGTAGCGATCACGGGGCATGTGATTAATCGTGAAAGGGGTTAT   43
SDS7         ---------------TATGGGGGCTGCTAGAGGAGCATGACGTATCGGTAAAATCTGCAA   45
E07          -------------CTTAAAGAAGTTCACCAGAGCAGCTGCCTAATCGATAAATTCTGAAA   47
Ag10         -------------CTTTTCTTGTTCCCTAAGAACAACATGACTTATCGGTACTTCTGCAA   47

Hg11         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   91
Ba01         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   92
Ni11         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   92
Pb06         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   92
SDS11        TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   98
SDS10-2      GA------------CGTGAAACCG----------GGCAGCAGAATTGACTTCGTTCAGTA   98
Cu12         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   94
SDS8         TCATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   97
Ag11         -CATACGGGGATAACGACTGTATGACGTGAAACCGGGCACAGTATTGACTTCGTTCAGTA   95
Hg10         -CATACGGGGATAACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   95
Cr07         CATATACGGGGATACGACTGTATGACGTGAAACCGGGCAGAGTATTGACTTCGTTCAGTA   96
Co11         GATACGACTGTATGACGTGA-AACCGGGAAGAGTAATA--------AGGTTCGTTCAGTA   95
Al11         TAACGACTGTATGACGTGAA-A-----CCGGGCAGAGTATTGAC-----TTCGTTCAGTA   93
E330         GCTTGGGAATGGG---AAAA-C-----GACA------------------------------   71
SDS3         AAATGAAATAAAG---CTTG-A-----TTACTAAAGAAAAATACGAGACGGGGTACATTG   94
SDS7         TAGAGATAGCCGC---TAAC-A-----TTAATAAAGAAAAATACGAGCCGTTATACATTG   96
E07          TAAAAATAGCCGC---TAAC-A-----TTAAAAAAAAAAAATACCAGCCGTTTTACTTTG   98
Ag10         TAAAGATACCCGC---AAAT-A-----TTAAAAAAAAAAACTAGCAGCCGTTATCCGTTG   98

Hg11         CAATGCGGCCGTTATCTTGTAAAA-ACATATCCACACGGGCTAGACCTCTACAGCCGAGC   150
Ba01         CAATGCGGCCGTTATCTTGTAAAA-ACATATCCACACGGGCTAGACCTCTACAGCCGAGC   151
Ni11         CAATGCGGCCGTTATCTTGTAAAA-ACATATCCACACGGGCTAGACCTCTACAGCCGAGC   151
Pb06         CAATGCGGCCGTTATCTTGTAAAA-ACATATCCACACGGGCTAGACCTCTACAGCCGAGC   151
SDS11        CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   157
SDS10-2      CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   157
Cu12         CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   153
SDS8         CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   156
Ag11         CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAAC   154
Hg10         CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   154
Cr07         CAATGCGGCCGTTATCTTGTAAAAAC-ATATCCACACGGGCTAGACCTCTACAGCCGAGC   155
Co11         CAATGCGGACGTTATCTTGTAAAAAAACATAGGACACGGGCTAGACCTCTACAGCCGAGC   155
Al11         CAATGCGGCCGTTATCTTGTAAAA-ACATATCCACACGGGCTAGACCTCTACAGCCGAGC   152
E330         ------------------------------------------------------------   71
SDS3         GAATTACGAAATCTGGTGTATGGA-AAATGTGCGAAAAACCTTGCGCGGAATGGGAAAAC   153
SDS7         GAATTACGAAATCTGGTGTATGGA-AAATGTGCGAAAAACCTTGCGCGGAATGGGAAAAC   155
E07          AAATTACAAATTCGGGGGTAGGGA-AAATGTGCAAAAAACCTTGGGGGAAAGGGAAAAAC   157
Ag10         TAATTACAAATTCGGGGGAAGGGA-AAAGGGGCAAAAACCCTGGGGGGAAAGGGAAAAAC   157

Hg11         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   204
Ba01         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   205
Ni11         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   205
Pb06         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   205
SDS11        GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   211
SDS10-2      GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   211
Cu12         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   207
SDS8         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   210
Ag11         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   208
Hg10         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   208
Cr07         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTCTGCTGAA   209
Co11         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCT-CGCTCCTTTGCTGAA   209
Al11         GCT-TTATATATTTTTTTTGCCGTTTCCTGTA----TCCGTCCTCGCTC-CTCTGCTGAA   206
E330         ----ATTGCTATTCAGACTCTCGCCGGATAAAAAAATGCAGGAATAAGAGATAAGGTAAA   127
SDS3         GACAATTGCTATTCAGCTGTACTCTCGCCGGATAAAAAAATGCACGGATTACTTGTTAAA   213
SDS7         GACAATTGCTATTCAGCTGTACTCTCGCCGGATAAAAAAATGCACCGATTACTTGTTAAA   215
E07          AACTTTTGTTTTTCTGTTGTCCTCTCGCCGGATAAAAAAATGCCCGGATTACTTGTAAAA   217
Ag10         AACATTTGCTTTTCTCCTGTCCCCTCGCCGGATAAAAAAATGCCCGGATTACTTGAAAAA   217
                  *   * **        *         *                     *   **
```

Figure 3.10 *van*A multiple-sequence alignments of enterococcal and non-enterococcal isolates. Clustal Omega was used to create multiple-sequence alignments. Asterisks denote identical residues

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Hg11          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   249
Ba01          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   250
Ni11          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   250
Pb06          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   250
SDS11         AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   256
SDS10-2       AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   256
Cu12          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   252
SDS8          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   255
Ag11          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   253
Hg10          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   253
Cr07          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCC----------------   254
Co11          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAAAGCCTTTTTCC----------------   254
Al11          AGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCCGGCTCGACTTCCTG-   265
E330          AAGAACCATGAATATGAAATCAACCATGTTGATGTAGCATTTTCAGCTTTGCATGGCAAG   187
SDS3          AAGAACCATGAATATGAAATCAACCATGTTGATGTAGCATTTTCAGCTTTGCATGGCAAG   273
SDS7          AAGAACCATGAATATGAAATCAACCTTGTTGATGTAGCATTTTCAGCTTTGCATGGTAAG   275
E07           AAAAACCATGATTATGAAATCCCCCTTGTTGATGTAGCTTTTTCATTTTTGGTTGGGAGG   277
Ag10          AAAAACCATAAATATAAAACCACCCTTGTTGATGTAGTTTTTTCATTTTTGGTGGGGAGG   277
              *                *         **         *****

Hg11          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   292
Ba01          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   293
Ni11          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   293
Pb06          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   293
SDS11         ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   299
SDS10-2       ------------------GGCTCGACTTCCTGATGAATACAAAAGATTCCGTACTGCAGCC   299
Cu12          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   295
SDS8          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   298
Ag11          ------------------GGCTCGACTTCCTGATGAATACCAAAGATTCCGTACTGCAGCC   296
Hg10          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   296
Cr07          ------------------GGCTCGACTTCCTGATGAATACAAAAGATTCCGTACTGCAGCC   297
Co11          ------------------GGCTCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCC   297
Al11          ----------AT----------------------GAATACGAAAGATTCCGTACTGCAGCC   294
E330          TCAGGTGAAGATGGATCCATACAAGGTCTGTTTGAATTGTCCGGTATCCCTTTTGTAGGC   247
SDS3          TCAGGTGAAGATGGATCCATACAAGGTCTGTTTGAATTGTCCGGTATCCCTTTTGTAGGC   333
SDS7          TCAGGTGAAGATGGATCCATCCATGGTCTGTGGGAATTGTCCCCGC--------------   321
E07           TCAGGGGAAGATGGATCCTTACGGGGTCTTTTTGATTTGCCCGTTTTCCTTTTTGGAGGC   337
Ag10          CGAGGGAAAGATGGATCCTTACAGGGTTTTTTTGAGTTCCCCGTTTTCCTTTTTGGAGGC   337
** *

Hg11          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   352
Ba01          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   353
Ni11          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   353
Pb06          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   353
SDS11         TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   359
SDS10-2       GGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   359
Cu12          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   355
SDS8          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   358
Ag11          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   356
Hg10          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   356
Cr07          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   357
Co11          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   357
Al11          TGATTTGGTCCACCTCGCCAACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGA   354
E330          TGCGATATTCAAAGCTCAGCA----------ATTTGTATGGACAAATCGTTGACATACAT   297
SDS3          TGCGATATTCAAAGCTCAGCA----------ATTTGTATGGACAAATCGTTGACATACAT   383
SDS7          ------------------------------------------------------------   321
E07           TGCAATATTCAAACTTCAGTT----------TTTTGTATGGACAAATTGTTAACTTACTT   387
Ag10          TGCATTATCCAAACTTCATCT----------TTTTGGATGGACAATTTGTGAACTTCCAT   387


Hg11          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   412
Ba01          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   413
Ni11          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   413
Pb06          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   413
SDS11         CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   419
SDS10-2       CCTCACAGCCCGAAACAGCCGGCTCAATTAAAATTTTGCTGTCATATTGTCTTGCCGATT   419
Cu12          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   415
SDS8          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   418
Ag11          CCTC------------------------------------------------------   360
Hg10          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   416
Cr07          CCTCACAGCCCGAAACAGCCGGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   417
Co11          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   417
Al11          CCTCACAGCCCGAAACAGCCTGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATT   414
E330          CGTTGCGAAAAATGCTGGGATAGCTACTCCCGCCTTTTGGGTTATTA----ATAAAGATG   353
SDS3          CGTTGCGAAAAATGCTGGGATAGCTACTCCCGCCTTTTGGGTTATTA----ATAAAGATG   439
SDS7          ------------------------------------------------------------   321
E07           CGTTGCAAAAAATGGGGGAATACCTCCCCCCGCTTTTGGGGTTATAA----AAAAAGATG   443
Ag10          CGTTGCAAAAAATGTTGGAACCGCCCCCCCCGCTTTTTGGGTTATAA----AAAAGGATG   443
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Figure 3.10 cont'd

```
Hg11          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   471
Ba01          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   472
Ni11          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   472
Pb06          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   472
SDS11         CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   478
SDS10-2       CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   478
Cu12          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   474
SDS8          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   477
Ag11          ------------------------------------------------------------   360
Hg10          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   475
Cr07          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   476
Co11          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   476
Al11          CAATTGCGTAGTCCAATTCGTCCGCG-CTATTGACTTTTTTCACACCGAAGGATGAGCCT   473
E330          ATAGGCCGGTGGCAGCTACGTTTACCTATCCTGTTTTTGTTAAGCCGGCGCGTTCAGGCT   413
SDS3          ATAGGCCGGTGGCAGCTACGTTTACCTATCCTGTTTTTGTTAAGCCGGCGCGTTCAGGCT   499
SDS7          ------------------------------------------------------------   321
E07           ATGGGCCGGGGGCACCTCCTTTCACTTTTCCTGTTTTTGTAAAGCCGGGGCGTTCAGGTT   503
Ag10          AGGGGCCGGGGGCACCTCCTTTTACTTTTCTTGTTTTTGTTAAGCCGGGGCGTCCAGGCT   503

Hg11          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   531
Ba01          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   532
Ni11          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   532
Pb06          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   532
SDS11         GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   538
SDS10-2       GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   538
Cu12          TTAACGC--------------------------------------------------------   481
SDS8          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   537
Ag11          ------------------------------------------------------------   360
Hg10          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   535
Cr07          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   536
Co11          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   536
Al11          GAACGCGCCGGCTTAACAAAAACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCT   533
E330          CATCCTTCGGTGTGAAAAAAGTCAATAGCGC-GGACGAA-TTGGACTACGCAATTGAATC   471
SDS3          CATCCTTCGGTGTGAAAAAA-------------------------------------------   519
SDS7          ------------------------------------------------------------   321
E07           CTTCCTTCGGGGAAAAAAAAGTCAATACCGC-GGACAAA-TTGAACTCCGCATTTGATTC   561
Ag10          CCTCCTTGGGGGGAAAAAAGTTCAAAAGGGC-GGACAAA-TGGTATTCCCTTTTTGAACC   561

Hg11          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   591
Ba01          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   592
Ni11          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   592
Pb06          TTATTAATAACCCAAAAGGCGGGA----------------------------------   556
SDS11         TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   598
SDS10-2       TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   598
Cu12          ------------------------------------------------------------   481
SDS8          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   597
Ag11          ------------------------------------------------------------   360
Hg10          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   595
Cr07          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   596
Co11          TTATTAATAACCCAAAAGGCGGGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTC   596
Al11          TTATTAATAACCCAAAAGGCGGGAGTAACTATCCCAGCATTTTTCGCAA------------   582
E330          GGCAAGACAATATGACAGCAAAATCTTAATTGAGCAGGCTGTTTCGGGCTGTGAGGTCGG   531
SDS3          ------------------------------------------------------------   519
SDS7          ------------------------------------------------------------   321
E07           GGCAAGACATTATAACAGCAAATTCTTATTTGAGCAGGTTGTTTCGGGTTGGGAGGTCGG   621
Ag10          GG--------------------------------------------------------   563

Hg11          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   650
Ba01          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   651
Ni11          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCCTT------------   639
Pb06          ------------------------------------------------------------   556
SDS11         AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   657
SDS10-2       AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   657
Cu12          ------------------------------------------------------------   481
SDS8          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   656
Ag11          ------------------------------------------------------------   360
Hg10          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   654
Cr07          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   655
Co11          AACGATTTGT-CCATACAAATTGCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACC   655
Al11          ------------------CG----------ATGTATGTCAACGA----------------   598
E330          TTGTGCGGTATTGGGAAACAGTGCCGC--GTTAGTTGTTGGCGAGGT--GGACCAAATCA   587
SDS3          ------------------------------------------------------------   519
SDS7          ------------------------------------------------------------   321
E07           TGGTGCGTTATGGAAACACGGTGCCTC--TTTAGTTGTTGGCGAGGA--GCACCAAATCG   677
Ag10          ------------------------------------------------------------   563
```

Figure 3.10 cont'd

```
Hg11          GGACAATTTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   710
Ba01          GGACAAATTCAAACAGACCTTGTATGG-------------------------------   678
Ni11          ---------------------------------------------------------   639
Pb06          ---------------------------------------------------------   556
SDS11         GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   716
SDS10-2       GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   716
Cu12          ---------------------------------------------------------   481
SDS8          GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   715
Ag11          ---------------------------------------------------------   360
Hg10          GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   713
Cr07          GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   714
Co11          GGAC-AATTCAAACAGACCTTGTATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTG   714
Al11          ---------------------------------------------------------   598
E330          GGC----TGCAGTACGGAATCTTTCGTATTCATCAGGAAG----------TCGAGCCGGA   633
SDS3          ---------------------------------------------------------   519
SDS7          ---------------------------------------------------------   321
E07           GGC----TGCATCACGGATTTTTTCTTATTCATCAGGACG----------TCCAGCCGGA   723
Ag10          ---------------------------------------------------------   563


Hg11          AAAAT-----------------------------------------------------   715
Ba01          ---------------------------------------------------------   678
Ni11          ---------------------------------------------------------   639
Pb06          ---------------------------------------------------------   556
SDS11         AAAATGCTACATCAACATGGTTGATTTCATATTC-ATGGTTCTTTTTAACAAGTAATCCG   775
SDS10-2       AAAATGCTACATCAACATGGTTGATTTCATATTC-ATGGTTCTTTTTAACAAGTAATCCG   775
Cu12          ---------------------------------------------------------   481
SDS8          AAAATGCTACATCAACATGGTTGATTTCATATTC-ATGGTTCTTTTTAACAAGTAATCCG   774
Ag11          ---------------------------------------------------------   360
Hg10          AAAATGCTACACCAACATGGTTGATTTCATATTCCATGGTTCTTTTTAACAAGTAATCCG   773
Cr07          AAAATGCTACATCAACATGGTTGATTTCATATTC-ATGGTTCTTTTTAACAAGTAATCCG   773
Co11          AAAATGCTACATCAACATGGTTGATTTCATATTC-ATGGTTCTTTTTAACAAGTAATCCG   773
Al11          ---------------------------------------------------------   598
E330          AAAAGGCTCTGAAAACGCAGTTATAACCGTTCCCGCAGA--CCTTTCAGCAGAGGAGCGA   691
SDS3          ---------------------------------------------------------   519
SDS7          ---------------------------------------------------------   321
E07           AGAAGGCTCTGAAAACGCATTTACCCCCCGTCCCCCGCCAACCTTCCAGCGGAGGAGCGG   783
Ag10          ---------------------------------------------------------   563


Hg11          ---------------------------------------------------------   715
Ba01          ---------------------------------------------------------   678
Ni11          ---------------------------------------------------------   639
Pb06          ---------------------------------------------------------   556
SDS11         TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAA-TAGCAATTGTCGTTTTCCCATTCC   832
SDS10-2       TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAAATAGCAATTGTCGTTTTCCCATTCC   833
Cu12          ---------------------------------------------------------   481
SDS8          TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAATAGCAATTGTCGTTTTCCCA--TTC   830
Ag11          ---------------------------------------------------------   360
Hg10          TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAATAGCAATTGTCGTTTTCCCATTCCC   831
Cr07          TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAATAGCATTGTCGT--TTTCCCATTCC   829
Co11          TGCATTTTTTT--ATCCGGCGAGAGTACAGCTGAATAGCATTTGTCGTTTTTCCCATTCC   831
Al11          ---------------------------------------------------------   598
E330          GGACGGATACAGGAAACG-GCAAAAAAAATATATAAAGCCGC-TCGGC-TGTAGAGGTCT   748
SDS3          ---------------------------------------------------------   519
SDS7          ---------------------------------------------------------   321
E07           AGCACGGTACCGGAAAACGGCAAAAAAAATTTTTATAAGGTGTTCGGTTTGTAGAGTCTC   843
Ag10          ---------------------------------------------------------   563


Hg11          ---------------------------------------------------------   715
Ba01          ---------------------------------------------------------   678
Ni11          ---------------------------------------------------------   639
Pb06          ---------------------------------------------------------   556
SDS11         GCGCAAGGTTTTTCGCACATTTTC--CA--TA--CAC-CAGATTTCGTAATTC----CAA   881
SDS10-2       GCGCAAGGTTTTTCGCACATTTTT--CCATAC--ACC-AAGATTTCGTAATTC----CAA   884
Cu12          ---------------------------------------------------------   481
SDS8          CGCGCAAGGTTTTTCGCACATTTT--CCATAC--ACCAG-AT-TTCGTAATTC----CAA   880
Ag11          ---------------------------------------------------------   360
Hg10          GCCCAGGGTTTCTCCCCACATTTT--CCCATA--CACCAGGATTTCGTAATTC-------   880
Cr07          GCGCAAGGGTTTTTCGCACATTTT--CCATAC--ACC-AGAT-TTCGTAATTT----CCA   879
Co11          CGCGCAAGGTTTTTCGCACATTTT--CCATAC--ACC-AGAT-TTCGTAATTC----CAA   881
Al11          ---------------------------------------------------------   598
E330          AGCCCGTGT--GGATATGTTTTTACAAGATAACGGCCGCATTG----TACTGAAACGAAG   802
SDS3          ---------------------------------------------------------   519
SDS7          ---------------------------------------------------------   321
E07           TCGCCCGGTGGTGATATTTTTTTTTACAAAATAACCGGCCTTTTTGGTACGGGAACGAAA   903
Ag10          ---------------------------------------------------------   563
```

Figure 3.10 cont'd

```
Hg11        ------------------------------------------------------------   715
Ba01        ------------------------------------------------------------   678
Ni11        ------------------------------------------------------------   639
Pb06        ------------------------------------------------------------   556
SDS11       TGTATA-ACGGCTCGTATTT-TTCTTTATTAATGTTAGCGGCTATCTCTATGCAGATTTT   939
SDS10-2     TGTATA-ACGGCTCGTATTTTTTCTTTATTAATGTTAGCGGCTATCTCTATGCAGATTTT   943
Cu12        ------------------------------------------------------------   481
SDS8        TGTATAACGGCTCGTATTTTTTCTTTTATTAATGTTAGCGGCTATCTCTATGCAGATTTT   940
Ag11        ------------------------------------------------------------   360
Hg10        ------------------------------------------------------------   880
Cr07        ATGTATAACGGCTC-GTATTTTTCTTTATTAATGTTAGCGGCTATCTCTATGCAGATTTT   938
Co11        TGTATAACGGCTCG-TATTTTTTCTTTATTAATGTTAGCGGCTATCTCCTATTTGCAGAT   940
Al11        ------------------------------------------------------------   598
E330        TCAATACTCTGCCCGGGTTTCACGTCATACAGTCGTATCCCCCGTA-----------ATG   851
SDS3        ------------------------------------------------------------   519
SDS7        ------------------------------------------------------------   321
E07         TATAAAT-----CCGTCTGGCCCGTGTTTTA----------------------------   929
Ag10        ------------------------------------------------------------   563

Hg11        ------------------------------------------------------------   715
Ba01        ------------------------------------------------------------   678
Ni11        ------------------------------------------------------------   639
Pb06        ------------------------------------------------------------   556
SDS11       AC-CGATACGTCA-----TGCTCCTCTGAGCAACCCC---CAAACAGTAT-TGCAACTTT   989
SDS10-2     ACCGGATACGTCA-----TGCTCCTCTGAGCAACCCC---CAAACAGTAT-TGCAACTTT   994
Cu12        ------------------------------------------------------------   481
SDS8        ACCGATACGTCATGC------TCCTCTGAGCAACCCC---CAAACAGTATTGC-ACTTAT   990
Ag11        ------------------------------------------------------------   360
Hg10        ------------------------------------------------------------   880
Cr07        ACCGATACGT-----CA-TGCTCCTCTGAGCAACCCC---CAAACAGTAT-TGCAACTTA   988
Co11        TTTAACCGATTACGTCATTGCTCCTCTGGAGCAACCCCCCAAAACAGTAATTGCAACTTT   1000
Al11        ------------------------------------------------------------   598
E330        ATGGGCCGCTGCAGGTATTGCACT------------TCCCCGAACTGAATGAACCGCTTT   899
SDS3        ------------------------------------------------------------   519
SDS7        ------------------------------------------------------------   321
E07         ------------------------------------------------------------   929
Ag10        ------------------------------------------------------------   563

Hg11        ----------------------------------        715
Ba01        ----------------------------------        678
Ni11        ----------------------------------        639
Pb06        ----------------------------------        556
SDS11       ATTTCTCTATTCATAAAAAAGGATAAA-------        1016
SDS10-2     AAGTTCTAATCTACTCTGTTCGTTGG--------        1020
Cu12        ----------------------------------        481
SDS8        TTT--CTCTATTCTAATATAAGGTGG--------        1014
Ag11        ----------------------------------        360
Hg10        ----------------------------------        880
Cr07        ATTTTCTTATTCTCAAGAGAAAATAGAAGTGGCA        1022
Co11        TTA----ATTTCCTAATTTCAGATAGAGAGCT--        1028
Al11        ----------------------------------        598
E330        --GATCGGATTAGCGGTAAAGGG-------GGAA        924
SDS3        ----------------------------------        519
SDS7        ----------------------------------        321
E07         ----------------------------------        929
Ag10        ----------------------------------        563
```

Figure 3.10 cont'd

3.5 Detection of D-Alanine-D-Lactate Ligase

Glycopeptides bind to the C-terminal D-alanine-D-alanine of late PG precursors and block the following steps in cell wall synthesis. The formation of modified PG precursor D-alanine-D-lactate due to the *van*A-encoded D-alanine-D-lactate ligase with the elimination of D-alanine-D-alanine ending precursor renders high-level resistance to vancomycin and teicoplanin antibiotics (Depardieu *et al.* 2007).

Therefore, in this study, the protein of D-alanine-D-lactate ligase was also detected by using SDS-PAGE and western blot analyses.

3.5.1 SDS-PAGE Analysis of D-Alanine-D-Lactate Ligase

The protein D-alanine-D-lactate ligase encoded by *van*A was analysed for all vancomycin- and teicoplanin-resistant isolates by using SDS-PAGE. All of the *van*A harboring vancomycin- and teicoplanin-resistant isolates contained one major band approximately at the 39 kDa position, which was the expected position of D-alanine-D-lactate ligase protein (Figure 3.11-14). SDS-PAGE analyses revealed that *van*A-encoded 39-kDa D-alanine-D-lactate ligase protein was not only carried by enterococcal isolates but also non-enterococcal ones.

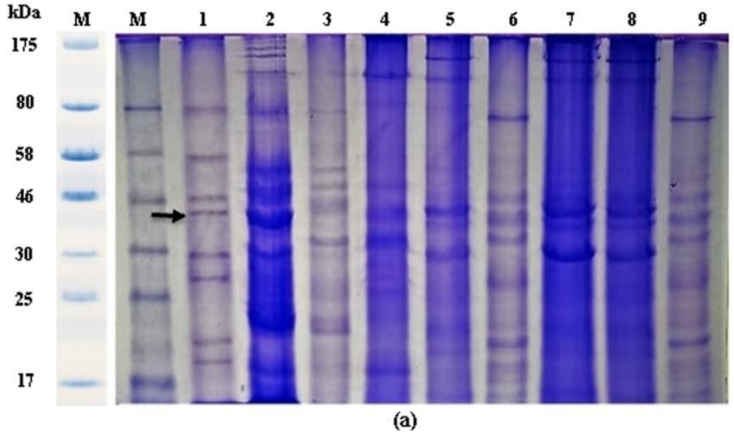

Figure 3.11 SDS-PAGE analysis of D-alanine-D-lactate ligase (39 kDa indicated by the black arrow): *E. faecium* E330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *P. plecoglossicida* Ag10 lane 3, *R. planticola* Ag11 lane 4, *E. faecalis* Cr07 lane 5, *P. koreensis* Cu12 lane 6. *P. koreensis* Hg10 lane 7, *P. koreensis* Hg11 lane 8, *E. faecalis* Pb06 lane 9. M, Colorplus Prestained Protein Marker (7-175 kDa) (Standard curve of the gel is given in Appendix D)

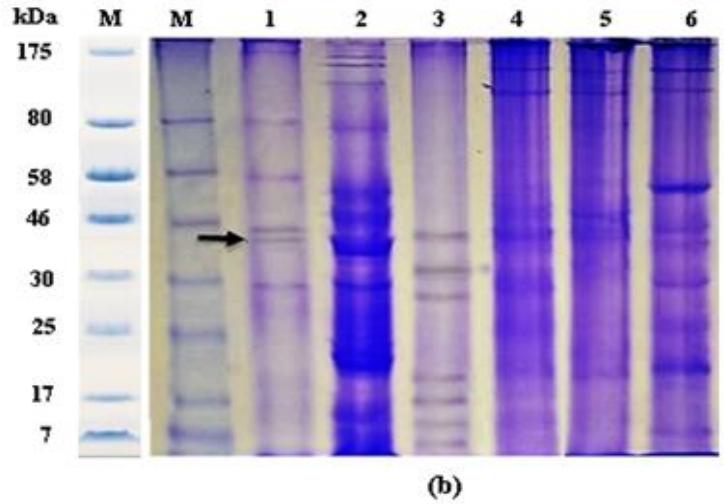

Figure 3.12 SDS-PAGE analysis of D-alanine D-lactate ligase (39 kDa indicated by the black arrow): *E. faecium* E330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *C. testosteroni* Ni11 lane 3, *P. fluorescens* SDS3 lane 4, *A. veronii* SDS7 lane 5, *P. baetica* SDS8 lane 6. M, Colorplus Prestained Protein Marker (7-175 kDa) (Standard curve of the gel is given in Appendix D)

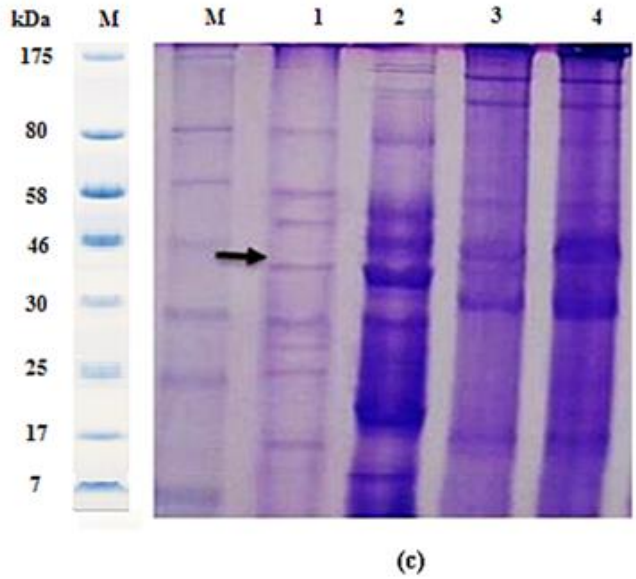

Figure 3.13 SDS-PAGE analysis of D-alanine D-lactate ligase (39 kDa indicated by the black arrow): *E. faecium* E330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *P. resinovorans* SDS10-2 lane 3, *P. kilonensi*s SDS11 lane 4. M, Colorplus Prestained Protein Marker (7-175 kDa) (Standard curve of the gel is given in Appendix D)

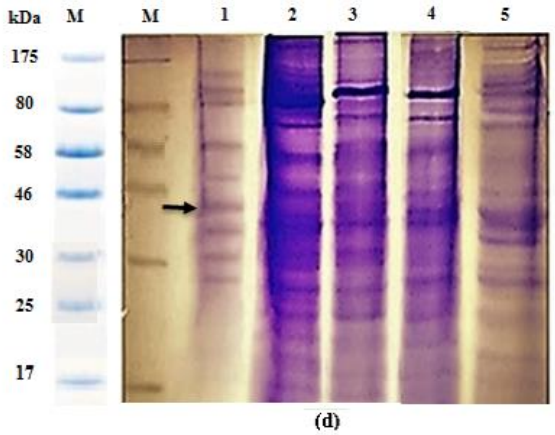

Figure 3.14 SDS-PAGE analysis of D-alanine D-lactate ligase (39 kDa indicated by the black arrow): *E.coli* DH5α (negative control) lane 1, *E. faecium* E330 (positive control) lane 2, S. *warneri* Co11 lane 3, *S. aureus* Ba01 lane 4, *S. aureus* Al11 *lane 5*. M, Colorplus Prestained Protein Marker (7-175 kDa) (Standard curve of the gel is given in Appendix D)

3.5.2 Western Blot Analysis of D-Alanine-D-Lactate Ligase

Expression of D-alanine-D-lactate ligase by the *van*A harboring surface water isolates was further checked through Western Blotting. The D-alanine-D-lactate ligase proteins were confirmed by using the mouse anti-human *van*A primary antibodies (US Biological Life Sciences, USA). Localization patterns of D-alanine-D-lactate ligase were performed by detecting with anti-rabbit HRP-conjugated IgG antibodies. 39 kDa D-alanine-D-lactate ligase protein bands were detected in all of the *van*A gene harboring enterococcal and non-enterococcal isolates, and the protein appeared at the same position (Figure 3.15-19). *Lactobacillus casei*, *Pediococcus pentosaceus*, and *Leuconostoc mesenteroides* are naturally resistant to glycopeptides and the terminus D-alanine–D-lactate also appears to be the same as in VRE (Billot-Klein *et al.* 1994; Handwerger *et al.* 1994).

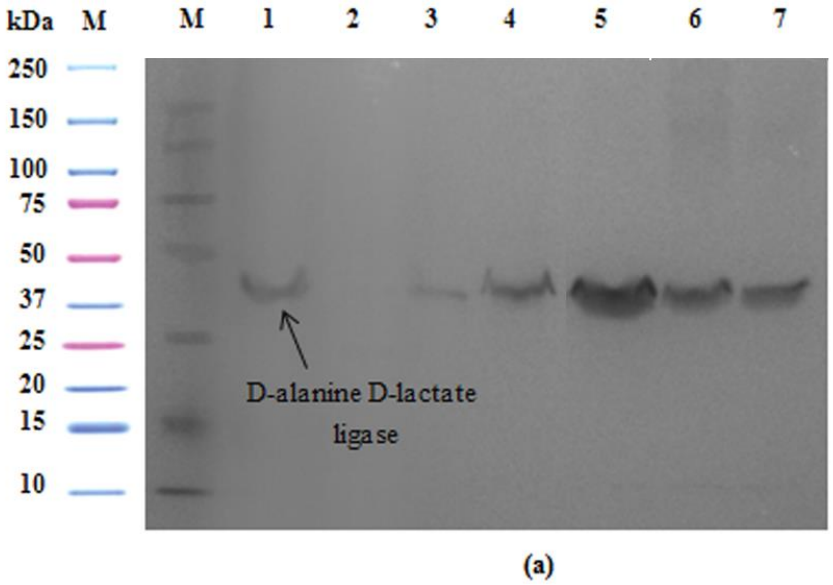

Figure 3.15 Western blot analysis of D-alanine-D-lactate ligase for *E. faecium* 330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *C. testosteroni* Ni11 lane 3, *E. faecalis* Pb06 lane 4, *P. fluorescens* SDS3 lane 5, *A. veronii* SDS7 lane 6, *P. baetica* SDS8 lane7. M, Precision Plus Protein WesternC Standards Marker (BioRad, USA). Arrow indicates 39 kDa D-alanine-D-lactate ligase protein

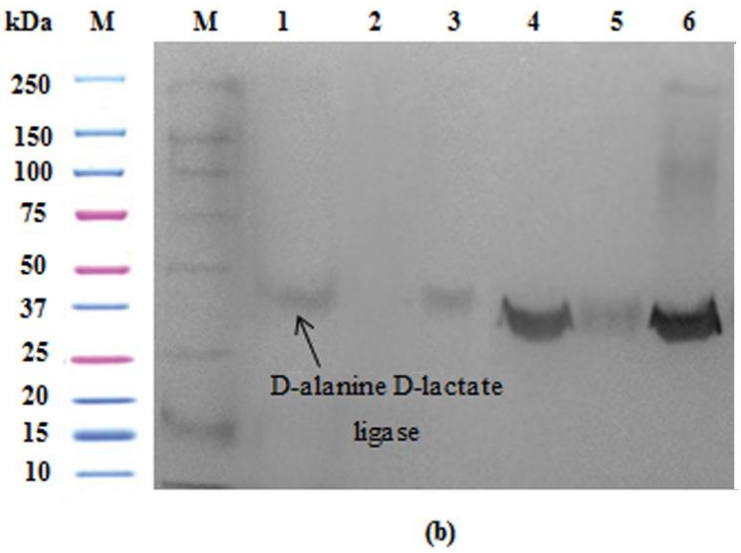

Figure 3.16 Western blot analysis of D-alanine-D-lactate ligase for *E. faecium* E330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *P. resinovorans* SDS10-2 lane 3, *P. kilonensis* SDS11 lane 4, *P. plecoglossicida* Ag10 lane 5, *R. planticola* Ag11 lane 6. M, Precision Plus Protein WesternC Standards Marker (BioRad, USA). Arrow indicates 39 kDa D-alanine-D-lactate ligase protein

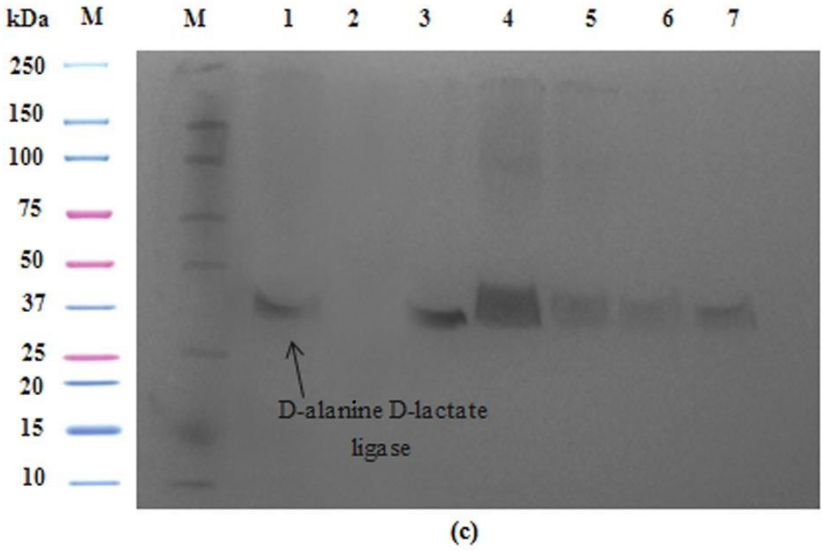

Figure 3.17 Western blot analysis of D-alanine-D-lactate ligase for *E. faecium* E330 (positive control) lane 1, *E. coli* DH5α (negative control) lane 2, *E. faecalis* Cr07 lane 3, *P. korensis* Cu12 lane 4, *P. korensis* Hg10 lane 5, *P. korensis* Hg11 lane 6, *E. faecalis* E07 lane 7. M, Precision Plus Protein WesternC Standards Marker (BioRad, USA). Arrow indicates 39 kDa D-alanine-D-lactate ligase protein

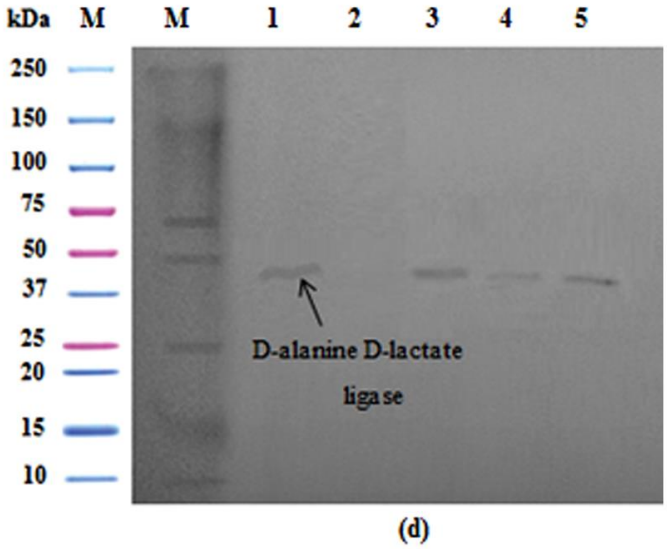

Figure 3.18 Western blot analysis of D-alanine-D-lactate ligase for *E. faecium* E330 (positive control) lane 1, *E.coli* DH5α (negative control) lane 2, *S. aureus* Al11 lane 3, *S. aureus* Ba01 lane 4, *S. warnerii* Co11 lane 5. M, Precision Plus Protein WesternC Standards Marker (BioRad, USA). Arrow indicates 39 kDa D-alanine-D-lactate ligase protein

3.6 Monitoring of *van*A harboring Bacteria in Surface Water by Fluorescent *in situ* Hybridization (FISH)

The determination of bacterial community composition and their activities in nature is fundamental but has long been a challenge to environmental scientists. FISH with rRNA-targeted oligonucleotide probes has provided information about absolute abundance of bacteria with defined phylogenetic affiliations and been applied to the investigation of community composition in lakes, oceans, activated sludge, drinking and river waters (Wagner *et al.,* 1993; Kalmbach *et al.,* 1997; Pernthaler *et al.,* 1997; Kenzaka *et al.,* 1998; Glockner *et al.,* 2000; Icgen and Yilmaz 2015). Traditionally FISH has been performed with DNA oligonucleotide probes. Such probes are usually 15-25 nucleotides long attached to a fluorochrome at the 5'end and target rRNA (16S rRNA or 23S rRNA). *In situ* monitoring of individual bacterial cells or consortium within complex microbial communities will be of great assistance in understanding the prevalence of antibiotic resistance in water bodies. Here, in this study, an oligonucleotide DNA probe targeting *van*A gene was also prepared to monitor vancomycin- and teicoplanin-resistant bacteria in surface waters through FISH.

3.6.1 Design of the *van*A Probe

A 25-mer-oligonucleotide *van*A-targeted oligonucleotide DNA probe was prepared by using the 909 bp *BamH*I-*Cla*I fragment of *van*A operon from *E. faecium* plasmids pVEF1 and pVEF2. The complete restriction sites of both plasmids pVEF1 (39626 bp) and pVEF2 (39714 bp) were obtained from GenBank with accession numbers of AM296544 and AM410096, respectively (Figure 3.19).

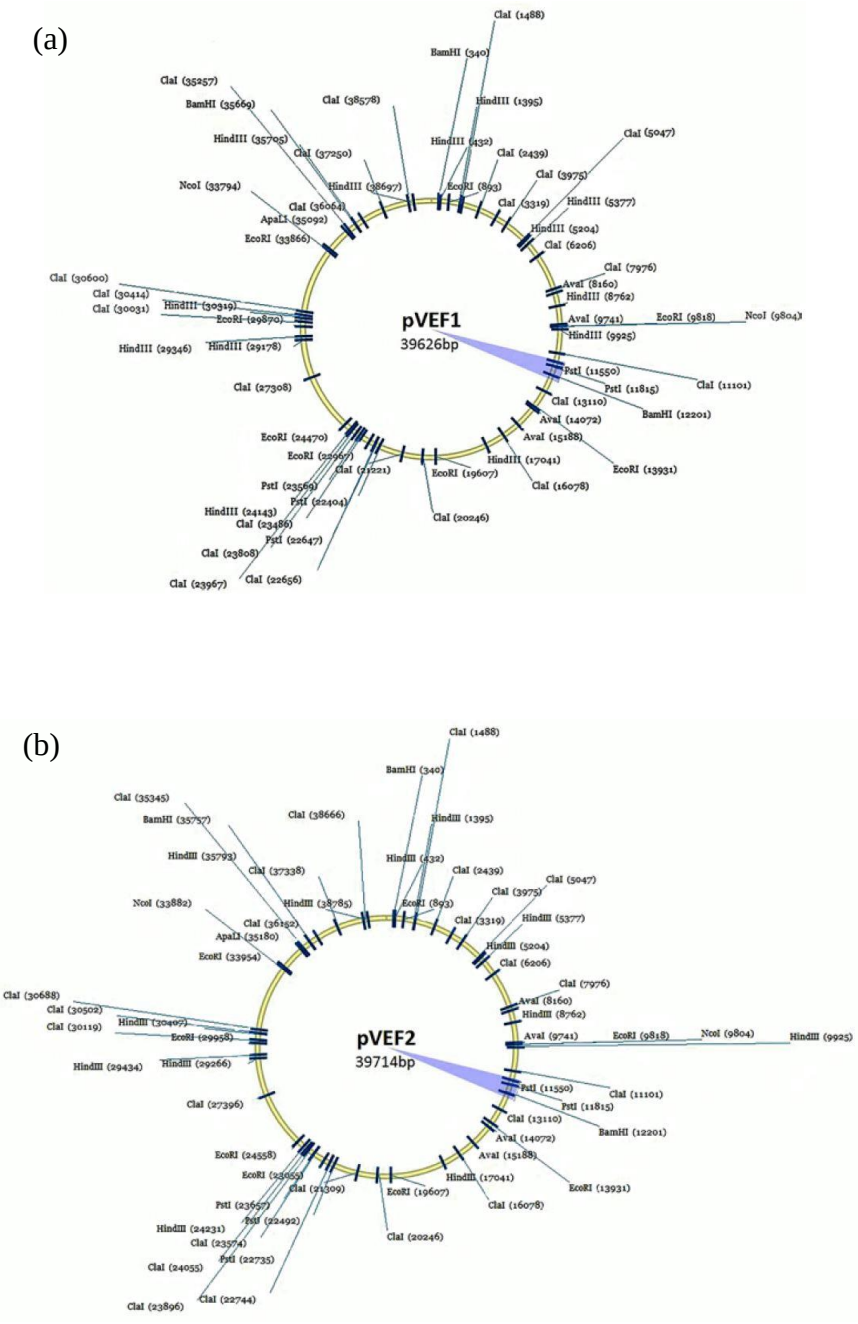

Figure 3.19 The complete sequence pVEF1 (a) and pVEF2 (b) plasmids of *E. faecium*. Shaded areas show *van*A operon

Open reading frame (ORF) and restriction site analyses were done on both plasmids by using tools in Vector NTI Express 1.2 (IBI, USA) (Figure 3.20).

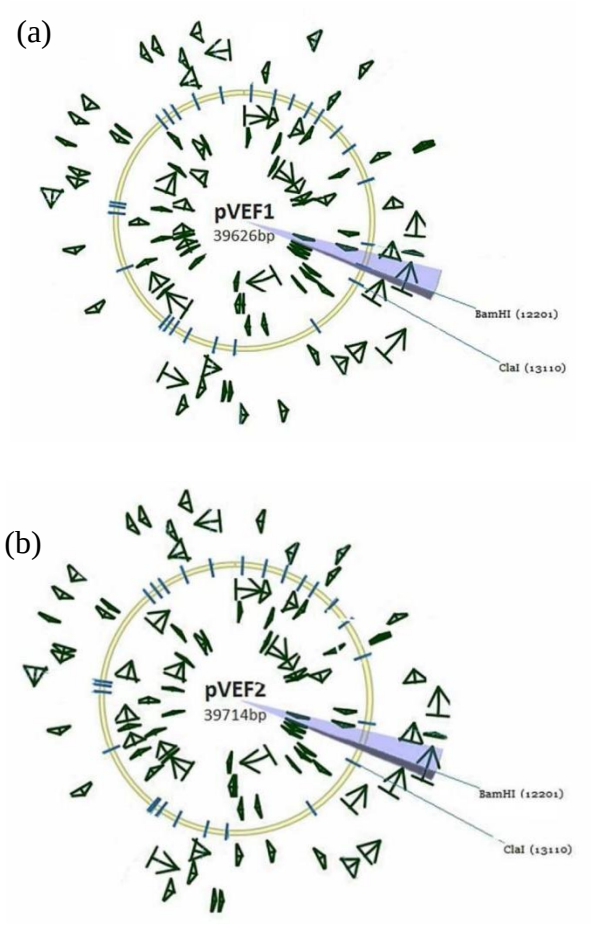

Figure 3.20 The entire 909 bp *BamH*I (12201) - *Cla*I (13110) fragment of *van*A operon from *E. faecium* pVEF1 (a) and pVEF2 (b) plasmids. Densely shaded areas show part of the *van*A operon used for probe selection

The *van*A hybridization probe search region was determined within *van*A operon in between 11489 and 12520 bp from pVEF1 and pVEF2 plasmids. From *van*A operon 20 sequences were selected and the selected sequences were subsequently confirmed for their 100 % specificity using BLAST (Table 3.2).

Table 3.2 Selected *van*A-targeted DNA probe sequence by using Vector NTI Express Software 1.2

	Sequence	Length (bases)	GC (%)	Site[a]	T_m (C°)
1	ATACTGTTTGGGGGTTGCTCAGAGG	25	52	22	28.1
2	TACTGTTTGGGGGTTGCTCAGAGGA	25	52	23	29.4
3	ACTGTTTGGGGGTTGCTCAGAGGAG	25	56	24	30.1
4	CTGTTTGGGGGTTGCTCAGAGGAGC	25	60	25	32.4
5	TTGCTCAGAGGAGCATGACGTATCG	25	52	36	29
6	TGCTCAGAGGAGCATGACGTATCGG	25	56	37	30.9
7	GCTCAGAGGAGCATGACGTATCGGT	25	56	38	29.2
8	CTCAGAGGAGCATGACGTATCGGTA	25	52	39	26.1
9	ATGTGCGAAAAACCTTGCGCGGAATGGGAA	30	50	151	41.7
10	TGTGCGAAAAACCTTGCGCGGAATGGGAA	29	51.7	152	42.0
11	GTGCGAAAAACCTTGCGCGGAATGGGAA	28	53.6	153	40.4
12	TGCGAAAAACCTTGCGCGGAATGGGAA	27	51.9	154	40.2
13	GCGAAAAACCTTGCGCGGAATGGGAA	26	53.8	155	38.5
14	CGAAAAACCTTGCGCGGAATGGGAA	25	52	156	36.2
15	GAAAAACCTTGCGCGGAATGGGAAAACG	28	50	157	37.7
16	AAAACCTTGCGCGGAATGGGAAAACG	26	50	159	36.1
17	AAACCTTGCGCGGAATGGGAAAACG	25	52	160	35.4
18	AACCTTGCGCGGAATGGGAAAACGA	25	52	161	35.9
19	ACCTTGCGCGGAATGGGAAAACGAC	25	56	162	35.7
20	CCTTGCGCGGAATGGGAAAACGACA	25	56	163	37.2

[a]*BamH*I-*Pst*I fragment digested *E. faecium* plasmid pVEF1 sites
T_m, melting temperature

Among these sequences, the sequence number 4 with the sequence of 5'-CTGTTTGGGGGTTGCTCAGAGGAGC-3' was selected by checking the length, % of GC base composition, melting temperature (Tm), intra-probe complementary regions, length of stretches and the location of the GC bases within the fragment. This selected 25 bp DNA fragment was labelled with FITC at the 5' end (Alpha DNA, Montreal, Canada) to use as a probe.

3.6.2 Determining Hybridization Stringencies for the Designed *van*A Probe

The FITC-labeled probe was first evaluated with *van*A-positive control of *E. faecalis* and *van*A-negative control of *E. coli* DH5α (Figure 3.21 and 3.22) isolates, respectively.

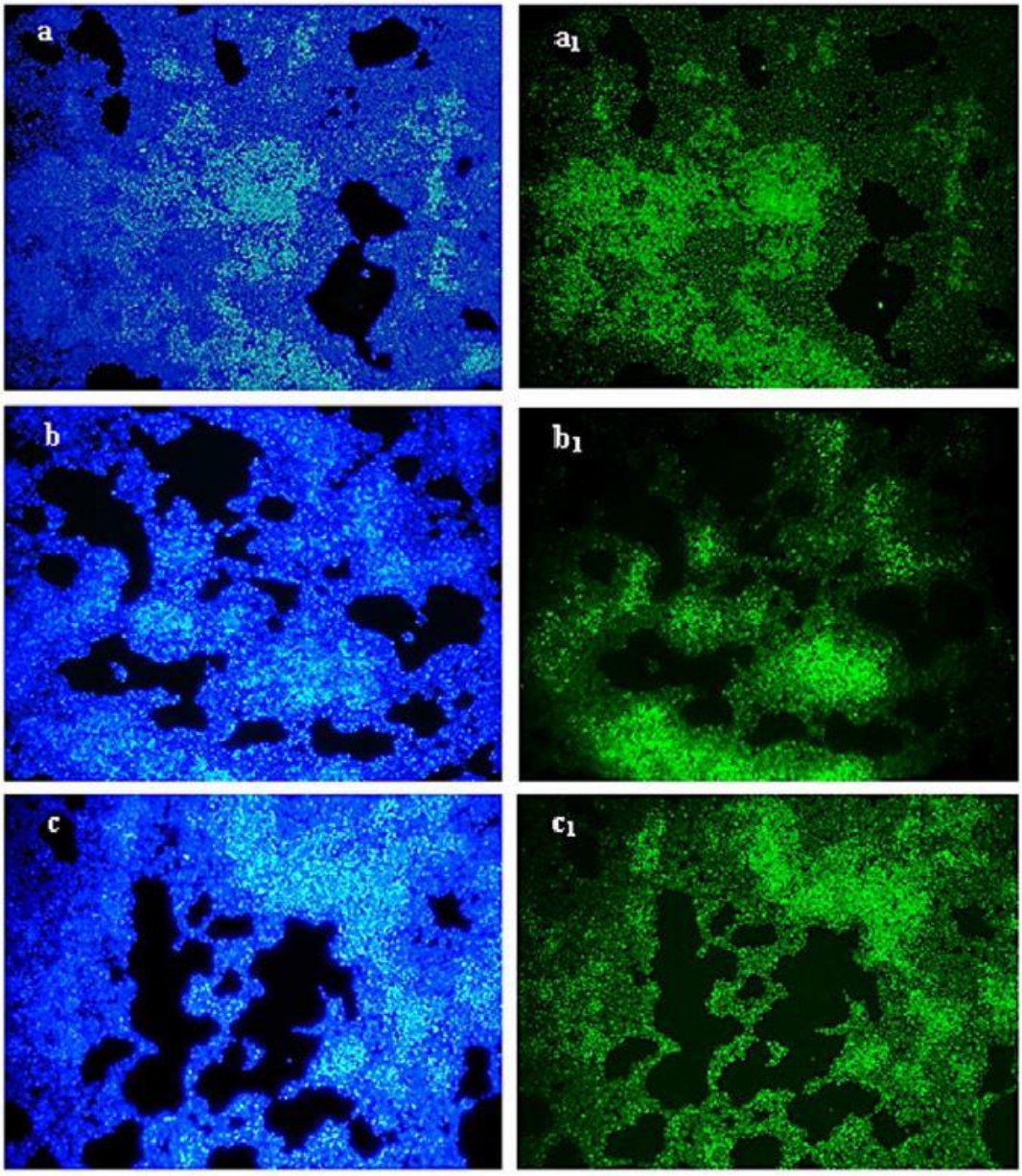

Figure 3.21 Representative hybridization stringency for *van*A harboring pure cultures of *E. faecalis* E07 at 45, 50, and 55% formamide concentrations, respectively. Total cell populations stained with DAPI (a,b,c) and their corresponding *van*A probe applied pure cultures (a_1,b_1,c_1)

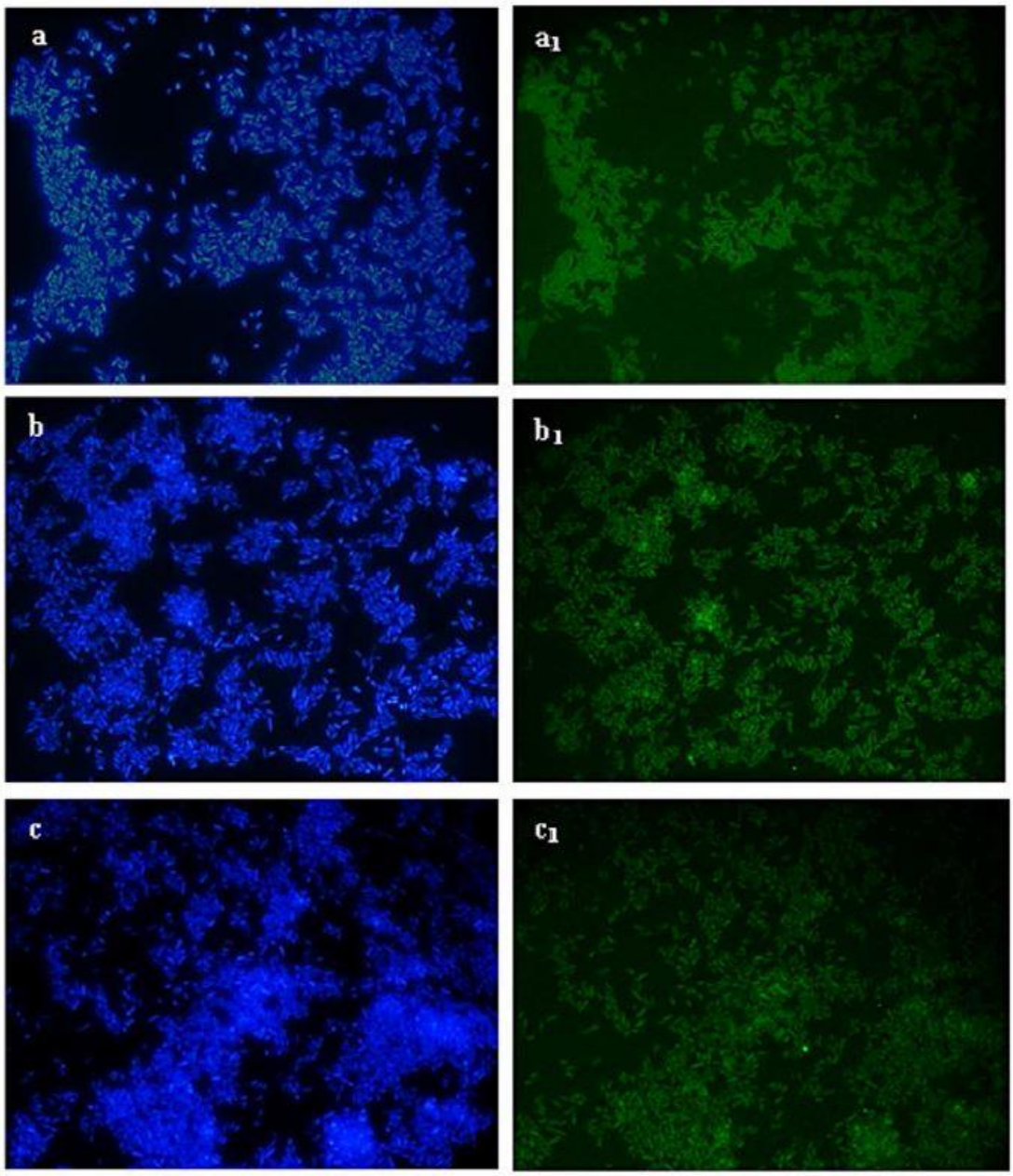

Figure 3.22 Representative hybridization results of *van*A-negative pure cultures of *E. coli* DH5α at 45, 50, and 55% formamide concentrations, respectively. Total cell populations stained with DAPI (a,b,c) and their corresponding *van*A probe applied pure cultures (a_1,b_1,c_1)

The hybridization stringency conditions of the prepared *van*A DNA probe was adjusted at changing salt and formamide concentrations with various temperatures (Table 3.3). Under the hybridization conditions of 46 °C, 55 % formamide and 0.020 M NaCl, the designed 25-mer FITC-labeled *van*A DNA probe was highly efficient up to 88.6 % ±1.97 ($p>0.05$) for the selection of *van*A harboring pure culture

isolates. Non-target bacteria only gave 2.89 % ±0.68 ($p>0.05$) hybridization with *van*A DNA probe. Strong linear relationships (R^2 = 0.99) between the signal intensity and the target *van*A gene was also observed at 55 % formamide concentration (Appendix E for measurements).

Table 3.3 The hybridization stringency adjustment for the prepared *van*A probe

			% of signal intensity for *vanA* gene after hybridization with *vanA* probe	
Hybridization conditions for *van*A probe used			*van*A $^+$ controls	*van*A $^-$ controls
Temperature (C°)	[Formamide] (%)	[NaCl] (M)	*E. faecalis* E07	*E. coli* DH5α
46	45	0.040	73.8±0.75	1.40±0.75
46	50	0.028	78.9±1.82	1.93±0.81
46	55	0.020	88.6±1.97	2.89±0.68

±, calculated standard errors
*van*A $^+$, positive
*van*A $^-$, negative

3.6.3 Monitoring Water Samples by Using Designed *van*A Probe

After adjusting hybridization stringency conditions, the designed *van*A DNA probe was next evaluated for monitoring of the water samples collected from river. River water samples were successfully monitored with the *van*A DNA probe prepared to determine the prevalence *van*A harboring bacterial isolates over the years of 2011 and 2012 (Figure 3.23 and 3.24), respectively. Both the pure cultures and the water samples used in the study were also checked with non-binding probe NON338 to detect auto-fluorescence and background interferences.

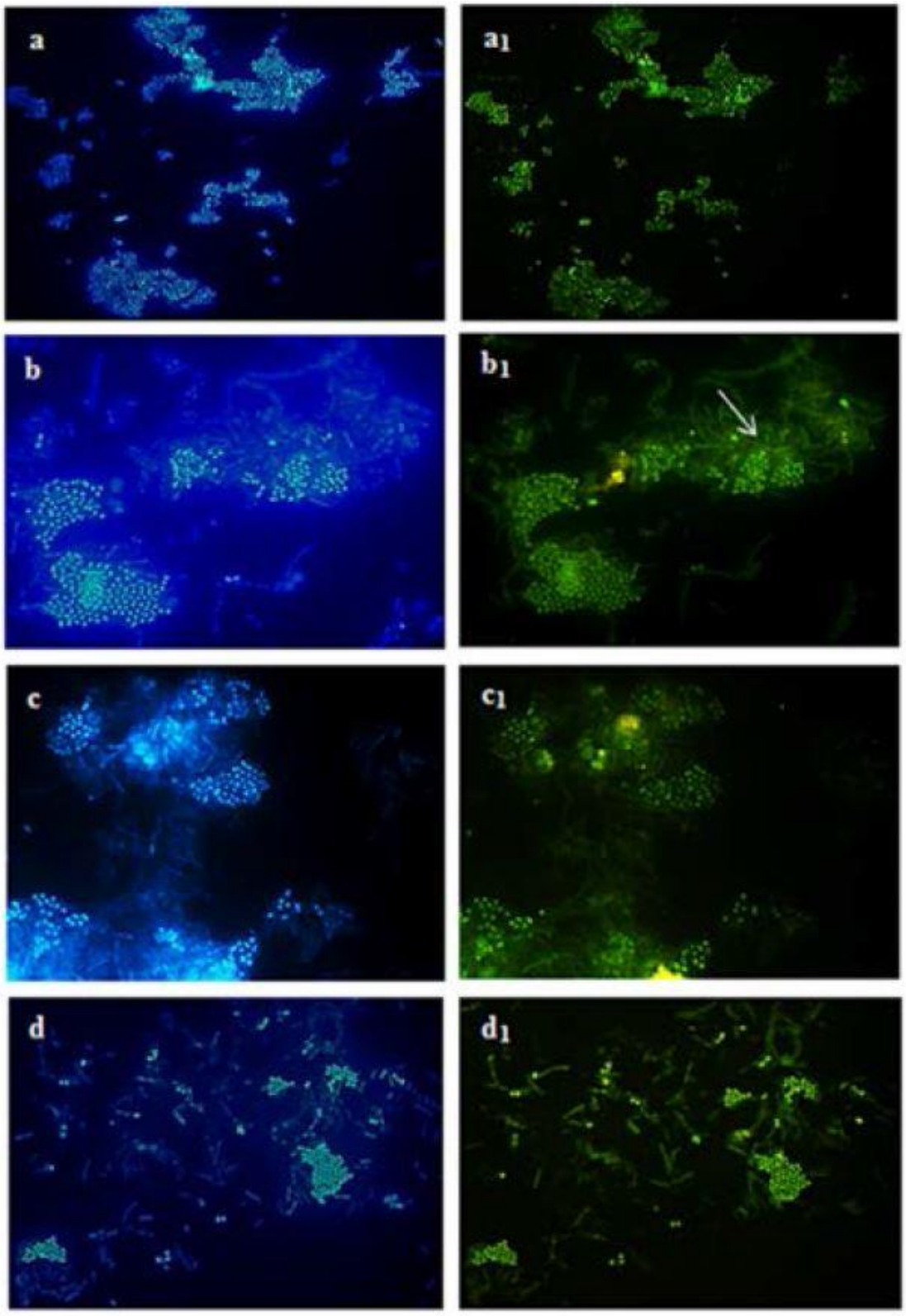

Figure 3.23 Representative hybridization results of river water samples for the year 2011 at 55% formamide concentrations, respectively. Total cell populations stained with DAPI (a,b,c,d) and their corresponding *van*A probe applied mixed cultures (a_1,b_1,c_1,d_1)

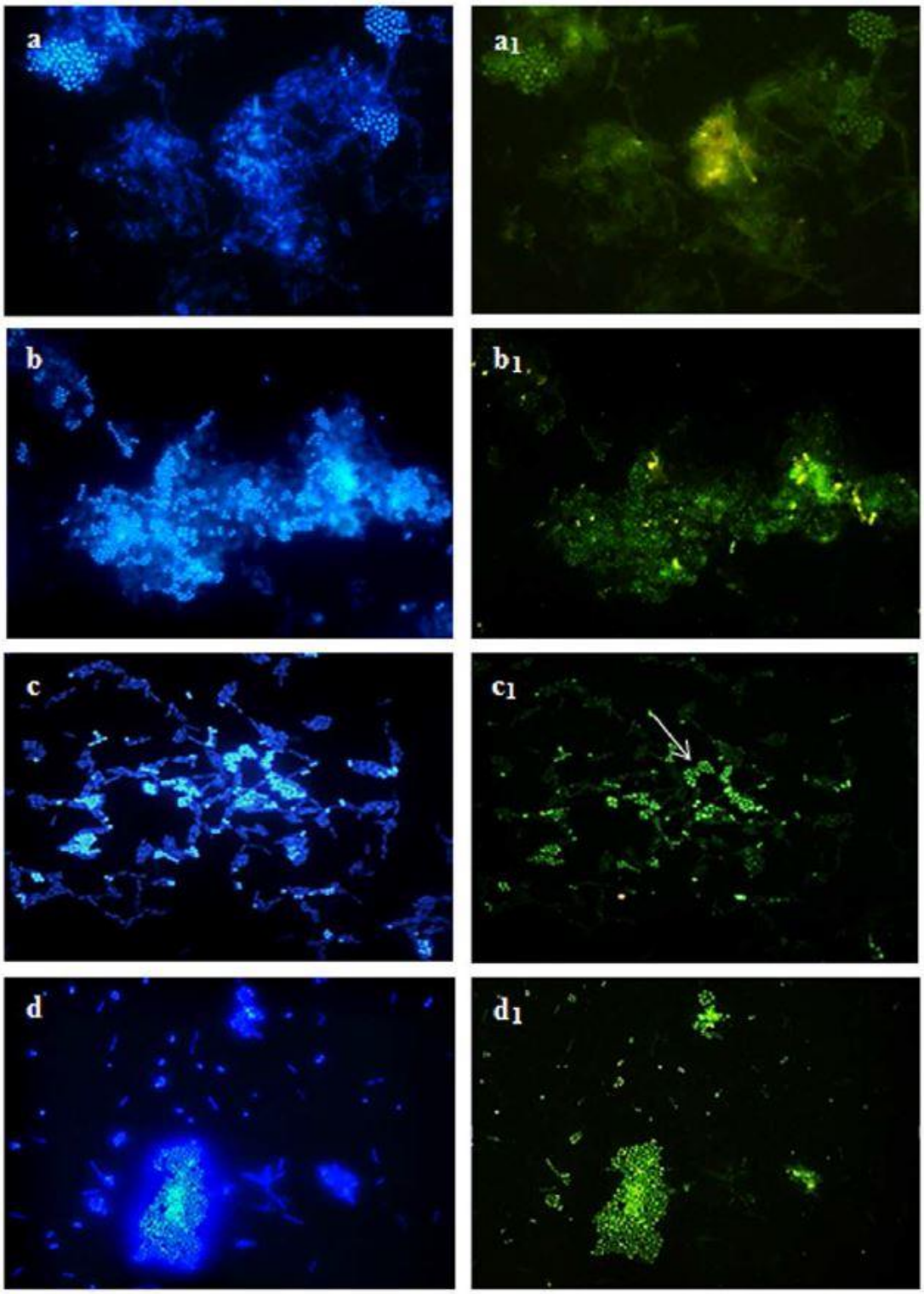

Figure 3.24 Representative hybridization results of river water samples for the year 2012 at 55% formamide concentrations, respectively. Total cell populations stained with DAPI (a,b,c,d) and their corresponding *van*A probe applied mixed cultures (a_1,b_1,c_1,d_1)

FISH is the method of choice for detecting specific nucleic acids in their native cellular environment. Since the first application of fluorescent probes for *in situ* detection of RNA, the technique has been improved and modified for a wide range of targets and probing methods (Levsky and Singer 2003; Volpi and Bridger 2008). Microorganisms can be identified, localized and quantified in almost every ecosystem with hybridization. In this study, water samples collected from river water were also checked for the in situ monitoring of prevalence of *van*A harboring bacterial isolates over the years of 2011-2012. % *van*A was determined by calculating the pixel areas (pp2) of DAPI and FITC images. The % of DAPI stained cells showed a seasonal patterns. The visualization of *van*A harboring bacterial isolates hybridizing with *van*A probe was shown in Figure 3.25.

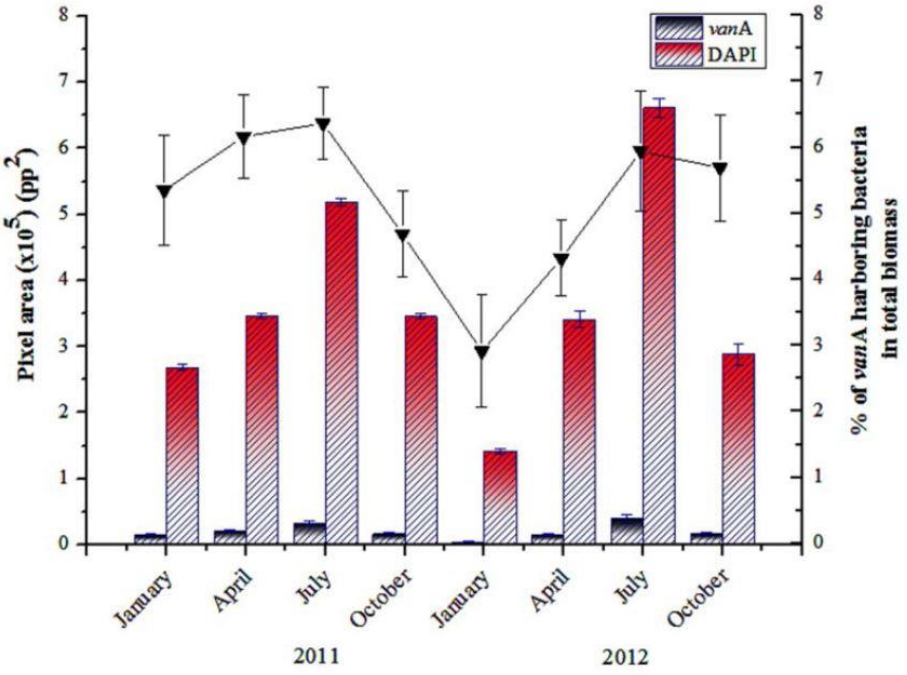

Figure 3.25 *In situ* distribution and abundance of % *van*A harboring bacteria (▾) in the total biomass within the water samples. Pixel area of total biomass determined with DAPI-stained cells (▨) and total *van*A harboring bacteria determined with FITC-labeled *van*A probe (▨). The error bars illustrate the calculated standard error

Population size (%) of *van*A harboring isolates in total biomass in between the years 2011 and 2012 was also calculated using average values of taken images for pixel

areas of FITC-labeled probe and DAPI-stained cells (Figure 3.19). The results indicated that *van*A harboring isolates had the lowest population size with 4.72 % ±0.67 ($p<0.05$) in October and the highest population size with 6.42 % ±0.66 ($p>0.05$) in July for the year of 2011 (Appendix E for measurements). There was significant differences ($p<0.05$) in the seasonal distribution of *van*A harboring isolates in January, April and October within the year of 2011. The *van*A harboring isolates had the highest population size with 5.94% ±0.91 ($p<0.05$) in July and the lowest population size with 2.91 % ±0.85 ($p<0.05$) in January for the year of 2012 (Appendix E for measurements).

In overall, for the year of 2012, there was significant difference in the seasonal distribution of *van*A harboring isolates within total biomass. As a result of the ANOVA test, it was determined that seasonal population shifts of *van*A harboring isolates varied during the years tested ($p<0.05$). Shapiro-Wilk test showed that the population of *van*A harboring isolates had a normal distribution ($p<0.05$) for the years tested except for July in 2011. The findings confirmed that the prepared *van*A DNA probe was also successful for *in situ* monitoring of the prevalence of *van*A harboring bacterial isolates in surface waters. Various targeted DNA probes have been designed for specific groups of bacteria and have been demonstrated to represent valuable tools for group- and species-specific hybridization studies of bacterial populations in complex communities such as biofilms marine sediments, microbial mats, anaerobic bioreactors, surface waters, and sea water without prior isolation of the target organisms (Amann *et al.* 2001; Daims *et al.* 2006; Icgen and Harrison 2006a, 2006b; Morozova *et al.* 2011; Bryukhanov *et al.* 2011; Yilmaz and Icgen 2014). Several other researchers also showed the seasonal succession of microorganisms by using FISH (Pernthaler *et al.* 1998; Pinhassi and Hagström 2000; Eilers *et al.* 2000; Klammer *et al.* 2002). However, the studies about *in situ* monitoring of antibiotic resistant bacterial isolates and their temporal succession are scarce. This type of studies is needed to unveil the prevalence of antibiotic resistant bacterial isolates in water bodies. Hence, here, we showed that FISH as a powerful technique can be successfully used for monitoring of antibiotic resistance genes harbouring bacteria in surface waters.

3.7 Conclusion

The dissemination of vancomycin- and teicoplanin-resistant and *van*A harboring bacteria appeared to be reservoired in surface waters and highly variable. Therefore, surface waters would need to be intensively monitored for not only vancomycin- and teicoplanin-resistant enterococcal species but also other potentially significant non-enterococcal counterparts like *Staphylococcus, Pseudomonas, Comamonas, Raoultella* and *Aeromonas* species. The sequence similarity values of partial *van*A gene from different origins indicated that the *van*A gene selectively maintained and transferred among the surface water isolates. Western blot analysis of the *van*A-encoded D-alanine-D-lactate ligase protein can be reliably used for the detection of both enterococcal and non-enterococcal *van*A harbouring isolates. In order to reduce the impact of high level vancomaycin and teicoplanin resistance in surface waters the *van*A gene dissemination and the contact of *van*A harbouring bacteria to human-linked environmental counterparts needs to be carefully evaluated.

This study also elucidated that monitoring vancomycin- and teicoplanin-resistant bacteria in surface waters was managed by using *van*A-targeted oligonucleotide DNA probe. The prevalence of *van*A harbouring bacteria in surface waters indicate that understanding the dynamics of these antibiotic resistant bacterial isolates in water bodies is important to improve the management and treatment of antibiotic resistance to avoid threats to public health. The *van*A-targeted oligonucleotide DNA probe prepared in this study displayed high efficiency for the monitoring of *van*A harboring bacterial isolates and could be useful to monitor vancomycin- and teicoplanin-resistant bacteria in surface waters. However, further studies are still needed to reveal the detection sensitivity of the *van*A-targeted oligonucleotide DNA probe in soil, sewage and WWTPs.

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APPENDIX A

DISC DIFFUSION TEST MEASUREMENTS

Table A.1 DD tests measurements in milimeters

Strains	Measurements					
	VAN			TEC		
	1st	2nd	3rd	1st	2nd	3rd
Ag10	10	9.5	10	9	8	8
Ag11	10	9	9.5	9	9	8.5
Al11	10	9	9	9	9	9
Ba01	10	9	9	9	8.5	8.5
Cr07	9	10	9.5	8	8	7.5
Co11	9	9	9	8	7.5	8
Cu12	10	10	9	9	8	8
E07	6	7	6	6	6	6
E330	6	6	6	6	6	6
Hg10	8	8	7	7	6	6.5
Hg11	8	8	8	7	7	8
Ni11	10	9	9	8	8	8
Pb06	9	8	9	7	8	8
SDS3	6	6	6	6	6	6
SDS7	6	6	6	6	6	6.5
SDS8	8	7	8	7	7.5	7
SDS10-2	8	7	7.5	7	6.5	7
SDS11	7	6	7	6	6	6

VAN, vancomycin; TEC, teicoplanin

APPENDIX B

MOLECULAR WEIGHT ESTIMATIONS OF PLASMID PROFILING

Distance (cm)	Fragment size (kDa)
0.4	215
4.2	60
5	54
7.3	23

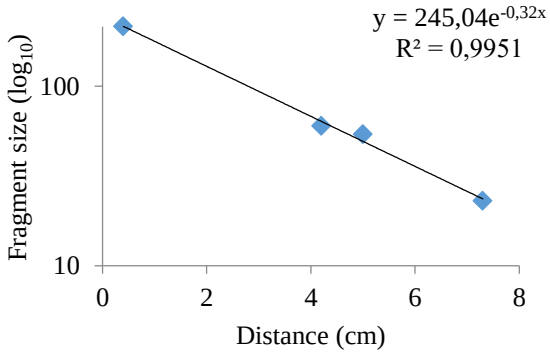

Figure B.1 Representative standard curve for molecular weight estimation of plasmid profiling gel

Distance (cm)	Fragment size (kDa)
0.5	215
2.55	140
4.32	60
7.05	23

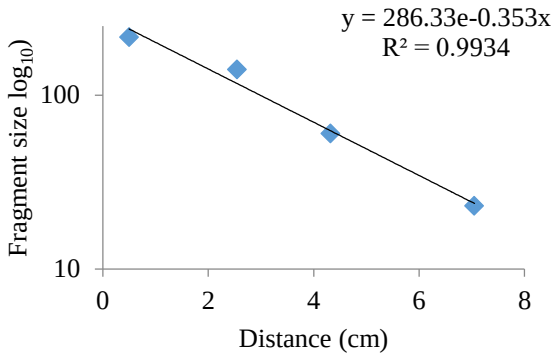

Figure B.2 Representative standard curve for molecular weight estimation of plasmid profiling gel

APPENDIX C

MOLECULAR WEIGHT ESTIMATIONS OF *van*A PCR ANALYSIS

Distance (cm)	Fragment size (kDa)
4.2	1517
4.9	1200
5.2	1000
5.4	900
5.6	800
5.8	700
6	600
6.3	517
6.8	400
7.2	300
7.7	200
8.4	100

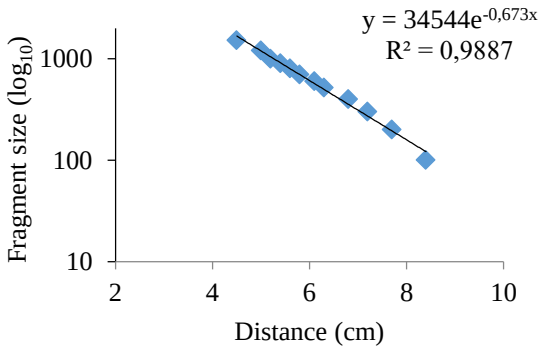

Figure C.1 Representative standard curve for molecular weight estimation of *van*A PCR analysis gel

Distance (cm)	Fragment size (kDa)
4.3	1517
4.9	1200
5.1	1000
5.4	900
5.6	800
5.8	700
6	600
6.3	517
6.8	400
7.2	300
7.7	200
8.6	100

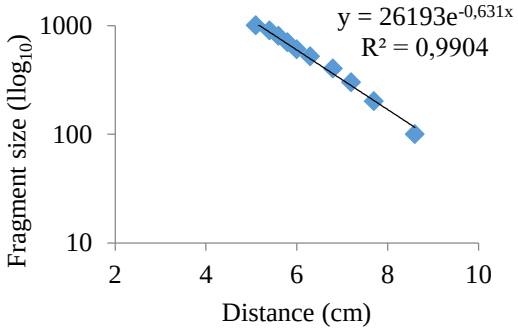

Figure C.2 Representative standard curve for molecular weight estimation of *van*A PCR analysis gel

APPENDIX D

MOLECULAR WEIGHT ESTIMATIONS OF D-ALANINE-D-LACTATE LIGASE ANALYSIS

Distance (cm)	Fragment size (kDa)
2.25	80
3.65	58
5.1	46
6.7	30
8.05	25
10.5	17

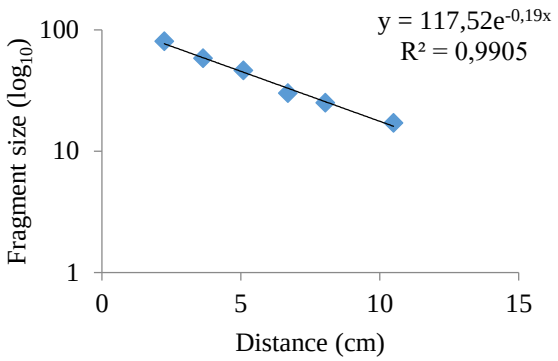

Figure D.1 Representative standard curve for molecular weight estimation of D-alanine-D-lactate ligase analysis gel

Distance (cm)	Fragment size (kDa)
2.5	80
4	58
5.4	46
7.25	30
8.8	25
10.85	17

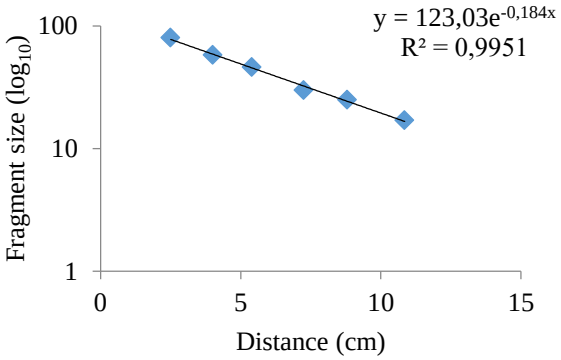

Figure D.2 Representative standard curve for molecular weight estimation of D-alanine-D-lactate ligase analysis gel

Distance (cm)	Fragment size (kDa)
2.1	80
3.4	58
4.8	46
6.6	30
8	25
10.6	17

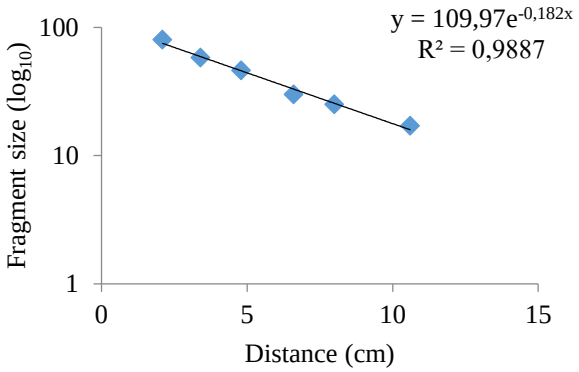

Figure D.3 Representative standard curve for molecular weight estimation of D-alanine-D-lactate ligase analysis gel.

Distance (cm)	Fragment size (kDa)
1	80
1.5	58
2.2	46
3.6	30
5.6	17

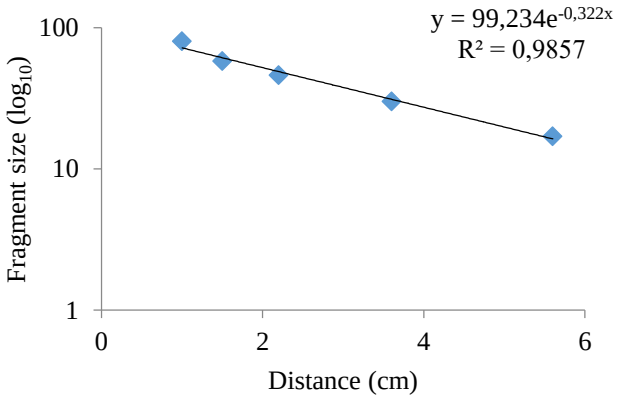

Figure D.4 Representative standard curve for molecular weight estimation of D-alanine-D-lactate ligase analysis gel

APPENDIX E

RAW DATA OF THE FISH ANALYSES

E. faecalis	Formamide	45%			50%			55%		
		Pixel Area (%)	FITC	DAPI	Pixel Area (%)	FITC	DAPI	Pixel Area (%)	FITC	DAPI
		74,82677782	345.036	461.113	77,97297856	358.218	459.413	89,33583225	439.007	491.412
		74,40057427	343.067	461.108	79,81468198	364.967	457.268	88,9537252	430.190	483.611
		72,42136586	345.602	477.210	78,51200056	357.082	454.812	87,83647035	434.000	494.100
		73,9761656	347.000	469.070	77,97653391	362.201	464.500	89,92090034	438.011	487.107
		74,20017201	345.105	465.100	76,5952971	364.108	475.366	90,30067038	429.023	475.105
		73,75556076	343.029	465.089	82,1220597	376.110	457.989	91,74286931	431.008	469.800
		72,75989808	346.081	475.648	81,0806685	377.107	465.101	88,50492968	428.101	483.703
		74,10150962	348.120	469.788	79,59174896	370.108	465.008	86,26622802	424.008	491.511
		74,49539079	347.004	465.806	77,15659047	361.170	468.100	84,89784094	422.000	497.068
Mean of Pixel Area (%)		73,88193498			78,98028442			88,63994072		
Standard Deviation		0,753112754			1,823731322			1,969528636		

Figure E.1 Image pixel area analysis of FITC/DAPI stained *E. faecalis* E07 pictures for various formamide concentrations

E.coli	Formamide	45%			50%			55%		
		% Pixel Area	FITC	DAPI	% Pixel Area	FITC	DAPI	% Pixel Area	FITC	DAPI
		3,209950848	18.240	568.233	2,488663289	22.298	895.983	3,796063652	23.569	620.880
		2,143200012	17.145	799.972	1,864024642	17.610	944.730	2,594671813	20.706	798.020
		1,135364478	9.026	794.987	2,75638561	21.722	788.061	2,805756125	22.138	789.003
		1,422815428	11.240	789.983	3,131478745	24.409	779.472	2,50399681	19.907	795.009
		1,011985328	8.045	794.972	1,214839107	11.039	908.680	2,10738865	21.009	996.921
		1,175697882	9.523	809.987	0,991249984	9.158	923.884	2,050220275	20.458	997.844
		0,68751461	5.500	799.983	2,537532352	21.089	831.083	4,004790703	35.879	895.902
		0,844131961	7.505	889.079	1,144086113	9.687	846.702	2,982113073	27.999	938.898
		0,942842749	7.505	795.997	1,327092511	13.014	980.640	3,120878049	31.989	1.025.000
Mean of Pixel Area (%)		1,397055922			1,939483595			2,885097683		
Standard Deviation		0,754632148			0,80506775			0,678814002		

Figure E.2 Image pixel area analysis of FITC/DAPI stained *E. coli* DH5α pictures for various formamide concentrations

	2011 JANUARY			2011 APRIL			2011 JULY			2011 OCTOBER		
	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI
	4.882558609	13,071	267,708	5.598841275	19,057	340,374	6.022499141	31,709	526,509	5.585326729	19,011	340,374
	6.407764805	17,079	266,536	6.221367025	21,307	342,481	7.30340783	37,659	515,636	5.07210619	17,371	342,481
	5.977570804	15,724	263,050	6.031606823	21,045	348,912	6.885758393	35,594	516,922	4.042280002	14,104	348,912
	4.442424018	11,971	269,470	5.668224612	19,489	343,829	5.814002848	30,132	518,266	5.668224612	19,489	343,829
	6.443493394	17,254	267,774	5.114986289	17,869	349,346	5.91887787	30,475	514,878	4.253376309	14,859	349,346
	5.794036242	15,533	268,086	6.842010751	23,623	345,264	5.953758895	30,914	519,235	3.945676352	13,623	345,264
	4.371740053	11,533	263,808	6.956699065	24,221	348,168	6.190317208	31,706	512,187	4.084522414	14,221	348,168
	4.520834527	12,620	279,152	6.806656563	23,355	343,120	6.133600284	32,351	527,439	4.766553975	16,355	343,120
	5.300964943	14,058	265,197	6.177102386	21,682	351,006	7.590892476	39,250	517,067	5.037520726	17,682	351,006
Mean of Pixel Area (%)	5.35			6.157499421			6.423679438			4.717287479		
Standard Deviation	0.836264551			0.631200792			0.661128793			0.666639936		

Figure E.3 Image pixel area analysis of FITC/DAPI stained pictures of the year 2011

	2012 JANUARY			2012 APRIL			2012 JULY			2012 OCTOBER		
	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI	Pixel Area (%)	*van* A	DAPI
	2.595330029	3,838	147,881	5.030427159	17,070	339,335	7.397485912	51,198	692,100	5.182272674	14,709	283,833
	1.689324904	2,397	141,891	4.080527879	13,945	341,745	6.452559094	42,017	651,168	5.158882476	15,053	291,788
	4.170329826	5,882	141,044	3.281077236	11,484	350,007	7.064041623	46,203	654,059	7.055903796	20,982	297,368
	3.404472254	4,875	143,194	4.454568829	15,419	346,139	5.740458297	37,374	651,063	6.531449999	19,125	292,814
	4.012519844	5,333	132,909	3.576316257	11,472	320,777	5.298638367	34,466	650,469	5.115150592	14,779	288,926
	2.226097516	3,153	141,638	4.355279308	14,939	343,009	5.861419493	38,278	653,050	5.184196692	15,384	296,748
	2.517943369	3,645	144,761	4.627810997	14,891	321,772	4.82226715	31,556	654,381	6.530528703	19,463	298,031
	3.275125846	4,717	144,025	4.743856606	16,409	345,900	5.96522737	39,923	669,262	5.606926698	16,675	297,400
	2.306694288	3,218	139,507	4.691559523	16,902	360,264	4.845648863	32,640	673,594	4.798453509	11,741	244,683
Mean of Pixel Area (%)	2.910870875			4.315713755			5.938638463			5.684862793		
Standard Deviation	0.848253192			0.57294483			0.905216755			0.806837985		

Figure E.4 Image pixel area analysis of FITC/DAPI stained pictures of the year 2012

	A(Y)	B(Y)	C(Y)	D(Y)	I1(Y)	J1(Y)	K1(Y)	**L1(Y)**
Long Name	January	April	July	October	Januray	April	July	October
Units	2011				2012			
Comments	vanA							
2	17079	21307	37659	17371	2397	13945	42017	15053
3	15724	21045	35594	14104	5882	11484	46203	20982
4	11971	19489	30132	19489	4875	15419	37374	19125
5	17254	17869	30475	14859	5333	11472	34466	14779
6	15533	23623	30914	13623	3153	14939	38278	15384
7	11533	24221	31706	14221	3645	14891	31556	19463
8	12620	23355	32351	16355	4717	16409	39923	16675
9	14058	21682	39250	16827	3218	16902	32640	11741
10								

Figure E.5 Pixel areas FITC stained for probe *van*A with respect to months of years 2011 and 2012

.

	E(Y)	F(Y)	G(Y)	H(Y)	I(Y)	J(Y)	K(Y)	L(Y)
Long Name	January	April	July	October	January	April	July	October
Units	2011				2012			
Comments	DAPI							
3	263050	348912	516922	348912	141044	350007	654059	297368
4	269470	343829	518266	343829	143194	346139	651063	292814
5	267774	349346	514878	349346	132909	320777	650469	288926
6	268086	345264	519235	345264	141638	343009	653050	296748
7	263808	348168	512187	348168	144761	321772	654381	298031
8	279152	343120	527439	343120	144025	345900	669262	297400
9	265197	351006	517067	351006	139507	360264	673594	244683
10								

Figure E.6 Pixel areas DAPI for probe *van*A with respect to months of years 2011 and 2012

	M(Y)	N(Y)	O(Y)	P(Y)	Q(Y)	R(Y)	S(Y)	T(Y)
Long Name	January	April	July	October	January	April	July	October
Units	2011				2012			
Comments	Biomass							
3	5.97757	6.03161	6.88576	4.04228	4.17033	3.28108	7.06404	7.0559
4	4.44242	5.66822	5.814	5.66822	3.40447	4.45457	5.74046	6.53145
5	6.44349	5.11499	5.91888	4.25338	4.01252	3.57632	5.29864	5.11515
6	5.79404	6.84201	5.95376	3.94568	2.2261	4.35528	5.86142	5.1842
7	4.37174	6.9567	6.19032	4.08452	2.51794	4.62781	4.82227	6.53053
8	4.52083	6.80666	6.1336	4.76655	3.27513	4.74386	5.96523	5.60693
9	5.30096	6.1771	7.59089	5.03752	2.30669	4.69156	4.84565	4.79845
10								

Figure E.7 Calculated biomass values harboring *van*A gene with respect to months of years 2011 and 2012

ANOVAOneWay (06.12.2015 14:58:09)

Notes

Input Data

Descriptive Statistics

	Sample Size	Mean	Standard Deviation	SE of Mean
January	9	5.34904	0.83626	0.27875
April	9	6.1575	0.6312	0.2104
July	9	6.42368	0.66113	0.22038
October	9	4.71729	0.66664	0.22221
January	9	2.91087	0.84825	0.28275
April	9	4.31571	0.57294	0.19098
July	9	5.93864	0.90522	0.30174
October	9	5.68486	0.80684	0.26895

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	7	85.23778	12.17683	21.65993	1.12133E-14
Error	64	35.97966	0.56218		
Total	71	121.21743			

Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are significantly different.

Figure E.8 One way ANOVA test for the measurements taken from the samples of 2011 and 2012

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
April 2011 January 2011	0.80846	0.35345	3.23474	0.31655	0.05	0	-0.29905	1.91597
July 2011 January 2011	1.07464	0.35345	4.29976	0.06345	0.05	0	-0.03287	2.18215
July 2011 April 2011	0.26618	0.35345	1.06502	0.99483	0.05	0	-0.84133	1.37369
October 2011 January 2011	-0.63176	0.35345	2.52774	0.63068	0.05	0	-1.73927	0.47575
October 2011 April 2011	-1.44021	0.35345	5.76248	0.00309	0.05	1	-2.54772	-0.3327
October 2011 July 2011	-1.70639	0.35345	6.8275	2.3057E-4	0.05	1	-2.8139	-0.59888
January 2012 January 2011	-2.43817	0.35345	9.75545	1.34205E-7	0.05	1	-3.54568	-1.33066
January 2012 April 2011	-3.24663	0.35345	12.99019	3.25479E-8	0.05	1	-4.35414	-2.13912
January 2012 July 2011	-3.51281	0.35345	14.05521	2.77917E-8	0.05	1	-4.62032	-2.4053
January 2012 October 2011	-1.80642	0.35345	7.22771	8.20002E-5	0.05	1	-2.91393	-0.69891
April 2012 January 2011	-1.03333	0.35345	4.13449	0.08464	0.05	0	-2.14084	0.07418
April 2012 April 2011	-1.84179	0.35345	7.36923	5.65528E-5	0.05	1	-2.9493	-0.73428
April 2012 July 2011	-2.10797	0.35345	8.43425	3.23859E-6	0.05	1	-3.21548	-1.00046
April 2012 October 2011	-0.40157	0.35345	1.60675	0.94619	0.05	0	-1.50908	0.70594
April 2012 January 2012	1.40484	0.35345	5.62096	0.00427	0.05	1	0.29733	2.51235
July 2012 January 2011	0.5896	0.35345	2.35905	0.70722	0.05	0	-0.51792	1.69711
July 2012 April 2011	-0.21886	0.35345	0.87569	0.99849	0.05	0	-1.32637	0.88865
July 2012 July 2011	-0.48504	0.35345	1.94071	0.86628	0.05	0	-1.59255	0.62247
July 2012 October 2011	1.22135	0.35345	4.88678	0.02071	0.05	1	0.11384	2.32886
July 2012 January 2012	3.02777	0.35345	12.11449	8.73507E-10	0.05	1	1.92026	4.13528
July 2012 April 2012	1.62292	0.35345	6.49353	5.34291E-4	0.05	1	0.51541	2.73044
October 2012 January 2011	0.33582	0.35345	1.34366	0.97959	0.05	0	-0.77169	1.44333
October 2012 April 2011	-0.47264	0.35345	1.89108	0.88102	0.05	0	-1.58015	0.63487
October 2012 July 2011	-0.73882	0.35345	2.9561	0.43214	0.05	0	-1.84633	0.36869
October 2012 October 2011	0.96758	0.35345	3.8714	0.13027	0.05	0	-0.13994	2.07509
October 2012 January 2012	2.77399	0.35345	11.0991	8.60143E-9	0.05	1	1.66648	3.8815
October 2012 April 2012	1.36915	0.35345	5.47814	0.00588	0.05	1	0.26164	2.47666
October 2012 April 2012	1.36915	0.35345	5.47814	0.00588	0.05	1	0.26164	2.47666
October 2012 July 2012	-0.25378	0.35345	1.01539	0.99615	0.05	0	-1.36129	0.85373

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

Figure E.9 Tukey test result for the measurements taken from the samples of 2011 and 2012

Normality Test (06.12.2015 14:56:07)

Notes

Input Data

Descriptive Statistics

	Sample Size	Mean	Standard Deviation	SE of Mean
January	9	5.34904	0.83626	0.27875
April	9	6.1575	0.6312	0.2104
July	9	6.42368	0.66113	0.22038
October	9	4.71729	0.66664	0.22221
January	9	2.91087	0.84825	0.28275
April	9	4.31571	0.57294	0.19098
July	9	5.93864	0.90522	0.30174
October	9	5.68486	0.80684	0.26895

NormalityTest

Shapiro-Wilk

	DF	Statistic	Prob<W
January	9	0.89066	0.20266
April	9	0.94001	0.58202
July	9	0.82223	0.03652
October	9	0.89587	0.22901
January	9	0.9426	0.60948
April	9	0.92123	0.40252
July	9	0.94229	0.60625
October	9	0.84708	0.06919

January: At the 0.05 level, the data was significantly drawn from a normally distributed population.
April: At the 0.05 level, the data was significantly drawn from a normally distributed population.
July: At the 0.05 level, the data was not significantly drawn from a normally distributed population.
October: At the 0.05 level, the data was significantly drawn from a normally distributed population.
January: At the 0.05 level, the data was significantly drawn from a normally distributed population.
If the probability value, value of Prob < W, is smaller than 0.05, the corresponding data is not significantly drawn from a normally distributed polulation at 0.05 level.

Figure E.10 Shapiro-Wilk test result for the measurements taken from the samples of 2011 and 2012