MONITORING METHICILLIN-RESISTANT BACTERIA IN RIVER WATER BY USING *MEC*A-SPECIFIC DNA PROBE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ELNAZ SEYEDMONIR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOCHEMISTRY

DECEMBER 2014

Approval of the thesis:

MONITORING METHICILLIN-RESISTANT BACTERIA IN RIVER WATER BY USING *MEC*A-SPECIFIC DNA PROBE

Submitted by **ELNAZ SEYEDMONIR** in partial fulfillment of the requirements for the degree of **Master of Science in Biochemistry Department, Middle East Technical University** by,

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

Prof. Dr. Orhan Adalı _____
Head of Department, **Biochemistry**

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

Examining Committee Members:

Prof. Dr. Candan Gürakan _____
Food Engineering Dept., METU

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

Assoc. Prof. Dr. Tuba Hande Ergüder Bayramoğlu _____
Enviromental Engineering Dept., METU

Assoc. Prof. Dr. Can Özen _____
Biotechnology Dept., METU

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

Date: December 23, 2014

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: Elnaz Seyedmonir

Signature

ABSTRACT

MONITORING METHICILLIN-RESISTANT BACTERIA IN RIVER WATER BY USING *MEC*A-SPECIFIC DNA PROBE

Seyedmonir, Elnaz
M.S., Department of Biochemistry
Supervisor: Assoc. Prof. Dr. Bülent İçgen

December 2014, 91 pages

Aquatic ecosystems represent important vehicles for the dissemination of not only antibiotic resistant bacteria but also antibiotic resistance genes. Of particular interest are methicillin-resistant staphylococci (MRS) harbouring *mec*A gene that confers their resistance to β-lactams. Therefore, in this study, water samples collected from different locations of a river impacted by surrounding facilities and domestic effluents were analysed to learn more about the occurrence of MRS and *mec*A gene. Out of 290, 12 surface water isolates displayed resistance to both cefoxitin and oxacillin (derivatives of methicillin) antibiotics. Cefoxitin/oxacillin-resistant surface water isolates were screened for the prevalence of *mec*A gene by a polymerase chain reaction method. All of the cefoxitin/oxacillin-resistant surface water isolates including 4 *Staphylococcus*, 7 *Pseudomonas*, and one *Aeromonas* species, identified by 16S rRNA sequencing, were found out to harbor *mec*A. Following Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blot analysis of penicillin-binding protein (PBP2a) encoded by *mec*A revealed that staphylococcal PBP2a-specific antibodies were unsuccessful in

detecting non-staphylococcal carriers. For this reason, *mec*A-specific DNA probe was also used to monitor all *mec*A harboring surface water isolates during the periods of two years in between 2011 and 2012 by using *in situ* fluorescent hybridization technique and image-analyzed microscopy. Our results indicated that the *mec*A-specific DNA probe might be a potential analytical tool in selecting and *in situ* monitoring of methicillin resistant isolates in surface waters. Once in the environment, bacteria of different origin come into physical contact and may exchange resistance genes with the indigenous bacterial population. Therefore, surface waters are not only hot spots for *mec*A harbouring staphylococcal isolates but also non-staphylococcal ones due to gene dissemination and require special scientific consideration.

Key words: MRSA, PBP2a, *mec*A, *mec*A probe, methicillin resistance, surface water

ÖZ

*MEC*A-SPESİFİK DNA PROBU KULLANARAK NEHİR SUYUNDA METİSİLİN DİRENÇLİ BAKTERİLERİN TAKİP EDİLMESİ

Seyedmonir, Elnaz
Yüksek Lisans, Biokimya Bölümü
Tez Yöneticisi: Doç. Dr. Bülent İçgen

Aralık 2014, 91 sayfa

Sucul ekosistemler antibiyotik dirençli bakteriler ile birlikte direnç genlerinin yayılması bakımından da büyük önem taşımaktadır. Bu direnç genleri arasından β-laktam antibiyotiklere karşı direnç sağlayan ve özellikle stafilokoklarda metisilin dirençliliğine (MRS) neden olan *mec*A geni toplum sağlığı açısından oldukça dikkat çekicidir. Bundan dolayı, bu çalışma ile sucul sistemlerde MRS ve *mec*A geni oluşumu hakkında daha fazla bilgi edinmek amacıyla şehirsel ve fekal kirliliğe maruz kalan Kırıkkale-Kızılırmak'ın farklı bölgelerinden su örnekleri alınarak incelenmiştir. Bu örneklerden elde edilen 290 saf bakteri kültüründen 12 tanesinin metisilin türevleri olan sefoksitin ve oksasiline karşı direnç gösterdikleri belirlenmiştir. Sefoksitin/oksasilin dirençliği gösteren bu izolatlarda metisilin dirençliliğinden sorumlu *mec*A geninin varlığı polimeraz zincir reaksiyonu yöntemi kullanılarak araştırılmıştır. 16S rRNA sekans analizi ile tanımlamaları sonucu 4 tanesinin *Staphylococcus*, 7 tanesinin *Pseudomonas* ve bir tanesinin *Aeromonas* cinsine ait türler olduğu olduğu belirlenen bu izolatların *mec*A genini taşıdıkları tespit edilmiştir. Sodyum Dodesil Sülfat Poliakrilamid Jel Elektroforez (SDS-PAGE) analizi yapıldıktan sonra, Western Blot analizi ile *mec*A geni tarafından kodlanan

penisilin bağlayıcı proteininin (PBP2a) belirlenmesi çalışmalarında kullanılan ve ticari olarak üretilen antikorların stafilokokkal PBP2a'ya özgü olduğu ve bunların tespitinde başırı ile sonuç verdiği, ancak söz konusu antikorların stafilokok olmayan ve *mec*A taşıyan bakterilerde bu gen tarafından kodlanan PBP2a proteininin tespitinde etkisiz olduğu belirlenmiştir. Ayrıca yüzey sularında 2011-2012 yılları arasında *mec*A taşıyan izolatlar, floresan işaretli *mec*A-specifik DNA probu kullanılarak floresan *in situ* hibridizasyon yöntemi ve görüntü analizi mikroskopu ile takip edilmiştir. Bu çalışma ile, yüzey sularında metisilin-dirençli bakterilerin yerinde izlenmesinde *mec*A-spesifik DNA probunun kullanılmasının potansiyel bir analitik yöntem olabileceği belirlenmiştir. Yapılan bu çalışma ile direnç genlerinin sucul ortamlarda farklı kökenli bakterilerin fiziksel teması ile birbirlerine aktarılabildiği *mec*A geni açısından gösterilmiştir. Bu nedenle yüzey sularında *mec*A geninin yayılımının sadece stafilokoklar ile sınırlı olmadığı, stafilokok dışındaki türlerde de olabileceğinden bilimsel değerlendirmelerde dikkate alınması gerektiği sonucuna varılmıştır.

Anahtar kelimeler: MRSA, PBP2a, *mec*A, *mec*A probu, metisilin dirençliliği, yüzey sular

To my parents and sisters, who always support me in all aspects of my life

To my husband, for his patience and support in my study

ACKNOWLEDGEMENTS

Most of all, I would like to thank my thesis advisor Assoc. Prof. Dr. Bülent İçgen a talented teacher and intelligent scientist. His critique and guidance has added substantial value to my research. I am so grateful for the time he carved out of his busy schedule to help me with every aspect of this research, for his careful guidance, and for his attentive teaching about research design and methods, scientific writing, and data analysis.

I would like to thank my committee members Prof. Dr. Candan Gürakan, Assoc. Prof. Dr. Tuba Hande Ergüder Bayramoğlu, Assoc. Prof. Dr. Can Özen and Assoc. Prof. Dr. Çağdaş D. Son for their advice and help during my thesis defence. I am indebted to other faculty members in the graduate school of natural and applied sciences; Prof. Dr. Orhan Adalı, head of biochemistry department, and Prof. Dr. Celal F. Gökçay for their valuable suggestions and concise comments on my education career.

I am highly thankful to Dr. Fadime Yilmaz for day-to-day lab support, enthusiastic support in planning of experiments, analysis of the experimental data and scientific interpretation of the results and also her husband Murat Artuç for data analysis and result interpretation. Thanks also to all the members of İçgen Team; Munire Selcen Ak, Lale Göksu, Mustafa Nakipoğlu, Huseyin Ulusoy and Salih Batuhan Salik for providing a good atmosphere in our lablatories at enviromental engineering department and for useful discussions and full of fun days.

I owe a lot to my parents, who encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true. I deeply

miss my father Mirhadi Seyedmonir, who is not with me to share this joy. I also thank my family who encouraged and supported me throughout my entire life.

Above all, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

This work was supported by the scientific and technological research council of Turkey (TUBITAK) through the support program for scientific and technological research projects (1001) with the project number 113Z198.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1 Antibiotic Resistance Overview

Ever since the introduction of penicillin during the Second World War antibiotics have been viewed as miracle drugs. After the introduction of penicillin, isolation of new antibiotics proceeded quickly and most of the major classes were isolated during the 1940s to 1960s (Walsh and Wright, 2005). Due to the application of these "miracle drugs" a decrease in deaths caused by infections was seen (Alanis, 2005). However, nowdays the miracle may be over because of the development of bacterial antibiotic resistance which threatens the earlier effective treatment of bacterial infections. Antibiotic resistance has been given a lot of attention during the last two decades both within the scientific community and in public media (Turlej *et al*., 2011; WHO, 2014).

Antibiotics work in variety of ways as summarized in Table 1.1. Some antimicrobial agents prevent bacterial cell wall synthesis. These agents include ß-lactam compounds such as penicillins (e.g. penicillin G, ampicillin and methicillin), cephalosporins and carbapenems, as well as monolactams and ß-lactamase inhibitors. ß-lactams inhibit the final stage of murein synthesis. This, by some undetermined mechanism, triggers murein hydrolases to lyse the cell (Mojica and Aga, 2011; Ba *et al*., 2014). A related group of antibiotics that prevent a different step in cell wall synthesis are the glycopeptides, vancomycin and teicoplanin. Other agents have an antibacterial effect by preventing protein synthesis. Representatives of this group

comprise the aminoglycosides, tetracyclines, lincosamides, macrolides and chloramphenicol which interfere with ribosome function (Moore, 2014). In addition, there are antibiotics that prevent DNA synthesis, involving quinolones/fluoroquinolones, quinoxalines, imidazoles and sulfonamides (Mojica and Aga, 2011; Moore, 2014).

Table 1.1 Antibiotic types and mechanism of action

Antibiotics Types	Mechanism of Action	Examples
Aminoglycosides	Inhibition of bacterial protein synthesis (bind to 30S ribosomal subunit)	Gentamicin, Amikacin
Beta-Lactams	Disruption of peptidoglycan synthesis (inactivate PBP_S^*)	Penicillins: Penicillin G, Amoxicillin Cephalosporins: Cefoxitin, Cefotaxime,Carbapenem: Imipenem
Fluoroquinolones/ Quinolones	Inhibition of bacterial DNA synthesis	Norfloxacin, Ciprofloxacin, Enoxacin, Ofloxacin
Glycopeptides	Bind to peptidoglycan precursor	Vancomycin, Teicoplanin
Imidazoles	Inhibition of bacterial DNA synthesis	Metronidazole
Ionophores	Block intracellular protein transport	Monensin, Lasalocid
Lincosamides	Inhibition of bacterial protein synthesis	Clindamycin, Lincomycin
Macrolides	Inhibition of bacterial protein synthesis (bind to 50S ribosomal subunit)	Erythromycin, Azithromycin, Tylosin
Peptides	Inhibition of bacterial cell wall synthesis	Bacitracin
Quinoxalines	Inhibition of bacterial topoisomerases (DNA synthesis)	Carbadox, Olaquidox
Sulfonamides	Block bacterial cell metabolism (by inhibiting enzymes)	Sulfadiazine, Sulfamethoxasole, Sulfapyridine
Tetracyclines	Inhibition of bacterial protein synthesis	Tetracycline, Chlortetracycline, Oxytetracycline, Doxycycline
Other	Inhibition of bacterial protein synthesis	Chloramphenicol

*Penicillin Binding Protein

1.1.1 Bacterial Resistance Mechanism

Mechanism of antimicrobial resistance categorizes in four general forms; enzymes that destroy or modify the antimicrobial substrate, target site alteration, bypass pathways that substitute for a metabolic pathway and barrier to penetration or efflux pumps that leave out the agent (Figure 1.1).

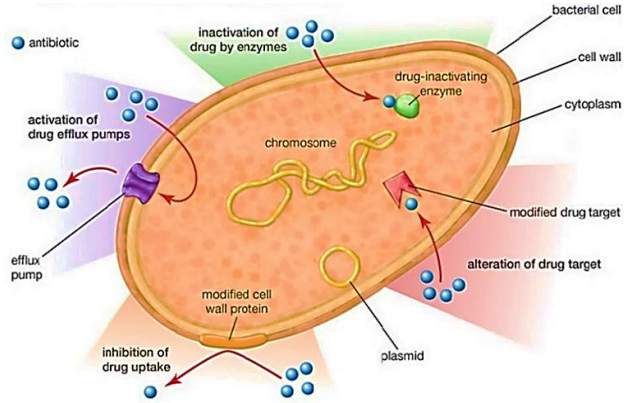

Figure 1.1 Mechanisms of antibiotic resistance (Morier, 2013).

1.1.2 Antibiotic Resistance and Resistance Genes as an Emerging Environmental Issue

Antibiotic resistant bacteria and antibiotic resistance genes are excreted into the environment primarily with feces of humans and animals that are treated with antibiotics. These bacteria end up in soil and surface water through the discharge of untreated or partially treated sewage (amongst others derived from potential 'hot spots' of antibiotic resistance such as health care centers, farms and slaughterhouses), application of activated sludge from wastewater treatment plants (WWTPs) as fertilizer of agricultural soil, and runoff of animal manure or excrement of pasture animals (Blaak *et al*., 2011).

Significantly, once in the environment, bacteria of different origin come into physical contact and may exchange resistance genes with the endogenous bacterial population (Genthner *et al*., 1988; Xu *et al*., 2007; WHO, 2014). Even though the resistant bacteria of human and animal source may die off in the environment, the endogenous environmental bacteria may pass the obtained resistance genes on to their progeny. Despite the generally believed negative influence of obtained antibiotic resistance on fitness of the bacteria (Andersson and Levin, 1999; Andersson and Hughes, 2010), these genes may exist in the environment for a long time (Andersson, 2003; Balcazar, 2014). Besides a collecting and mixing vessel of resistance genes of anthropogenic origin, the environment is also considered to be a natural reservoir of resistance genes. The presence of these resistance genes, and also genes encoding proteins that originally have alternative biochemical functions but can easily change into resistance proteins in the case of selective pressure, are believed as the origin of resistance in bacterial populations (Allen *et al*., 2010; Balcazar, 2014).

1.1.3 Sources and Transport of Antibiotic Resistance Genes in the Environment

The existence of antibiotic residues in terrestrial and aquatic systems, arising mainly from discharges from municipal WWTPs and land application of animal wastes, is now well documented in the literature (Blaak *et al*., 2011). Many animal confinement operations produce manure that contains antibiotics since animals get these drugs in feed rations, either as growth promoters or as therapeutic substances. Treated animals excrete antibiotic metabolites and some non-metabolized antibiotics, which are then introduced to agricultural lands through repeated fertilization with animal manure (Mojica and Aga, 2011). As depicted in Figure 1.2, veterinary antibiotics are introduced into the soil from manure application. Through surface runoff and leaching, antibiotics and their metabolites can be carried to surface water and groundwater. Correspondingly, human antibiotics from the wastewater of WWTPs are injected directly into surface water (Mojica and Aga, 2011).

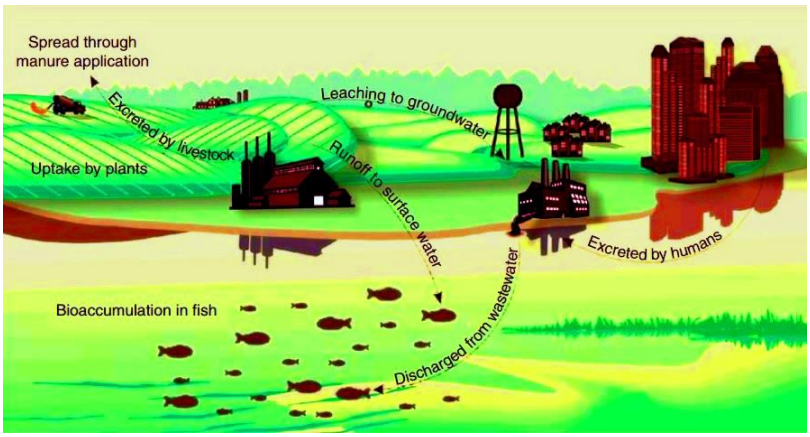

Figure 1.2 Sources and transport of human and veterinary antibiotics in the environment (Mojica and Aga, 2011).

1.2 Mobile Genetic Elements, Horizontal and Vertical Gene Transfers

Mobile genetic elements (MGEs) were first represented in the maize genome in the late 1940s (McClintock, 1950) and are essential ways for transfer of genetic information among prokaryotes and eukaryotes. MGEs are characteristically identified as fragments of DNA that encode a variety of virulence and resistance determinants and also the enzymes that mediate their own transfer and merging into new host DNA (Frost *et al*., 2005; McCarthy *et al*., 2014). Transfer of MGEs between cells is named lateral or horizontal gene transfer (HGT). In HGT, the fragment of DNA has ability to transfer from prokaryote-to-prokaryote, prokaryote-to-eukaryote, or eukaryote-to-eukaryote (Malachowa and DeLeo, 2010; Lindsay, 2014) (Figure 1.3). MGEs may consist of insertion sequences, transposons, phages, plasmids, pathogenicity islands, and chromosome cassettes (Alibayov *et al*., 2014). These segments of DNA are largely propagated by vertical gene transfer (VGT), which is explained as the transmission genetic information from parent to progeny cell (Malachowa and DeLeo, 2010).

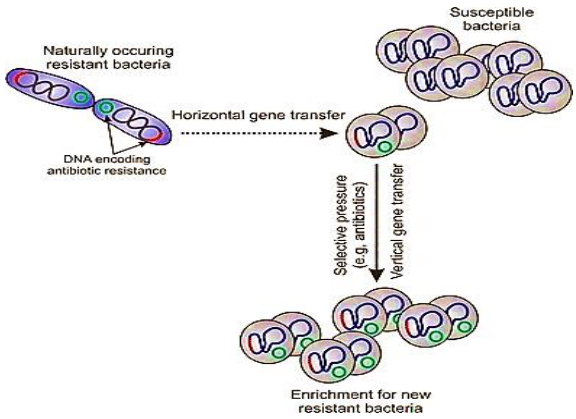

Figure 1.3 HGT and VGT (Malachowa and DeLeo, 2010).

Bacteria acquire genetic information from other cells or the surrounding environment in three ways as shown in Figure 1.4: transformation through uptake of free DNA from the environment, transduction by the help of bacteriophages, and conjugation through direct contact between bacterial cells (Balcazar, 2014; Bbosa *et al*., 2014).

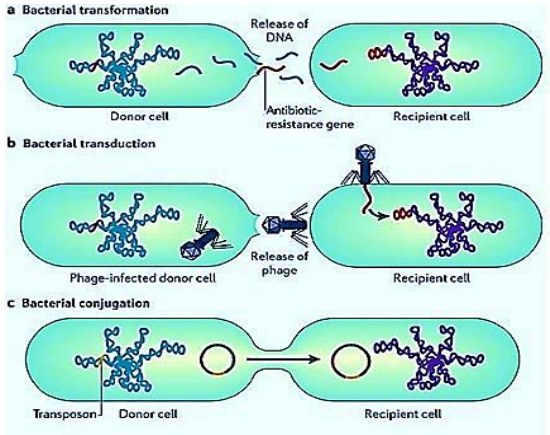

Figure 1.4 Bacterial transformation (a), transduction (b), conjugation (c) (Bbosa *et al*., 2014).

1.3 *Staphylococcus aureus*

Staphylococci were viwed and cultured for the first time by Robert Koch (1843-1910) and Louis Pasteur (1822-1895) but first detailed studies were published by a Scottish surgeon Sir Alexander Ogston (1844-1929) a few years later (Ogston, 1880; Ogston, 1882). He observed clusters of ring-shaped organisms under the microscope and exhibited their effective role in abscess configuration. The Latin name *Staphylococcus* was given by Sir Ogston to this family of pus forming bacteria based on their appearance under the microscope. Shortly after Sir Ogston's discovery another surgeon, a German Anton J. Rosenbach (1842-1923), was able to isolate and grow *S. aureus*. He named the species after the yellowish color of the colony (from Latin: aurum "gold"). In 1928, the bacteriologist Sir Alexander Fleming (1881-1955) observed that *S. aureus* could not grow in the presence of the mold *Penicillium notatum* (Fleming, 1929). However, it was not until 10 years later that penicillin was purified and large enough quantities could be produced to begin treatment trials. The first animal experiments were conducted in Oxford in 1939 and the results were published by the Lancet next year. The first human trial soon followed (Abraham *et al*., 1941). By the fall of 1943 enough penicillin was available to fulfill the huge demand of the allied war efforts (Riley, 1972). Before the introduction of penicillin rate of death in *S. aureus* bacteremia had exceeded 80% in that time (Skinner and Keefer, 1941).

1.3.1 Antimicrobial Resistance in *S. aureus*

The first report of *S. aureus* resistance to penicillin through the acquisition of genes yielding beta-lactamase (penicillinase) came out even before the widespread production of this antibiotic (Rammelcamp and Maxon, 1942). The appearance of penicillin resistance led to the introduction of the semisynthetic penicillinase-stable antistaphylococcal penicillins. The first of them, methicillin, was introduced for the medical care of *S. aureus* infections in 1961, being followed by other derivatives involving oxacillin, cloxacillin and dicloxacillin. By the late 1960s more than 80% of

S. aureus isolates had developed resistance to penicillin (Lowy, 2003; Todar, 2013). Thus, penicillin was no longer an alternative for the antimicrobial treatment of *S. aureus* infections (Todar, 2013; WHO, 2014).

1.3.2 Mobile Genetic Elements of *S. aureus*

S. aureus is one of the most noticeable causes of nosocomial- and community-acquired bacterial infections throughout the world (Chambers and DeLeo, 2009; McCarthy *et al*., 2014). Although the basis for this diseases is multifactorial and mainly dependent on host susceptibility, heterogeneity of *S. aureus* strains likely plays a role in this process. Heterogeneity among *S. aureus* strains improved in part as a result of its interaction with the mammalian host. Numerous putative and examined virulence factors, genes responsible directly for host adaptation, and toxins, are placed on *S. aureus* MGEs (Baba *et al*., 2002; Lindsay, 2014). As shown in Figure 1.5, *S. aureus* possesses many types of MGEs, including plasmids, transposons (*T*n), insertion sequences (*IS*), bacteriophages, pathogenicity islands (*PI*), and staphylococcal chromosome cassettes (SCC) (Malachowa and DeLeo, 2010; Alibayov *et al*., 2014; Balcazar, 2014; McCarthy *et al*., 2014). It is remarkable that most genes encoded by MGEs remain under the control of global regulators located within the core genome (Malachowa and DeLeo, 2010; Alibayov *et al*., 2014).

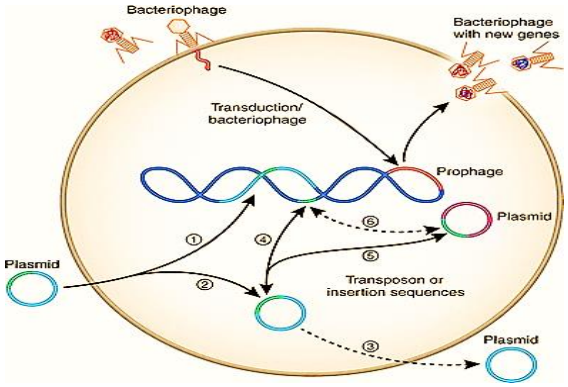

Figure 1.5 Acquisition of MGEs by *S. aureus* (Malachowa and DeLeo, 2010). 1. Incorporation of plasmids or plasmid elements into genomic DNA, 2. Plasmids can be maintained as free circular DNA, 3. Suicide plasmid, 4. Transfer of a *Tn* or an *IS* between plasmid and genomic DNA, 5. Transfer of a *Tn* or an *IS* between plasmids within the cell, 6. Transfer of a *Tn* or an *IS* from genomic DNA to another plasmid.

1.4 Methicillin-resistant *S. aureus* (MRSA)

The first informational work of methicillin resistance in *S. aureus* came into view soon after the introduction of penicillinase-stable penicillins (Jevons, 1961). At the beginning, MRSA strains were encountered only in the hospitals, but in the late 1990s first virulent community-acquired MRSA (CA-MRSA) clones, characterized by the presence of the toxin Panton-Valentine Leukocidin (PVL), appeared quickly and unexpectedly (Turlej *et al*., 2011). They rapidly spread through the word, initially only in the community, but later on also in the healthcare facilities, displacing in some countries typical hospital-acquired MRSA (HA-MRSA) (Paterson *et al*., 2014; WHO, 2014).

Methicillin resistance in staphylococci is caused by the *mec*A gene which encodes an altered 78 kDa penicillin-binding protein 2a (PBP2a) with a low affinity for β-lactam antibiotics such as penicillin, anti/staphylococcal penicillins including methicillin, and cephalosporins (Chambers, 1997; Ba *et al*., 2014; Paterson *et al*.,

2014). Recently, a new *mec*A gene homologue (*mec*ALGA251) has also been discovered to carry methicillin resistance (Garcia-Alvarez *et al*., 2011). It was recently renamed as *mec*C (Ito *et al*., 2012; Laurent *et al.*, 2012; Petersen *et al*., 2013; Paterson *et al*., 2014). The Staphylococcal cassette chromosome *mec* (SCC*mec*) is a family of large mobile genetic elements that include the *mec*A gene (Ito *et al*., 2001; Ito *et al*., 2014). The *mec*A harboring MRSA strains are considered to have emerged by means of horizontally transferred SCC*mec* from coagulase-negative *Staphylococcus* (CoNS) (Wielders *et al*., 2002; Lindsay, 2014). A majority of nosocomial CoNS, the foremost of them *S. epidermidis*, has been resistant to methicillin for years due to *mec*A gene (Archer and Climo, 1994). Earlier studies demonstrated that there was a considerable homology between *S. aureus mec*A and the *mec*A genes found in the coagulase-negative *S. sciuri* group, isolated from animals and foodstuffs (Couto *et al*., 1996). More recent studies have revealed that of the 3 *mec*A-positive *S. sciuri* species groups (*S. sciuri*, *S. vitilinus*, and *S. fleurettii*), the *mec*A gene of *S. fleurettii* shows *in vitro* methicillin resistance and has the highest homology (> 99%) with the *mec*A gene of MRSA strain N315 (Tsubakishita *et al*., 2010). This obviously suggests that *S. fleurettii* rather than *S. sciuri* might have been the origin of *mec*A (Moellering, 2012). On the other hand, a transfer of DNA containing the *mec*A code from *S. epidermidis* to *S. aureus* has also been witnessed *in vivo* (Wielders *et al*., 2001; McCarthy *et al*., 2014).

1.4.1 Staphylococcal Chromosome Cassette *mec* (SCC*mec*) Composition

SCC*mec* elements, recognized in almost all MRSA strains, belong to special type of the staphylococcal MGEs coding for meticillin-resistance and named as staphylococcal chromosome cassette *mec* (SCC*mec*) (Ito *et al*., 2014). In *S. aureus* strains, SCC*mec* elements always integrate sequence specifically at the unique site of the chromosome, *attBscc* (bacterial chromosomal attachment site). The *attBscc* is placed near the origin of replication, at the 3' end of *orfX*, coding for an open reading frame X of unknown function, well conserved among both MRSA and MSSA strains

(Hiramatsu *et al*., 2001; Ito *et al*., 2001; Ito *et al*., 2014). The connection site contains a core 15-bp sequence, called the integration site sequence (*ISS*) that is essential for *ccr*-mediated recombination (IWG-SCC, 2009). After integration of SCC*mec* into the chromosome, *ISS* is discovered in direct repeat sequences at left and right SCC*mec*/chromosomal junctions of the merged SCC*mec* element. Various SCC*mec* elements share similar backbone structure, that consists of (i) *mec* complex, composed of *mec*A operon, (ii) *ccr* gene complex, composed of chromosome cassette recombinase (*ccr*) gene(s) and (iii) three regions bordering the *ccr* and *mec* complexes, designated as joining (J) regions. The composition of almost all SCC*mec* elements identified so far in *S. aureus* can be demonstrated as follow: (*orfX*)J3-*mec*-J2-*ccr*-J1 (Turlej *et al*., 2011; Ito *et al*., 2014). The exception constitute SCC*mec*VII and a newly described SCC*mec*IX, with the *ccr* gene complex positioned between J3 and J2 regions and the *mec* gene complex between J2 and J1 regions (Li *et al*., 2011; Turlej *et al*., 2011; Ito *et al*., 2014).

1.4.2 The *mec* Gene Complex

Two evolutionary altered lineages of *mec* gene complexes have been described in *S. aureus*. The first one, which encompasses the abundant majority of known and well characterized *mec* gene complexes, has been observed in MRSA isolates of human origin since the nineties. The prototype of this lineage is the *mec* gene complex designated as class A, composed of an intact *mec* operon, the hyper-variable region (HVR) and the insertion sequence *IS*431 (Ito *et al*., 2001; Turlej *et al*., 2011). The *mec* operon includes *mec*A gene and located upstream of *mec*A its regulatory genes: *mec*R1 and *mec*I, coding for the signal transducer and the repressor, respectively. Differences between class A *mec* gene complex and other *mec* gene complexes of this lineage, described to date, result mainly from insertions of *IS* elements, *IS*1272 or *IS*431, into the region of *mec*A regulatory genes, causing complete removal of *mec*I and, different in size, partial deletions of *mec*R1. Depending on the structural diversity of *mec*I-*mec*R1 region, five major classes of

mec gene complexes, of the said lineage, have been defined by IWG-SCC (IWG-SCC, 2009): Class A, which contains intact *mec* gene complex: *IS*431-*mec*A-*mec*R1-*mec*I; Class B, where *mec*R1 is truncated by insertion sequence *IS*1272: *IS*431-*mec*A-Δ*mec*R1-*IS*1272; Class C1, where *mec*R1 is truncated by insertion sequence *IS*431 having the same direction as the *IS*431 downstream of *mec*A: *IS*431-*mec*A-Δ*mec*R1-*IS*431; Class C2, where *mec*R1 is truncated by insertion sequence *IS*431 having the reverse direction to the *IS*431 downstream of *mec*A: *IS*431-*mec*A-Δ*mec*R1-*IS*431; and Class D, where *mec*R1 is partly deleted but there is no *IS* element downstream of Δ*mec*R1: *IS*431-*mec*A-Δ*mec*R1 (Turlej *et al*., 2011). Four classes of the *mec* complex and four different *ccr* allotypes define at present eight SCC*mec* types (I–VIII) (Figure 1.6). Nonetheless, SCC*me* types can be further separated into subtypes depending on differences in the J regions (Malachowa and DeLeo, 2010; Ito *et al*., 2014).

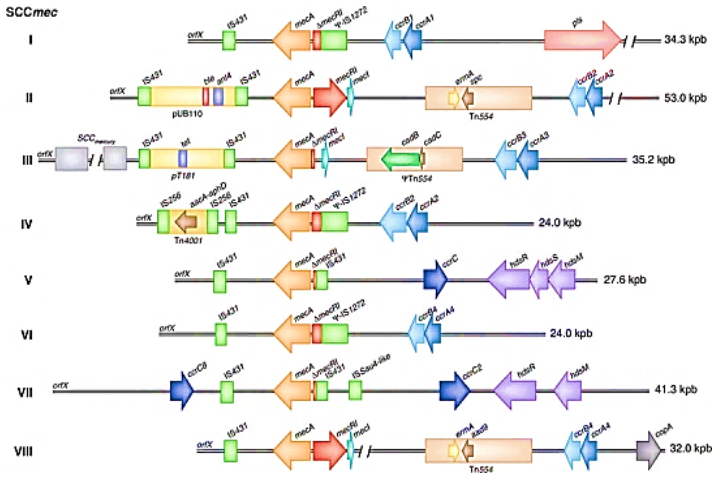

Figure 1.6 Comparison of *S. aureus* SCC*mec* types (Malachowa and DeLeo, 2010). Class A SCC*mec* contains a complete *mec*A regulon (*mec*1-*mec*R1-*mec*A). Class B and class C SCC*mec* contain regulatory genes that are disrupted by *IS*, *IS*1272-Δ*mec*R1-*mec*A and *IS*431-Δ*mec*R1-*mec*A, respectively.

1.4.3 *mec*A Gene and β-lactam Resistant Mechanism

MRSA has a finely-tuned, "on-demand system" that turns *mec*A expression on in the presence of β-lactam drugs, while keeping expression turned off in the lack of these drugs (Le, 2013). This regulation is performed by proteins whose genes are also discovered on the SCC*mec* component. In the absence of β-lactams the expression of *mec*A is kept in check by the protein mecI. MecI attaches to the DNA promoter regioncof *mec*A and inhibits gene transcription. Nevertheless, in the existence of β-lactam drugs the bacteria require PBP2a around in order to stay alive. In this situation, expression of *mec*A is turned on through the action of the cell surface protein mecR1 whose job is to keep an eye out for β-lactams (Le, 2013). When mecR1 notices the presence of β-lactams, it instructs the bacterial cell to analysis the mecI inhibitor. This permits expression of the *mec*A gene that is critical for the bacteria's survival to happen. Another gene on the SCC*mec* (*mec*R2) was recognized (Arede *et al*., 2012), as it turns out the MecR2 protein adjusts *mec*A expression. When MRSA bacteria encounter β-lactam drugs it starts ramping up the production of MecR2 protein. MecR2 sequentially knocks the MecI inhibitor protein off of the *mec*A gene promoter, thereby increasing *mec*A expression (Arede *et al*., 2012) (Figure 1.7). In the presence of β-lactams, mecR1 is activated and prompts the transcription of *mec*A and *mec*R1-*mec*I-*mec*R2; the anti-repressor mecR2, destabilizes mecI-dimers, disturbing their joining to the *mec*A promoter and fostering their proteolytic inactivation, resulting in a sustained induction of *mec*A transcription; when depletion of β-lactam occurs, mecR1 is no longer activated and a steady state is established consisting of stable mecI-dimers bound to the *mec*A promoter (and protected from proteolysis) and residual copies of *mec*R1 at the cell membrane; the remaining free mecR2 molecules are most likely degraded by the cellular protein turnover pathway (Arede *et al*., 2012; Le, 2013).

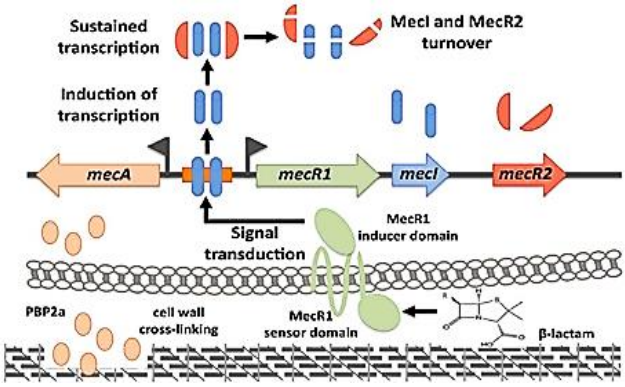

Figure 1.7 Model for the *mec*A induction by *mec*R1-*mec*I-*mec*R2 in the presence of ß-lactam antibiotics (Arede et al., 2012).

1.5 Molecular Based Methods for Detection of *mec*A Gene

1.5.1 Detection by Polymerase Chain Reaction (PCR)

The use of molecularly based techniques for finding and estimation of microorganisms are an integral part in microbiology laboratories nowadays. A plenty of various nucleic acid based systems exists for detection and assessment of antimicrobial resistance and mechanisms, widely reviewed by Fluit *et al*., (2001). PCR first presented in 1988 by Mullis and co-workers (Saiki *et al*., 1988), as the "golden standard". Compared to cultivation-based methods, PCR suggests a fast, sensitive and easy mean for finding of resistance genes and is particularly beneficial for slow-growing and uncultivable microorganisms. This is vital for evaluating microorganisms in non-clinical settings, such as wastewater and soil, as it has been estimated that >99 % of the bacteria are uncultivable (Amann *et al*., 1995). Furthermore, PCR identifies nucleic acids rather than living cells hence there is a threat of "free" nucleic acids or nucleic acids from dead cells giving false-positives (Wolffs *et al*., 2005; Kallea *et al*., 2014). The basis for PCR is the detection and synthesis of a specific DNA/RNA template (Kallea *et al*., 2014). The template is

recognized with two short synthetic and sequence-specific oligonucleotide primers. The primers also act as the beginning points for the synthesis, which is carried out by polymerases. The first and greatest used polymerase is *Taq* DNA polymerase, isolated from *Thermus aquaticus*, but another commonly used is the high fidelity *Pfu* DNA polymerase, isolated from *Pyrococcus furiosus* (Elshawadfy *et al*., 2014). In addition, to polymerase and primers, deoxyribonucleotide triphosphates (dNTP), which are building blocks of the synthesized DNA strands, are required. The PCR is carried out by temperature cycling, starting with high temperature for separation of the double stranded helical DNA molecule (denaturing), after which the temperature is decreased to let primers attach (annealing) and then increased, usually to around 72 ºC, to permit the polymerase to lengthen the primers from the 3´end using the dNTPs (extension). This process is usually repeated through 30-40 cycles, which will allow for an exponential increase in the amount of DNA produced, with each newly formed product acting as a template during the remaining cycles. PCR is a sensitive technique where the stability and efficiency of the reaction are influenced by many restrictions; DNA/RNA concentration/quality, primer concentration/quality, dNTP concentration, type and concentration of polymerase, buffer type, cycling parameters, type of tubes/wells and PCR instrument (Saiki *et al*., 1988; Kallea *et al*., 2014; NCBI, 2014). Furthermore, after completion it necessitates a separation of the formed PCR-product, usually by agarose gel electrophoresis with GelRed staining (Olerup SSP, Sweden), to confirm that the template has been amplified and that it is of predictable size (NCBI, 2014).

1.5.2 Penicillin Binding Protein 2a (PBP2a)

1.5.2.1 Detection by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Peptidoglycan is the major constituent of the bacterial cell wall, which sustains cell shape, resists intracellular pressure and acts as a platform for anchored macromolecules such as proteins and polysaccharides (Dramsi *et al*., 2008; Sewell

and Brown, 2014).This macromolecular network is made of glycan chains of alternating N-acetylglucosamine and N-acetylmuramic acid (MurNAc) that are cross-linked by short peptides bound to the MurNAc (Helassa *et al*., 2012). The peptidoglycan is polymerized from a precursor (lipid II) that is synthesized in the cytoplasm and flipped to the outer face of the plasma membrane (Mohammadi *et al*., 2011; Helassa *et al*., 2012). PBPs catalyze the polymerization of glycan chains (transglycosylation) and the peptidic cross-linking of glycans strands (transpeptidation). PBPs have been divided into three classes: Class A PBPs are bifunctional with both glycosyltransferase (GT) and transpeptidase (TP) activity. Class B PBPs have only one well-known catalytic domain with potential TP activity. Class C PBPs have hydrolytic activitiesand participate in peptidoglycan maturation and cell separation (Sauvage *et al*., 2008; Helassa *et al*., 2012; Sewell and Brown, 2014). The domain with hydrolytic or TP activity is the target of b-lactams. The enzymatic GT activity of the class A PBPs constitutes a promising alternative target (Sauvage *et al*., 2008; Helassa *et al*., 2012).

PBP2a is a peptidoglycan transpeptidase that, in cooperation with the transglycosylase domain of PBP2 of *S. aureus*, can catalyze cell wall biosynthesis in the existence of β-lactam antibiotics, thus enabling survival and growth of the bacteria (Kim *et al*., 2012; Ba *et al*., 2014; Sewell and Brown, 2014). PBP2a is encoded by the imported *mec*A gene, which is integrated into the *S. aureus* chromosome as portion of a huge heterologous mobile genetic element SCC*mec* (Kim *et al*., 2012; Ba *et al*., 2014; Ito *et al*., 2014; Sewell and Brown, 2014).

1.5.2.2 Detection by SDS-PAGE

SDS-PAGE separates proteins according to their molecular weight, based on their variance rates of movement through a gel under the influence of an applied electrical field (Rath *et al*., 2009). A gel made by crosslinked polymerization of two organic monomers, acrylamide and the crosslinking agent, N, N'-methylenbisacrylamide. The solubilizing denaturing agents like SDS are broadly

applied in the separation of proteins by gel electrophoresis. SDS has high affinity for proteins and endorses protein denaturation. In addition the negatively charged detergent molecules coat the proteins and cover their native charge. As a result, the proteins separated by SDS-PAGE migrate according to their molecular weight regardless of charge (Laemmli, 1970). Following electrophoresis, the gel may be stained with Coomassie Brilliant Blue or silver stain to visualize the separated proteins. After staining, different proteins appear as distinct bands within the gel according to their sizes and thus by molecular weights (Rath *et al*., 2009).

1.5.2.3 Detection by Western Blot Analysis

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is applied to specifically distinguish its antigen) was presented by Towbin, *et al*., (1979) and is now a routine technique for protein analysis (Gibbons, 2014). The specificity of the antibody-antigen interaction enables a single protein to be recognized in the midst of a complex protein mixture. Western Blotting is commonly applied to positively detect a specific protein in a complex mixture and to gain qualitative and semi-quantitative data about that protein (Mahmood and Yang, 2012; Gibbons, 2014). The first step in a Western Blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively (Jensen, 2012; Mahmood and Yang, 2012; Gibbons, 2014). The most sensitive detection methods use a chemiluminescent

substrate that, when combined with the enzyme, produces light as a byproduct (Towbin *et al*., 1979; Jensen, 2012) The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane (Pierce, 2004).

Detailed procedures for detection of a Western Blot vary broadly (Mahmood and Yang, 2012). One common variation involves direct vs. indirect detection as shown in Figure 1.8. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is also labeled with an enzyme or fluorescent dye. This detection method is not widely used as most researchers prefer the indirect detection method for a variety of reasons. In the indirect detection method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase (Pierce, 2004; Mahmood and Yang, 2012).

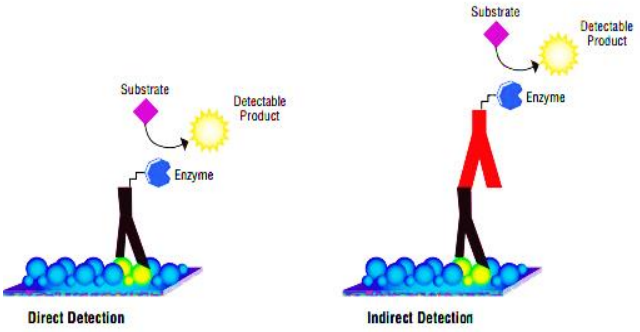

Figure 1.8 Direct and indirect detection method for desired product in Western Blotting (Pierce, 2004).

1.5.3 Detection by Fluorescent *in situ* Hybridization (FISH)

The introduction of fluorescence *in situ* hybridization (FISH) almost 30 years ago marked the beginning of a novel era for the study of chromosome structure and function (Volpi and Bridger, 2008). FISH with rRNA targeted oligonucleotide probes allows the characterization of bacterial population in environmental samples. In bacterial work, FISH probes are usually designed to distinguish DNA coding for 16S rRNA (Bidinenko *et al*., 1998), providing reliable information in phylogenetic studies (Amann *et al*., 1995). FISH with polynucleotide DNA probes and FISH with oligonucleotide probes targeted to mRNA has also been explained (Trebesius *et al.,* 1994; DeLong *et al.*, 1999). FISH analysis is extremely vital for microbial ecology to solve the problem of impossibility in cultivating most of the cells present at a habitat (Hu *et al*., 2014), and to allow *in situ* identification of microorganisms (Amann *et al*., 1992). The identification of bacteria from both pure and mixed cultures representing the population of an environmental sample can be established with FISH (Amann *et al*., 1991; Korzeniewska and Harnisz, 2012). The techniques have been used broadly for the detection and quantification of *mec*A harboring bacteria in diverse environmental samples and clinical setting. Specificity of probe binding to the target site depends on the hybridization and washing conditions. Hybridization probes are added to a defined, stringency determining buffer at saturation concentrations to maximize probe binding. During hybridization the samples are incubated at elevated temperature in an airtight vessel saturated with water and formamide vapours of additional hybridization buffer to avoid concentration effects due to evaporation. The washing step is performed at a slightly higher temperature and serves mainly to rinse off excess probe molecules at conditions that prevent unspecific binding (Korzeniewska and Harnisz, 2012). The diversification of the original FISH protocol (Figure.1.9) into the impressive number of procedures available these days has been promoted through the years by a number of interconnected factors, such as the improvement in sensitivity, specificity, and resolution of the technique, brought about by a better understanding of the chemical and physical properties of nucleic

acids and chromatin, together with the advances in the fields of fluorescence microscopy and digital imaging, and the growing availability of genomic and bioinformatics resources (Volpi and Bridger, 2008; Hu *et al*., 2014). As Volpi and Bridger have reviewed, there is abundant FISH application such as ACM-FISH, armFISH, CARD-FISH, catFISH, CB-FISH, CO-FISH and etc.

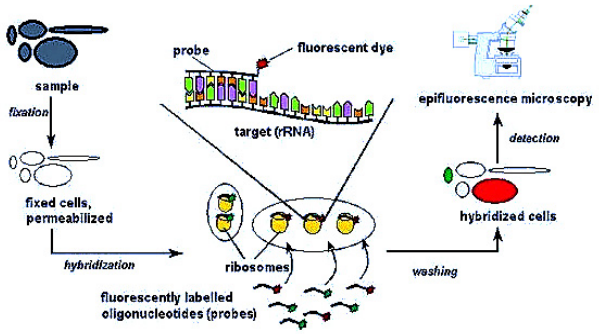

Figure 1.9 FISH procedure (Glockner *et al*., 1996)

1.6 Aim of the Study

The *mec*A gene is widely disseminated among *S. aureus* and other staphylococcal species, and its expression is essential for the methicillin-resistant phenotype (Tsubakishita *et al*., 2010). The spread of resistance and virulence in staphylococci occur via mobile genetic elements like genomic islands, bacteriophages, pathogenicity islands, chromosomal cassettes, plasmids, insertion sequences and transposons (Baba *et al*., 2002; Holden *et al*., 2004; Gill *et al*., 2005; Colomer-Lluch *et al*., 2011; Maslanova *et al*., 2013; Ito *et al*., 2014). Horizontal *mec*A transfer could contribute to the worldwide dissemination of MRS (Hanssen *et al*., 2004). MRSA strains appear to have evolved independently many times by gene transfer of the *mec*A gene into different strains of methicillin-susceptible *S. aureus*

(Duquette and Nuttall, 2004). Recently, *mec*A gene homologues that are only distantly related to *mec*A have been identified in the genomes of some staphylococci related bacterial species, too (Ito *et al*., 2012). The similarity of the *mec*A sequences in non-staphylococcal genera of *Proteus*, *Morganella*, and *Enterococcus* indicates that *mec*A is more widely distributed in the environment than previously revealed and also lead to the potential for SCC*mec* transfer among differing bacterial genera (Kassem *et al*., 2008). Despite continual increases in the community prevalence of MRSA, there is still speculation on the role of the aquatic environment as a reservoir of staphylococci and other potentially pathogenic bacteria that harbor *mec*A. Because of these concerns, we investigated the occurrence of MRS and *mec*A in river water impacted by industrial and agricultural pollution from the surrounding facilities and domestic effluents from the city. The transfer of *mec*A from staphylococcal to non-staphylococcal bacteria might frequently occur in surface water. These findings makes the *mec*A gene a biomarker molecule for the monitoring of methicillin-resistant isolates. Therefore, this study also aimed at monitoring of *mec*A harboring surface water isolates by using *mec*A-specific DNA probes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample Collection

Water samples were collected from 12 different locations of the river Kızılırmak 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, Turkey. For microbial analysis, water samples were put into sterile screw capped bottles aseptically, kept in an icebox containing ice packs and taken immediately to the laboratory. A quantity of 1 mL of water from each of the collected samples was dissolved in 9 mL sterile distilled water and serial dilutions were made. Each dilution was plated on Luria Bertani (LB) agar plates by the standard pour plate method (Wohlsen *et al*., 2005). Plates were incubated at 30°C for 3 days and colonies differing in morphological characteristics were selected. After the growth of different microorganisms on the plate, each bacterial colony on the basis of its morphological characteristics was picked up and further purified by repeated streaking on nutrient agar (NA) plates. Each bacterial culture was then inoculated in nutrient broth, incubated and glycerol stocks were made and frozen at -70°C for long term storage. For isolation and purification, strains were routinely grown in LB medium at 30°C (Maier *et al*., 2009).

2.2 Culture Media

All media were prepared in the laboratory depending on the manufacturers' instruction.

2.2.1 Blood Agar

Blood agar (Merck KGaA, Darmstadt, Germany) was prepared by suspending 40 g of blood agar base powder in 1 L of distilled water. Then, the solution was mixed and sterilized by autoclaving for 15 min at 121°C. After that the solution was cooled to 45-50°C and 5-8% sterile defibrinated blood was added. Finally, the solution was mixed well before pouring to plates. The poured blood plates were stored in the refrigerator at 4°C.

2.2.2 Luria Bertani Agar

LB agar was prepared by weighting 10 g tryptone, 5 g yeast extract, 10 g sodium NaCl, 15 g agar and 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).

2.2.3 Mueller-Hinton Agar

Mueller-Hinton (MH) agar was used for antibiotic susceptibility testing. MH agar (Oxoid, Basingstoke, U.K.) was prepared by suspending 38 g in 1 L of distilled water. The solution was boiled for 1 min to dissolve the medium completely. The pH of the solution was adjusted to 7.3± 0.1. The solution was sterilized by autoclaving at 121°C for 15 min and kept in a refrigerator at 4°C.

2.2.4 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. After that, the

solution was mixed well and then poured in petri dishes. Finally, the media was stored in a refrigerator at 4°C.

2.2.5 Nutrient Broth

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

2.3 Selection of Methicillin-Resistant Surface Water Isolates

Two procedures namely, disk diffusion (DD) method and minimum inhibitory concentrations (MIC) were used for determining the antimicrobial susceptibility of bacteria (CLSI, 2006).

2.3.1 Disk Diffusion (DD)

For the selection of methicillin-resistant surface water isolates, antibiotic susceptibility testing was done by Kirby Bauer DD method as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). All the isolates were subjected to oxacillin (OXA) DD test using a 1 μg disc and cefoxitin (FOX) DD test by using 30 μg disc on MH agar (Oxoid, Basingstoke, U.K.) with 4% NaCl. The plates were incubated at 30°C for 24 h and zones of inhibition were measured. An inhibition zone of ≥13 mm was considered as susceptible and ≤ 10 mm as resistant for OXA. An inhibition zone of ≥ 22 mm was considered as susceptible and ≤ 21 mm as resistant for FOX (Brown *et al*., 2005; Rao Venkatakrishna *et al*., 2011).

2.3.2 Minimum Inhibitory Concentration (MIC)

MIC testing was carried out for both antibiotics FOX and OXA by agar dilution method. The MIC values of ≤ 2 μg mL^{-1} were assumed as susceptible and

the MIC values of ≥ 6 μg mL^{-1} were assumed as resistant for both antibiotics (Brown *et al*., 2005).

2.4 Gram Staining

Cell morphology, shape and arrangement were observed after performing gram staining of overnight grown bacterial culture. Reagents of gram staining were crystal violet as a primary stain, gram's iodine as a mordant, ethyl alcohol or acetone as a decolorizer and safranin as a secondary stain (Gerhardt *et al*., 1994). Crystal violet solution was prepared by suspending 20 g of crystal violet (90%) in 200 mL of 95% ethanol. After the solution was mixed well, 8 g of ammonium oxalate and 800 mL of distilled water were added. The mixed solution was filtered before using. Iodine solution was prepared by suspending 3.3 g of iodine and 6.6 of potassium iodide in 1 L of distilled water. Decolorizer solution was prepared by mixing 500 mL of acetone and 500 mL of 95% ethanol. Safranin solution was prepared by mixing 2.5 g of safranin O in 100 mL of 95% ethanol and 900 mL of distilled water. Safranin solution was filtered before using.

Gram staining protocol was performed according to Gerhardt (1994). A thin smear from bacterial suspension was prepared on a clean slide and dried by air flow. In order not to wash off during the staining, bacterial smear was heat fixed. After fixation, first, the smear stained with crystal violet solution for 1-2 min and rinsed gently with tap water. Then, the smear was flooded with iodine solution for 60 sec. After rinsing with tap water, it was decolorized with alcohol-acetone solution until violet color was disappeared. Following rinsing with tap water, the smear was counter-stained with safranin solution for 30 to 60 sec. After that it was rinsed with distilled water again, air dried and examined under the microscope with an oil immersion lens.

2.5 Genomic DNA Extraction

Genomic DNA extraction was performed 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 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 dissolved in 4.5 mL double distilled water, inhibitor removal buffer in which added 20 mL of absolute ethanol to 3 mL volume (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6), wash buffer in which added 80 mL of absolute ethanol to 20 mL volume (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. Additional equipment and reagents were absolute ethanol, absolute isopropanol, standard tabletop microcentrifuge, sterilized microcentrifuge tubes, lysozyme (10 mg mL^{-1} in 10 mM Tris-HCl, pH 8.0) and phosphate buffered saline (PBS). 1X PBS was prepared by dissolving 8 g of sodium chloride (137 mM NaCl), 0.2 g of potassium chloride (2.7 mM KCl), 1.44 g of sodium hydrogen phosphate (10 mM Na_2HPO_4) and 0.24 g of potassium dihydrogen phosphate (1.8 mM KH_2PO_4) in 800 mL of distilled H_2O. The pH was adjusted to 7.4 with 1 N hydrochloric acid. The total volume was completed to 1 L with additional distilled H_2O. Solution was sterilized by autoclaving at 121°C for 20 min (CSH Protocols, 2006).

The extraction of DNA was carried out according to High Pure PCR Template Preparation Kit (Roche, Germany) protocol. Isolated bacteria were grown in 50 mL of NB and incubated while 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 min at 3,000× g. Supernatants were discarded and cell pellet resuspended in 200 µL of PBS. 5 µL of lysozyme were added to gram-positive bacteria and incubated at 37°C for 15 min. After that 200 µL of binding buffer and 40 µL of reconstituted proteinase K were added to the sample material and mixed immediately and incubated at 70°C for 10 min. Then 100 µL of 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 and centrifuged for 1 min at 8,000× 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 min at 8,000× g. The flow through liquid and the collection tube were discarded and 500 µL of wash buffer added to the filter tubes and centrifuged for 1 min at 8,000× g and discarded the flow through. After repeating the previous step, the tubes were centrifuged for 10 sec at 12,500× 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 and centrifuged for 1 min at 8,000× g to elute 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 min. The amount of DNA was measured by Qubit Flourometre (Invitrogen, USA) and stored at -20°C.

2.6 Agarose Gel Electrophoresis

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 H_2O and stirred and stored at room temperature (pH 8.3). 1X TBE buffer was also prepared by diluted 100 mL of 10X TBE buffer in 900 mL of distilled water (CSH Protocols, 2006). The DNA preparations were electrophoresed in 1% agarose gels with 1X TBE running buffer at 45 mA and 90 V for 1.30 h at room temperature in Mini-Sub Cell GT (Bio-Rad, USA) apparatus. The gels were stained with GelRed (Olerup SSP, Sweden). Lambda DNA/*Eco*RI+*Hind*III ladder (Thermo Scientific, USA) was used as DNA size marker. Monitoring of DNA bands were done under UV light.

2.7 Screening for *mec*A Harboring Surface Water Isolates by Polymerase Chain Reaction (PCR)

FOX/OXA-resistant surface water isolates were further subjected to the detection of *mec*A gene by using polymerase chain reaction (PCR). Extraction of DNA from the FOX/OXA-resistant surface water isolates was performed by using High Pure PCR Template Preparation Kit (Roche, Germany) as described previously. The *mec*A gene was amplified by using *mec*A specific primers as shown in Table 2.1. Optimization was carried out by changing the amount of DNA template and $MgCl_2$ concetration at varying annealing temperatures. PCR was performed in 50 µL of a reaction mixture containing DNA (100 ng), 200 µM each of deoxynucleoside triphosphates (dNTP), 1.5 mM $MgCl_2$, 5X Taq buffer, 20 pmol each primer and 2.5 units of Taq polymerase (Fermantas, Germany). Amplifications were performed using a Thermal Cycler (Bio-Rad T-100, USA) with the following optimal conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 1 min, followed by final extension at 72°C for 4 min. The PCR products were electrophoresed on a 1% agarose gel and stained with GelRed (Olerup SSP, Sweden). Gene Ruler 100 bp DNA ladder (Fermentas, Germany) was used as DNA size marker. The reference marker possessed 10 bands with the following sizes; 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Table 2.1 Primers used for amplification of *mec*A gene

***mec*A Primers**	**Sequences (5′ to 3′)**	**Amplicon Size (bp)**	**Annealing Temperature (°C)**	**References**
*mec*A1F	AAAATCGATGGTAAAGGTTGG	533	53	Pereira *et al*., 2014
*mec*A2R	AGTTCTGCAGTACCGGATTTG	533	53	Pereira *et al*., 2014
*mec*AF	TGGCTATCGTGTCACAATCG	310	55	Schmidt *et al*., 2014
*mec*AR	CTGGAACTTGTTGAGCAGAG	310	55	Schmidt *et al*., 2014
*mec*A P4F	TCCAGATTACAACTTCACCAGG	162	54	Buntaran *et al*., 2013
*mec*A P7R	CCACTTCATATGTTGTAGG	162	54	Buntaran *et al*., 2013

2.8 Identification of *mec*A-harboring Surface Water Isolates by 16S rRNA Sequencing

Identification of *mec*A harboring surface water extracted were done by 16S rRNA sequencing. Genomic DNA was isolated from *mec*A-positive surface water isolates and analyzed as indicated previously. Bacterial 16S rDNA was amplified by using the universal bacterial 16S rRNA primers, 27F 5'-AGAGTT TGATCCTGGCTCAG-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 $MgCl_2$ and dNTPs at a concentration of 2 mM, as well as 1.5 U of Taq 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 55°C for 1 min, extension at 72°C for 1 min, and final extension at72°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 3730× 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 tools 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 choosing ClustalW tab in MEGA program. Phylogenetic trees were constructed by Neighbor-Joining method. The sequential distances between the strains according to their pairwise differences were also computed.

2.9 Sequencing of the *mec*A Amplicons

The *mec*A amplicons (162 bp) were also purified with the QIAquick PCR Purification Kit (QIAGEN, Toronto, Canada). Then each *mec*A amplicons were sequenced with the same primers for *mec*A with 3730×1 DNA synthesizer (Applied Biosystems, USA). Sequence alignment was carried out by using the NCBI basic local alignment search tools BLASTn program. A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic tree for *mec*A gene from different species was created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction).

2.10 Penicillin Binding Protein 2a (PBP2a) Extraction

Membrane fractions were prepared from the *mec*A-positive isolates following the method described previously (Kim *et al*., 2012). 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 min, washed with 25 mL of cold PBS (preparation described previously at section 2.5), with 1 mM ß-mercaptoethanol and Protease Inhibitor Cocktail Tablets (Invitrogen, UK), centrifuged at 8000× g for 10 min, resuspended in 2.5 mL of PBS, and kept cold on ice. Cells were broken by sonication, the lysate was cleared by centrifugation at 8000× g for10 min 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 BSA Standart Kit (Bio-Rad, USA).

2.11 Detection of PBP2a

2.11.1 SDS-PAGE Analysis of PBP2a

The PBP2a preparations were detected by SDS-PAGE according to Laemmli (1970). First, stock solutions were prepared as shown in Table 2.2. Then 20 µL of supernatant protein were 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 min. Prepared samples were stacked in a 4% acrylamide stacking gel and separated in a 12% acrylamide resolving gel (Tabale 2.3). 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). The reference marker possessed 8 proteins with the following sizes; 175, 80, 58, 46, 30, 23, 17 and 7 kDa, respectively. A standard curve was constructed for each acrylamide gel using the molecular weights of standard marker and the distances migrated by each in the gel. Then the distance that each of the proteins migrated within the same gel was measured and used to calculate the molecular weight of each protein in the isolates from the standard curve.

Table 2.2 Stock solution of SDS-PAGE

Stock Solution	Preparation
Acrylamide solution (30% acrylamide, 0.8% bisacrylamide, 100 mL)	30 g of acrylamide and 0.8 g of N'N'-bis-methylene-acrylamide were dissolved in 100 mL of double-distilled water. Solution was stored up to 3 month 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 ddH_2O 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, 100 mL)	6 g of Tris-HCl was suspended in 80 mL of ddH_2O 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 month 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 2.3 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.

2.11.2 Western Blot Analysis of PBP2a

2.11.2.1 Transferring of PBP2a from SDS-PAGE Gel to Polyvinylidene Difluoride Membrane

For western blot analysis of PBP2a, 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 tansfer 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 PBP2a 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 PBP2a from SDS-PAGE gel to PVDF membrane was performed at 1.3 mA and 25 V for 7 min.

2.11.2.2 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).

2.11.2.3 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 rabbit anti-PBP2a primary antibody (RayBiotech, USA) diluted 1:1000 in TBST. Then the membrane was washed with TBST and then incubated with the goat anti-rabbit immunoglobulin G (IgG), (H+L) horseradish peroxidase (HRP)-conjugated secondary antibody (Milipore, USA) diluted 1:10000 in TBST for 2 h at room temperature (Wu et *al*., 2001).

2.11.2.4 Treatment of PVDF Membrane with Horseradish Peroxidase (HRP) Conjugate Substrate

After three times of washing with TBST, the membrane was treated using horseradish peroxidase (HRP)-conjugate Substrate Kit solution (Bio-Rad, USA) according to the manufacturer's instructions. The membrane was immersed in the color developement 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 developement 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.

2.12 Monitoring of *mec*A-harboring Bacteria in Surface Water by Fluorescent *in situ* Hybridization (FISH) Method

2.12.1. Sample Preparation and Fixation

FISH method was applied to detect directly *mec*A-positive bacteria among whole bacterial population in the water samples. Microbial samples from culture medium and surface water were processed according to the protocol of Amman *et*

al., (1990). Methicillin-resistant *S. aureus* ATCC3359, methicillin-sensitive *S. aureus* ATCC25922 and *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 10,000 rpm for 2 min. 500 mL of water samples collected in between the years of 2011 and 2012 were also centrifuged at 10,000 rpm for 10 min. The supernatants were removed and the pellets were resuspended in 1 mL of 1X PBS (preparation described at 2-5). After centrifugation, the supernatants were discarded and samples were washed twice in this manner. 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 further FISH analyses.

2.12.2 Sample Dehydration and Permeabilization

10 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 min. Dry slides were dehydrated by dipping them into glass jaws containing 50, 80 and 100% ethanol 3 min 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 min. The slides were washed 3 times with distilled water, then once in absolute ethanol and air-dried (Nielsen *et al*., 2009).

2.12.3 Hybridization of Fixed Samples with Oligonucleotide Probes

The hybridization protocol for samples from culture medium and also from 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 described in Table 2.4. 50 mL of washing buffer were also prepared by replacing formamide by NaCl as indicated in Table 2.5.

Table 2.4 Composition of the hybridization buffer at various formamide concentration

5 M NaCl (µL)	1 M Tris-HCl (µL)	dH_2O (µL)	Formamide (%)	Formamide (µL)	10% SDS* (µL)
360	40	1600	0	0	2
360	40	1500	5	100	2
360	40	1400	10	200	2
360	40	1300	15	300	2
360	40	1200	20	400	2
360	40	1100	25	500	2
360	40	1000	30	600	2
360	40	900	35	700	2
360	40	800	40	800	2
360	40	700	45	900	2
360	40	600	50	1000	2
360	40	500	55	1100	2

*SDS, Sodium Dodecyl Sulfate

Table 2.5 Composition of the washing buffer corresponding to the formamide concentrations in hybridization buffer

Formamide (%)	5 M NaCl (µL)	1 M Tris-HCl (µL)	0.5 M EDTA* (µL)	10% SDS** (µL)
0	9000	1000	0	50
5	6300	1000	0	50
10	4500	1000	0	50
15	3180	1000	0	50
20	2150	1000	500	50
25	1490	1000	500	50
30	1020	1000	500	50
35	700	1000	500	50
40	460	1000	500	50
45	300	1000	500	50
50	180	1000	500	50
55	100	1000	500	50

*SDS, Sodium Dodecyl Sulfate; **EDTA, Ethylenediaminetetraacetic acid

The probes EUB338 (Bact338), EUB338 II (SBACT P 338) and EUB338 III (SBACT V 338) were used to identify the domain Bacteria (Amman *et al*., 1990; Daims *et al*., 1999). To detect non-specific binding of EUB338, probe NONEUB (NON338) (non-Bact338) was also used as a negative control (Wallner *et al*., 1993). *mec*A I, *mec*A II and *mec*A III probes were used to identify the bacteria harboring *mec*A in surface water (Table 2.6). All probes were 5′ end fluorescein isothiocyanate (FITC) labeled (Alpha DNA, Montreal, Canada). DAPI (4′, 6′-diamidino-2 phenylindole) staining was applied to record all living organisms in the samples. Reference strains of methicillin-resistant *S. aureus* ATCC3359 as a positive control and methicillin-sensitive *S. aureus* ATCC25922 as a negative control were used for the optimization of the assay by changing concentration of formamide/ NaCl. *E. coli* DH5a strain was also used as a negative control for the stringency check of each species specific probes during all FISH applications. Hybridization of probes was processed according to Amman (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.

2.13.4 Microscopic Evaluation

*mec*A harboring bacteria were observed by a Leica DM 5000B fluorescence microscope equipped with A and I3 two filter sets: A filter was used for total microorganisms in water samples stained by DAPI and I3 for *mec*A-positive bacteria hybridized with FITC-labelled *mec*A III probe. Capturing of triplicate images were done by using CCD camera. The captured digital images were saved in Leica QWin Plus software and processed with Microsoft Photoshop to remove the blur areas.

Each image was separately counted in terms of the pixel areas of green region conferred by FITC-labelled *mec*A III probe and blue region conferred by DAPI. The population of *mec*A harboring bacteria were determined depending on the quantification of pixel areas of images by using the blow equation (Baker and Irvin 2007);

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

Biomass (%) of *mec*A harboring bacteria were calculated using average values of taken images for pixel areas of FITC probe and DAPI. For removing autofluorescence and background interference, the pixel areas were subtracted by the areas of non-binding probe (NON338) just before the calculation. The oligonucleotide *mec*A III probe images were assumed as the total amount of *mec*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 *mec*A probe with positive and negative pure culture controls, the river water samples were screened for the *mec*A harboring isolates by using FISH.

2.13.5 Statistical Analyses

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; Yilmaz and Icgen, 2014).

Table 2.6 *mec*A-specific and 16S rRNA-targeted oligonucleotide probes used in the study

Prob	Specificity	Sequences (5′ To 3′)	Length (bases)	GC (%)	Tm* (°C)	References
*mec*A I	*mec*A gene	GTGGAATTGGCCAATACAGGAACAGCATA	29	76.6	84	Uhl *et al*., 2006
*mec*A II	*mec*A gene	AGATCTTATGCAAACTTAATTGGCAAATCC	27	72.7	80	Uhl *et al*., 2006
*mec*A III	*mec*A gene	GAGATAGGCATCGTTCCAAAGAATGTA	30	73.2	76	Uhl *et al*., 2006
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	18	66.7	55	Amman *et al*., 1990
EUB338 II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	18	66.7	55	Daims *et al*., 1999
EUB338 III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	18	66.7	55	Daims *et al*., 1999
NONEUB	Negative control	ACTCCTACGGGAGGCAGC	18	66.7	55	Wallner *et al*., 1993

*Tm, melting temperatu

CHAPTER 3

RESULT AND DISCUSSION

3.1 Selection of Methicillin-Resistant Surface Water Isolates

A total of 290 surface water isolates collected were tested for their methicillin resistance. Methicillin resistance was detected by FOX/OXA DD and MIC tests. Methicillin-resistant *S. aureus* ATCC33591 and methicillin-sensitive *S. aureus* ATCC25922 isolates were used as positive and negative controls, respectively. Out of 290, 12 surface water isolates displayed resistance to both antibiotics FOX and OXA antibiotics. These isolates were considered as methicillin-resistant and designated as Al11, Ba01, Co11, Li12, SDS4, 6, 7, 8, 10, 10-2, 10-3 and 11 (Table 3.1). The DD zone values of isolates varied in between 6 and 16 mm for FOX while it differed from 6 to 10 mm for OXA. The MIC of methicillin-resistant isolates was also determined. The MIC of isolates varied in between 128 and ≥512 μg mL^{-1} for FOX while it varied in between 32 and ≥512 μg mL^{-1} for OXA (Table 3.1). FOX/OXA resistance implies resistance to all penicillins, cephems, imipenem, β-lactam/β-lactamase inhibitor combinations such as ampicillin/sulbactam, amoxacillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid (Moreillon *et al*., 2005; Bbosa *et al*., 2014). Several studies have also identified antibiotic-resistant bacteria in surface waters (Ash *et al*., 2002; Pruden *et al*., 2006; Balcazar, 2014). However, the antibiotic resistance in surface water isolates to newer antibiotics like FOX and OXA that are currently being used to treat humans has not been well-documented. Out of 12, 4 surface water isolates, namely, Al11, Ba01,

Co11 and Li12 were found to be gram-positive, cocci, and the rest of the isolates, namely, SDS4, 6, 7, 8, 10, 10-2, 10-3 and 11 were found to be gram-negative, bacilli, as a result of gram staining.

Table 3.1 Staphylococcal and non-staphylococcal methicillin-resistant surface water isolates used in the study

Strain Designations	Sampling Coordinates	Cefoxitin DD Test[a] (mm)	Oxacillin DD Test[b] (mm)	Cefoxitin MIC[c] (µg mL^{-1})	Oxacillin MIC[c] (µg mL^{-1})	*mec*A PCR Analysis	PB2a SDS-PAGE Analysis	PB2a Western Blot Analysis	16S rRNA Sequencing (Homology %)	EMBL Access No
Al11	39º50'28.41''N, 33º28'02.13''E, 686 m	6	8	128	32	+	+	+	*Staphylococcus aureus* (99%)	KJ395360
Ba01	39º22'16.39''N, 33º 26'49.26''E, 890 m	6	6	128	32	+	+	+	*Staphylococcus aureus* (99%)	KJ395371
Co11	39º50'28.41''N, 33º28'02.13''E, 686 m	10	6	≥512	≥512	+	+	+	*Staphylococcus warneri* (99%)	KJ395373
Li12	39º57'22.98''N, 33º25'04.35''E, 679 m	6	10	128	32	+	+	+	*Staphylococcus aureus* (99%)	KJ395370
SDS4	39º28'39.46''N, 33º24'26.73''E, 740 m	16	6	≥512	≥512	+	+	-	*Pseudomanas koreensis* (97%)	KJ937669
SDS6	39º34'34.39''N, 33º26'11.61''E,763 m	6	6	≥512	≥512	+	+	-	*Pseudomonas fluorescens* (99%)	KJ937670
SDS7	39º37'02.34''N, 33º26'38.26''E,773 m	6	6	≥512	≥512	+	+	-	*Aeromonas veronii* (97%)	KJ937671
SDS8	39º39'53.04''N, 33º28'55.46''E, 852 m	6	6	≥512	≥512	+	+	-	*Pseudomanas baetica* (99%)	KJ937672
SDS10	39º48'38.97''N, 33º29'14.57''E, 684 m	6	6	≥512	≥512	+	+	-	*Pseudomanas migulae* (96%)	KJ937673
SDS10-2	39º48'38.97''N, 33º29'14.57''E, 684 m	6	6	≥512	≥512	+	+	-	*Pseudomanas resinovorans* (93%)	KJ937675
SDS10-3	39º48'38.97''N, 33º29'14.57''E, 684 m	6	6	≥512	≥512	+	+	-	*Pseudomanas corrugata* (93%)	KJ937676
SDS11	39º50'28.41''N, 33º28'02.13''E, 686 m	6	6	≥512	≥512	+	+	-	*Pseudomanas kilonensis* (99%)	KJ937677

DD, disc diffusion; MIC, minimal inhibitory concentration; +, positive result; -, negative result; PCR, Polymerase Chain Reaction; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; 16S rRNA, 16S ribosomal RNA; EMBL, European Molecular Biology Laboratory;

[a]mean values of triplicate measurements ≥ 22 mm was considered as susceptible and ≤ 21 mm resistant for cefoxitin (CLSI 2006);

[b]mean values of triplicate measurements ≥ 13 mm was considered as susceptible and ≤10 mm as resistant for oxacillin (CLSI 2006);

[c]mean values of triplicate measurements interpreted as susceptible if the MIC was ≤ 2 µg mL^{-1}and resistant if the MIC was ≥ 6 µg mL^{-1} (Brown *et al*., 2005).

.

3.2 Detection of *mec*A Gene among Methicillin-Resistant Isolates

Although the DD and MIC tests known as conventional susceptibility tests are commonly used in routine microbiological laboratories, the results of these tests can be sometimes unreliable (Ryffel *et al*., 1994). The antibiotic resistance patterns were influenced by the environmental factors (such as the temperature, pH and concentration of NaCl in the medium) and antibiotic pressure. Therefore FOX/OXA-resistant isolates found in our study were further characterized by screening the *mec*A gene through PCR. Several PCR methods have been developed to detect the *mec*A gene (Murakami *et al*., 1991; Tokue *et al*., 1992; Velasco *et al*., 2014); however, in our study three pairs of PCR primers were used to detect *mec*A gene among methicillin-resistant isolates. After electrophoresis of genomic DNA in 1% agarose gels, DNA bands were visualized under UV light (Figure 3.1.).

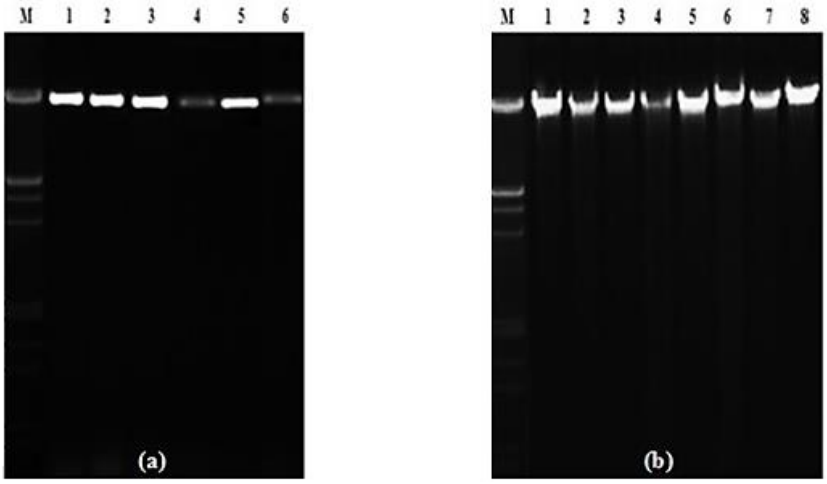

Figure 3.1 Agarose gel electrophoresis of genomic DNA extracted from surface water; *S. aureus* ATCC33591 (methicillin-resistant positive control) lane 1, *S. aureus* ATCC25922 (methicillin-sensitive negative control) lane 2, Al11 lane 3, Ba01 lane 4, Co11 lane 5, Li12 lane 6 (a) SDS4 lane 1, SDS6 lane 2, SDS7 lane 3, SD*S*8 lane 4, SDS10 lane 5, SDS10-2 lane 6, SDS10-3 lane 7, SDS11 lane 8 (b). M, Marker Lambda DNA/*Eco*RI+*Hind*III (125-21226 bp).

The *mec*A amplicons were only obtained with *mec*A P4F 5'-TCCAGATTACAACTTCACCAGG-3' and *mec*A P7R 5'-CCACTTCATATGTTGTAGG-3' primers. We did not obtain any amplicons with the *mec*A1F 5'-AAAATCGATGGTAAAGGTTGG-3', *mec*A2R 5'-AGTTCTGCAGTACCGGATTTG-3' and *mec*AF 5'-TGGCTATCGTGTCACAATCG-3', *mec*AR 5'-CTGGAACTTGTTGAGCAGAG-3' primers. In order to avoid nonspecific amplification products, PCR conditions were optimized by modifying reaction conditions including the annealing temperature and $MgCl_2$ concentration. Various annealing temperatures (50-60°C) were examined for staphylococcal and non-staphylococcal species. The gradient temperature in between 53-54°C appeared to be sufficient to get PCR product for both staphylococcal and non-staphylococcal species (Figure 3.2).

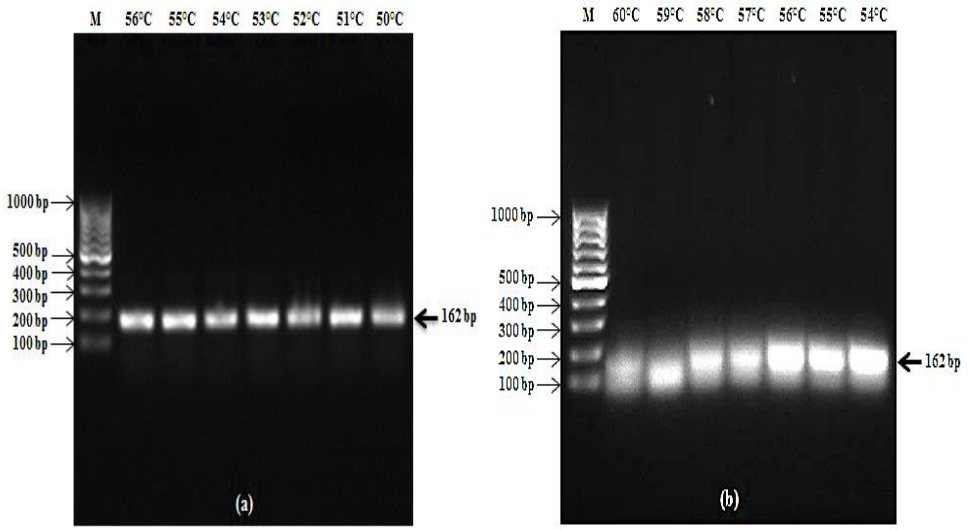

Figure 3.2 Optimization of PCR conditions at various annealing temperatures for; *S. aureus* ATCC33591 (a), SDS10-3 (b). M, Marker Gen Ruler (100-1000 bp).

The changing concentration of $MgCl_2$ between in 1-3 mM was also optimized for both staphylococcal and non-staphylococcal species. The optimal $MgCl_2$ concentration was found to be 1.5 mM for staphylococcal species and 3 mM for non-staphylococcal species as shown in Figure 3.3.

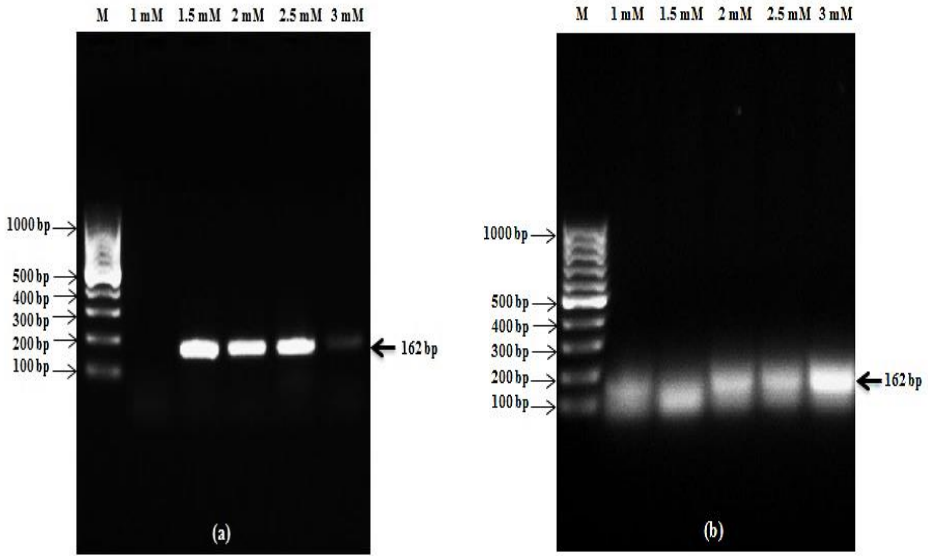

Figure 3.3 Optimization of PCR conditions at $MgCl_2$ concentrations of 1-3 mM for; *S. aureus* ATCC33591 (a) and SDS10-3 (b). M, Marker Gen Ruler (100-1000 bp).

Optimal PCR conditions were 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 $MgCl_2$ and dNTPs at a concentration of 2 mM, as well as 1.5 U of Taq polymerase and buffer used (Fermentas, Germany). Methicillin-resistant *S. aureus* isolates ATCC33591 and methicillin-sensitive *S. aureus* ATCC25922 were used as *mec*A-positive and *mec*A-negative controls, respectively. Presence of *mec*A gene was confirmed in all of the methicillin-resistant surface water isolates by the amplification products with the expected sizes of 162 bp (Figure 3.4). All of the

methicillin-resistant and *mec*A-positive isolates were coherently detected. The presence of the *mec*A gene is usually linked to the methicillin resistance (Gradelski *et al*., 2001; Velasco *et al*., 2014). Although methicillin resistance can be difficult to detect, because the *mec*A-positive strains can differ in their level of expression of resistance, our results were in good agreement with the conventional susceptibility tests used. Methicillin resistance, due to the *mec*A-encoded PBP2a, renders resistance to all the β-lactam antibiotics which are the most important group of antibiotics in the treatment of staphylococcal infections. The dissemination of *mec*A gene and hence the methicillin resistance pose a great difficulty in selecting antimicrobial agents for the management of these infections (Chambers, 2001).

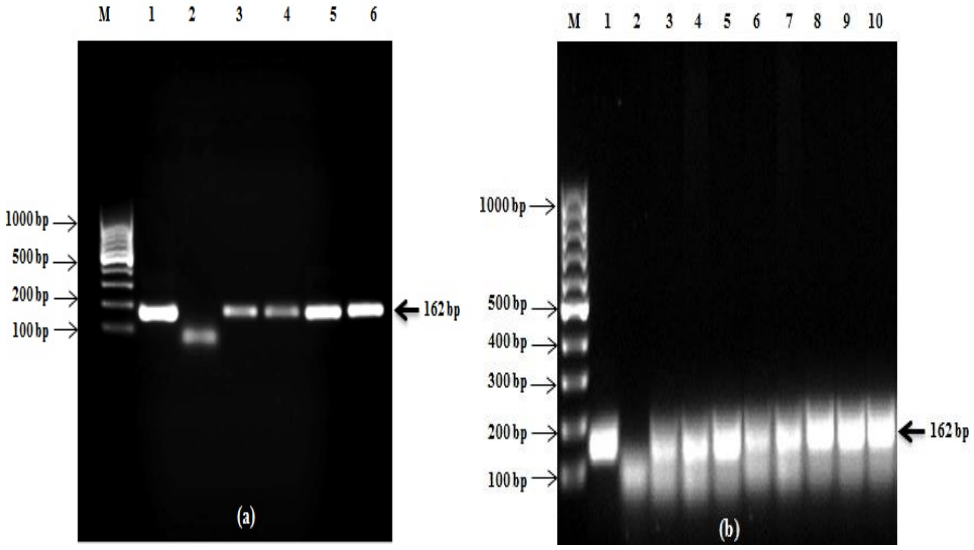

Figure 3.4 The *mec*A gene harboring surface water isolates; Staphylococcal isolates: *S. aureus* ATCC33591 (positive control) lane 1, *S. aureus* ATCC25922 (negative control) lane 2, Al11 lane 3, Ba01 lane 4, Co11 lane 5, and Li12 lane 6 (a). non-staphylococcal isolates: ATCC33591 (positive control) lane 1, *S. aureus* ATCC25922 (negative control) lane 2, SDS4 lane 3, SDS6 lane 4, SDS7 lane 5, SD*S*8 lane 6, SDS10 lane 7, SDS10-2 lane 8, SDS10-3 lane 9, SDS11 lane 10 (b). M, Marker Gen Ruler (100-1000 bp).

A standard curve was constructed for each gel using the molecular weights of standard markers and the distance they migrated in the gel. The standard curve was used to calculate the molecular weight of each *mec*A gene detected in the surface water isolates (Figure 3.5).

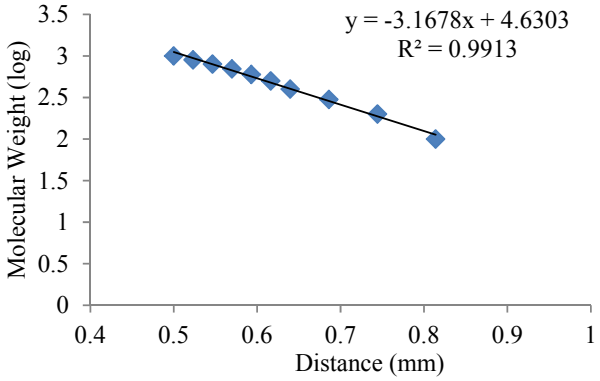

Figure 3.5 Representative standard curve for molecular weight estimation of amplified *mec*A gene.

3.3 Identification of *mec*A harboring Isolates by 16S rRNA Sequencing

Among 12 methicillin-resistant and *mec*A-positive surface water isolates only four of them identified as MRS and three were found out to be MRSA after 16S rRNA sequencing (Table 3.1). The only MRS isolate Co11 showed 99% homology with *S. warneri* and affiliated with an accession number of KJ395373. The MRSA isolates Al11, Ba01 and Li12 displayed 99% homologies with *S. aureus* and affiliated with accession numbers of KJ395360, KJ395371 and KJ395370, respectively. The rest of the isolates contained 7 *Pseudomonas* species designated as SDS4, 6, 8, 10, 10-2, 10-3, 11 (*P. koreensis*, *P.* fluorescens, P. *baetica*, *P. migulae*, *P. resinovorans*, *P. corrugata* and *P. kilonensis*, respectively) and one *Aeromonas veronii* species designated as SDS7 were shown with their respective percent homologies and EMBL access numbers in Table 3.1. Phylogenetic trees of 12

isolates were constructed as given in Figure 3-6,11. Earlier findings of El Kharroubi *et al*., (1991), Fitzgerald *et al*., (2001), Kassem, (2008), Baba *et al*., (2009), and Ito *et al*., (2012) were also confirmed by our study that the *mec*A gene was not only carried in MRS but also in non-staphylococcal isolates. Moreover, to our knowledge, this is the first study which has reported the presence of *mec*A gene in *Pseudomonas* and *Aeromonas* species. Water and soil are the primary sources of *Pseudomonas* species. Species of *Aeromonas*, ubiquitous and autochthonous inhabitants of aquatic environments, are also widespread in natural habitats such as soil, fresh and brackish water, sewage and wastewater (Araoju *et al*., 1991). One means by which these species may develop methicillin resistance in surface waters is through *mec*A gene transfer. Bacteria originally sensitive to methicillin may acquire *mec*A gene from *mec*A carrier organisms. As the antibiotic resistance genes are commonly carried on transferable genetic elements (Roy, 1999; Alibayov *et al*., 2014). Varying in their physical location such as chromosome, plasmid or transposons, antibiotic resistance genes can undergo horizontal gene transfer (Lindsay, 2014). Therefore, it is possible that *mec*A gene may be selectively maintained and transferred among the surface water isolates. The acquisition of *mec*A gene may aid in the spread and maintenance of methicillin-resistant bacteria in these waters. Improving the understanding of the distribution of methicillin-resistant bacteria as well as the *mec*A gene in bacteria isolated from surface waters will improve our ability to assess the effects of these waters may have on human health as suggested by Pertel *et al*., (2008).

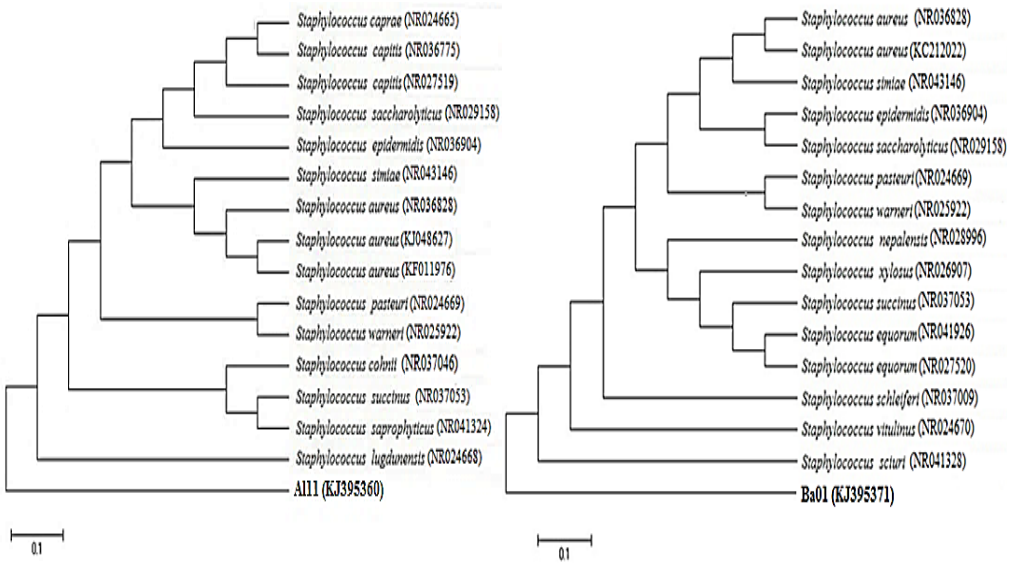

Figure 3.6 Phylogenetic trees of *S. aureus* (Al11-Ba01) isolates. The scale bar indicates substitutions per base pair.

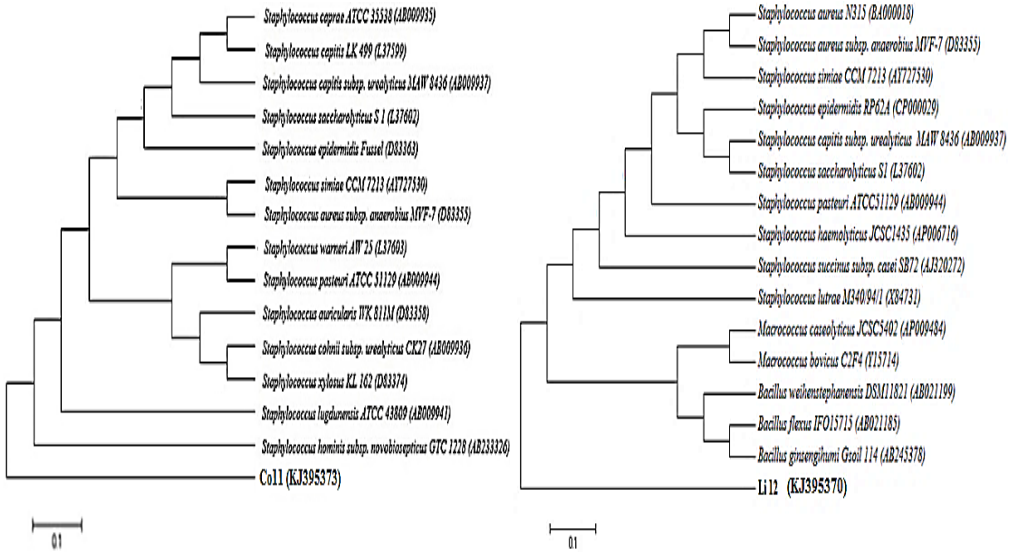

Figure 3.7 Phylogenetic trees of *S. warnerii* (Co11) and *S. aureus* (Li12) isolates. The scale bar indicates substitutions per base pair.

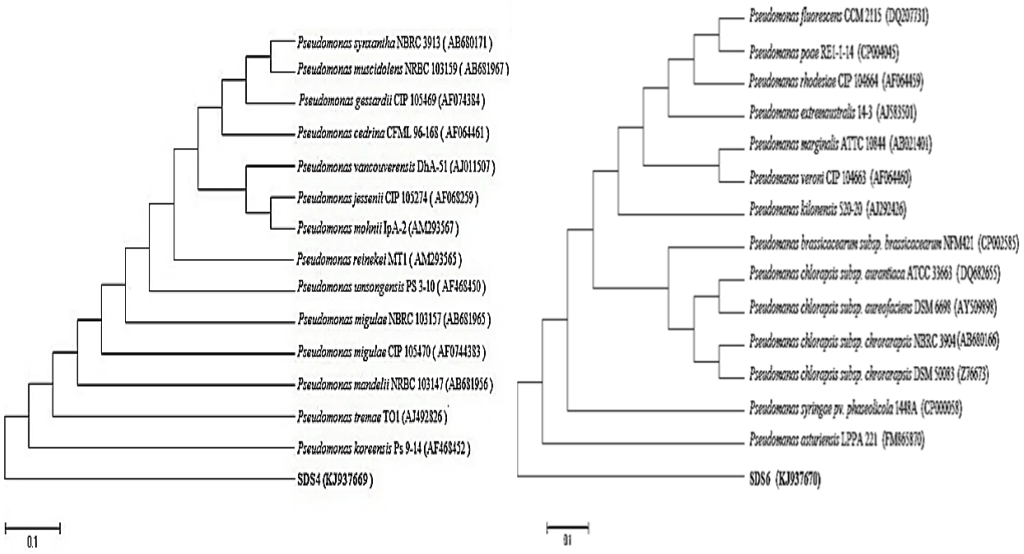

Figure 3.8 Phylogenetic trees of *P. koreensis* (SDS4) and *P. fluorescens* (SDS6) isolates. The scale bar indicates substitutions per base pair.

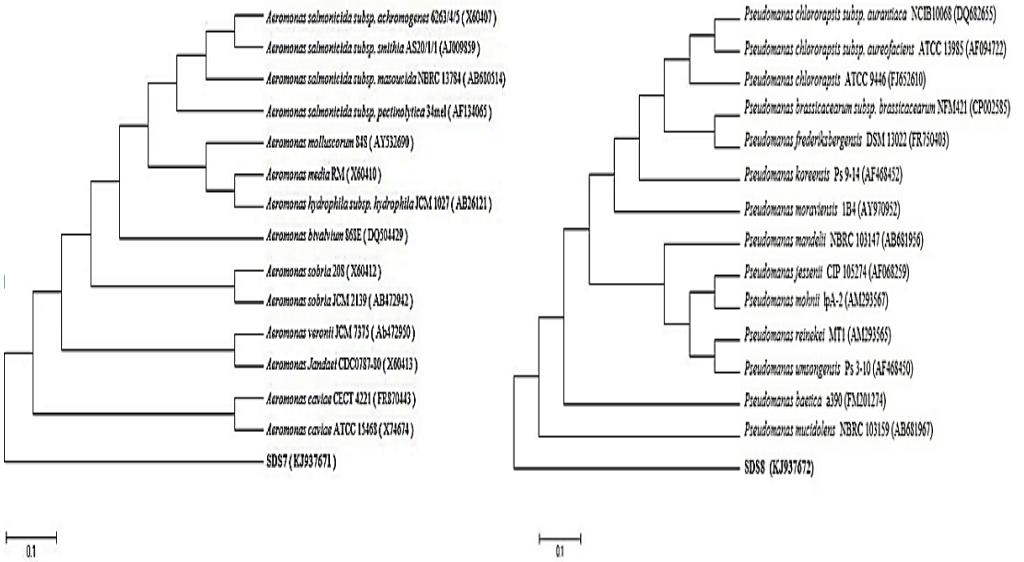

Figure 3.9 Phylogenetic trees of *A. veronii* (SDS7) and *P. baetica* (SDS8) isolates. The scale bar indicates substitutions per base pair.

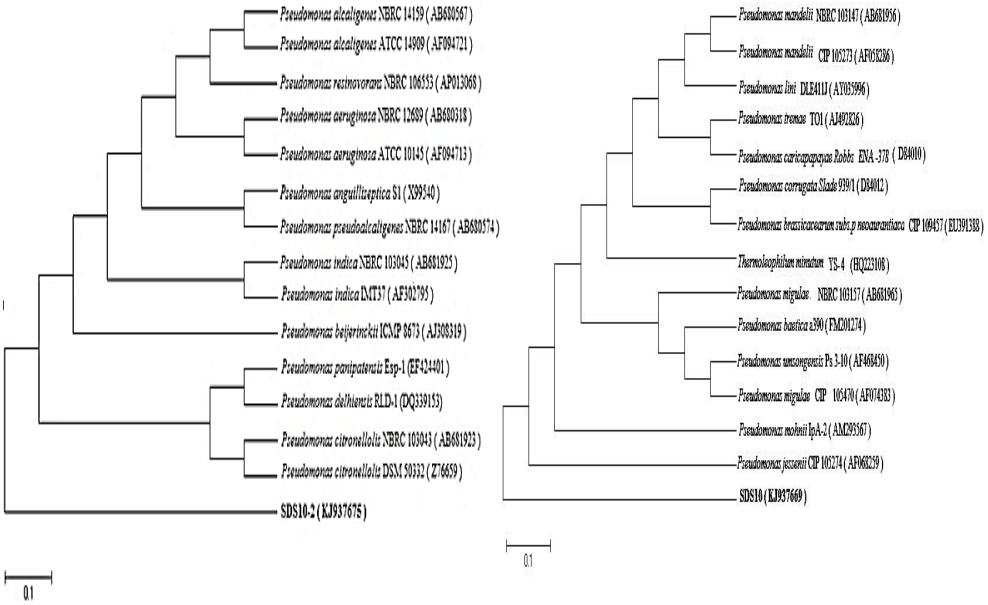

Figure 3.10 Phylogenetic trees of *P. migulae* (SDS10) and *P. resinovorans* (SDS10-2) isolates. The scale bar indicates substitutions per base pair.

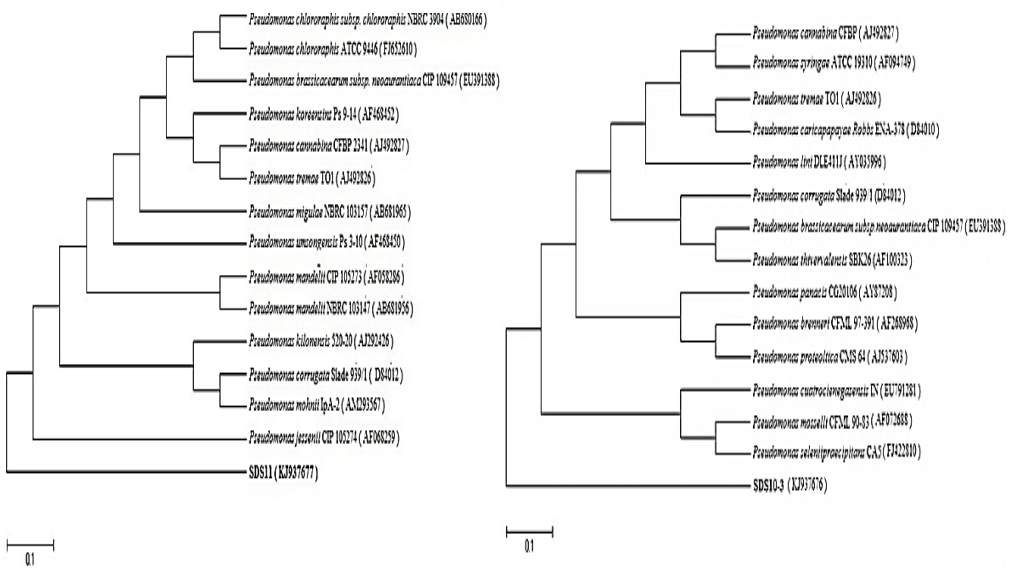

Figure 3.11 Phylogenetic trees of *P. corrugata* (SDS10-3) and *P. kilonensis* (SDS11) isolates. The scale bar indicates substitutions per base pair.

3.4. Sequencing of the *mec*A Amplicons

The homology of *mec*A genes from different bacteria was also confirmed by the sequencing of *mec*A genes obtained. The distance matrix generated highlighted the distances among the sequences aligned (Figure 3.12).

```
SDS8            -CATTCCCCCGCA---CCTGTTTGCCATCTT-TACCATCGATTTTA-TAACT---TGTTT 51
SDS10-3         -CCTTCCCCGGCA---CTGTTTTGTTATCGGGTACCATCCATTTTA-TAACT---TGTTT 52
SDS11           --CTTAGCCGACA---CTG-TTTGCTAACGGTTACCATCGATTTTA-TAACT---TGTTT 50
Ba01            --CCACGTCAGCAA--CTGTTTTGC-AACGGTTACCATCGATTTTA-TAACT---TGTTT 51
SDS10           ----CGGCCGGCGGA-CCTGTTTGCTCACGGTTACCATCGATTTTA-TAACT---TGTTT 51
SDS10-2         --CGTGACGG-CATC-TA-GTTTGCCACGG-TTACCATCGATTTTA-TAACT---TGTTT 50
Al11            --CGAGAGCG-CAACGTGTGTTTGCCACCT-TTACCATCGATTTTA-TAACT---TGTTT 52
SDS7            CTTGGGGT---CATC-TGG-TTTGCCACCT-TTACCATCGATTTTA-TAACT---TGTTT 50
SDS4            --CGGGGTAGACATC-TGGGTTTGCCATCGGTTACCATCGATTTTA-TAACT---TGTTT 53
SDS6            -CCCGAGGCGGAA---CTTGTTTGCC-CCGGTTACCATCGATTTTA-TAACT---TGTTT 51
Co11            --CTCGGGTGGA----------TATTAA-----ATGATTGGGTTAAATAACAAAACATTA 43
Li12            --CAAAGGTGAAA---------TGTGCA-----ATGATTGGGTTAA-TAACAAAACATTA 43
                                      *          *  **    ** * ****      **

SDS8            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 104
SDS10-3         TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 105
SDS11           TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 103
Ba01            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 104
SDS10           TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 104
SDS10-2         TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 103
Al11            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 105
SDS7            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 103
SDS4            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 106
SDS6            TATCGTCTAATGTTTTGTTATTTAACCCA--ATCATTGCTGTTAA-----TATTTTTTGA 104
Co11            GACGATAAAACAA---GTTATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGG 100
Li12            GACGATAAAACAA---GTTATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGG 100
                 *   *  **      *****  ** * *   * *  * **  **     **  * ***

SDS8            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 133
SDS10-3         GTTGAACCTGGTG----AAGTTGTAATCTGGAA 134
SDS11           GTTGAACCTGGTG----AAGTTGTAATCTGGAA 132
Ba01            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 133
SDS10           GTTGAACCTGGTG----AAGTTGTAATCTGGAA 133
SDS10-2         GTTGAACCTGGTG----AAGTTGTAATCTGGAA 132
Al11            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 134
SDS7            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 132
SDS4            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 135
SDS6            GTTGAACCTGGTG----AAGTTGTAATCTGGAA 133
Co11            GGTGGTTACAACGTTACAAGATATGAAGTGGA- 132
Li12            GGTGGTTACAACGTTACAAGATATGAAGTGGA- 132
                * **        *    *** * * *  ****
```

Figure 3.12 *mec*A multiple-sequence alignments of staphylococcal and non-staphylococcal isolates. CLUSTAL W2 was used to create multiple-sequence alignments. Asterisks denote identical residues.

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 for *mec*A gene from different species was created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction). The overlapping and non-overlapping sequences of several *mec*A genes from different species confirmed that the *mec*A gene was not only harbored and conserved among staphylococcal species but also non-staphylococcal ones (Figure 3.13).

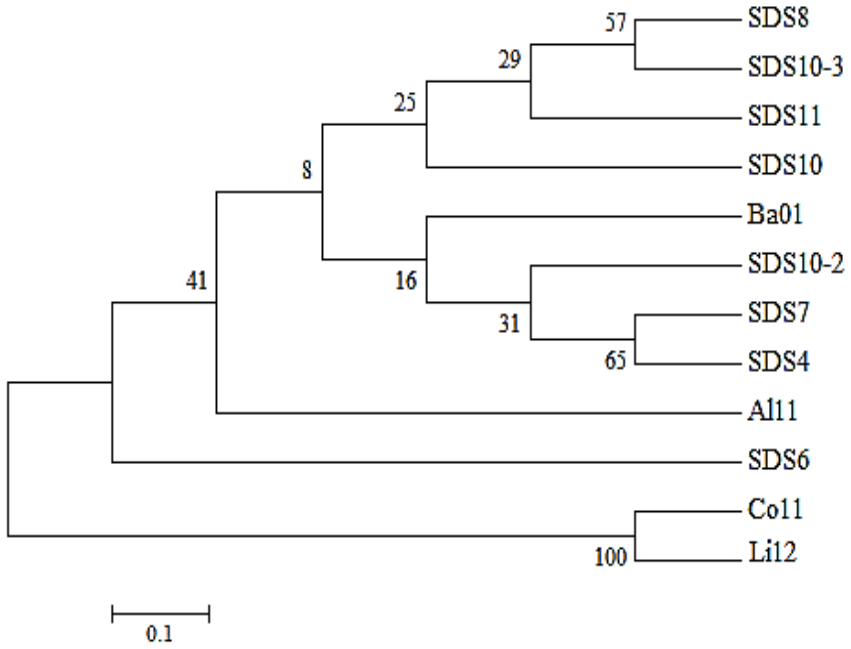

Figure 3.13 Phylogenetic tree of *mec*A gene from staphylococcal and non-staphylococcal isolates. The phylogenetic tree was constructed based on common partial sequences (162 bp) by using the NJ method with 1000 bootstrapped data sets. The scale bar indicates substitutions per base pair.

The phylogenetic tree of partial *mec*A sequences obtained from staphylococcal and non-staphylococcal isolates showed sequence similarity values of 8% –100% (Figure 3.13). The most closely related sequences being those of *S. warneri* Co11 and *S. aureus* Li12. The results indicated that the *mec*A gene sequence similarity among surface water isolates from staphylococcal and non-staphylococcal origins were related. These results confirmed the findings of Garcia-Alvarez *et al*., (2011) who also showed the transmission of the *mec*A gene among the isolates. The *mec*A genes present in the species identified sofar have high percentage of sequence identity indicating the phylogenetic relationship of these genes, too (Garcia-Alvarez *et al*., 2011; Ito *et al*., 2012; Kim *et al*., 2013).

3.5. Detection of PBPs

3.5.1. SDS-PAGE Analysis of PBP2a

PBPs are enzymes that assemble the peptidoglycan on the external face of the plasma membrane of bacteria (Sauvage *et al*., 2008; Ba *et al*., 2014). For the detection of PBPs, the proteins isolated from *mec*A harbouring isolates were analysed by SDS-PAGE. All of the *mec*A harboring isolates including 4 *Staphylococcus*, 7 *Pseudomonas*, and one *Aeromonas* species contained one major band approximately at the 75 kDa position, which was the expected position of PBPs (Figure 3.14). SDS-PAGE analysis results indicated that PBP2a encoded by *mec*A was not only carried by staphylococcal isolates but also non-staphylococcal ones. Different strains of several important species such as *Bacillus subtilis*, *S. aureus*, *Enterococcus faecium*, *Neisseria meningitides*, *Streptococcus pneumonia, Aquifex aeolicus*, and *Escherichia coli* are also shown to resist β-lactams by expressing PBPs by Popham and Setlow, (1995); Yuan *et al*., (2008); Zapun *et al*., (2008); Sung *et al*., (2009); Kim *et al*., (2012).

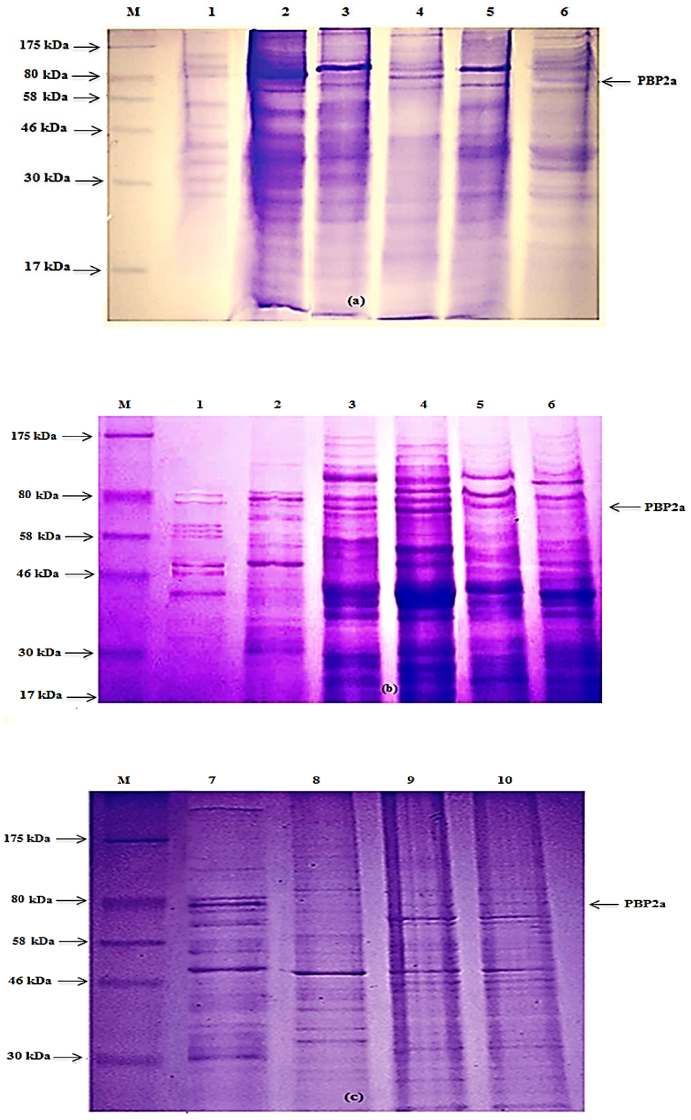

Figure 3.14 SDS-PAGE analysis of PBP2a for; staphylococcal isolates: *S. aureus* ATCC25922 (negative control) lane 1, *S. aureus* ATCC33591 (positive control) lane 2, *S. aureus* Al11 lane 3, *S. aureus* Ba01 lane 4, *S. warneri* Co11 lane 5, *S. aureus* Li12 lane 6 (a). non-staphylococcal isolates: *S. aureus* ATCC25922 (negative control) lane 1, *S. aureus* ATCC33591 (positive control) lane 2, *P. koreensis* SDS4 lane 3, *P. fluorescens* SDS6 lane 4, *A. veronii* SDS7 lane 5, *P. baetica* SDS8 lane 6, *P. migulae* SDS10 lane 7, *P. resinovorans* SDS10-2 lane 8, *P. corrugata* SDS10-3 lane 9, *P. kilonensis* SDS11 lane 10 (b,c). M, Colorplus Prestained Protein Marker (7-175 kDa).

A standard curve was constructed for each acrylamide gel using the molecular weights of standard marker and the distances migrated by each in the gel. Then the distance that each of the proteins migrated within the same gel was measured and used to calculate the molecular weight of each protein bands on the gel (Figure 3.15).

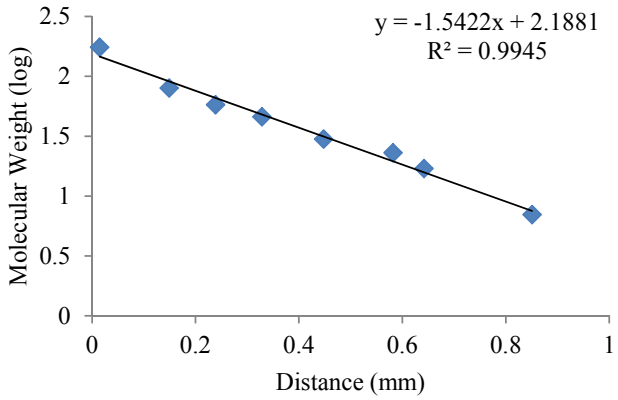

Figure 3.15 Representative standard curve for molecular weight estimation of PBP2a

3.5.2. Detection of PBP2a by Western Blot Analysis

Expression of PBPs by the *mec*A harboring isolates was further checked by Western Blotting. The presence of PBP2a were analysed by using the rabbit anti-PBP2a antibodies. Localization patterns of PBP2a were performed by detecting with anti-rabbit HRP-conjugated IgG antibodies. 75 kDa PBP2a bands were only detected in all of the *mec*A harboring staphylococcal isolates, and the protein appeared at the same position (Figure 3.16a). The localization specificity was consistent with previous study done by Kim *et al*., 2012. However, the PBPs extracted from non-staphylococcal isolates gave negative results with Wetsern Blotting (Figure 3.16b). *S. aureus* normally produces four PBPs enzymes that are anchored on the outer surface of the bacterial cell wall, where it can be easily recognized by antibodies (Roth *et al*., 2006). However, Stegger *et al.,* (2012) indicated that the degree of nucleotide

divergence between the homologous *mec* genes pose a potential public health problem, because they may give negative results when using current diagnostic tests to detect PBP2a. Our findings also confirm that, the PBP2a and the antibodies raised against the staphylococcal PBP2a might be used easily to develop tests for detection of methicillin-resistant and *mec*A harboring staphylococcal isolates but not the non-stapphylococcal counterparts with different degree of homologies in their *mec*A gene sequences.

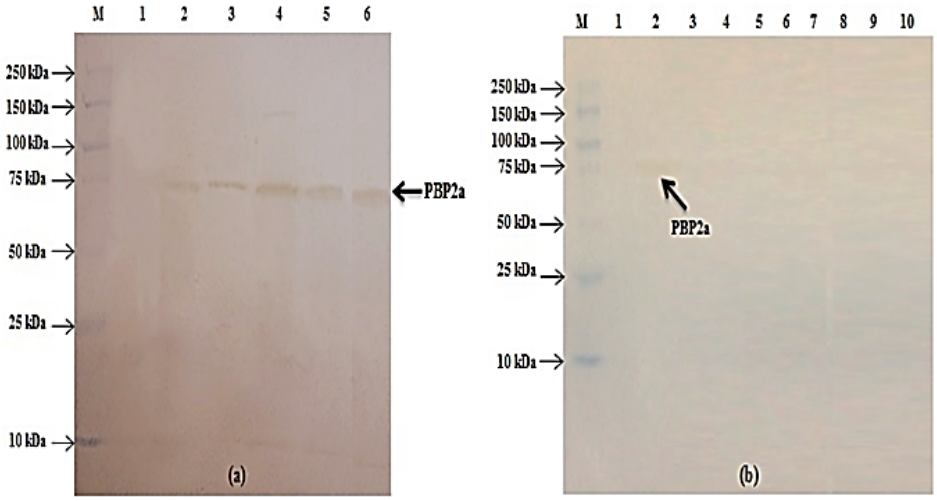

Figure 3.16 Western Blot Analysis of PBP2a for; staphylococcal isolates: *S. aureus* ATCC25922 (negative control) lane 1, *S. aureus* ATCC33591 (positive control) lane 2, *S. aureus* Al11 lane 3, *S. aureus* Ba01 lane 4, *S. warneri* Co11 lane 5, *S. aureus* Li12 lane 6 (a). non-staphylococcal isolates: *S. aureus* ATCC25922 (negative control) lane 1, *S. aureus* ATCC33591 (positive control) lane 2, *P. koreensis* SDS4 lane 3, *P. fluorescens* SDS6 lane 4, *A. veronii* SDS7 lane 5, *P. baetica* SDS8 lane 6, *P. migulae* SDS10 lane 7, *P. resinovorans* SDS10-2 lane 8, *P. corrugata* SDS10-3 lane 9, *P. kilonensis* SDS11 lane 10 (b). M, Precision Plus Protein WesternC Standards Marker (10-250 kDa).

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

3.6.1 Determining Hybridization Stringencies for each Probe Used

The DNA region from *S. aureus* strain NCTC10442 containing type-I SCC*mec* carrying the *mec*A gene (Genbank accession number AB033763) were identified previously (Uhl *et al*., 2006). From this region three different sequences depicted were used in the study to prepare *mec*A-targeted oligonucleotide probes shown previously in Table 2.6. The *mec*A probes prepared, namely *mec*A I, II and III, were first used in hybridization studies with *mec*A harboring pure cultures of *S. aureus* ATCC33591 to optimize striengency conditions at varying formamide and NaCl concentrations (Table 3.2). The hybridization results of *mec*A harboring pure cultures with *mec*A I, II, III probes with 45, 50 and 55% formamide and 0.040, 0.028 and 0.020 M NaCl concentrations at 46°C were shown in Figure 3.17, 3.18 and 3.19, respectively.

Table 3.2 Hybridization stringency conditions for three *mec*A probes used with *mec*A-positive and negative control pure cultures at 46°C

	Measured % of signal intensities at given hybridization conditions for each *mec*A probes used								
	45% Formamide + 0.040 M NaCl			**50% Formamide + 0.028 M NaCl**			**55% Formamide + 0.020 M NaCl**		
	***mec*A I**	***mec*A II**	***mec*A III**	***mec*A I**	***mec*A II**	***mec*A III**	***mec*A I**	***mec*A II**	***mec*A III**
***S. aureus* ATCC33591 *mec*A**	77.1±0.98	65.9±1.63	76.4±0.79	85.7±1.05	79.3±1.51	80.9±2.18	88.9±0.15	81.6±1.71	92.1±1.57
***S. aureus* ATCC25922 *mec*A**	1.86±0.94	2.52±1.01	2.96±1.52	2.84±0.85	3.66±1.09	3.81±0.57	2.14±0.35	2.66±0.63	3.98±0.67
***E. coli* DH5α *mec*A**	2.04±0.57	2.50±0.26	3.08±0.46	3.24±1.84	2.5±0.36	3.80±1.54	1.40±0.75	1.93±0.81	2.89±0.68

±, calculated standard errors;
*mec*A^{+}, *mec*A-positive control;
*mec*A^{-}, *mec*A-negative control.

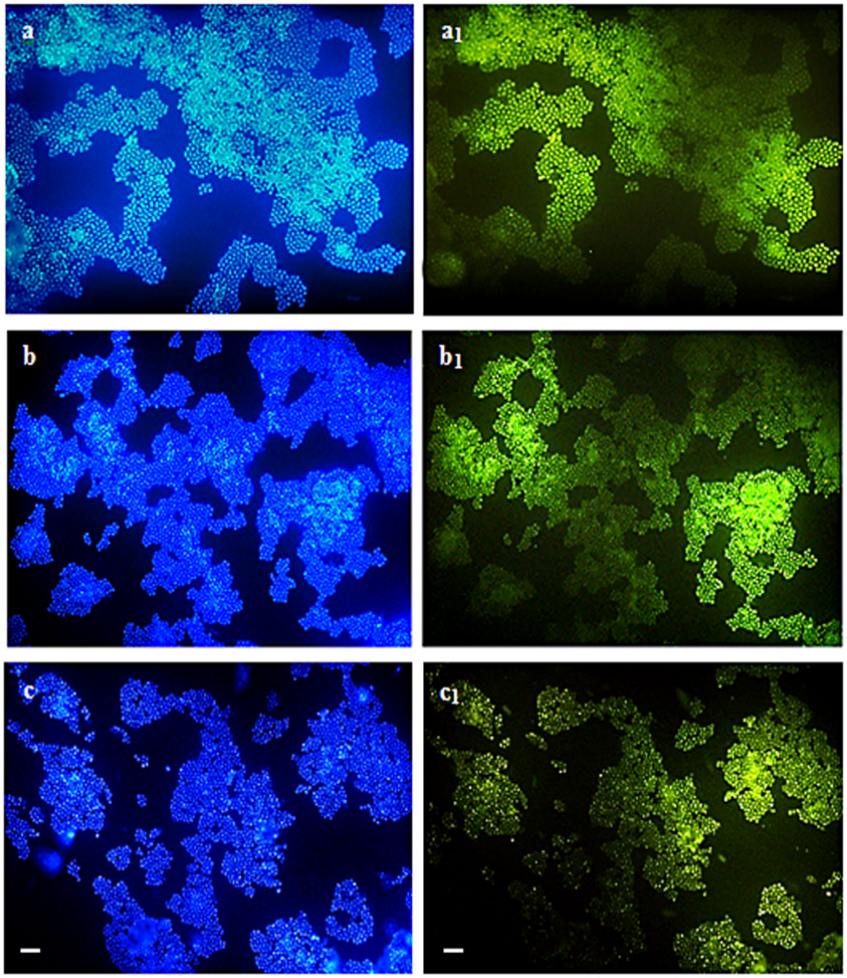

Figure 3.17 Hybridization stringency for *mec*A harboring pure culture of *S. aureus* ATCC33591 at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *mec*A I probe applied to pure cultures (a_1, b_1, c_1). Bar, 10μm and applies to all photomicrographs.

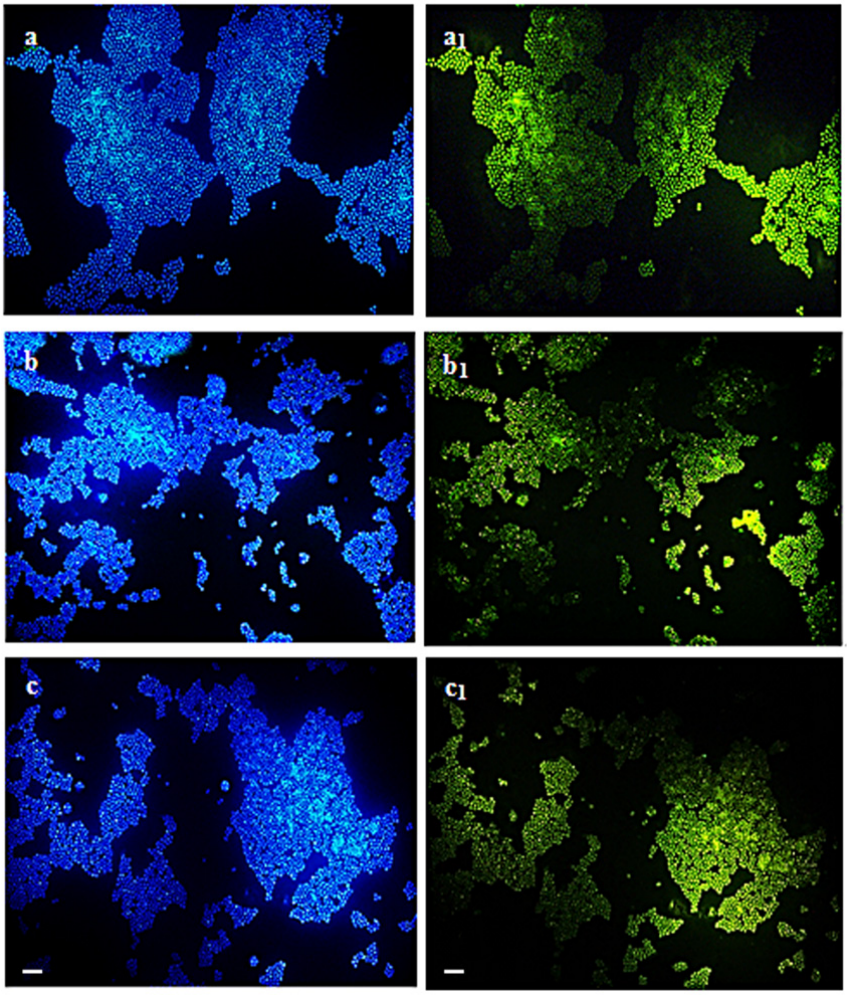

Figure 3.18 Hybridization stringency for *mec*A harboring pure cultures of *S. aureus* ATCC33591 at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *mec*A II probe applied to pure cultures (a_1, b_1, c_1). Bar, 10µm and applies to all photomicrographs.

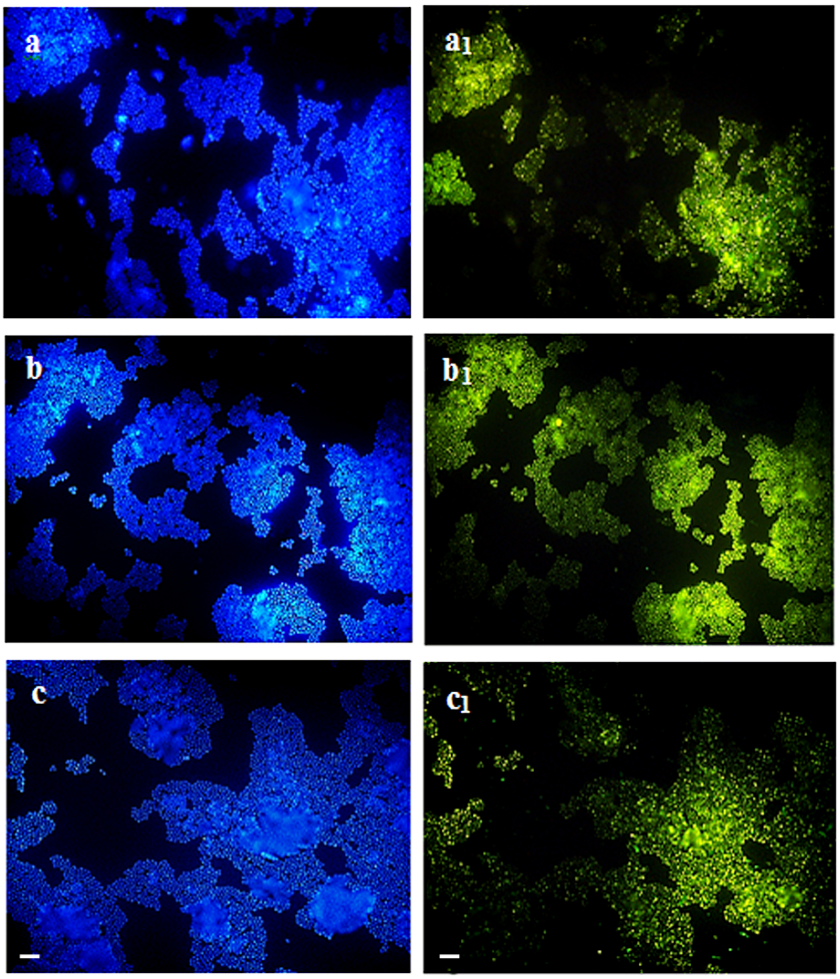

Figure 3.19 Hybridization stringency for *mec*A harboring pure cultures of *S. aureus* ATCC33591 at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *mec*A III probe applied to pure cultures (a_1, b_1, c_1). Bar, 10µm and applies to all photomicrographs.

As shown in Table 3.2, the percentage of hybridization for *mec*A harboring pure cultures of *S. aureus* ATCC33591 hybridizing with *mec*A I, II, III probes at 45, 50 and 55% formamide and 0.040, 0.028 and 0.020 M NaCl concentrations at 46°C was estimated by calculating the pixel areas (pp^2) of DAPI and FITC images of this strain with the Leica QWin Plus software. Under the hybridization conditions of 55% formamide and 0.020 M NaCl at 46°C, *mec*A III probe showed the highest efficiency with 92.1%±1.57 ($p<0.05$) for the detection of *mec*A harboring pure culture isolates. The specificity of the *mec*A III probe was also confirmed by applying to *mec*A-negative controls of *S. aureus* ATCC25922 (Figure 3.20) and *E. coli* DH5α (Figure 3.21) isolates with the low intensities of 3.98%±0.67 ($p<0.05$) and 2.89%±0.68 ($p<0.05$), respectively.

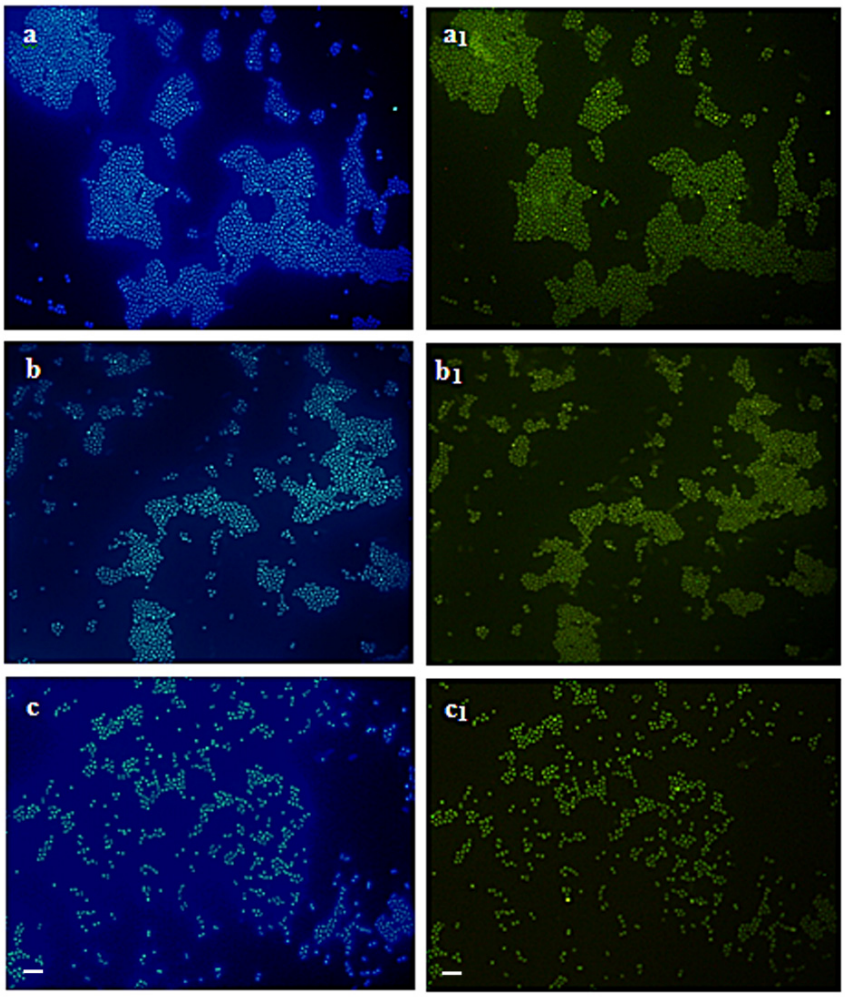

Figure 3.20 Hybridization results of *mec*A-negative pure cultures of *S. aureus* ATCC25922 at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *mec*A III probe applied to pure cultures (a_1, b_1, c_1). Bar, 10µm and applies to all photomicrographs.

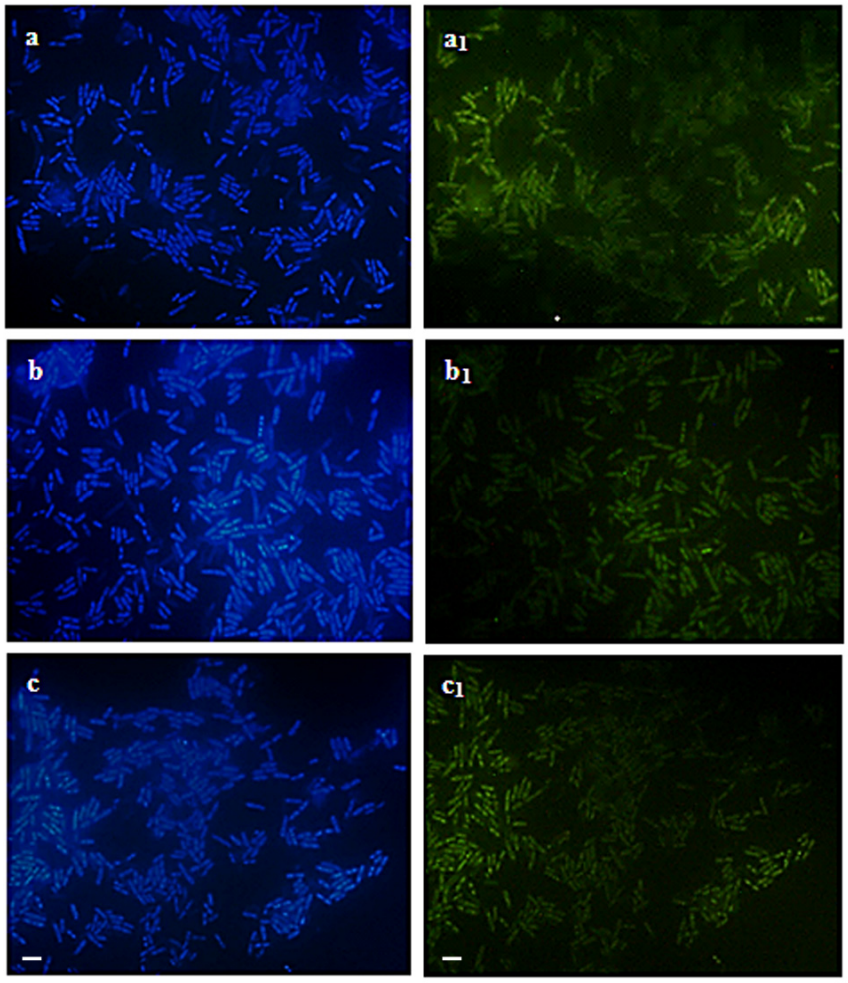

Figure 3.21 Hybridization results of *mec*A-negative pure cultures of *E. coli* DH5α at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *mec*A III probe applied to pure cultures (a_1, b_1, c_1). Bar, 10µm and applies to all photomicrographs.

3.6.2 Probing Water Samples at Pre-determined Hybridization Stringencies

After determining hybridization stringency conditions, the selected *mec*A III probe was also evaluated for their specificity with fixed water samples collected from the river. Water samples were also successfully hybridized by using *mec*A III to monitor *mec*A harboring various bacteria inhabiting river water as shown in Figure 3.22 and 3.23. Both the pure cultures and the water samples used in the study were also checked for auto-fluorescence and background interferences by using non-binding probe NON338. The amounts of auto-fluorescence and background interferences were found to be higher in water samples then the pure cultures used (Figure 3.22, 3.23). Low-stringency washing conditions (higher salt and lower temperature) increases sensitivity, however, these conditions can give nonspecific hybridization signals and high background. High-stringency washing conditions (lower salt and higher temperature, closer to the hybridization temperature) can reduce background and only the specific signal will remain. The hybridization signal and background can also be affected by probe length, purity, concentration, sequence and target contamination (Icgen *et al*., 2007).

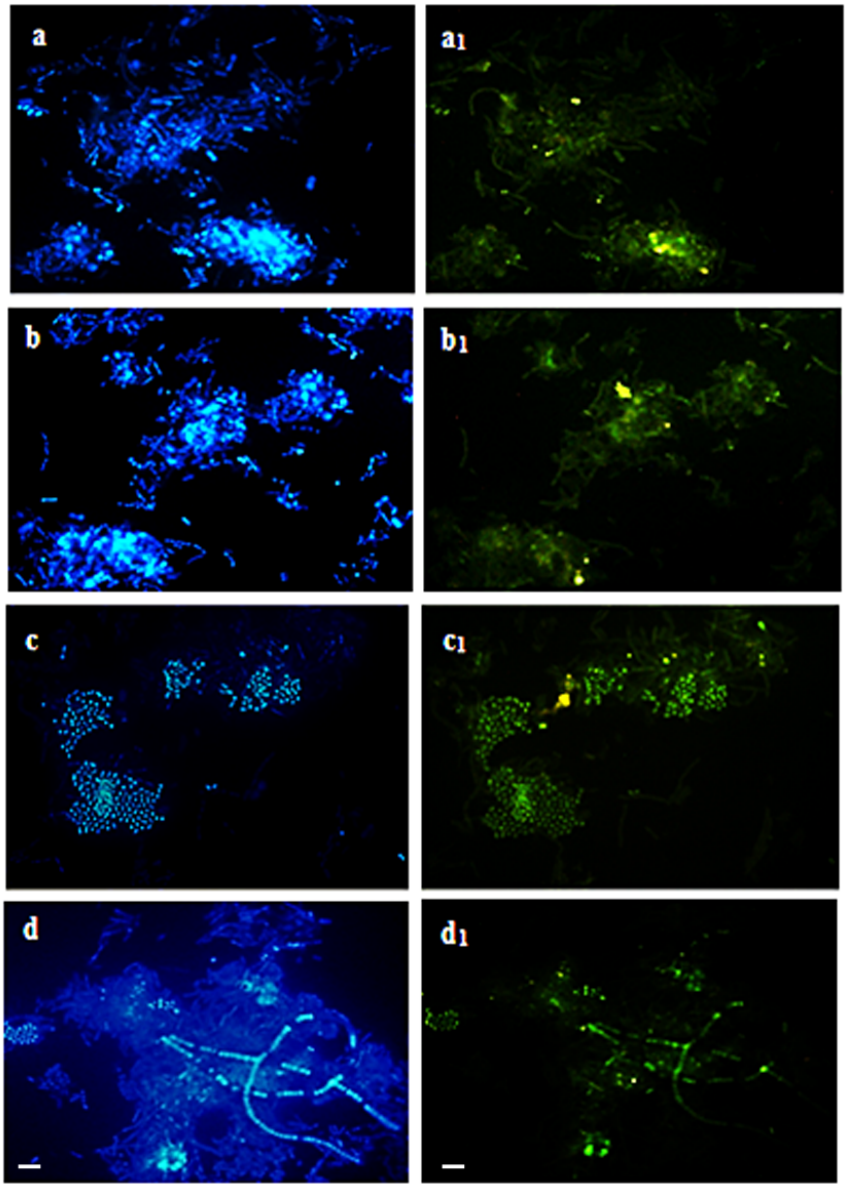

Figure 3.22 Hybridization results of water samples in January (a-a_1), April (b-b_1), July (c-c_1) and October (d-d_1) of the year 2011 at 55% formamide and 0.020 M NaCl concentrations. Total cell populations stained with DAPI (a, b, c, d) and their corresponding *mec*A III probe applied to mixed cultures (a_1, b_1, c_1, d_1). Bar, 10µm and applies to all photomicrographs.

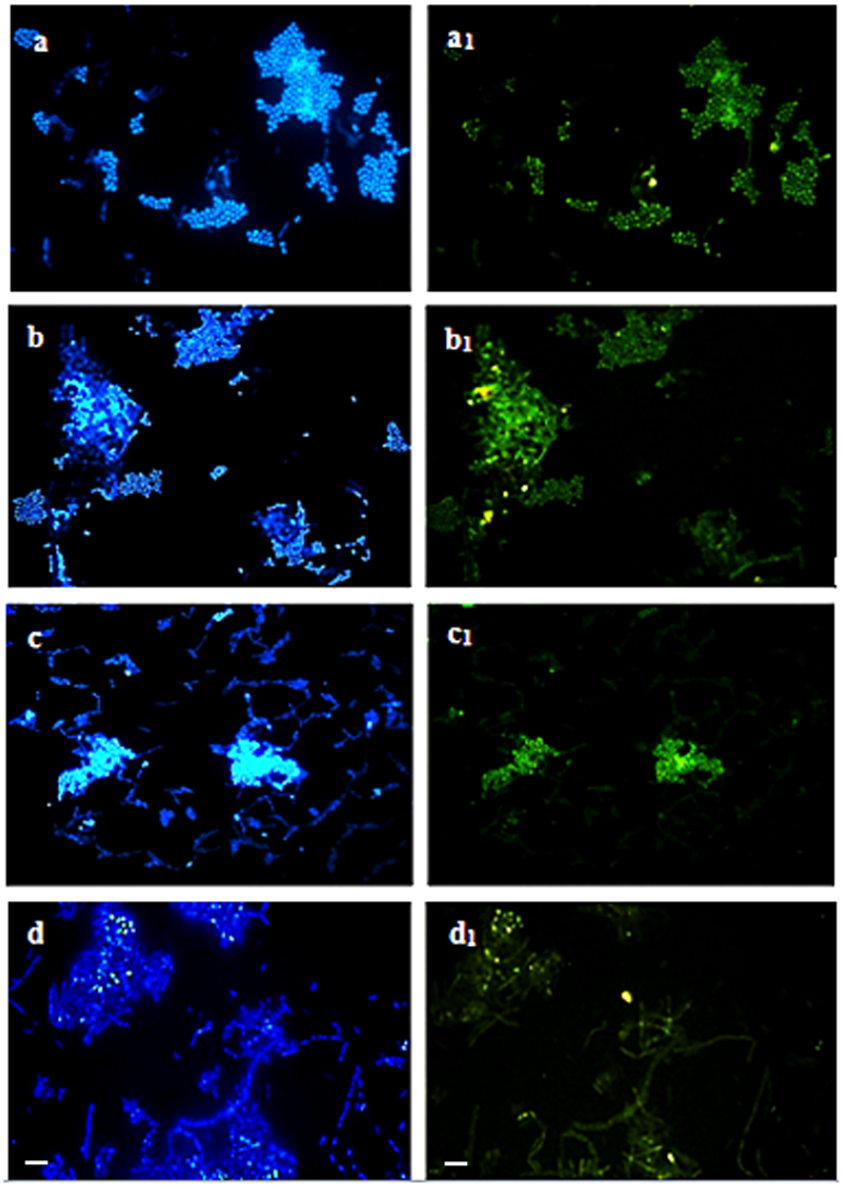

Figure 3.23 Hybridization results of water samples in January (a-a_1), April (b-b_1), July (c-c_1) and October (d-d_1) of the year 2012 at 55% formamide and 0.020 M NaCl concentrations. Total cell populations stained with DAPI (a, b, c, d) and their corresponding *mec*A III probe applied to mixed cultures (a_1, b_1, c_1, d_1). Bar, 10µm and applies to all photomicrographs.

3.6.3 Monitoring *mec*A Harboring Bacteria Inhabiting in River Water with *mec*A III Probe

The paraformaldehyde-fixed water samples collected from river water were also checked for monitoring of prevalence of *mec*A harboring bacteria over the years of 2011-2012. The population of *mec*A harboring surface water bacteria was determined by calculating the pixel areas (pp^2) of DAPI and FITC images. The percentage of DAPI stained cells depicted a seasonal patterns. The visualization of *mec*A harboring strains hybridizing with *mec*A III probe was shown in Figure 3.24. The percentage of population size of *mec*A harboring surface water bacteria 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 (Figure3.24).

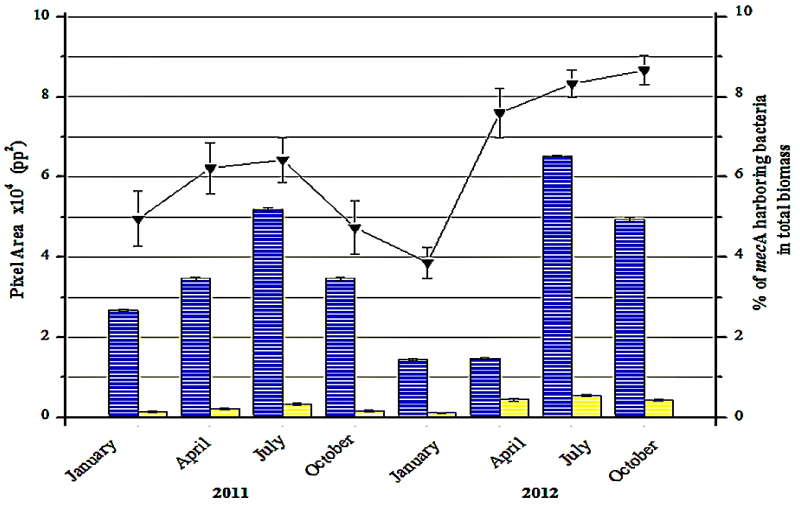

Figure 3.24 *In situ* distribution and abundance of *mec*A harboring bacteria population within the total biomass in water samples (▼). Pixel area of total biomass determined with DAPI-stained cells (▬) and total *mec*A harboring bacteria determined with FITC-labeled *mec*A III probe (▬). The error bars illustrate the calculated standard error between ± 0.02-0.70.

The result showed that the population of total bacteria ($2.67\times10^4\pm0.02\times10^4$ PP^2) and *mec*A harboring bacteria ($0.143\times10^4\pm0.02\times10^4$ PP^2) was the lowest in January of 2011. Also the population of total bacteria ($5.19\times10^4\pm0.05\times10^4$ PP^2) and *mec*A harboring bacteria ($0.33\times10^4\pm0.03\times10^4$ PP^2) was the highest in July of 2011. As indicated in the year of 2011, the population of bacteria had the similar pattern in the year of 2012; total bacteria ($1.43\times 10^4\pm0.02\times10^4$ PP^2) and *mec*A harboring bacteria ($0.01\times10^4\pm0.01\times10^4$ PP^2) had the lowest population size in January of 2012. Moreover, the population of total bacteria ($6.52\times10^4\pm0.02\times10^4$ PP^2) and *mec*A harboring bacteria ($0.54\times10^4\pm0.02\times10^4$ PP^2) was the highest in July of 2011. The results of *mec*A harboring surface water bacteria biomass (%) indicated that *mec*A harboring surface water bacteria had the population size with 4.90%±0.68 in January, 6.16%±0.63 in April, 6.36%±0.55 in July and 4.69%±0.66 in October for the year of 2011. Overall, for the year of 2011, there was significant difference in the seasonal distribution of *mec*A harboring surface water bacteria within total biomass ($p<0.05$). The *mec*A harboring surface water bacteria had the population size with 3.82%±0.38 in January, 7.52%±0.61 in April, 8.24%±0.34 in July and 8.58%±0.36 in October for the year of 2012. There was also significant differences ($p<0.05$) in the seasonal distribution of *mec*A harboring surface water bacteria in April, July and October within the year of 2012. As a result of the ANOVA test, it was determined that seasonal population shifts of *mec*A harboring surface water bacteria varied during the years tested ($p<0.001$). Shapiro-Wilk test showed that the population of *mec*A harboring surface water bacteria had a normal distribution ($p<0.05$) for the years tested. The findings confirmed that the *mec*A probe III could be successfully used to monitor prevalence *mec*A harboring bacteria inhabiting surface waters. Molecular tools allow to study bacterial diversity in the environment, the area which the research were previously limited by the classical microbiological methods disadvantages. For this reason they become more and more popular in microbiological and technological laboratories. FISH is a relatively new technology utilizing fluorescently labelled DNA probes to detect genes or groups of organisms

in enviromental samples. The advantage in using water samples it is the possibility to detect spatial distribution of microorganisms at the aquatic environment (Amann *et al*., 2001). When working with culture samples, it is possible to detect only cultivable species, the minority of microorganisms present in that environment. Moreover, FISH technique makes possible to visualize the morphology of the cells *in situ* and supply important information about identification of bacterial groups of cells without culturing (Santos *et al*., 2010; Hu *et al*., 2014). FISH technique has been used to detect and quantify the presence or absence of specific DNA/rRNA sequences. FISH fluorescent probes show a high degree of specificity to complementary sequences and therefore have been applied in numerous fields of research (Hu *et al*., 2014).

3.7 Conclusion

The prevalence of methicillin-resistant and *mec*A harboring bacteria appeared to be shed in surface waters and highly variable. Therefore, surface waters would need to be intensively monitored for not only methicillin-resistant staphylococcal species but also other potentially significant non-stapylococcal counterparts like *Pseudomonas* and *Aeromonas* species. The homologous patterns of *mec*A gene from different origins indicated that the *mec*A gene selectively maintained and transferred among the surface water isolates. The *mec*A-encoded non-staphylococcal PBPs could not be detected by using staphylococcal PBP2a-spesific antibodies which were found to be highly efficient in staphylococcal species. The methicillin-resistant and *mec*A harboring surface water isolates were believed to require much broader approach for detection and scientific consideration. Our results also indicated that the *mec*A-specific DNA probe might be a potential analytical tool in selecting and *in situ* monitoring of methicillin resistant isolates in surface waters.

REFERENCES

Abraham, E., Chain, C., Fletcher, A., *et al*. (1941). Further observations on penicillin. *Lancet, ii*, 177-188.

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research*, *36*(6), 697-705.

Alibayov, B., Baba-Moussa, L., Sina, H., Zdenkova, K., and Demnerova, K. (2014). *Staphylococcus aureus* mobile genetic elements. *Molecular Biology Reports, 41*(8), 5005-5018.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology*, *8*(4), 251-259.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990a). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, *56*, 1919-1925.

Amann, R. I., Devereux, R., Key, R., and Stahl, D. A. (1991). Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Applied and Environmental Microbiology*, *58*(2), 614-623.

Amann, R. I., Fuchs, B. M., and Behrens, S. (2001). The identification of microorganisms by fluorescence *in situ* hybridisation. *Current Opinion in Biotechnology*, *12*, 231–236.

Amann, R. I., Krumholz, L., and Stahl, D. A. (1990b). Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, *172*, 762-770.

Amann, R. I., Ludwig, W., and Scheleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1), 143-169.

Amann, R. I., Zarda, B., Stahl, D. A., and Scheleifer, K. H. (1992). Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology*, *58*(9), 3007-3011.

Andersson, D. I. (2003). Persistence of antibiotic resistant bacteria. *Current Opinion in Microbiology*, *6*(5), 452-456.

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*, *8*(4), 260-271.

Andersson, D. I., and Levin, B. R. (1999). The biological cost of antibiotic resistance. *Current Opinion in Microbiology*, *2*(5), 489-493.

Araoju, R. M., Arribas, R. M., and Pares, R. (1991). Distribution of *Aeromonas* species in waters with different level of pollution. *Journal of Applied Bacteriology*. *71*, 182-186.

Archer, G., and Climo, M. (1994). Antimicrobial susceptibility of coagulase-negative staphylococci. *Antimicrobial Agents and Chemotherapy, 38*(10), 2231-2237.

Arede, P., Milheirico, C., de Lencastre, H., Oliveira, D. C. (2012). The anti-repressor mecR2 promotes the proteolysis of the mecA repressor and enables optimal expression of ß-lactam resistance in MRSA. *PLOS Pathogens*, *8*(7), e1002816.

Ash, R. J., Mauck, B., and Morgan, M. (2002). Antibiotic resistance of gram-negative bacteria in rivers, United States. *Emerging Infectious Diseases*, *8*(7), 713-716.

Ba, X., Harrison, E. M., Edwards, G. F., Holden, M. T., Larsen, A. R., Petersen, A., Skov, R. L., Peacock, S. J., Parkhill, J., Paterson, G. K., and Holmes, M. A. (2014). Novel mutations in penicillin-binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the *mec* gene. *Journal of Antimicrobial Chemotherapy*, *69*(3), 594-597.

Baba, T., Kuwahara-Arai, K., Uchiyama, I., Takeuchi, F., Ito, T. and Hiramatsu, K. (2009). Complete genome sequence of *Macrococcus caseolyticus* strain JCSC5402, reflecting the ancestral genome of the humanpathogenic staphylococci. *Journal of Bacteriology*, *191*(4), 1180-1190.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K. I., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., and Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*. *359*, 1819–1827.

Baker, K., and Irvin, S. (2007). The variation of nitrifying bacterial population sizes in a sequencing batch reactor (SBR) treating low, mid, high concentrated synthetic wastewater. *Journal of Environmental Engineering and Science*, *6*(6), 651–663.

Balcazar, J. L. (2014). Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathogens*, *10*(7), e1004219.

Bbosa, G., Mwebaza, N., Odda, J., Kyegombe, D., and Ntale, M. (2014) Antibiotics/antibacterial drug use, their marketing and promotion during the post-antibiotic golden age and their role in emergence of bacterial resistance. *Health, 6*, 410-425.

Bidnenko, E., Mercier, C., Tremblay, J., Tailler, P., and Kulakaukas, S. (1998). Estimation of the state of the bacterial cell wall by fluorescent *in situ* hybridization. *Applied and Environment Microbiology*, *64*(8), 3059-3062.

Blaak, H., Van Rooyen, S. R., Schuijt, M. S., Docters Van Leeuwen, A. E., Van Den Berg, H. H. J. L., Lodder-Verschoor, F., Italiaander, R., Schets, F. M., and De Roda Husman, A. M. (2011). Prevalence of antibiotic resistant bacteria in the rivers Meuse, Rhine and New Meuse. RIVM report 703719071. *National Institute for Public Health and the Environment*, Bilthoven, The Netherlands.

Brown, D. F., Edward, D. I., Hawkey, P. M., Morrison, D., Ridgway, D. L., and Towner, K. J. (2005). Guidelines for the laboratory diagnosis and susceptibility testing of methicillin resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, *56*, 1000-1018.

Buntaran, L., Hatta, M., Sultan, A. R., Dwiyanti, R., and Sabir, M. (2013). Scc*mec* type II gene is common among clinical isolates of methicillin-resistant *Staphylococcus aureus* in Jakarta, Indonesia. *BMC Research Notes*, *23*, 106-110.

Chambers, H. F. (1997). Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clinical Microbiology Reviews, 10*(4), 781-791.

Chambers, H. F. (2001). The changing epidemiology of *Staphylococcus aureus*. *Emerging Infectious Diseases journal*, *7*(2), 178-182.

Chambers, H., and DeLeo, F. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, *7*(9), 629-641.

Clinical and Laboratory Standards Institute (CLSI) (2006). Performance standards for antimicrobial disc diffusion tests; approved standards. 9th edition. CLSI Document M2-M9. Wayne Pa: Clinical and Laboratory Standards Institute.

Cold Spring Harbor Protocols (CSH PROTOCOLS) (2006). Retrieved October 10, 2014 from http://cshprotocols.cshlp.org/site/recipes/nav_p.dtl

Colomer-Lluch, M., Jofre, J., and Muniesa, M. (2011). Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE*, *6*(3), e17549.

Couto, I., de Lencastre, H., Severina, E., *et al*. (1996). Ubiquitous presence of a *mec*A homologue in natural isolates of *Staphylococcus sciuri*. *Microbial Drug Resistance*, *2*(4), 377-391.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H., and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, *22*(3), 434-444.

DeLong, E. F., Taylor, L. T., Marsh, T. L., and Preston, C. M. (1999). Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent *in situ* hybridization. *Applied and Environmental Microbiology*, *65*(12), 5554-5563.

Dramsi, S., Magnet, S., Davison, S., and Arthur, M. (2008). Covalent attachment of proteins to peptidoglycan. *FEMS Microbiology Reviews, 32*(2), 307–320.

Duquette, R., and Nuttall, T. J. (2004). Methicillin-resistant *Staphylococcus aureus* in dogs and cats: an emerging problem? *Journal of Small Animal Practice*, *45*(12), 591–597.

El Kharroubi, A., Jacques, P., Piras, G., Van Beeumen, J., Coyette, J., and Ghuysen, J. M. (1991). The Enterococcus hirae R40 penicillin-binding protein 5 and the methicillin-resistant *Staphylococcus aureus* penicillin-binding protein 2' are similar. *Biochemical Journal*, *280*(Pt 2), 463–469.

Elshawadfy, A. M., Keith, B. J., Ee Ooi, H., Kinsman, T., Heslop, P., and Connolly, B. A. (2014). DNA polymerase hybrids derived from the family-B enzymes of

Pyrococcus furiosus and *Thermococcus kodakarensis*: improving performance in the polymerase chain reaction. *Frontiers in Microbiology*, *5*, 224.

Fitzgerald, J. R., Sturdevant, D. E., Mackie, S. M., Gill, S. R., and Musser, J. M. (2001). Evolutionary genomics of *Staphylococcus aureus*: Insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(15), 8821–8826.

Fleming, A. (1929). On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. *British Journal of Experimental Pathology*, *10*(3), 226-236.

Fluit, A. C., Visser, M. R., and Schmitz, F. J. (2001). Molecular detection of antimicrobial resistance. *Clinical Microbiology* Reviews, *14*(4), 836-871.

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005).Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology, 3*(9), 722–732.

Garcia-Alvarez, A., Holden, M., Lindsay, H., *et al*. (2011). Methicillin resistant *Staphylococcus aureus* with a novel *mec*A homologue in human and bovine populations in the UK and Denmark: a descriptive study. *The Lancet Infectious Diseases, 11*(8), 595-603.

Genthner, F. J., Chatterjee, P., Barkay, T., and Bourquin, A. W. (1988). Capacity of aquatic bacteria to act as recipients of plasmid DNA. *Applied and Environmental Microbiology*, *54(*1), 115-117.

Gerhardt, P., Murray, R. G. E., Wood, W. A., and Krieg, N. R. (1994). Methods for General and Molecular Bacteriology. *ASM Press*, Washington, D. C.

Gibbons, J. (2014). Western Blot: Protein Transfer Overview. *North American Journal of Medical Sciences*, *6*(3), 158–159.

Gill, S. R., Fouts, D. E., Archer, G. L. *et al*. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm producing methicillin-resistant *Staphylococcus* epidermidis strain. *Journal of Bacteriology*, *187*, 2426–2438.

Glockner, F. O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K., and Schleifer, K. H. (1996). An *in situ* hybridization protocol for detection and identification of planktonic bacteria. *Systematic and Applied Microbiology*, *19*, 403-4

Gradelski, E., Valera, L., Aleksunes, L., Bonner, D., and Fung-Tomc, J. (2001). Correlation between the genotype and phenotypic categorization of staphylococci based on the methicillin susceptibility and resistance. *Journal of Clinical Microbiology, 39*, 2961-2963.

Hanssen, A. M., Kjeldsen, G. G., and. Sollid, J. U. (2004). Local variants of staphylococcal cassette chromosome *mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci: evidence of horizontal gene transfer? *Antimicrobial Agents and Chemotherapy*, *48*, 285–296.

Helassa, N., Vollmer, W., Breukink, E., Vernet, T., and Zapun, A. (2012). The membrane anchor of penicillin-binding protein PBP2a from *Streptococcus pneumonia* influences peptidoglycan chain length. *FEBS Journal*, *279*, 2071–2081.

Hiramatsu, K., Cui, L., Kuroda, M., and Ito, T. (2001). The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends. in Microbiology*, *9*(10), 486–493.

Holden, M. T., Feil, E. J., Lindsay, J. A., *et al*. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proceedings of the National Academy of Sciences*, *101*, 9786–9791.

Hu, L., Ru, K., Zhang, L., Huang, Y., Zhu, X., Liu, H., and Miao, W. (2014). Fluorescence *in situ* hybridization (FISH): an increasingly demanded tool for biomarker research and personalized medicine. *Biomarker Research*, *2*, 3.

Icgen, B., Moosa, S., and Harrison, S. T. L. (2007). A study of the relative dominance of selected anaerobic sulphate-reducing bacteria in a continuous bioreactor by fluorescence *in situ* hybridization. *Microbial Ecology*, *53*(1), 43-52.

Ito, T., Hiramatsu, K., Tomasz, A., *et al*. (2012). Guidelines for reporting novel *mec*A gene homologues. *Antimicrobial Agents and Chemotherapy*. *56*(10), 4997-4999.

Ito, T., Katayama, Y., Asada, K., *et al*. (2001). Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 45*(5), 1323-1336.

Ito, T., Kuwahara-Arai, K., Katayama, Y., Uehara, Y., Han, X., Kondo, Y., and Hiramatsu, K. (2014). Staphylococcal Cassette Chromosome *mec* (SCC*mec*) analysis of MRSA. *Methods in Molecular Biology*, *1085*, 131-148.

IWG-SCC. (2009). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. *Antimicrobial Agents and Chemotherapy*, *53*(12), 4961–4967.

Jensen, E. C. (2012). The basics of Western Blotting. *The Anatomical Record*, *295*, 369–371.

Jevons, M. (1961). “Celpedin”-resistant staphylococci. *British Medical Journal*, *1*(5219), 124–125.

Kallea, E., Kubistab, M., and Rensing, C. (2014). Multi-template polymerase chain reaction. *Science Direct*, *2*, 11-29.

Kassem, I., Sigler, V., and Esseili, M. (2008). Occurrence of *mec*A in nonstaphylococcal pathogens in surface waters. *Journal of Clinical Microbiology*. *46*, 3868-3869.

Kim, C., Milheirico, C., Gardete, S., Holmes, M. A., Holden, M. T., de Lencastre, H., and Tomasz, A. (2012). Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the β-lactam-resistant phenotype. *The Journal of Biological Chemistry*, *287*, 36854-36863.

Korzeniewska, E., and Harnisz, M. (2012). Culture-dependent and culture-independent methods in evaluation of emission of *Enterobacteriaceae* from sewage to the air and surface water. *Water, Air, and Soil Pollution*, *223*(7), 4039–4046.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, *227*, 680–685.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences*, *82*, 6955-6959.

Laurent, F., Chardon, H., Haenni, M., *et al*. (2012). MRSA harboring *mec*A variant gene *mec*C, France. *Emerging Infectious Diseases journal, 18*(9), 1465-1467.

Le, V. (2013). Drug resistance in MRSA is finely-tuned. *PLOS Blogs*, Retrieved October 10, 2014 from http://blogs.plos.org/publichealth/2013/03/05/drug-resistance-in-mrsa-is-finely-tuned/

Li, S., Skov, R. L., Han, X., *et al*. (2011). Novel types of staphylococcal cassette chromosome *mec* elements identified in CC398 methicillin resistant *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy*, *55*(6), 3046–3050.

Lindsay, J. A. (2014). *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. *International Journal of Medical Microbiology*, *304*(2), 103-109.

Lowy, F. (2003). Antimicrobial resistance: the example of *Staphylococcus aureus*. *The Journal of Clinical Investigation*, *111*(9), 1265-1273.

Mahmood, T., and Yang, P. C. (2012). Western Blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences*. *4*(9), 429-434.

Maier, R. M., Pepper, I. L., Gerba, C. P., and Gentry, T. J. (2009). A textbook of environmental microbiology, third edition. *Elsevier Science*, San Diego, CA.

Malachowa, N., and DeLeo, F. R. (2010). Mobile genetic elements of *Staphylococcus aureus*. *Cellular and Molecular Life Sciences*, *67*(18), 3057–3071.

Maslanova, I., Doskar, J., Varga, M., Kuntova, L., Muzik, J., Maluskova, D., Ruzickova, V., and Pantucek, R. (2013). Bacteriophages of *Staphylococcus aureus* efficiently package various bacterial genes and mobile genetic elements including SCC*mec* with different frequencies. *Environmental Microbiology Reports*, *5*, 66–73.

McCarthy, A. J., Loeffler, A., Witney, A. A., Gould, K. A., Lloyd, D. H., and Lindsay, J. A. (2014). Extensive horizontal gene transfer during *Staphylococcus aureus* co-colonization *in vivo*. *Genome Biology and Evolution*, *6*(10), 2697-2708.

McClintock, B. (1950). The Origin and Behavior of Mutable Loci in Maize. *Proceedings of the National Academy of Sciences of the United States of America*, *36*(6), 344–355.

Moellering, R. (2012). MRSA: the first half century. *Journal of Antimicrobial Chemotherapy*, *67*(1), 4-11.

Mohammadi, T., Van Dam, V., Sijbrandi, R., *et al*. (2011). Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *EMBO Journal*, *30*(8), 1425–1432.

Mojica, E. R. E., and Aga, D. S. (2011). Antibiotics pollution in soil and water: potential ecological and human health issues. *Elsevier B.V*.

Moore, D. (2014). Antibiotic classification & mechanism. Retrieved October 22, 2014 from http://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism

Moreillon, P., Que, Y. A., and Glauser, M. P. (2005). *Staphylococcus aureus* (including Staphylococcal toxic shock) in Mandell, Doughlas and Bennett's. Principles and practice of infectious disease, 6th edition, *Churchill Livingstone,* 2321-2351.

Morier, D. (2013). Antibiotic resistance. *Encyclopedia Britannica*. Retrieved November 17, 2014 from http://www.britannica.com/EBchecked/topic/1027479/antibiotic-resistance

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe, S. (1991). Identification of methicillin-resistant strains of Staphylococci by polymerase chain reaction. *Journal of Clinical Microbiology*, *29*, 2240-44.

National Center for Biotechnology Information (NCBI) (2014). Polymerase chain reaction (PCR). *NCBI*, Retrieved August 10, 2014 from http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/

Nielsen, P., Daims, H., and Lemmer, H. (2009). FISH handbook for biological wastewater treatment: identification and quantification of microorganisms in activated sludge and biofilms by FISH. *IWA Publishing,* London, New York.

Ogston, A. (1880). Uber abscesse. *Archiv fur Klinische Chirurgie*, *25*, 588-600.

Ogston, A. (1882). Micrococcus poisoning. *Journal of Anatomy and Physiology*, *17*, 24-58.

Paterson, G. K., Harrison, E. M., and Holmes, M. A. (2014). The emergence of *mec*C methicillin-resistant *Staphylococcus aureus*, *Trends in Microbiology*, *22*(1), 42–47.

Pereira, V. C., Riboli, D. F. M., and da Cunha, M. de L. R. de S. (2014). Characterization of the clonal profile of MRSA isolated in neonatal and pediatric intensive care units of a University Hospital. *Annals of Clinical Microbiology and Antimicrobials*, in press.

Pertel, P. E., Bernardo, P., Fogarty, C., Matthews, P., Northland, R., Benvenuto, M., Thorne, G. M., Luperchio, S. A., Arbeit, R. D., and Alder, J. (2008). Effects of prior effective therapy on the efficacy of daptomycin and ceftriaxone for the treatment of community-acquired pneumonia. *Clinical Infectious Diseases*, *46*, 1142-1151.

Petersen, A., Stegger, M., Heltberg, O., *et al*. (2013). Epidemiology of methicillin resistant *Staphylococcus aureus* carrying the novel *mec*C gene in Denmark corroborates a zoonotic reservoir with transmission to humans. *Clinical Microbiology and Infection*, *19*(1), E16–E22.

Pierce, (2004). Overview of secondary antibodies. *Thermo Scientific*, Retrieved March 12, 2014 from http://www.pierce-antibodies.com/products/secondary-antibodies/

Popham, D. L., and Setlow, P. (1995). Cloning, nucleotide sequence, and mutagenesis of the Bacillus subtilis ponA operon, which codes for penicillin-binding protein (PBP) 1 and a PBP-related factor. *Journal of Bacteriology*, *177*(2), 326-335.

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environmental Science & Technology*, *40*(23), 7445-7450.

Rammelcamp, C., and Maxon, T. (1942). Resistance of *Staphylococcus aureus* to the action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine, 51*, 386-389.

Rao Venkatakrishna, I., Bhat Kishore, G., Kugaji Manohar, S., Pai, V., and Shantaram, M., (2011). Detection of methicillin resistance in *Staphylococcus aureus*:

comparison of disc diffusion and MIC with *mec*A gene detection. *International Journal of Pharmacy and Biological Sciences*, *1*(4), 518-521.

Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G., and Deber, C. M. (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(6), 1760–1765.

Riley, H. (1972). The story of penicillin (Alexander Fleming). *The Journal of the Oklahoma State Medical Association*, *65*(3), 107-119.

Roy, P. H. (1999). Horizontal transfer of genes in bacteria. *Microbiology Today, 26*, 168-170.

Ryffel, C., Strassle, A., Kayser, F. H. and Berger-Bachi, B. (1994). Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents of Chemotherapy*, *38*, 724–8.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., and Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, *239*(4839), 487–491.

Santos de Jesus, A. F., Batista, L. L. F., Torres Lima, J. B., *et al*. (2010). Evaluation of the fluorescence *in situ* hybridization technique for detection of eubacteria and sulfate-reducing bacteria from samples of water in oil fields. *Chemical Engineering Transactions*, *20*, 139–144.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., and Charlier, P. (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*, *32*, 234-258.

Schmidt, V. M., Williams, N. J., Pinchbeck, G., Corless, C. E., Shaw, S., McEwan, N., Dawson, S., and Nuttall, T. (2014). Antimicrobial resistance and characterisation

of staphylococci isolated from healthy Labrador retrievers in the United Kingdom. *BMC Veterinary Research*, *10*, 17.

Sewell, E. W. C., and Brown, E. D. (2014). Taking aim at wall teichoic acid synthesis: new biology and new leads for antibiotics. *The Journal of antibiotics*, *67*(1), 43-51.

Skinner, D., and Keefer, C. (1941). Significance of bacteremia caused by *Staphylococcus aureus*. *Archives of Internal Medicine, 68*(5), 851–875.

Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., Laurent, F., Teale, C., Skov, R., and Larsen, A. R. (2012). Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mec*A or the new *mec*A homologue *mec*A (LGA251). *Clinical Microbiology and Infection*, *18*(4), 395-400.

Sung, M. T., Lai, Y. T., Huang, C. Y., Chou, L. Y., Shih, H. W., Cheng, W. C., Wong, C. H., and Ma, C. (2009). Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 8824-8829.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, *30*, 2725-2729.

Todar, D. (2013). Bacterial resistance to antibiotics. *Todar's Online Textbook of Bacteriology*. Retrieved November 15, 2014 from http://textbookofbacteriology.net/resantimicrobial.html

Tokue, Y., Shoji, S., Satoh, K., *et al*. (1992). Comparison of a polymerase chain reaction assay and a conventional microbiologic method for detection of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*; *36*, 6-9.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(9), 4350–4354.

Trebesius, K., Amann, R. I., Ludwig, W., Muhlegger, K., and Schleifer, K. H. (1994). Identification of whole fixed bacterial cells with nonradioactive 23S rRNA targeted polynucleotide probes. *Applied and Environmental Microbiology*, *60*, 3228-3235.

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., and Hiramatsu, K. (2010). Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrobial Agents and Chemotherapy*, *54*(10), 4352-4359.

Turlej, A., Hryniewicz, W., and Empel, J. (2011). Staphylococcal cassette chromosome *mec* (SCC*mec*) classification and typing methods: an overview. *Polish Journal of Microbiology*, *60*(2), 95–103.

Uhl, J. R., Rochester, M. N., Franklin, R., Cockerill, III., and Rochester, M. N. (2006). Detection of *mec*A-containing *Staphylococcus* spp. Patent N0: US 7074599 B2.

Velasco, V., Sherwood, J. S., Rojas-Garcia, P. P., and Logue, C. M. (2014). Multiplex Real-Time PCR for Detection of *Staphylococcus aureus*, *mec*A and Panton-Valentine Leukocidin (PVL) Genes from Selective Enrichments from Animals and Retail Meat. *PLoS ONE, 9*(5), e97617.

Volpi, E. V., Bridger, J. M. (2008). FISH glossary: an overview of the fluorescence *in situ* hybridization technique. *BioTechniques*, *45*(4), 385–409.

Walsh, C., and Wright, G. (2005). Introduction: antibiotic resistance. *Chemical Reviews*, *105*(2), 391-392.

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms, *Cytometry Part A*, *14*(2), 136-143.

Wielders, C., Fluit, A., Brisse, S., *et. al*. (2002). *mec*A gene is widely disseminated in *Staphylococcus aureus* population. *Journal of Clinical Microbiology*, *40*(11), 3970-3975.

Wielders, C., Vriens, M., Brisse, S., *et al*. (2001). In-vivo transfer of *mec*A DNA to *Staphylococcus aureus*. *Lancet*, *357*(9269), 1674-1675.

Wohlsen, T., Bates, J., Vesey, G., Robinson, W. A., and Katouli, M. (2005). Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. *Letters in Applied Microbiology*, *42*(2006), 350–356.

Wolffs, P., Norling, B., and Radstrom, P. (2005). Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *Journal of Microbiological Methods*, *60*, 315-323.

World Health Organization (WHO) (2014). Antimicrobial resistance: global report on surveillance. WHO Press, Geneva, Switzerland.

Wu, S. W., de Lencastre, H., and Tomasz, A. (2001). Recruitment of the *mec*A gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*. *Journal of Bacteriology*, *183*(8), 2417–2424.

Xu, H., Davies, J., and Miao, V. (2007). Molecular characterization of class 3 integrons from *Delftia* spp. *Journal of Bacteriology*, *189*, 6276-6283.

Yilmaz, F., and Icgen, B. (2014). Characterization of SDS-degrading *Delftia acidovorans* and *in situ* monitoring of its temporal succession in SDS-contaminated surface waters. *Environmental Science and Pollution Research*, *21*(12), 7413-7424.

Yuan, Y., Fuse, S., Ostash, B., Sliz, P., Kahne, D., and Walker, S. (2008). Structural analysis of the contacts anchoring moenomycin to peptidoglycan glycosyltransferases and implications for antibiotic design. *ACS Chemical Biology*, *3*, 429-436.

Zapun, A., Contreras-Martel, C., and Vernet, T. (2008). Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiology Reviews*, *32*(2), 361-385.