An Investigation into the Efflux Mechanism against Quaternary Ammonium Compounds as a Contribution of Bacterial Resistance

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DECLARATION

I declare that the dissertation hereby submitted for the qualification *Magister Scientiae* (Microbiology) at the University of the Free State is my own independent work and has not been previously submitted by me for a qualification at/in another University/faculty.
Furthermore, I concede copyright to the University of the Free State.

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Marisa Coetzee

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ABC	Adenosine triphosphate-binding cassette
APEC	Avian pathogenic *E. coli*
ATP	Adenosine triphosphate
BC	Benzalkonium chloride
bp	Base pairs
cDNA	Complementary DNA
Cp	Crossing point
Ct	Cycle threshold
DDAC	Didecyldimethylammonium chloride
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ds	Double stranded
E	PCR efficiency
EDTA	Ethylenediaminetetraacetic acid
FI	Field isolate
gDNA	Genomic DNA
GOI	Gene of interest
hrs	Hours
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
MATE	Multidrug and toxic compounds extrusion
MDR	Multidrug resistance
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
MS	Mass-spectrometry
OD	Optical density
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PMF	Proton motive force

QAC	Quaternary ammonium compound
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
SDS	Sodium dodecyl sulphate
SMR	Small multidrug resistance family
SPE	Solid phase extraction
Ta	Annealing temperature
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Tm	Melting temperature
TMS	Transmembrane segments
TSB	Tryptic soy broth
UV	Ultraviolet

Chapter 1 Literature Review: Bacterial Resistance against Quaternary Ammonium Compounds

1.1 Introduction

There are several ways to control infectious diseases in poultry. Currently, control mainly involves the use of antibiotics, vaccinations and biosecurity through disinfection. Antibiotics are used therapeutically to control bacterial infections and have also been used as growth promoters. Vaccination is mainly used to control viral infections and some bacterial infections (Joerger, 2003). Biosecurity is used to minimise the spread of bacteria. However, potential problems exist with controlling bacterial diseases (Bragg & Plumstead, 2003). Biosecurity programmes are not always in place to effectively control the spread of bacteria. Bacteria have the ability to develop resistance through the acquisition of genetic elements (McDonnell & Russell, 1999) resulting in an increase in resistance to antibiotics. An additional contributing factor to resistance is the widespread and unrestricted use of the antibiotics (Bragg & Plumstead, 2003). Restrictions or bans have been placed on the use of antibiotics as growth promoters in animals (Joerger, 2003). In the European Union and the USA the use of antibiotics as growth promoters have been banned (Dibner & Richards, 2005). Thus, there is a need for alternative methods to control bacterial diseases in animal productions. Some of the recognised alternatives include; bacteriocins, small antimicrobial peptides and bacteriophages (Joerger, 2003).

Vaccines still remains an effective way to control diseases. However, potential problems are associated with the use of vaccines (Bragg & Plumstead, 2003). These include, breaks in the vaccine cold chain, incorrect vaccine application, use of incorrect vaccines for a particular disease and the ability of the pathogen to change its antigenic expression (Bragg & Plumstead, 2003).

The spread of bacteria can also be controlled using disinfectants. In the poultry industry, it is mostly just ensured that the poultry houses are as pathogen-free as possible before the chicks are placed in the poultry house. However, after this, little emphasis is placed on continuous disinfection and biosecurity (Bragg & Plumstead, 2003). Antibiotics are then used to control diseases; however this is also the cause of the antibiotic resistance problem.

The use of disinfectants is envisaged to be the last resort in the fight against bacterial infections. Disinfectants have a broad-spectrum antimicrobial activity and generally act on several targets in microbial cells (Bridier *et al*., 2011; Russell, 2003). Studies done in poultry pens indicated that a proper disinfectant programme can result in a significant reduction in microbial populations which, resulted in better control of infectious diseases (Bragg & Plumstead, 2003).

Quaternary ammonium compounds (QACs) are widely used disinfectants (McBain *et al*., 2004; Brannon, 1997). However, QAC resistance has already been seen in *Staphylococcus aureus* and other staphylococci species (Bjorland *et al*., 2001). This is mainly due to the acquisition of *qac* resistance genes, some of which have already been identified (Bjorland *et al*., 2005; Anthonisen *et al*., 2002; Bjorland & Sunde, 2001; Bjorland *et al*., 2001; Bjorland *et al*., 2003; Heir *et al*., 1998; Heir *et al*., 1999a, b). These genes have been isolated from various sources, such as in medical environments and food production (Bjorland *et al*., 2005). The current concern is whether there is a link between antibiotic and QAC resistance (Hegstad *et al*., 2010), since some QACs are used in the same environments as antibiotics (Hegstad *et al*., 2010; Sidhu *et al*., 2001). Co-resistance could have serious consequences for industries where the use of disinfectants is important. Thus, it is important that the correct usage of disinfectants is implemented, to ensure that bacteria do not survive and produce more resistant clones (Hegstad *et al*., 2010; McBain *et al*., 2002).

This review introduces information on quaternary ammonium compounds. It focuses on their antimicrobial activity, mechanisms of resistance with focus on bacterial resistance against the QACs. A brief overview of techniques used are discussed such as; real-time PCR and liquid chromatography-mass spectrometry (LC-MS) is provided. Real-time PCR will be used to determine the presence of the *qac* resistance genes and the expression of one of the *qac* genes will be quantified by measuring the difference in mRNA production under different QAC concentrations. Liquid chromatography-mass spectrometry will be used to study the expulsion of the QACs out of the bacterial cells.

1.2 Quaternary Ammonium Compounds

Quaternary ammonium compounds are widely used in the clinical, industrial (McBain *et al*., 2004, Brannon, 1997) and veterinary environments to control the spread of bacteria (Bjorland *et al*., 2005). QACs are favoured in hygiene products and disinfectants because of their broad-spectrum antimicrobial and surfactant properties (McBain *et al.,* 2004; Bloomsfield, 2002; Gilbert & McBain, 2003). The molecular structure of surfactants (surface-active agents) consists out of a: hydrocarbon or hydrophobic group and a hydrophilic or

polar group. Depending on the charge or the absence of ionization, surfactants can be classified into cationic, anionic, nonionic, or ampholytic compounds (McDonnell & Russell, 1999). QACs are mostly classified as cationic surfactants (McDonnell & Russell, 1999).

The structure of QACs generally contains one quaternary nitrogen that is associated with at least one major hydrophobic substituent (Gilbert & Moore, 2005). The general structures of QACs are $NR_1R_2R_3R_4^+X$ as illustrated in Figure 1.1. Hydrogen atoms, plain alkyl groups, or alkyl groups substituted with other functional groups are represented by R, and an anion is represented by X (Hegstad *et al*., 2010). The antimicrobial activity of QACs has been described since 1916, (Jacobs *et al*., 1916). However, they have only been actively used since the 1930s, mainly by surgeons for pre-operative hand cleansing (Hegstad *et al.,* 2010; Buffet-Bataillon *et al.,* 2012). It was found that long-chain QACs between 8 and 18 carbons with one of the radicals replaced with an aliphatic group possess germicidal activity (Hegstad *et al*., 2010; McDonnell & Russell, 1999).

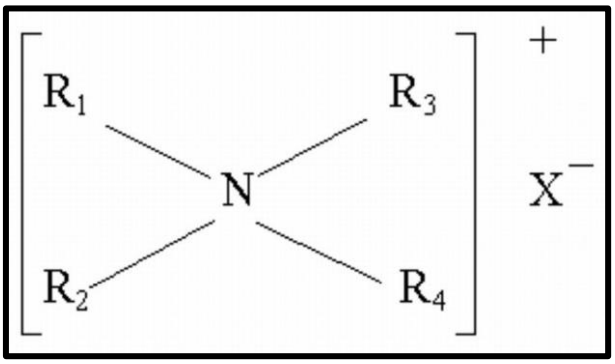

Figure 1.1. The general structure of quaternary ammonium compounds. R represents hydrogen atoms, alkyl groups or substituted alkyl groups. X represents an anion (Schmidt, 2003).

1.2.1 Antimicrobial Activity

QACs have broad-spectrum antimicrobial properties. They are reported to have antimicrobial effects on vegetative bacteria, yeast, molds, algae, and viruses (Hegstad *et al.,* 2010; Fredell, 1994). However, they are only sporostatic, and can therefore not inhibit the germination process of the spores, but only the development of the vegetative cell (McDonnell & Russell, 1999; Russell, 1990). Gram-positive bacteria and algae growth are generally more affected by QACs compared to Gram-negative bacteria and molds (Hegstad *et al*., 2010). QACs are also reported to possess mycobacteriostatic action; therefore they are able to inhibit the growth of mycobacteria (McDonnell & Russell, 1999).

Several environmental factors can play a role on the activity of the QAC, affecting the lethality and inhibitory effects of the QACs (Hegstad *et al*., 2010; Fredell, 1994). Biofilm producing microorganisms can reduce the efficacy of the QAC due to the organic material and high microbial densities (Hegstad *et al*., 2010; Grönholm *et al*., 1999). Temperature, exposure time, chelators and water pH have all been reported to influence the efficacy (Hegstad *et al*., 2010; Ferreira *et al*., 2011). Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), make cell aggregates less compact, therefore enhancing the lethal effects of QACs (Langsrud *et al*., 2003a) As temperature and exposure time increase, the antimicrobial activity increases (Hegstad *et al*., 2010). The type of water also plays a role; water with an excess of mineral salts decreases the activity, since high levels of mineral salts seem to inactivate the QACs (Hegstad *et al*., 2010).

The antimicrobial activity of the QAC is determined by the N-alkyl chain length (n4-18) of the compound (Gilbert & Moore, 2005; Hegstad *et al*., 2010; Buffet-Bataillon *et al*., 2012). It was found that compounds with chain lengths between n=12-14, display optimal antimicrobial activity against Gram-positive bacteria and yeasts. Optimal activity against Gram-negative bacteria can be achieved with chain length n=14-16 (Gilbert & Moore, 2005; Hegstad *et al*., 2010; Buffet-Bataillon *et al*., 2012). It is reported that a compound is virtually inactive when chain length is less than 4 or more than 18 (Gilbert & Moore, 2005; Hegstad *et al*., 2010; Buffet-Bataillon *et al*., 2012; Daoud *et al*., 1983; Gilbert & Al-Taae, 1985).

1.2.2 Mode of action

The mode of action involves the interaction of the QACs with the cell membranes of the microbial cells. This causes disruption and leakage of the cellular content (Gilbert & Moore, 2005; McBain *et al*., 2004). At various concentrations of QACs different types of interactions with the cell membrane are reported (Gilbert & Moore, 2005). Low concentrations of QACs bind to anionic sites on the membrane surface through ionic and hydrophobic bonds. This causes the cells to lose osmoregulatory capability, leaking potassium ions and protons into the environment (Gilbert & Moore, 2005). Intermediate concentration levels of QACs disrupt membrane-located physiologies such as respiration, solute transport, and cell wall biosynthesis (Gilbert & Moore, 2005). High concentrations of QACs kill the cells by solubilisation of the membrane to release the content of the cell to the environment (Gilbert & Moore, 2005).

Gilbert and Moore (2005) proposed the following model for the mode of action of QACs at a molecular level, illustrated in Figure 1.2, segments a-f. The positively charged quaternary nitrogen of the QAC interacts with the head groups of the acidic-phospholipids in the membrane as seen in Figure 1.2 segment b. The hydrophobic tail then integrates into the

hydrophobic membrane core Figure 1.2 segments b and c. At low concentrations which are approximately minimum inhibitory concentrations (MIC), the QAC binds to the membrane, increasing the surface pressure of the exposed leaflet. This causes the membrane fluidity and phase transition temperature to decrease. The membrane undergoes a transition from a fluid to a liquid crystalline state and then loses many of its osmoregulatory and physiological functions as seen in Figure 1.2 segment d. At high concentrations, the solutions of QACs form mixed micellular aggregates that solubilise hydrophobic membrane components i.e. lipid A, phospholipids, etc. as seen in Figure 1.2 segment e and f, eventually resulting in cell lysis (Gilbert & Moore, 2005). Lethality occurs through the generalized and progressive leakage of cytoplasmic materials (McBain *et al.,* 2004). A study done by Jansen and co-workers (2013) using NanoSAM (Scanning Auger Microscopy) showed that treatment with QACs cause nodule formation on the cell walls and thereby causing leakage of the cellular content.

1.3 Bacterial Resistance

Microorganisms differ in their responses to antibiotics and disinfectants (McDonnell & Russell, 1999), because of differences in cellular structures, composition, and physiology (McDonnell & Russell, 1999). In literature, there are two major mechanisms of resistance to disinfectants described (Russell, 1998). One mechanism is decreased susceptibility to a specific agent due to a natural property of the cell. This is called intrinsic resistance which is mostly demonstrated by Gram-negative bacteria (Hegstad *et al*., 2010). The second mechanism is named acquired resistance which result from mutations of normal cellular genes, or by acquiring foreign genetic information that allows the microorganism to survive harsh environments (Hegstad *et al.,* 2010; White & McDermott, 2001).

A disinfectant is only effective if it can reach and interact with its bacterial target site(s). Therefore the disinfectant must cross the outer layers of the cell. Depending on the microorganism Gram stain property, the nature and composition of these layers may act as a permeability barrier, reducing the uptake of the disinfectant (McDonnell & Russell, 1999). Gram-positive bacteria seem to be generally more susceptible to biocide action than Gram-negative bacteria (White & McDermott, 2001). This is due to the lack of an outer membrane, which restricts the entry of disinfectants (White & McDermott, 2001; Paulsen *et al*., 1997). *Pseudomonas aeruginosa* is an example of Gram-negative bacteria that is extremely resistant to many disinfectants, due to its lipopolysaccharide layer composition which prevents the disinfectant from reaching the outer membrane (Adair *et al*., 1971; Méchin *et al*., 1999). Another form of intrinsic resistance is the formation of biofilms by some bacterial cells, resulting in reduced sensitivity towards the disinfectants (McDonnell & Russell, 1999;

Gilbert *et al*., 1990; Campanac *et al*., 2002; Brown & Gilbert, 1993). One possibility of the decrease in sensitivity could be based on a chemical interaction between the disinfectant and the biofilm, modulation of the microenvironment, production of degradative enzyme and neutralising chemicals or genetic exchange between cells in a biofilm (McDonnell & Russell, 1999).

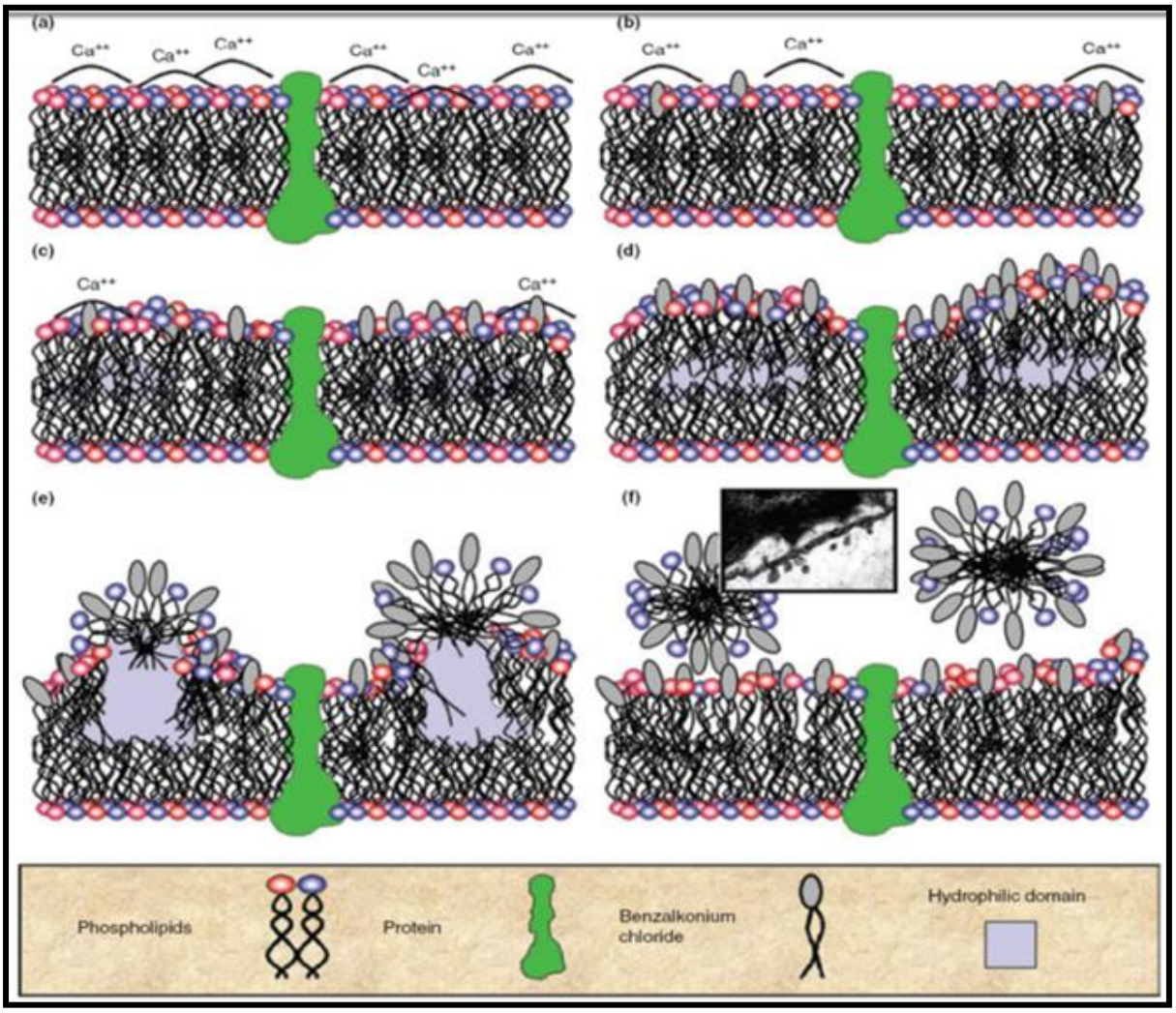

Figure 1.2. The mode of action of quaternary ammonium compounds at a molecular level. The segments (a-f) show progressive adsorption of the quaternary headgroup to the acidic phospholipids in the membrane with increasing QAC exposure/concentration. The membrane fluidity decreases and this creates hydrophilic voids in the membrane. The protein is disturbed with an eventual lysis of the cell, and solubilisation of phospholipids and proteins into QAC/phospholipid micelles. The micrograph shows vesicle being formed on the outer membrane by QAC treatment (Gilbert & Moore, 2005).

Acquired resistance can occur through mutation or by the acquisition of genetic material in the form of plasmids or transposons (McDonnell & Russell, 1999). Resistant clones can emerge when microbial communities are exposed to sub-inhibitory concentrations or long-term exposure to QACs with low chemical reactivity (Hegstad *et al.,* 2010, Buffet-Bataillon *et al.,* 2012; McBain *et al*., 2002). This can lead to changes in susceptibility to different antimicrobial agents (Hegstad *et al.,* 2010, Buffet-Bataillon *et al.,* 2012; McBain *et al*., 2002). Even though disinfectant resistance can be obtained through mutations in normal cellular genes, resistance is mainly acquired through the acquisition of antimicrobial resistance genes (Hegstad *et al*., 2010).

1.3.1 QAC resistance genes

Disinfectants based on QACs have various applications in veterinary medicine and play an important role in the control of bacterial infections, and therefore diseases (Bjorland *et al.,* 2001; 2005). However, bacteria have the ability to adapt to their environment and survive in the presence of disinfectants (White & McDermott, 2001).

Bacteria have the ability to acquire plasmid-encoded multidrug resistance genes that can give the bacterium the ability to counteract the effects of some antimicrobial agents (Hegstad *et al*., 2010; Ioannou *et al.,* 2007). This is particularly seen in *Staphylococcus aureus* (Ioannou *et al.,* 2007). QAC resistance genes have already been identified from staphylococci, isolated from various sources (Bjorland *et al*., 2005; Anthonisen *et al*., 2002; Bjorland & Sunde, 2001; Bjorland *et al*., 2001; Bjorland *et al*., 2003; Heir *et al*., 1998; Heir *et al*., 1999a,b). It was found that these resistance genes are generally plasmid-borne (Bjorland *et al*., 2005; Russell, 1997). They encode proteins responsible for the expulsion of hydrophobic compounds including QACs, intercalating dyes and cationic biocides (Bjorland *et al.,* 2005). The *qac* resistance genes are found on mobile genetic elements, such as plasmid and transposons, and their location on these genetic mobile elements allows them to interact between various staphylococci species (Hegstad *et al*., 2010).

The *qac* resistance genes termed *qacA* and *qacB* are homologous and are found on large plasmids (Bjorland *et al*., 2005; Alam *et al*., 2003; Littlejohn *et al*., 1991; Paulsen *et al*., 1995). Whereas the genes termed *smr, qacG, qacH,* and *qacJ* are found on plasmids smaller than 3kb (Bjorland *et al*., 2005; Alam *et al*., 2003; Littlejohn *et al*., 1991; Paulsen *et al*., 1995). The resistance genes found on the small plasmids are commonly found in gene cassettes and encode proteins which form part of the small multidrug resistance (SMR) family (Bjorland *et al*., 2005; Alam *et al*., 2003; Littlejohn *et al*., 1991; Paulsen *et al*., 1995). The *qacA* and *qacB* genes encode proteins that form part of the Major Facilitator

Superfamily (MFS) (Heir *et al*., 1999a). All the proteins encoded by the resistance genes are membrane embedded (Langsrud *et al*., 2003b).

1.3.2 Link between antibiotic and disinfectant resistance

Since biocides have been used longer in clinical environments, it is of great concern if there is a link between biocide resistance and antibiotic resistance. Disinfectant and antibiotic resistance genes are sometimes found on the same genetic units (Hegstad *et al*., 2010). The *qac* resistance genes are commonly found on class 1 integrons, isolated from clinical and environmental bacteria (Gillings *et al*., 2009a). However, clinical class 1 integrons were shown to generally carry one to six gene cassettes, including gene cassettes encoding antibiotic resistance (Gillings *et al*., 2009b; Recchia & Hall, 1995; Partridge *et al*., 2001). Therefore the *qac* resistance genes are often closely linked to gene cassettes encoding antibiotic resistance (Gillings *et al*., 2009a; White & McDermott, 2001; Gaze *et al*., 2005). The integrons are collectors of resistance cassette and can readily pick up new additional resistance determinants on plasmids, which is an efficient vehicle to travel among different species (Hegstad *et al*., 2010; Gillings *et al*., 2009a). It is thought that the use of biocides causes the initial selective pressure, introducing resistance to biocides mediated by *qac* genes and the spread of the class 1 integron, which is responsible for a major part of antibiotic resistance (Hegstad *et al*., 2010; Gillings *et al*., 2009a). Therefore, it seems that the increasing use of QACs promotes the fixation of other novel genetic elements. Also, the presence of QAC determinants and other resistance determinants on the same mobile elements may contribute to the transfer of resistance into other bacteria (Hegstad *et al*., 2010).

In addition, some efflux pumps can mediate cross-resistance since they export both QACs and other antimicrobial agents (Hegstad *et al*., 2010). Different resistance mechanisms are linked on the same genetic unit, such as the same plasmid, transposon or integron, or a combination of both (Hegstad *et al*., 2010). Some efflux systems are able to accommodate both QACs and other antimicrobials, due to the resistance determinants being on the same genetic unit. QACs can select for organisms expressing these efflux systems that are able accommodate other antimicrobial agents, and therefore might confer resistance to antibiotics (Hegstad *et al*., 2010; Carson *et al*., 2008).

1.4 Efflux pumps

Efflux is the pumping of a solute out of a cell which lowers the concentration of the solutes inside the cell (Piddock, 2006). If the solute is an antimicrobial agent, the organism becomes less susceptible to the antimicrobial (Piddock, 2006). Active efflux is a primary mechanism of

disinfectant resistance in bacteria (Hassan *et al*., 2010). Efflux pumps can either be substrate specific or have a wide range of substrates, as in the case of multidrug resistance pumps (Piddock, 2006).

Multidrug resistance (MDR) proteins confer resistance to a wide range of substrates including toxic compounds, antibiotics and QACs (Buffet-Bataillon *et al*., 2012). Antibiotics and biocides are actively expelled out of the bacterial cell by these proteins (Buffet-Bataillon *et al*., 2012). These efflux systems are capable of pumping out a broad range of chemically and structurally unrelated compounds in an energy-dependent manner, without altering the compound (Kumar & Schweizer, 2005).

The multidrug resistance proteins can be divided into two major classes of transporters based on bioenergetics and structural criteria (Putman *et al*., 2000). The multidrug transporters are divided into primary and secondary transporters as illustrated in Figure 1.3 (Putman *et al*., 2000). The primary transporters utilise the free energy captured during ATP hydrolysis to pump solutes out of the cell (Putman *et al*., 2000). Secondary multidrug transporters generate a transmembrane electrochemical gradient using either protons or sodium ions to drive the expulsion of solutes out of the cell (Putman *et al*., 2000).

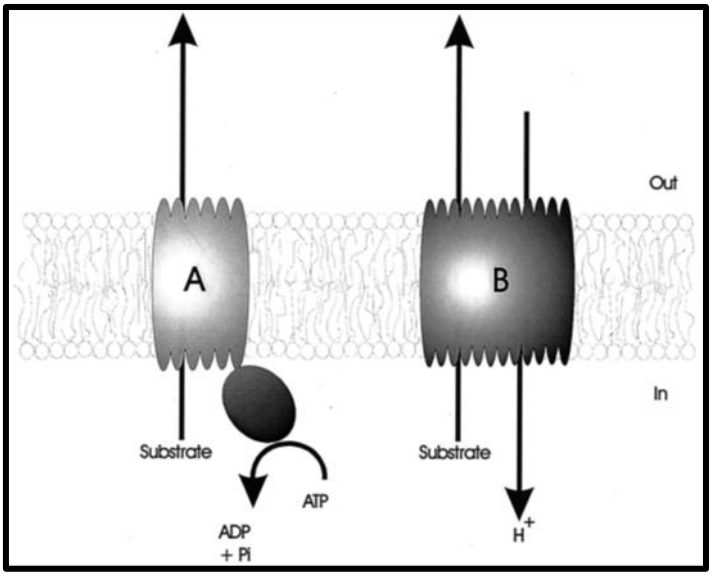

Figure 1.3. A schematic presentation of the two major classes of multidrug transporters. The protein A is an example of a primary transporter belonging to the ABC-type multidrug transporter. The protein B is an example of a secondary multidrug transporter, making use of proton gradients as an energy source (Putman *et al*., 2000).

There are currently five families of multidrug efflux transporters recognised namely: (1) The adenosine triphosphate (ATP)-binding cassette (ABC) transporters; (2) The small multidrug resistance (SMR) family; (3) The major facilitator superfamily (MFS); (4) The resistance-nodulation-division (RND) family and (5) The multidrug and toxic compounds extrusion (MATE) family as depicted in Figure 1.4 (Kumar & Schweizer, 2005; Buffet-Bataillon *et al*., 2012; Putman *et al*., 2000). The ABC transporters are the only family that belongs to the class primary transporters and the remaining four families belong to class secondary transporters (Putman *et al*., 2000). The MFS, SMR and RND families are H^+/solute antiporters, whereas MATE is a Na^+/solute antiporter. These families of multidrug transporters also include proteins involved in PMF (proton motive force)-dependent transport processes or other functions (Putman *et al*., 2000).

The classes can be further divided into single component and multicomponent pumps (Kumar & Schweizer, 2005). Single component pumps transport their substrates across the cytoplasmic membrane, whereas multicomponent pumps, found in Gram-negative bacteria, function with a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP) as seen in Figure 1.4, allowing the efflux of substrates across the entire cell envelope (Kumar & Schweizer, 2005). Gram-positive bacteria have single component pumps which are located in the cytoplasmic membrane (Kumar & Schweizer, 2005).

The SMR transporters are the smallest secondary drug efflux proteins, and are about 107 amino acid residues in length, with only 4 predicted transmembrane segments (TMS) as seen in Figure 1.5 (Putman *et al*., 2000; Marquez, 2005). These proteins may function as homo-oligomeric or hetero-oligomeric complexes because of their small size (Putman *et al*., 2000; Marquez, 2005; Paulsen *et al*., 1993; Paulsen *et al*., 1995).

These transporters are capable of extruding a wide range of structurally different compounds (Putman *et al*., 2000; Lewis *et al*., 1997; Nikaido, 1996; Paulsen *et al*., 1996a; van Veen *et al*., 1998). This is possible because substrates share physical characteristics, such as a degree of hydrophobicity, an amphiphilic nature, and a positive or neutral charge (Putman *et al*., 2000). The majority of substrates utilized by multidrug resistance pumps are positively charged (Putman *et al*., 2000). Therefore, it is thought that the intramembraneous acidic residue may be involved in substrate binding (Putman *et al*., 2000). Multiple-sequence analysis showed that there are conserved amino acid sequence motifs throughout the transporters of MFS, SMR and RND families (Putman *et al*., 2000). This indicates that these motifs play a structural or functional role in the transporters and may be directly involved in drug and proton translocation (Putman *et al*., 2000). In the SMR family, the charged residue in motif A is glutamic acid and predicted to be located in TMS (Figure 1.5) (Putman *et al*.,

2000). As the substrate binds to the hydrophobic binding pocket, two of the three glutamate residues deprotonate. The transporter then undergoes a conformational change, altering the binding site and exposing the drug to the exoplasmic face of the membrane (Borges-Walmsley *et al*., 2003; Putman *et al*., 2000). Upon release of the disinfectant, the glutamic acid residues are protonated again (Borges-Walmsley *et al*., 2003; Putman *et al*., 2000). This is an attractive model, since the glutamate trimer catalyses the coupling of the substrate export to proton import (Borges-Walmsley *et al*., 2003).

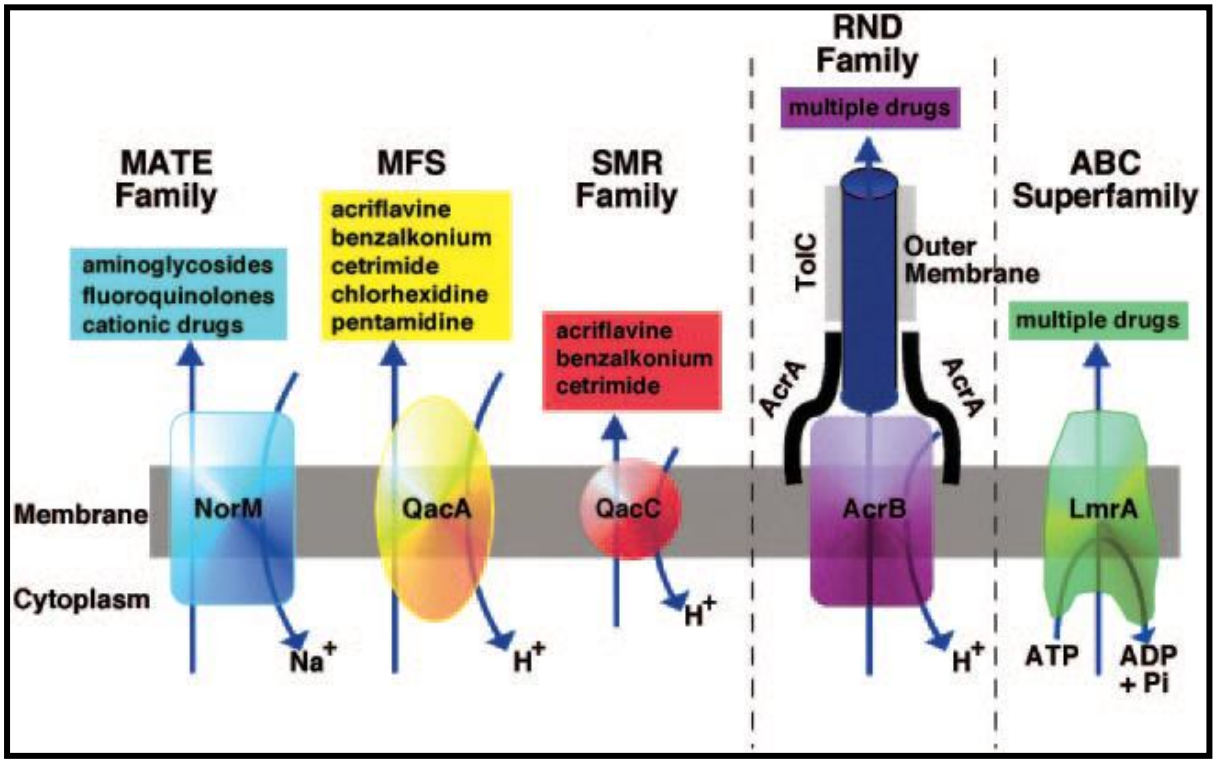

Figure 1.4. A diagrammatic comparison of the five families of efflux pumps. The MATE, MFS, SMR and RND families are secondary transporters using H^+/Na^+ gradient, whereas ABC family belongs to the primary transporter class and uses ATP. The RND family are multi-component pumps found in Gram-negative bacteria (Piddock, 2006).

Studies have indicated that a single antimicrobial can cause the overexpression of multidrug efflux systems, stimulating the resistance to a broad range of compounds (Hassan *et al*., 2010). Hassan and co-workers (2010) indicated that these multidrug transporters could easily accommodate new classes of biocides upon minor mutational changes (Hassan *et al*.,

2010; Paulsen *et al*., 1996b). Therefore it is important for the correct use of biocides to prevent resistance against biocides (Hassan *et al*., 2010).

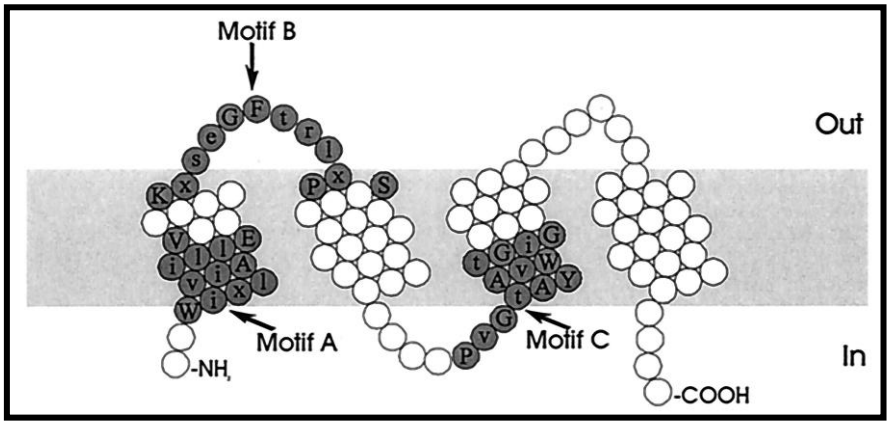

Figure 1.5. Structural model for multidrug transporters of the SMR family. It is a transmembrane protein, consisting of 4 TMS. Multiple alignments of amino acid sequences revealed conserved amino acid sequence motifs. These conserved regions are found throughout the multidrug transporters of MFS, SMR, and RND families. This suggests that these motifs play an important structural and functional role in the transporters. The residues constituting the conserved sequence motifs are shaded (Putman *et al*., 2000).

1.5 Liquid chromatography-mass spectrometry

There are numerous techniques described in literature to analyse cationic surfactants (Martínez-Carballo *et al*., 2007). The techniques include gas chromatography, nuclear magnetic resonance, thin layer chromatography coupled with flame ionisation detection, liquid chromatography (LC) and capillary electrophoresis (Martínez-Carballo *et al*., 2007; Schmitt, 2001; Heinig *et al*., 1997). Although the use of these techniques have been published for the analysis of quaternary ammonium compounds, difficulties still exists (Castro *et al*., 2001). This is due to the properties of QACs (Martínez Vidal *et al*., 2004). Frequently used for the analysis of QACs is liquid chromatography with either conductivity detection or indirect ultraviolet (UV) detection (Castro *et al*., 2000; Martínez-Carballo *et al*., 2007). However, there where inherent disadvantages based on the lack of analytical specificity, resulting in identification and quantification difficulties, especially in complex matrices (Castro *et al*., 2000). This disadvantage was overcome by using mass spectrometry (MS) as the method of detection (Castro *et al*., 2000). The use of MS as detection method

requires a trace enrichment step before chromatographic analysis to increase analytical specificity (Castro *et al*., 2000).

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) have been successfully used in the past to extract and purify the analyte (Martínez-Carballo *et al*., 2007; Radke *et al*., 1999; Castro *et al*., 2000). These methods are usually used to clean the sample before using a chromatographic or analytical method to quantify the amount of the analyte (Żwir-Ferenc & Biziuk, 2006). Both these methods involve the partitioning of solutes between two phases. The LLE consists of two immiscible liquid phases, whereas the SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase (Żwir-Ferenc & Biziuk, 2006). Treating the sample with these procedures allows for the purification and concentration of analytes from solutions (Żwir-Ferenc & Biziuk, 2006). Solid phase extraction is frequently used due to its simplicity and economy in terms of solvents needed (Castro *et al*., 2000). It is a versatile procedure and has successfully been applied in environmental analysis (Martínez Vidal *et al*., 2004). It is recommended for the isolation of quaternary ammonium herbicides (Martínez Vidal *et al*., 2004; Pico *et al*., 2000). The general procedure involves the loading of the sample onto the SPE sorbent, washing away contaminants and then eluting the desired analyte with another solvent (Żwir-Ferenc & Biziuk, 2006; Camel, 2003).

Liquid chromatography (LC) is a separation technique used in life sciences to separate mixtures into their individual components. This enables the identification and measurement of these components (Agilent Technologies, 2001). Liquid chromatography is suitable for non-volatile and thermally fragile molecules (Agilent Technologies, 2001). It is used to separate a wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins (Agilent Technologies, 2001).

Different detectors are used with LC to generate data. The traditional detectors included refractive index, electrochemical, fluorescence and ultraviolet-visible (UV-Vis) detectors (Agilent Technologies, 2001). Mass spectrometers are used as detectors for LC, since it is more sensitive and specific. The traditional detectors generate two-dimensional data; therefore the data represents signal strength as a function of time (Agilent Technologies, 2001). Three dimensional data are generated by detectors such as fluorescence and diode-array UV-Vis as well as mass spectrometers (Agilent Technologies, 2001). The data incorporates the signal strength together with spectral data for each time point (Agilent Technologies, 2001; Pitt, 2009). The data can provide information on molecular weight, structure, identity, quantity, and purity of a sample. The mass spectral data generated by MS

add specificity that increases confidence in the results of both qualitative and quantitative analysis (Agilent Technologies, 2001; Pitt, 2009).

Mass spectrometers ionise the analyte molecules to a charged state (Agilent Technologies, 2001; Pitt, 2009). The ions or any fragmented ions that are produced during the ionisation process are analysed. The ions can be identified based on their mass-to-charge ratio (*m/z*) (Agilent Technologies, 2001; Pitt, 2009). The key components in this process are the ion source, which generates the ions, and the mass analyser, which sorts the ions (Agilent Technologies, 2001; Pitt, 2009). Different technologies are available for the ionisation and ion analysis; therefore there are many different types of mass spectrometers with different combinations of these two processes. Also each combination has advantages and disadvantages depending on the type of information that is needed (Agilent Technologies, 2001; Pitt, 2009).

It is important to be able to quantify QAC levels in drinking water since QACs are commonly used in herbicides (Castro *et al*., 2000, 2001). The European Union set a general concentration limit of pesticides allowed in drinking water to 0.1 µg l^{-1}, therefore a sensitive technique is used to quantify the levels present in the water (Castro *et al*., 2001). Studies have shown that LC-MS is reliable enough to determine the lower limits in drinking water (Castro *et al*., 2001). Some studies have also been done to detect QACs in the marine environment (Bassarab *et al*., 2011). Studies indicated that LC-MS can also detect metabolites of pesticides showing a larger number of pesticide metabolites occurring in the environment (Reemtsma *et al*., 2013). Therefore LC-MS has become a widely accepted technique for analysing environmental samples for QACs because of the greater detection sensitivity compared with alternative types of LC detection (Bassarab *et al*., 2011).

1.6 Real-Time PCR

Real-time polymerase chain reaction (Real-time PCR) is a technique of choice that has been widely used for the quantification of gene expression (Pfaffl, 2001). This is because this technique is highly sensitive and allows for the quantification of genes present in low copy number (Pfaffl, 2001). It is easy to perform and provides the necessary accuracy and produces reliable and rapid quantification results (Pfaffl, 2001). Real-time PCR collects the data throughout the PCR run, thus combining amplification and detection into a single step (Fraga *et al*., 2008). This is achieved by monitoring the amplification of the target deoxyribonucleic acid (DNA) using fluorescent technology (Fraga *et al*., 2008). Two general amplicon detection chemistries are available (Arya *et al*., 2005). These include double-stranded (ds) DNA intercalating dyes and fluorescent probes (Arya *et al.*, 2005). The

simplest and most cost-effective technique is the use of SYBR® Green I fluorescence dye, which intercalates into dsDNA (Pfaffl, 2001, Arya *et al*., 2005). SYBR® Green is the reporter dye in the system, and the fluorescent signal it generates is monitored during the PCR process. SYBR® Green I works by binding to the minor groove of the dsDNA and this DNA-dye complex result in a dramatic increase in fluorescence output (Shipley, 2006). Therefore, as the double stranded PCR product accumulates, the level of fluorescence increases, representing the accumulation of template (Peters *et al*., 2004). The disadvantage of intercalating dyes is that any double stranded product will be detected including primer-dimers and therefore false positive can occur (Peters *et al*., 2004; Yin *et al*., 2001). Thus, intercalating dyes are non-specific, therefore good primer design and quality control during the assay development is necessary (Shipley, 2006).

More specific and accurate ways of detecting accumulation of target amplicons is by use of fluorescent probes (Peters *et al.,* 2004, Arya *et al*., 2005). These probes are sequence specific oligonucleotides coupled to fluorescent dyes, therefore eliminating false positives due to unspecific product formation (Peters *et al.,* 2004, Arya *et al*., 2005). However, probes are more expensive than the intercalating dyes (Peters *et al.,* 2004, Arya *et al*., 2005).

Polymerase chain reaction can be divided into four phases, namely the linear ground phase, early exponential phase, log-linear phase or exponential phase and the plateau phase as outlined in Figure 1.6 (Wong & Medrano, 2005; Tichopad *et al*., 2003). The linear ground phase is during the beginning of the PCR reaction and fluorescence detected is not above background (Wong & Medrano, 2005). During this phase, baseline fluorescence is calculated (Wong & Medrano, 2005). In the early exponential phase, the amount of fluorescence increases to a level that is significantly higher than the background levels. The cycle where this occurs is known as the cycle threshold (Ct) or the crossing point (Cp) (Wong & Medrano, 2005). This value is representative of the starting copy number in the original template and is used to calculate experimental results (Wong & Medrano, 2005).

The greater the amount of the target DNA in the starting material, the faster a significant increase in fluorescence is detected, yielding a lower Cp (Wong & Medrano, 2005). The PCR reaches optimal amplification period during the log-linear phase with PCR product doubling after each cycle in ideal reaction conditions (Wong & Medrano, 2005). When reaction components become limited and therefore no more amplification is achieved this is called the plateau phase and fluorescence intensity can no longer be used for data calculation (Wong & Medrano, 2005).

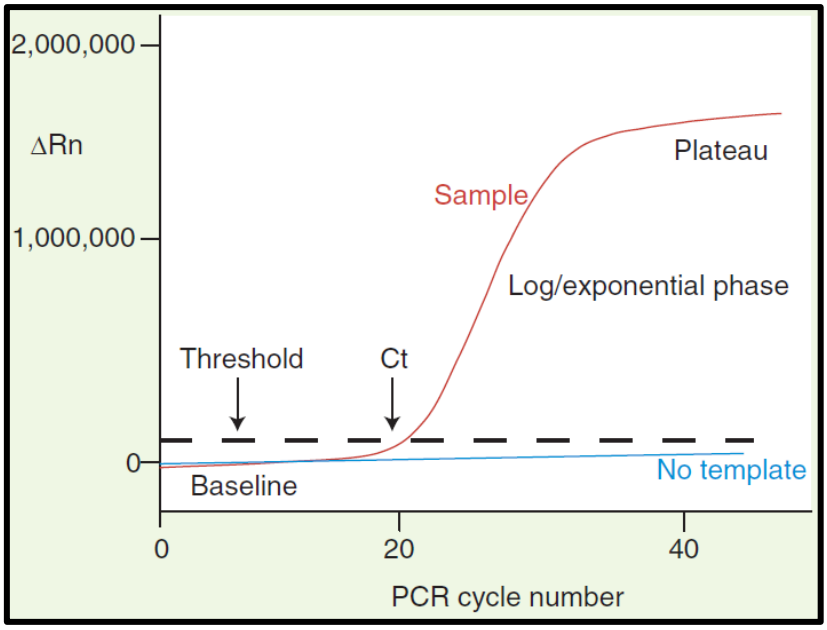

Figure 1.6. The four phases of a PCR amplification curve, the linear ground, early exponential, log-linear, and plateau phases. The PCR amplification curve shows the accumulation of fluorescent emission at each reaction cycle. The background signal, cycle threshold (Ct) and amplification efficiency can be calculated using the phases. ΔRn is calculated as the difference in Rn values of a sample and either no template control or background, and thus represents the magnitude of signal generated during PCR (Wong & Medrano, 2005; Arya *et al*., 2005).

Melt curve analysis can be done at the end of the PCR to analyse the products that were generated during the PCR process and to confirm whether or not a single product was amplified. Thus, primer-dimers or any non-specific products can be detected. During melt curve analysis the fluorescent signal is monitored. As the temperature increases and the PCR product melts, SYBR® Green is released into the solution. The fluorescence intensity therefore decreases. The software of the instrument then plots the change in fluorescence over temperature and a peak showing the Tm of the PCR products is produced (Ponchel, 2006; Keer, 2008). Any primer-dimers or non-specific products that were formed during the PCR process will generally have a lower Tm than the specific product formed in the PCR (Figure 1.7); therefore the products can be distinguished from each other (Keer, 2008).

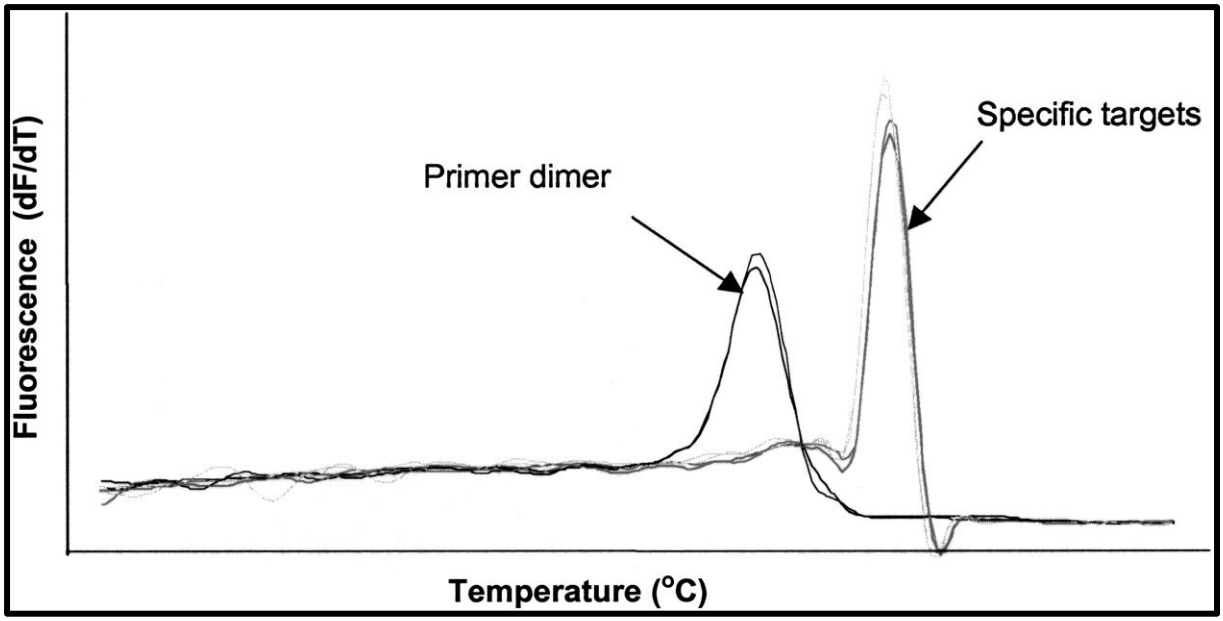

Figure 1.7. A schematic representation of melt curve analysis after the PCR process to differentiate specific PCR products from primer-dimers. The primer-dimers or non-specific PCR products will have a lower melting temperature than the specific PCR product (Keer, 2008).

1.6.1 Quantitative real-time PCR

Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample (Shipley, 2006). There are two types of quantification methods that can be used to quantify real-time PCR results. These methods are known as absolute quantification and relative quantification (Pfaffl, 2001). Absolute quantification is based on an internal or an external calibration curve and is used when it is necessary to determine the absolute transcript copy number (Livak & Schmittgen, 2001; Pfaffl, 2001). Relative quantification is based on the relative expression of a target gene versus a reference gene and can be used to investigate the physiological changes in gene expression (Livak & Schmittgen, 2001). This is done by measuring the difference in mRNA production. However, the RNA has to be converted to cDNA to be detected by real-time PCR. Relative quantification requires certain equations for quantifying gene expression (Livak & Schmittgen, 2001).

When using absolute quantification, a standard curve must be generated using serially diluted standards of known concentrations (Wong & Medrano, 2005; Heid *et al*., 1996). This produces a linear relationship between Cp and the initial amount of total RNA or cDNA

(Wong & Medrano, 2005; Heid *et al*., 1996). Therefore, the concentration of the unknown sample can be determined using their Cp values (Wong & Medrano, 2005; Heid *et al*., 1996). This method assumes that the amplification efficiencies of the standards and samples are approximately the same (Wong & Medrano, 2005; Souaze *et al*., 1996).

During relative quantification gene expression is measured based on an external standard or reference sample, known as a calibrator (Wong & Medrano, 2005; Livak & Schmittgen, 2001). The calibrator or reference control is usually a non-treated sample such as RNA from normal tissue or a sample at time zero in a time-course study (Arya *et al*., 2005; Wong & Medrano, 2005). The results are expressed as a target / reference ratio (Wong & Medrano, 2005). Several mathematical models have been established to calculate the expression of the target gene to the reference gene (Pfaffl, 2004). These calculations are based on the Cp values (Pfaffl, 2004).

The amplification efficiency of the PCR reaction is important for relative quantification (Pfaffl, 2004). The relative quantification can be calculated using one of two models either with efficiency correction or without (Pfaffl, 2004). Therefore, the relative expression ratio of a target gene is calculated, based on its real-time PCR efficiency (*E*) or a static efficiency of 2 (Pfaffl, 2004). An amplification efficiency of 2 means the PCR product doubles with every cycle in the exponential phase (Pfaffl, 2004; Gibson *et al*., 1996). However, many PCRs do not have ideal efficiencies. Therefore, relative quantification can also be calculated with efficiency correction so that the starting concentration is not overestimated (Pfaffl, 2004). The amplification efficiency can be calculated from a standard curve: $E=10^{-(1/slope)}$ (Wong & Medrano, 2005). However, calculating amplification efficiency from a standard curve may overestimate the efficiency because the efficiency can change from being stable in the early exponential phase with gradual declination to zero (Wong & Medrano, 2005). Calculating the efficiency from a standard curve is not reflective of these changes in efficiency (Wong & Medrano, 2005). Since the PCR results are based on the Cp, which are determined early in a reaction, the differences in amplification efficiencies generate only minor differences in Cp values (Wong & Medrano, 2005). There are alternative methods in calculating amplification efficiencies using the raw data collected during a PCR reaction (Wong & Medrano, 2005). Consequently, the data collected during the exponential phase can be log-transformed and then plotted with the slope of the regression line representing the amplification efficiency (Wong & Medrano, 2005). Calculating the amplification efficiency from the raw data is reportedly more accurate than calculating it from a standard curve (Wong & Medrano, 2005).

The PCR product quantification can be done either using the standard curve method or the comparative delta-delta Ct ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001; Wong & Medrano,

2005). With the standard curve method, the quantity of each experimental sample is first determined using a standard curve and then expressed relative to a single calibrator sample (Wong & Medrano, 2005). The calibrator is designated as 1-fold, with all experimentally derived quantities reported as an *n*-fold difference relative to the calibrator (Wong & Medrano, 2005). This is the simplest method of quantification, since there are no preparations of exogenous standards required. The calibrator samples do not have to be quantified and it is not based on complex mathematics (Wong & Medrano, 2005). This method is applied when amplification efficiencies differ between reference and target genes (Wong & Medrano, 2005). It does however require normalisation of the results because it does not incorporate an endogenous control (Wong & Medrano, 2005).

The comparative delta-delta Ct ($2^{-\Delta\Delta Ct}$) method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample (Livak & Schmittgen, 2001; Wong & Medrano, 2005). This method can be used with or without correction of amplification efficiency. However, when using it with amplification efficiency correction, the efficiencies generated must be approximately equal, to eliminate errors that can be generated using this method (Livak & Schmittgen, 2001; Wong & Medrano, 2005).

With the Pfaffl model, the gene quantification and normalisation is combined into a single calculation. It incorporates the efficiencies of the target and reference genes to correct for differences between the two assays (Pfaffl, 2001; Wong & Medrano, 2005). The relative expression ratios are defined by the expression of the gene of interest (GOI) in one specific sample compared to the reference sample (Schefe *et al*., 2006).

The GOI must be normalised to minimize errors that could result from minute differences in starting amount of template, the quality of template or the differences in efficiency of cDNA synthesis and PCR amplification (Wong & Medrano, 2005). Normalisation corrects for sample-to-sample variations. The GOI is normalised to a control gene with unchanging expression levels regardless of the experimental conditions (Wong & Medrano, 2005). However, there is no gene that meets this standard in experimental condition (Wong & Medrano, 2005). Housekeeping genes were thought to have stable expression and are usually used as controls (Wong & Medrano, 2005). However, it has been shown that these housekeeping genes are prone to certain experimental condition. Thus the use of one housekeeping gene for normalisation can falsely bias results (Wong & Medrano, 2005). It is thus necessary to validate the expression stability of the housekeeping gene to the experimental conditions (Wong & Medrano, 2005).

Real-time PCR is a sensitive method that could be used to determine the quantification of genes present in low copy number (Pfaffl, 2001). Therefore this method can be used to quantify the expression of the *qac* resistance genes. Previous studies to determine the expression of *qac* resistance genes included determining the expression of the *qacA* and *qacB* gene by monitoring the expression of the *qacR* gene, which regulates the expression of these genes, using an assay with luciferase reporter (Smith *et al*., 2008). It was shown that the expression increased in the presence of disinfectants. Another study determining which internal controls to use, determined the expression of the *qacA* and the *qacR* genes using quantitative real-time PCR (Theis *et al.,* 2007). It was reported that the expression of the *qacA* gene increased with the increase in concentration of the substrates used (Theis *et al*., 2007). Therefore, quantitative real-time PCR is suitable for analysing gene expression (Theis *et al*., 2007) and is one of the best methods to determine gene expression, since it is sensitive and reliable (Pfaffl, 2001).

1.7 Introduction to study

Bacterial infections are a major problem in the poultry industry. These infections are controlled through the use of antibiotics. Due to the increase in antibiotic resistance and the restrictions placed on the use of antibiotics in animals, the poultry industry is slowly heading for a post antibiotic era.

The use of disinfectants could possibly be the last resort in the fight against bacterial infections. Quaternary ammonium compounds are widely used disinfectants, especially in medical and food industry, and over the last decade are more frequently found in domestic cleaning products. The QACs cause perturbation of the cytoplasmic and outer membrane lipid bilayer and lethality occurs through the generalised and progressive leakage of cytoplasmic materials.

Bacteria have the ability to acquire resistance and resistance against the QACs have already been seen, especially in staphylococci. This is due to bacteria acquiring genetic units, such as plasmids, transposons or integrons, which contain resistance genes that code for proteins that confer resistance to antimicrobials. These proteins are multidrug transport proteins and therefore expel the QAC out of the bacterial cell.

However, the correct use of disinfectants would not cause more resistant clones to be produced. It was shown that a continuous disinfection programme can cause a significant decrease in microbial population, controlling bacterial infections (Bragg & Plumstead, 2003). The resistance to QACs has to be investigated in order to prevent similar problems associated with antibiotic resistance.

1.8 Aims of the study

The overall aim of this study was to understand bacterial resistance to QACs. The objectives included the screening of field isolates for *qac* genes, determining if the presence of these genes affected the minimum inhibitory concentration (MIC) of the QAC based products. It is reported that the resistance mechanism to QACs is an efflux mechanism. Therefore, exportation of the QAC out of the bacterial cell was studied using liquid chromatography-mass spectrometry (LC-MS). The structure of the QAC was also determined with LC-MS after the QAC was exported out of the cell, determining if there are any changes in the structure of the QAC. Real-time PCR was used to relatively quantify the expression of the *qacJ* gene in the presence of different QAC concentrations.

Chapter 2 Determination of the minimum inhibitory concentration of quaternary ammonium compounds and detection of the genes conferring QAC resistance in bacteria

2.1 Introduction

The restriction placed on antibiotic use in animals, has prompted the use of alternatives for the control of bacterial infections (Joerger, 2003; Nelson *et al*., 2007). The use of disinfectants may be the last resort in the fight against bacterial infections, and if they are not used properly the consequences could be similar to those seen with antibiotic resistance. Disinfectants are being incorporated more in the agricultural industry, especially quaternary ammonium compound (QAC)-based disinfectants, to control bacterial growth in the environment. However, bacteria are capable of acquiring resistance against antibiotics, heavy metals and QACs (White & McDermott, 2001). When bacteria are able to tolerate these substances, they have the ability to survive harsh environments where these substances are used (White & McDermott, 2001; Hegstad *et al*., 2010).

Resistance against QACs has already been seen in the clinical, industrial and veterinary environments, and several resistance genes against QACs have been identified, especially in *Staphylococcus aureus* with several genes already isolated (Ioannou *et al*., 2007). Most of the *qac* resistance genes code for proteins that are part of the Small Multidrug Resistance (SMR) family, whereas larger genes have also been identified that code for proteins which are part of the Major Facilitator Superfamily (MFS) (Bjorland *et al*., 2005; Heir *et al*., 1999a). All these genes code for membrane embedded multidrug transporters that are capable of pumping out the antimicrobial and lowering the concentration of the antimicrobial inside the cell (McBain *et al*., 2004).

In this study four genes were selected which code for proteins within the SMR family. These genes are *smr*, *qacJ*, *qacH* and *qacG*, which have been identified in food-borne, clinical and veterinary isolates (Bjorland *et al*., 2005; Smith *et al*., 2008). Efflux is the major resistance mechanism that bacteria use against disinfectants (Hassan *et al*., 2010). The proteins encoded by these genes are very small, and are able to transport a wide variety of substrates (Bjorland *et al*., 2003). These genes are found on different plasmids and can be transferred to other bacteria. The sequences of these genes are available on GenBank.

The concentration of QACs used in industry is much higher than the reported concentration, known as the minimum inhibitory concentration (MIC) (White & McDermott, 2001). Upon exposing microbial communities to sub-inhibitory levels of QACs or QAC with low chemical reactivity, bacteria could potentially display changes in their susceptibility. Thus, prolonged exposure to the QAC can result in more resistant clones (Hegstad *et al*., 2010). This could also lower the susceptibility of the strain towards other antimicrobial agents.

In this study, two QACs were chosen to represent different generations of quaternary ammonium compounds; Benzalkonium chloride (BC) a first generation QAC and Didecyldimethylammonium chloride (DDAC) a fourth generation QAC. It is expected that there would be a higher tolerance towards BC since it has been used longer. DDAC is a fourth generation QAC therefore it is newer than BC. Virukill® was also used in this study, representing a commercially available QAC which contains DDAC as its active ingredient.

The aim of this chapter was to screen for the presence of *qac* resistance genes in field isolates obtained from poultry pens, as well as to determine the MICs for various QACs against the field isolates. This was done in order to relate the presence of resistance genes in field isolates to the MIC of different QACs.

Staphylococcus aureus strains known to have the individual *qac* resistance genes were used to screen for the presence of the gene when cultivated in the absence or presence of a QAC. This was done to determine if there is any difference when the cells were cultivated in the absence of and presence of a QAC.

2.2 Materials and Methods

2.2.1 Bacterial strains

Bacterial strains with known QAC resistance genes were obtained from Prof Bjorland, Department of Production Animal Clinical Sciences, Norwegian School of Veterinary Science, Oslo Norway. These strains were identified as *S. aureus*, known to contain different quaternary ammonium compound (*qac)* resistance genes. The *S. aureus* ATCC 25923 strain was obtained from the University of the Free State bacterial culture collection. Field isolates were isolated from poultry pens at the animal house at the University of the Free State, during a study conducted in 2010. Pure cultures were stored. All bacterial strains were routinely cultivated in tryptic soy broth (TSB) (30 g l^{-1}) at 37 °C for 18 hrs.

All bacterial strains were stored in microbanks (Prolab). The beads suspended in a cryopreservative solution were inoculated with single colonies and the excess

cryopreservative solution was then aspirated. The bacterial cells are bound to the porous beads and the microbanks were stored at -20 °C. The bacterial cells were reconstituted by inoculating a bead into the growth media or by streaking on an agar plate and were incubated at 37 °C for 18 hrs. Table 2.1 shows the different bacterial strains used in this study as well as the source of the strains.

Table 2.1. Description and source of bacterial strains used in this study.

Strain	Strain description	Source
VB1_qacG	Resistant (*qacG*)	Norwegian School of Veterinary Science, Oslo Norway
VB2_qacH	Resistant (*qacH*)	Norwegian School of Veterinary Science, Oslo Norway
VB3_qacJ	Resistant (*qacJ*)	Norwegian School of Veterinary Science, Oslo Norway
VB4_smr	Resistant (*smr*)	Norwegian School of Veterinary Science, Oslo Norway
Staphylococcus aureus ATCC 25923	Susceptible	University of the Free State culture collection
FI1	Gram-positive cocci	Poultry pens
FI2	Gram-positive cocci	Poultry pens
FI3	Gram-positive cocci	Poultry pens
FI4	Gram-positive cocci	Poultry pens
FI5	Gram-positive cocci	Poultry pens
Avian pathogenic *Escherichia coli* (APEC)	Gram-negative rod	Poultry pens

2.2.2 Identification of field isolates

2.2.2.1 Identification of field isolates using the 16S rDNA gene amplification

2.2.2.1.1 Genomic DNA (gDNA) isolations

Field isolates were cultivated in TSB overnight at 37 °C. Cells were then harvested for DNA extraction using an alkaline lysis method as described by Labuschagne & Albertyn (2007). The cells were lysed by resuspending in 500 µl lysis buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1% SDS) with final concentration of 35 µg ml^{-1} Lysostaphin (Sigma-Aldrich), followed by incubation at 37 °C for 20 min to activate the Lysostaphin, so as to disrupt the cell membrane. The samples were vortexed for 4 min and cooled on ice for 4 min. A volume of 275 µl ammonium acetate (7 M, pH 7.0) was added and incubated at 65 °C for 5 min followed by 5 min on ice, to denature proteins. After incubation, 500 µl chloroform was added, thereby dissolving the proteins. Subsequently, the samples were vortexed and thereafter centrifuged at 20 000 x *g* for 5 min at 4 °C. The DNA remains in the aqueous top phase, whereas the cell debris and proteins are in the organic phase or interface. The top phase containing the DNA was transferred to a clean 1.5 ml microcentrifuge tube (Eppendorf) and the DNA was precipitated by adding 1 volume of isopropanol per volume supernatant. The samples were centrifuged at 20 000 x *g* for 5 min at 4 °C. The supernatant was discarded and the pellet was washed with 70% ethanol, dried and re-dissolved in 50 µl TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) containing 10 µg ml^{-1} RNase. This was incubated at 37 °C for 1 hrs to digest any residual RNA.

2.2.2.1.2 Amplification of the 16 rDNA gene

The 16S rDNA gene was amplified using universal 16S rDNA primer pair 27F and 1492R, as listed in Table 2.2. Each PCR reaction consisted of 2 µl (40 ng) DNA template, 1 µl of 10 mM dNTP mix, 1 µl of 10 mM primer, 5 µl 10 x ThermoPol Reaction Buffer and 1 U of *Taq* DNA polymerase (New England Biolabs Inc.) made up to 50 µl with sterile Milli-Q water. Reaction was thermocycled using a G-storm Thermal cycler (Vacutec) starting with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 90 s. A final elongation step of 72 °C for 1 min was performed to allow complete elongation of product. Results were visualised on a 1% agarose gel, stained with 0.3 mg $µl^{-1}$ ethidium bromide and electrophoresed in TAE buffer (0.1 M Tris, 0.05 M EDTA [pH 8.0] and 0.1 mM glacial acetic acid) at 90 V for 35 min. Amplified fragments were observed under ultraviolet (UV) illumination (Biorad Gel Doc™ EZ Imager system).

2.2.2.1.3 Purification of PCR amplicons from agarose gels

Amplicons with bands sizes of 1500 bp were excised from the gel and purified using Illustria™ DNA and Gel Band Purification Kit (GE Healthcare) following the manufacturer's protocol without modifications. Each agarose gel piece was weighed and 10 µl of the Capture buffer type 3 was added for each 10 mg of gel, mixed and incubated at 60 °C for 15-30 min until the gel dissolved. The capture buffer changes the pH to optimise DNA binding to the silica membrane. The capture buffer sample mix was then transferred to GFX Microspin column and collection tube, and incubated for 1 min and centrifuged for 30 s at 16 000 x *g*. The spin column was washed with 500 µl Wash buffer type 1 containing absolute ethanol, to remove salts and other contaminants. The column was then centrifuged at 16 000 x *g* for 30 s. After the wash and dry step the DNA was eluted using elution buffer type 6, which consists of sterile nuclease-free water. The purified DNA was store at -20 °C, until later use.

2.2.2.2 DNA sequencing reactions

DNA sequencing reactions were performed using the BigDye terminator v3.1 Kit (Applied Biosystems). The sequencing PCR was carried out in a total volume of 40 µl for sequencing of each 16S rDNA gene. The reaction volume consisted of 4 µl premix, 1 µl of 10 mM sequencing primer, 20 ng DNA template, 6 µl Sequencing buffer and Milli-Q water to a final volume of 40 µl. Reactions were thermocycled starting with an initial denaturation step at 96 °C for 1 min. Twenty five cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and elongation at 60 °C for 4 min were carried out and then cooled at 4 °C.

The sequencing PCR for the resistance genes was carried out in a total volume of 10 µl. The reaction consisted of 10 ng PCR product, 0.5 µl premix, 1 µl (3.2 mM) sequencing primer per reaction (Table 2.2) (each reaction only contains one primer), 2 µl sequencing buffer and Milli-Q water to a final volume of 40 µl. The reactions were thermocycled at the same conditions for 16S rDNA amplification reactions.

Post reaction clean-up was performed for both 16S rDNA and resistance genes sequencing PCR products using the EDTA/ethanol precipitation method. The 16S rDNA sequencing PCR reaction was transferred to a 1.5 ml microcentrifuge tube containing 10 µl (125 mM) EDTA and 120 µl absolute ethanol. The mixture was vortexed for 5 s and precipitated at room temperature for 15 min, followed by centrifugation at 20 000 x *g* at 4 °C for 15 min. This was done in order to remove unincorporated BigDye terminators, preventing "BigDye blobs". The supernatant was completely aspirated and the pellet was washed with 60 µl of

70% ice cold ethanol and centrifuged at 4 °C for 5 min at 20 000 x *g.* The supernatant was completely aspirated and pellet was dried and sent in for sequencing

For the resistance genes, the sequencing PCR reaction was adjusted to 20 µl, transferred to a 1.5 ml microcentrifuge tube containing 5 µl (125 mM) EDTA and 60 µl absolute ethanol. The rest of the post reaction clean-up was done the same as for 16S rDNA.

Sequencing was performed with the capillary sequencer 3130xl ABI Genetic Analyzer (Applied Biosystems) at the University of the Free State, Department of Microbial, Biochemical and Food Biotechnology. Complete overlapping of complementary sequences, editing and consensus construction was performed using Geneious Pro v5.4.4 (Biomatters Ltd). Analysed sequences were compared to known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov).

2.2.3 Plasmid extractions

The *S. aureus* strains obtained from Prof Bjorland were cultivated in TSB for time periods of 2 hrs, 4 hrs, 8 hrs and 24 hrs. Cells were harvested for plasmid DNA extraction using the Zyppy™ Plasmid Miniprep Kit (Zymo Research). The plasmid DNA was extracted following the manufacturer's protocol with modification in the lysis step. The pelleted cells were resuspended in 600 µl MilliQ water and 35 µg ml^{-1} Lysostaphin was added. This was incubated at 37 °C for only 5 min, to prevent complete lysis of the cells, thereby ensuring extraction of plasmid DNA and not genomic DNA. After incubation, 100 µl of 7X Lysis Buffer was added and mixed by inverting the tube. The reaction was neutralized by the addition of 350 µl cold Neutralization buffer, changing the colour of the reaction from blue to yellow indicating complete neutralisation. This mixture was centrifuged at 16 000 x *g* for 4 min, and the supernatant was transferred to a Zymo-Spin™ II column. This was centrifuged for 15 s followed by the addition of 200 µl Endo-Wash buffer to remove denatured proteins from the resin, and centrifuged again for 15 s. The Zyppy™ Wash Buffer was added and the column was centrifuged for 30 s. The plasmid DNA was then eluted using the Zyppy™ elution buffer (10 mM Tris-HCl, pH 8.5 and 0.1 mM EDTA).

2.2.4 Amplification of the quaternary ammonium compound resistance genes

Genomic DNA was extracted from bacterial strains as described above (section 2.2.2.1.1) using the method described by Labuschagne & Albertyn (2007). PCRs were performed on the gDNA to screen for quaternary ammonium chloride (*qac)* resistance genes: *smr, qacJ, qacG,* and *qacH.* The PCRs were performed using the primers listed in Table 2.2. The PCRs were carried out in a total reaction volume of 50 µl, consisting of 5 µl DNA template, (0.2 mM) dNTP mix, (1 mM) primer, 5 µl 10 x ThermoPol Reaction Buffer and 1 U of *Taq* DNA

polymerase (New England Biolabs Inc.) made up to 50 µl with sterile Milli-Q water. Reactions were thermocycled on a G-storm Thermocycler (Vacutec) starting with an initial denaturation step at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at different annealing temperatures for the different primer sets for 30 s (see annealing temperatures listed in Table 2.2) and elongation at 72 °C for 1 min. This was followed by a final elongation step at 72 °C for 5 min and cooling at 4 °C. Results were visualised on a 1% agarose gel, stained with 0.3 mg μl^{-1} ethidium bromide and electrophoresed in TAE buffer (0.1 M Tris, 0.05 M EDTA [pH 8.0] and 0.1 mM glacial acetic acid) at 90 V for 35 min. Amplified fragments were observed under ultraviolet (UV) illumination (Biorad Gel Doc™ EZ Imager system).

Table 2.2. Sequence information of primer pairs used in the screening of the various QAC resistance genes.

Gene amplified	**Primer name**	**Primer sequence**	**Tm (°C)[1]**	**Ta (°C)[2]**
Smr	qacC rev	5'-AACGAAACTACGCCGACTATG-3'	54.9	51.9
Smr	qacC forw	5'-AAACAATGCAACACCTACCAC-3'	55.1	
qacJ	qacJ rev	5'-CGTTAAGAAGCACAACACC-3'	51.6	44
qacJ	qacJ forw	5'-GCGATTATAACTGAAATAATAGG-3'	47.1	
qacH	qacH rev	5'-GTGTGATGATCCGAATGTGTT-3'	53.1	50
qacH	qacH forw	5'-CAGTGAAGTAATAGGCAGTGC-3'	53.4	
qacG	qacG rev	5'-CTCAATTGCAACAGAAATAATCG-3'	50.7	47
qacG	qacG forw	5'-GGCTTTCACCAAATACATTTAAG-3'	50.5	
16S rDNA	27f	5'-AGAGTTTGATCMTGGCTCAG-3'	53.2	48
16S rDNA	1492	5'-ACCTTGTTACGACTT-3'	43.1	

[1]T_m: melting temperature of primer; [2]Ta: annealing temperature of primer pair (T_m-5^{o}C)

2.2.5 Determination of the MIC of QACs against bacterial strains

The minimum inhibitory concentration (MIC) of the QACs didecyldimethylammonium chloride (DDAC) (Lonza Int.), benzalkonium chloride (BC) (Fluka) and Virukill® (ICA International Chemicals) was determined against bacterial field isolates, as listed in Table 2.1, using a microtiter assay. Each well of the microtiter plate contained 100 µl Mueller Hinton broth (Merck), with a 100 µl of the QAC added to the first well of each row. Two (2)-fold serial dilutions of the QAC starting at a concentration of 10 µg ml^{-1} and diluting to 0.02 µg ml^{-1} were made. The last two wells in a row were for positive and negative controls. The bacterial field isolates were cultivated overnight in TSB and were diluted to an optical density (OD_{600nm}) reading of 0.1. The diluted cultures were then inoculated into the Mueller Hinton broth containing QAC in the microtiter plate and incubated at 37 °C for 16 hrs. The MIC was determined as the lowest concentration without growth. The MIC assays were performed in triplicate.

Minimum inhibitory concentrations were also determined for a contact time of 20 min. This was also determined using a microtiter plate containing 100 µl Mueller Hinton broth (Merck), with a 100 µl of the QAC added to the first well of each row. Again two-fold serial dilutions of the QAC starting at a concentration of 1000 µg ml^{-1} BC, 250 µg ml^{-1} DDAC and 25 µg ml^{-1} Virukill® were made. The bacterial field isolates were cultivated overnight in TSB and were diluted to an OD_{600nm} of 0.1. The plates were inoculated and incubated at 37 °C for 20 min, after which 20 µl of the culture was inoculated in 200 µl Mueller Hinton broth and incubated at 37 °C overnight. The lowest concentration where no growth was observed was recorded as the MIC. This assay was also done in triplicate.

2.3 Results

2.3.1 Screening for *qac* resistance genes

Bacterial field isolates were screened for the *qac* resistance genes *smr*, *qacJ*, *qacH* and *qacG,* present in the genome. Genomic DNA (gDNA) was extracted from the field isolates and PCRs were done to detect the genes. Since the PCR products for these genes, except *smr* gene, are approximately the same size, multiplex PCR was difficult to perform. Therefore, the PCR for each gene was performed individually. The expected band sizes were taken as being positive for the gene, and sequencing was done on four of the bands to confirm that it was indeed the gene. The expected band sizes for *smr*, *qacJ*, *qacH* and *qacG* are 157 bp, 280 bp, 289 bp and 296 bp, respectively.

Banding patterns visualised on a 1% agarose gel of the field isolates are depicted in Figure 2.1. All the field isolates screened, contained the *smr* gene. Faint bands were observed for the *qacJ* gene for all of the field isolates. The differences in the band intensities between the field isolates are due to the DNA not being standardized. The *qacH* gene is present in FI1, FI2, FI3 and FI4, but seems to be absent in FI5. The *qacG* is present in four of the field isolates but not in FI4. This indicates that a bacterial strain can contain more than one resistance gene.

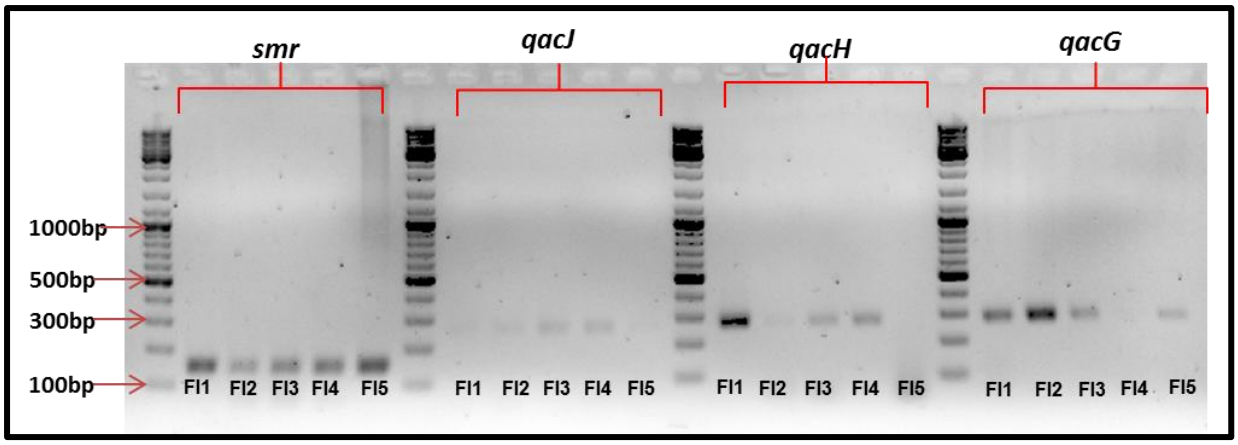

Figure 2.1. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *smr*, *qacJ*, *qacH* and *qacG* genes in the field isolates. Band sizes of approximately 157 bp, 280 bp, 289 bp and 296 bp were achieved during amplification of *smr*, *qacJ*, *qacH* and *qacG* genes, respectively. M – Fermentas GeneRule™ DNA Ladder Mix.

One PCR product for each gene was sequenced to confirm that it is indeed a *qac* resistance gene being amplified. For the *qacH*, the PCR product of FI1 was used, *qacG* FI2, whereas for *qacJ* FI4 was sequenced. For the *smr* gene FI6 was used. The amplified products were identified using NCBI blast. The results are tabulated in Table 2.3.

Table 2.3. Blast results obtained for *qac* genes isolated and sequenced from FI1, FI2, FI4 and FI6.

Gene	Accession No.	Query coverage (%)	Gene identification with NCBI BLAST search	Identity (%)	E-value
smr	AY121858.1	100	smr	100	$3e^{-35}$
qacJ	EU622635.1	100	qacJ	100	$3e^{-78}$
qacH	EU622634.1	100	qacH	99	$4e^{-90}$
qacG	Y16944.1	100	qacG	100	$2e^{-95}$

2.3.2 Minimum inhibitory concentration

The MIC for different QACs was determined with the field isolates listed in Table 2.1. The QACs used were benzalkonium chloride (BC) (Sigma-Aldrich), didecyldimethylammonium chloride (DDAC) (Lonza Int.) and Virukill® (ICA International Chemicals). The MIC test was performed in a microtiter plate, similar to the MIC test for antibiotics, and the MIC was determined for a contact time of 16 hrs as well as 20 min to the QACs. This shows the MIC of the QACs for long-term exposure and short-term exposure normally used for a disinfectant.

The long-term exposure MIC results are presented in a bar graph illustrated by Figure 2.2 for better visualisation. In this study the ATCC 25923 strain was used as the susceptible strain, and a MIC higher than the MIC of the ATCC strain was deemed resistant. Some field isolates were more susceptible to DDAC than to BC. A very low MIC was determined for Virukill®. Field isolate 5 was very tolerant to BC, but displayed lower tolerance towards DDAC and the lowest tolerance to Virukill®. However, FI5 was still more tolerant toward most of the QACs than the rest of the field isolates. Field isolate 2 showed a higher tolerance to BC than DDAC and Virukill®. Field isolates 1 and 4 showed a higher tolerance to DDAC. This was not expected since DDAC is a fourth generation QAC. When compared to the ATCC strain all the field isolates were resistant to the QACs.

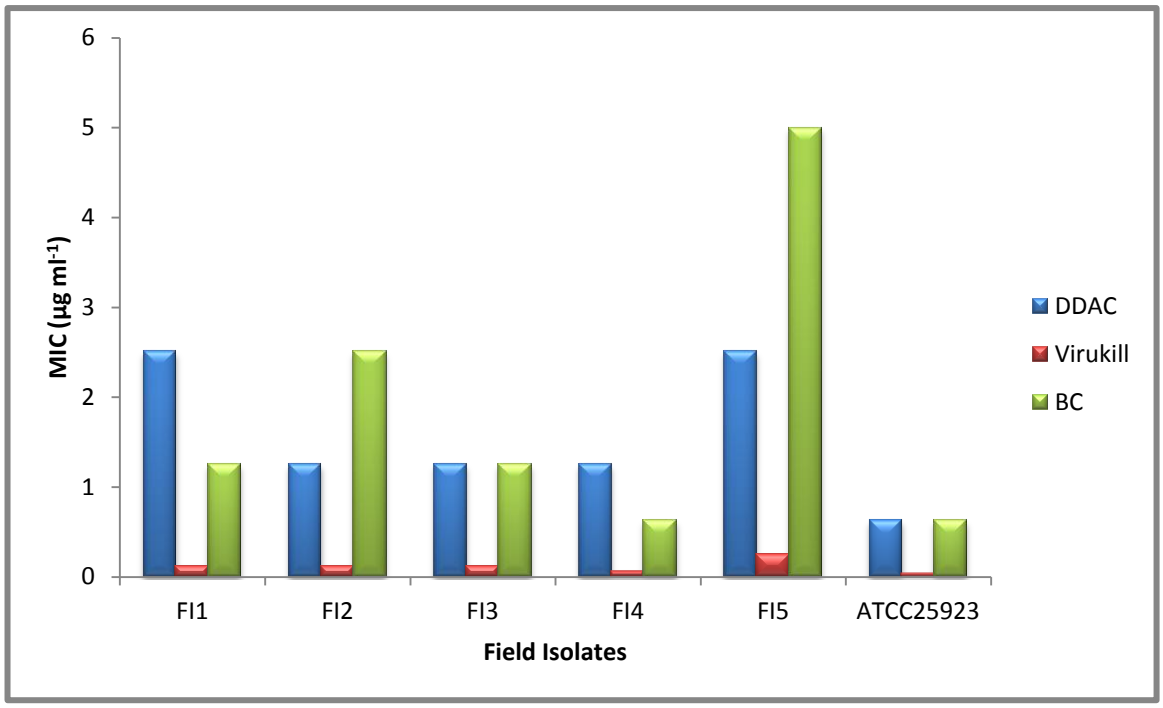

Figure 2.2. Minimum inhibitory concentration (MIC) for field isolates tested against QACs didecyldimethylammonium chloride, Virukill® and benzalkonium chloride with 16 hrs contact time.

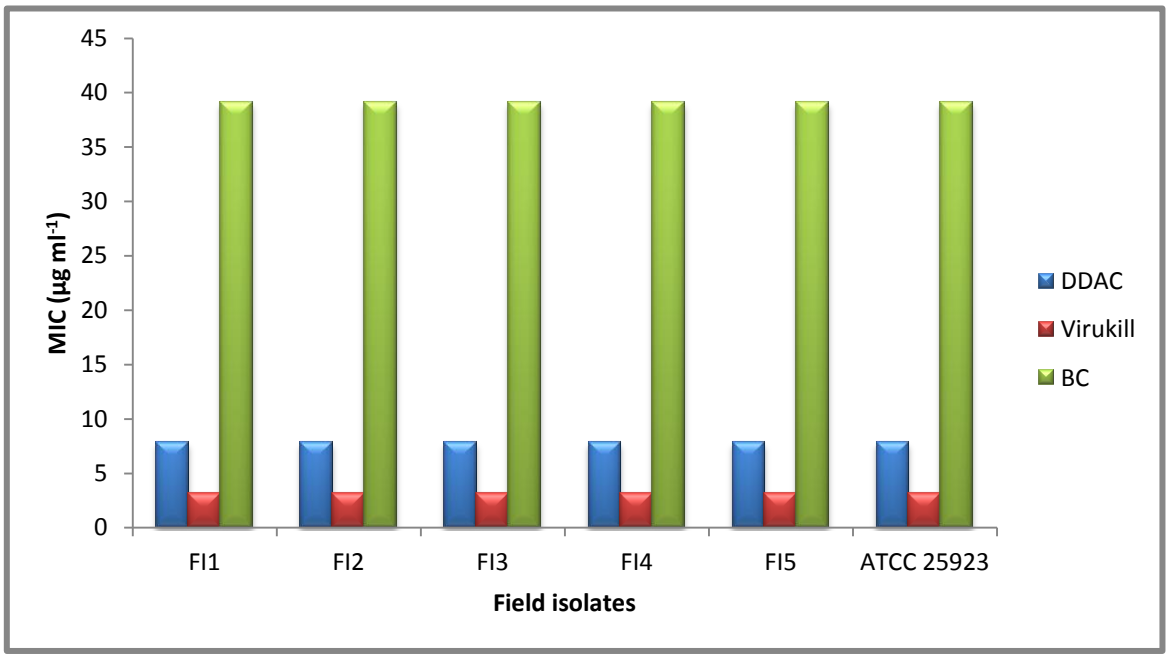

Figure 2.3. Minimum inhibitory concentration (MIC) for field isolates tested against QACs didecyldimethylammonium chloride, Virukill® and benzalkonium chloride with 20 min contact time.

The 20 min contact time exposure MIC results are presented in Figure 2.3. The MIC determinations for the different QACs were the same for the different field isolates. The concentrations were higher for the 20 min contact time than for the 16 hrs contact time. Again the MIC showed that the field isolates were more resistant to BC, whereas a lower MIC was recorded for DDAC and Virukill®, with Virukill® having the lowest MIC.

2.3.3 Identification of field isolates

The field isolates were identified using 16S rDNA sequencing. The PCR products of approximately 1500 bp were sequenced. The sequences were analysed using nucleotide BLAST tool from NCBI and the results are shown in Table 2.4. The bacterial strains were identified as different strains of *Enterococcus* (Table 2.4).

Table 2.4. Blast results for field isolates obtained from poultry pens.

Field isolate	Accession No.	Query coverage (%)	16S identification with NCBI BLAST search	Identity (%)	E-value
FI1	KF060267.1	96	*Enterococcus casseliflavus*	99	$1e^{-177}$
FI2	KF254553.1	99	*Enterococcus gallinarum*	95	0.0
FI3	KF525785.1	100	*Enterococcus gallinarum*	100	0.0
FI4	AB819042.1	98	*Enterococcus faecium*	97	0.0
FI5	EF204315.1	96	*Enterococcus faecium*	98	0.0

2.3.4 Comparison when bacterial strains are grown in the absence and presence of DDAC

Plasmid DNA was extracted from the strains obtained from Prof Bjorland cultivated in the absence and presence of DDAC. The *smr*, *qacJ*, *qacH* and *qacG* genes were amplified in these strains. The *smr* gene was amplified in all of the strains in the absence of DDAC, see Figure 2.4. The same size gene was observed in the *E. coli* strain, which is not unexpected since there are also *qac* resistance genes in Gram-negative bacteria that are similar to the

smr gene. There was also a larger fragment in the *E. coli* strain amplified. The *smr* gene was detected in the ATCC strain, which is the susceptible control strain. Different degrees of band intensities were obtained which was expected since the DNA was not standardised. The *smr* was also present in all of the strains in the presence of DDAC (Figure 2.5). The bands seems to be brighter in VB4_smr strain, however the DNA was not standardised.

Similar results are observed with the amplification of the other genes, with the difference being that in the absence of DDAC (Figure 2.6, Figure 2.8, Figure 2.10), the different genes are only detected in that specific strain. In the presence of DDAC (Figure 2.7, Figure 2.9, Figure 2.11), the genes are detected in almost all the strains, with brighter bands in the respective strains. However this is not conclusive since the DNA was not standardised before the PCR. It seems that the *E. coli* and ATCC strains also contain the different genes, except the *qacJ* gene. This again shows that it is possible for one strain to have more than one resistance gene.

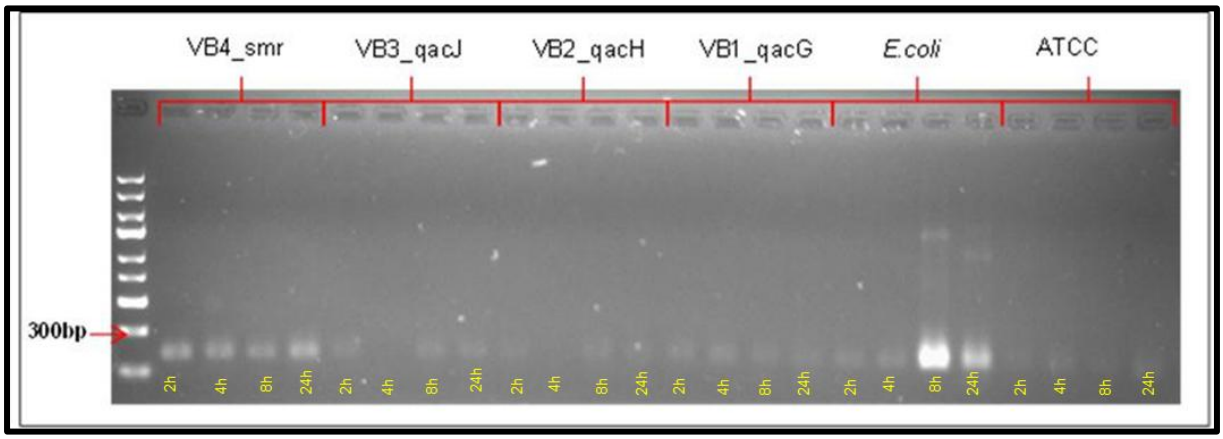

Figure 2.4. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *smr* gene using qacC forw and qacC rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the absence of DDAC. The expected band size of 157 bp was achieved during amplification of all the strains. The band was also detected in the *E.coli* strain, an additional larger sized fragment was also observed for the *E.coli* strain.

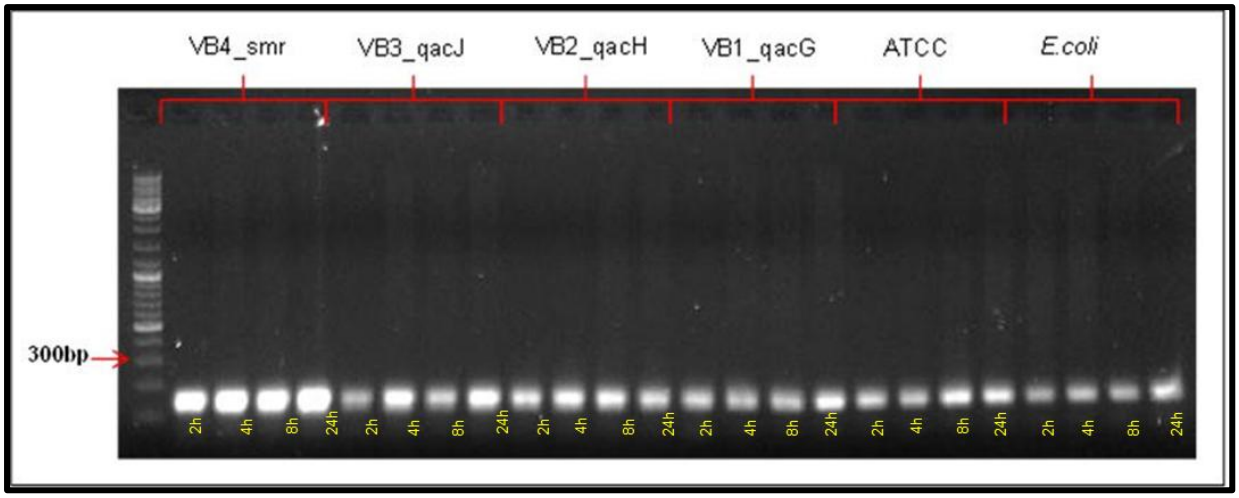

Figure 2.5. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *smr* gene using qacC forw and qacC rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the presence of DDAC. The expected band size of 157 bp was achieved during amplification of all the strains. The band was also detected in the *E.coli* strain.

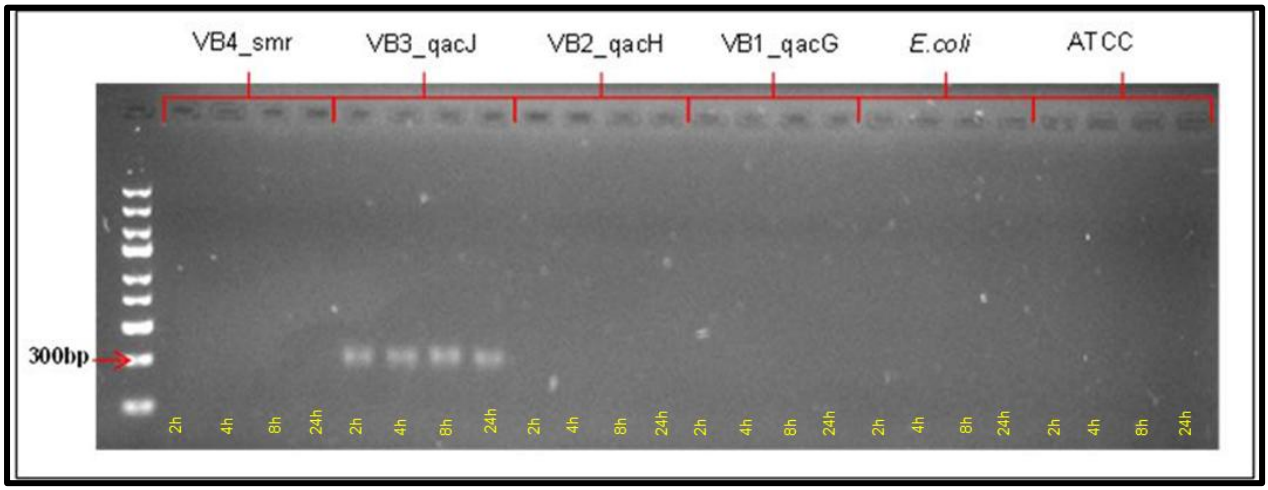

Figure 2.6. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacJ* gene using qacJ forw and qacJ rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the absence of DDAC. The expected band size of 280 bp was achieved during amplification only in VB3_qacJ strain.

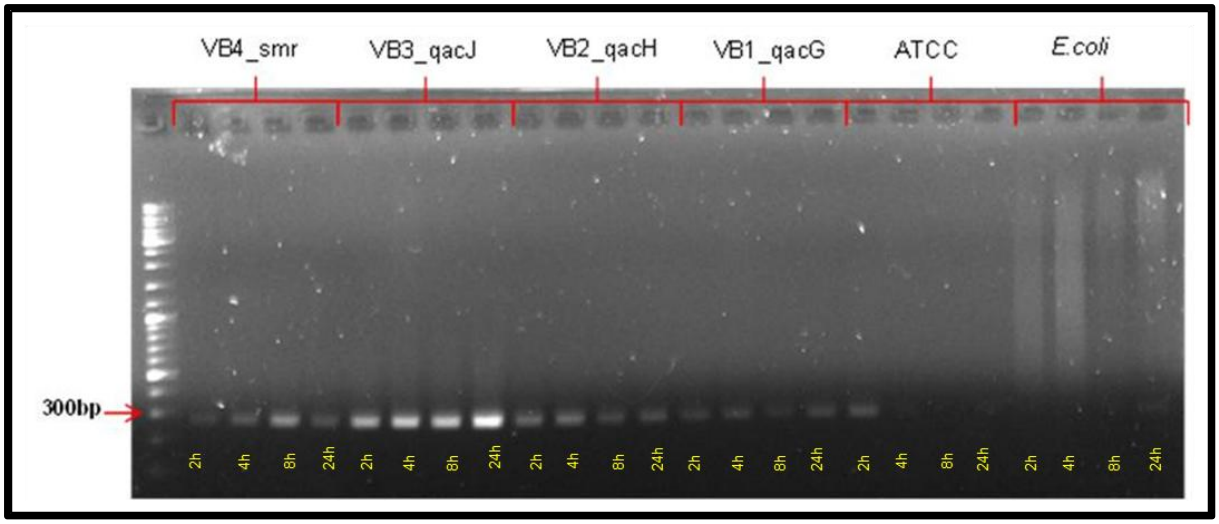

Figure 2.7. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacJ* gene using qacJ forw and qacJ rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the presence of DDAC. The expected band size of 280 bp was achieved during amplification in all the strains, however very faint bands are seen for the ATCC and *E.coli* strains.

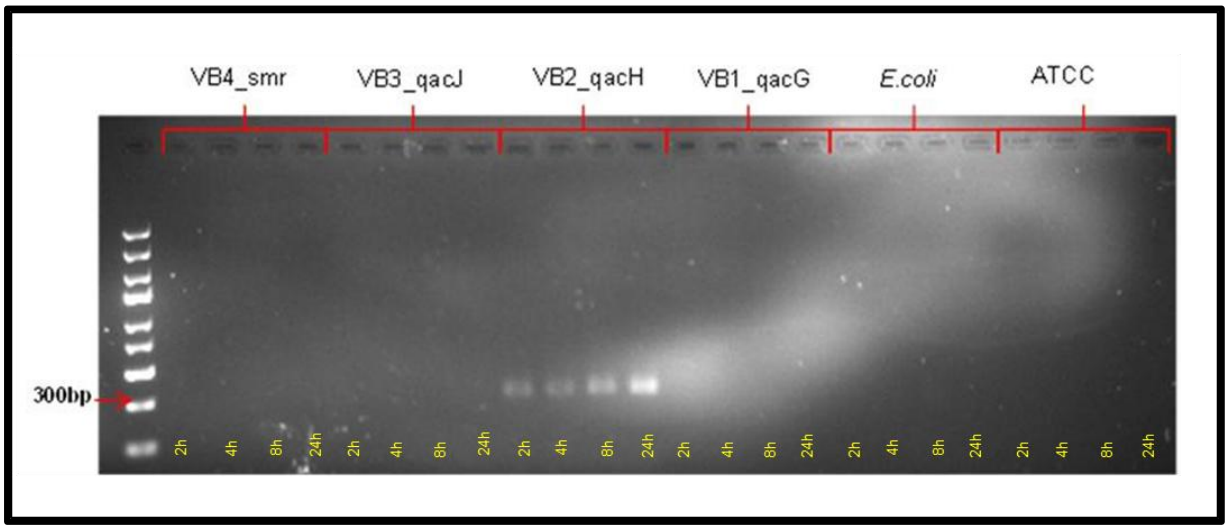

Figure 2.8. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacH* gene using qacH forw and qacH rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the absence of DDAC. The expected band size of 289 bp was achieved during amplification only in VB2_qacH strain.

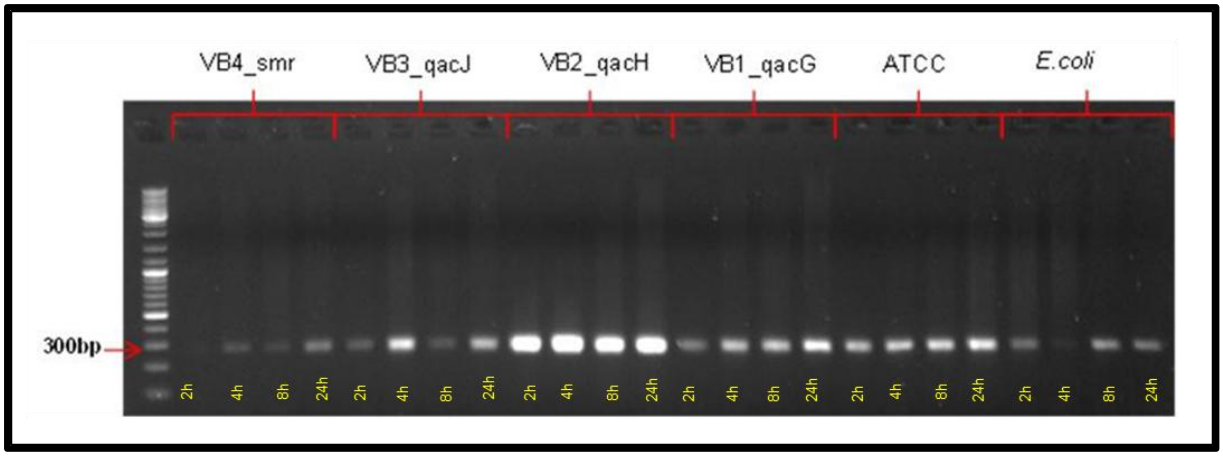

Figure 2.9. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacH* gene using qacH forw and qacH rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the presence of DDAC. The expected band size of 289 bp was achieved during amplification in all the strains.

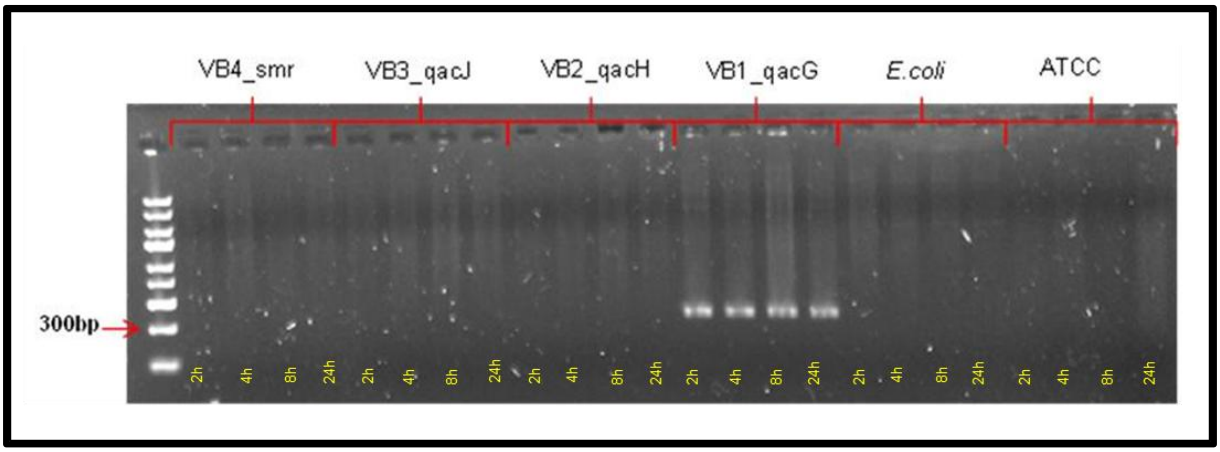

Figure 2.10. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacG* gene using qacG forw and qacG rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the absence of DDAC. The expected band size of 296 bp was achieved during amplification only in the VB1_qacG strain.

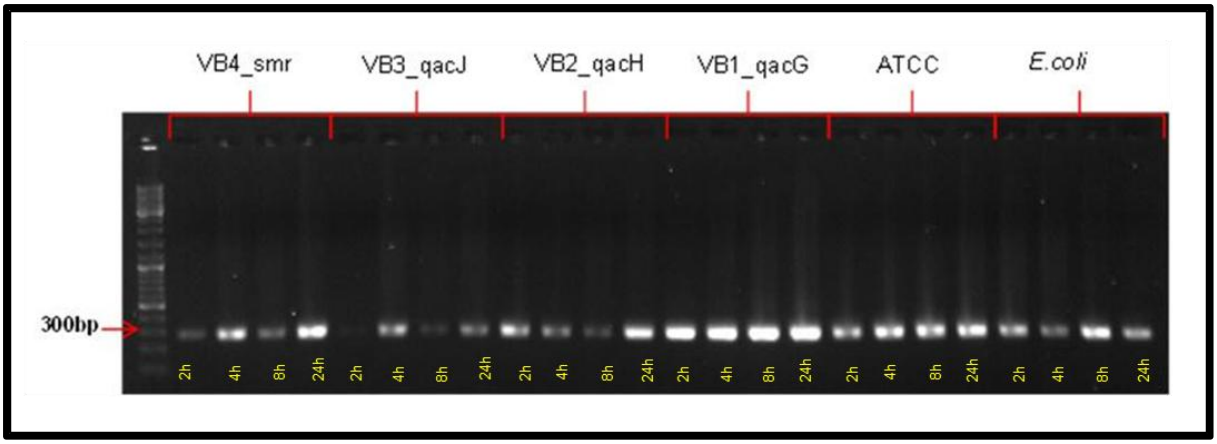

Figure 2.11. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *qacG* gene using qacG forw and qacG rev primers. The PCR products are from plasmid DNA extracted from cells cultured in the presence of DDAC. The expected band size of 296 bp was achieved during amplification in all the strains.

2.4 Discussion

Bacterial resistance against QACs has already been reported and studied in *S. aureus* strains (McBain *et al*., 2004). The resistance is thought to be due to the acquisition of plasmids containing the resistance genes (Hegstad *et al*., 2010). These resistance genes code for proteins that are able to export the QAC out of the bacterial cell (McDonnell & Russell, 1999). As these are multidrug transporter proteins, they have a wide range of substrates that can bind to them (McDonnell & Russell, 1999). In this study, the genes *smr*, *qacJ*, *qacH* and *qacG*, coding for the small multidrug resistance (SMR) proteins were studied. The proteins expel the QACs via a proton-driven efflux system (Piddock, 2006). They actively transport toxins and disinfectants out of the cell, decreasing the concentration of the compounds inside the cell (Piddock, 2006). They are also one of the smallest transporters to actively transport compounds (Putman *et al*., 2000; Marquez, 2005).

Field isolates obtained from poultry pens were screened for the respective *qac* resistance genes *smr*, *qacJ*, *qacH* and *qacG*. Upon amplification, products were then sequenced and identified. The strains were also sequenced and were identified as different species and strains of *Enterococcus*. From the results it was shown that one strain can contain more than one resistance gene, with some field isolates containing more resistance genes than the other strains. This correlates with studies done by Gillings and co-workers (2009a, b), showing that *qac* gene cassettes are found on class 1 integrons. They have recovered *qac* gene cassettes from environmental samples, making this the most common cassette type to

be recovered. This indicates that microorganisms can possess the *qac* resistance genes even in the absence of QACs. Gilbert and McBain (2003) also suggested that the efflux pumps help protect the microorganism against toxins in natural ecosystems. However, it remains uncertain what the natural targets are for these *qac* efflux systems (Gillings *et al*., 2009a).

The minimum inhibitory concentration of the QACs to the field isolates was determined in order to correlate the number of resistance genes present to the MIC. The MICs for a contact time of 16 hrs indicated that there was an increase in tolerance in some of the field isolates when compared to the susceptible strain ATCC 25923. However, one would expect that the field isolates would be highly tolerant towards the QACs since they have multiple QAC resistance genes. The number of *qac* resistance genes present could not be related to MIC. For example, FI5 showed higher tolerance to all the QACs than the other field isolates although not all resistance genes screened for, were detected compared to the other field isolates, where more genes was detected. Therefore, it could be hypothesised that a greater number of resistance genes present does not necessarily indicate higher resistance. The proteins coded for by these genes have a wide range of substrates. Some of the MIC results obtained were unexpected. Isolates FI1 and FI4 showed a higher tolerance towards DDAC with isolate FI3 showing the same tolerance towards DDAC and BC. This is unexpected because DDAC is a fourth generation QAC, therefore the newest QAC. Thus one would expect that it would have a lower MIC than BC. It is expected that Virukill® would have the lowest MIC, since DDAC is the active ingredient in Virukill®. It is claimed by the manufacturers of Virukill® that the DDAC used as the active ingredient in Virukill® has been modified and that these modifications have increased efficacy but reduced toxicity (Bragg, personal communication, 2012). The results obtained in these experiments clearly indicate that the efficacy of Virukill® is superior to that of the DDAC. Isolates FI2 and FI5 gave the expected results with the strains being more tolerant to BC.

The MIC was also determined for these QACs with a 20 min contact time. This contact time was used since it is the contact time normally regarded as a standard contact for use of disinfectants in the poultry industry. The MIC recorded with the 20 min contact time for the different QACs are higher than with the 16 hrs contact time. This was as expected, and one of the main factors influencing the efficacy of a disinfectant is contact time. The longer the contact time, the more effective the disinfectant will be. However, these concentrations are still much lower than the concentration used in the industry.

The *S. aureus* strains obtained from Prof Bjorland (Norway) were screened for the resistance genes, *smr*, *qacJ*, *qacH* and *qacG*, in the absence and presence of DDAC. In the

absence of DDAC the genes were only amplified in the respective strains known to contain the individual genes except for the *smr* gene which was amplified in all of the strains, even in the susceptible strain ATCC 25923. Furthermore the gene was amplified in the *E. coli* strain, which is expected since there is a *qac* resistance gene very similar to the *smr* gene found in Gram-negative bacteria. However, another gene was amplified in the *E. coli* strain, which is larger than the gene similar to *smr* gene that was amplified. Gram-negative organisms have a more complex cell membrane, and more proteins or larger proteins might be involved in the efflux, since the efflux pumps need to transport the QAC through two membranes (Kumar & Schweizer, 2005). Therefore the larger band observed in *E. coli* might be another *qac* resistance gene, which have similar sequences to the *smr* gene, and the primers are able to bind. However, no additional tests were conducted to identify this gene. When the *E. coli* strain was cultivated in the presence of DDAC the bigger band was not visible. It is possible that this larger gene was on another plasmid that was in *E.coli*, however it might have been lost, and therefore the gene was not detected.

When DDAC was present in the growth media, the different genes were amplified in most of the strains at different sampling time points, even in the susceptible strain. It is expected that the genes would be detected regardless of DDAC being present. Therefore, the expression of gene mRNA needs to be investigated. During a PhD study done, Jansen (2012) showed that in the absence of DDAC resistance genes were only detected in their respective strains when amplifying with conventional PCR. However, when amplifying with real-time PCR which is more sensitive, more resistance genes were detected in the strains.

It could be hypothesised that the plasmid containing the resistance gene was lost when there was no selective pressure from the DDAC and that the respective gene from that strain had the gene integrated into the genome. It is possible that some genomic DNA was also extracted with the plasmid DNA; therefore it is possible that the gene was integrated into that strain. However, when there is selective pressure the plasmids are retained by the bacteria and the different genes can be detected using PCR. It seems that the genes of the respective strains could be inherent to that strain, thus it is present even when there is no pressure from a QAC.

It is important to be able to screen field isolates for the presence of resistance genes, and to determine if the bacteria are becoming more tolerant to the disinfectants used. Screening bacteria for the presence of *qac* resistance genes in the poultry pens are vital, since disinfectants and antibiotics are frequently used in these environments. It is also assumed that there is a link between antibiotic resistance and QAC resistance (Hegstad *et al*., 2010). It is thus important to monitor the progress of the resistance and to try and avoid using the

two antimicrobials in conjunction. If the microorganism comes in contact with both it is possible for it to obtain resistance to one or both. It is also important that special care should be taken when using antimicrobials and that the correct dosage must be used, since exposing bacteria to sub-lethal levels might produce resistant clones which can survive (Hegstad *et al*., 2010).

These *qac* resistant genes are seen in different bacterial species, and there are differences in the sizes of these genes. However, most of these genes form proteins that are part of the SMR family (Bjorland *et al*., 2005). There are conserved regions in the amino acid sequences which can be used to identify these genes. The major problem with these genes are that they are found on mobile elements, such as plasmids and transposons, and some of these mobile elements can be transferred to the same species or different bacterial species (Hegstad *et al*., 2010). Therefore, these genes are able to spread quickly between bacterial species and confer resistance. Correct disinfectant use is crucial to controlling the bacterial population and may minimize the use of antibiotics. Therefore, knowledge of the correct use of antimicrobials is of utmost importance.

Chapter 3 Determining the expression of *qacJ* gene in *S. aureus* using Real-Time PCR

3.1 Introduction

Bacteria have the ability to acquire resistance genes, allowing them to survive harsh environments (White & McDermott, 2001; Ioannou *et al*., 2007). This can cause an increased tolerance against antimicrobial agents, including quaternary ammonium compounds (QACs). The *qac* resistance genes, code for multidrug efflux pumps which form part of the small multidrug resistance (SMR) family (Bjorland *et al*., 2005). These proteins are capable of expelling structurally diverse compounds out of the bacterial cell (Bjorland *et al*., 2005). The genes coding for the proteins forming part of the SMR family are *smr*, *qacJ*, *qacH* and *qacG* (Bjorland *et al*., 2005). These genes have been found in clinical and environmental samples (Hegstad *et al*., 2010). Limited knowledge is available on these *qac* resistance genes, as they have not been extensively studied.

Real-time PCR has been widely used for quantification of gene expression and relative quantification can be used to determine the relative expression of a target gene (Pfaffl, 2001). Quantitative real-time PCR could therefore be used to quantify the expression of the *qac* resistance genes because the relative change in mRNA expression levels can be measured. Physiological changes in gene expression can be investigated as RNA is transiently expressed. This means that the expression levels can vary according to the cell type, developmental stage, physiology and pathology (Scott Adams, 2006).

Relative quantification requires equations to determine the relative expression of the gene (Livak & Schmittgen, 2001). The changes in the sample gene expression are measured based either on an external standard or reference sample, also known as a calibrator (Livak & Schmittgen, 2001; Wong & Medrano, 2005). Usually the calibrator is from an untreated sample and therefore the expression of the genes can be compared to the calibrator (Arya *et al*., 2005; Wong & Medrano, 2005). The target gene is then normalised using reference genes, to compensate for the variation between samples, allowing comparison amongst samples (Wong & Medrano, 2005). Reference genes are utilised because they are usually needed for cell maintenance or they are constitutively expressed (Wong & Medrano, 2005). The expression of at least three reference genes are used since it is a more reliable basis of normalisation (Pfaffl, 2006).

The relative quantification ratio can be calculated using three general procedures (Pfaffl, 2006). Firstly, it can be calculated without any efficiency correction; therefore the amplification efficiency would be taken as 2, meaning that with each cycle the amount of target gene amplified doubles (Livak & Schmittgen, 2001). The other two procedures include efficiency correction, either with one or multiple samples, or models based on multiple samples and multiple reference genes (Pfaffl, 2004).

In this chapter it was hypothesised that the expression of the *qacJ* gene would increase with the increase in didecyldimethylammonium chloride (DDAC) concentration. Relative quantitative real-time PCR was used to measure the relative expression of the *qacJ* gene against a set of reference genes in the presence of different QAC concentrations. In this study, the Pffafl method was used to determine the relative expression of the *qacJ* gene, using efficiency correction and normalisation based on multiple reference genes. The expression for the *qacJ* gene was determined for a QAC tolerant *Staphylococcus aureus* strain VB3_qacJ and a QAC susceptible *S. aureus* ATCC 25923 strain.

3.2 Materials and Methods

3.2.1 Cultivation of *Staphylococcus aureus*

Staphylococcus aureus strains (QAC resistant strain VB3_qacJ and the QAC susceptible strain ATCC 25923) were routinely cultivated in tryptic soy broth (TSB) for approximately 18 hrs. Subsequently, Mueller Hinton broth was inoculated with these strains and grown for 3 hrs, after which different concentrations of DDAC were added to the cultures. The concentrations of DDAC used were; 0.00 µg ml^{-1} (control), 0.01 µg ml^{-1} DDAC; 0.1 µg ml^{-1} DDAC and 1 µg ml^{-1} DDAC. After another 2 hrs, the cells were harvested and total RNA was extracted in duplicate. The sample names are given in Table 3.1.

Table 3.1. Description of the samples extracted from different growth conditions.

Sample	Sample Description
J 0	VB3_qacJ strain in absence of DDAC (used as calibrator)
J 0.01	VB3_qacJ strain in 0.01µg ml^{-1} DDAC
J 0.1	VB3_qacJ strain in 0.1µg ml^{-1} DDAC
J 1	VB3_qacJ strain in 1µg ml^{-1} DDAC
ATCC 0.01	ATCC strain in 0.01µg ml^{-1} DDAC
ATCC 0.1	ATCC strain in 0.1µg ml^{-1} DDAC
ATCC 1	ATCC strain in 1µg ml^{-1} DDAC

3.2.2 Total RNA extraction

Total RNA was extracted using the RNeasy® Minikit (Qiagen), following the manufacturers protocol for RNA extraction from yeast cells with slight modifications for application on Gram-positive bacteria. The RNeasy® Minikit columns have a silica-based membrane which has selective binding properties allowing RNA to bind to the column, contaminants to be washed away, and then RNA to be eluted from the column. Cells were harvested in a 1.5 ml microcentrifuge tube (Eppendorf) by centrifugation (Eppendorf Centrifuge 5417R) at 1000 x *g* for 5 min at 4 °C and the supernatant was removed. The bacterial cells were lysed using enzymatic lysis, therefore the cells were resuspended in Buffer Y1, a yeast lysis buffer (1 M sorbitol; 0.1 M EDTA, pH 7.4; 0.1% β-mercapto-ethanol) containing 35 µg ml^{-1} Lysostaphin. Incubation was carried out for 20 min at 37 °C, after which 350 µl buffer RLT (complete buffer composition is unknown) was added and the samples were vortexed. Buffer RLT contains a high concentration of guanidine isothiocyanate which aids in the disruption and lysis of cells and it supports the binding of RNA to the silica membrane, which is positively charged, of the column. It also contains β-mercapto-ethanol, which effectively inactivates the RNases in the lysate, to ensure the isolation of intact RNA. If insoluble material was still visible, the sample was centrifuged for 2 min at full speed (20817 x *g*). Only the supernatant was used in the subsequent steps. The lysate was homogenised by adding 250 µl of 100% ethanol, which provided the appropriate binding conditions. The sample was transferred to the RNeasy spin column which captures the RNA, and centrifuged at 8000 x *g* for 20 s. Buffer RW1 (complete buffer composition is unknown) was then added to the spin column

and centrifugation was carried out at 8000 x *g* for 15 s to wash the spin column membrane. Buffer RW1 contains guanidine salts and ethanol, thereby acting as a stringent washing buffer that efficiently removes biomolecules e.g. carbohydrates, proteins and fatty acids that are non-specifically bound to the column.

An on-column DNase digestion was performed to eliminate possible gDNA contamination. DNase I incubation mix (10 µl DNase I, 70 µl buffer RDD) was added directly to the RNeasy spin column and was incubated at room temperature for 15 min. The spin column was then washed with Buffer RW1 to remove the DNase I. Buffer RPE (complete buffer composition) was then added and centrifuged at 8000 x *g* for 15 s to wash the spin column membrane, and this step was repeated however, centrifugation was carried out for 2 min to wash and dry the spin column membrane. Buffer RPE is a mild washing buffer that removes traces of salts. The column was placed in a clean 1.5 ml collection tube; 30 µl RNase-free water was added to the spin column and centrifuged for 1 min at 8000 x *g* to elute the RNA. The RNA was stored at -80 °C, for later use.

3.2.3 Analysis of the concentration and purity of RNA, using a Nanodrop ND-1000 spectophotometer

The concentration and purity of the total RNA extracted, using the method described above, was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The apparatus was blanked with RNase free water and measurements were performed by dropping 2 µl of total RNA containing sample onto the Nanodrop. The concentrations were recorded in ng μl^{-1}. The RNA purity was assessed based on the A_{260} / A_{280} ratio, with a ratio of ≥ 1.8, being accepted as RNA devoid of contaminants such as proteins.

3.2.4 Preparation of cDNA

First-strand cDNA was synthesised using the high capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer's protocol, without modification. The RNA was reverse transcribed to form cDNA. The reverse transcription reaction was performed on 500 ng total RNA. The reverse transcription reaction mix contained 10 µl RT buffer (2x), 1 µl enzyme mix (20x), 500 ng total RNA, and nuclease-free water to a final volume of 20 µl. The RT buffer contained both random hexamers and oligo-dT primers and the enzyme mix contained the reverse transcription enzyme. The oligo-dT primers produce cDNA that begins at the poly-A tail, while the random hexamers anneal at a variety of positions on the mRNA and produce a variety of products. Since reverse transcriptase does not usually reach the 5' end of long mRNAs, random primers are beneficial, increasing the probability that the 5' ends would be converted to cDNA. The reaction mix was incubated at 37 °C for 60 min for

the reverse transcription to occur and the enzyme was inactivated at 95 °C for 5 min followed by cooling at 4 °C. The intact cDNA was stored at -20 °C for later use. The concentration of the cDNA was measured using a Nanodrop ND-1000 spectrophotometer, following the methods as outlined in section 3.2.3.

3.2.5 Primers

Three different sets of primers for *qacJ* were used to optimise the primers, to obtain the set with the lowest Cp value (Table 3.2). The Cp value is the cycle number where fluorescence increases to a much higher level than the baseline levels. Set A was previously used in Chapter 2. The combination of primers AB, were used in the rest of the study (Table 3.2).

All primers used for this study are shown in Table 3.3 as well as their primer sequences. Primer sequences for the reference genes were obtained from literature (Theis *et al*., 2007). The three reference genes used, formed part of a previous study (Jansen, 2012). These primers were based on the Pyrroline-s-carboxylate reductase (*proC*), DNA gyrase A (*gyrA*) and transcription termination factor Rho (*rho*) genes. These genes are involved in amino acid biosynthesis, replication and transcription, respectively.

Table 3.2. *qacJ* primers tested to determine which set of primers gives optimum results. The primers shown in bold were used in the rest of the study.

qacJ	Primer sequence	Tm (°C)
A Forward	5’-GCGATTATAACTGAAATAGG-3’	47.1
A Reverse	**5’-CGTTAAGAAGCACAACACC-3’**	51.6
B Forward	**5’-GGCCAACATTAGGCACACTTA-3’**	60.6
B Reverse	5’-TGACTTGATCCAAAAACGTTA-3’	54.8
C Forward	5’-GGCCAACATTAGGCACACTT-3’	53.8
C Reverse	5’-CGTTAAGAAGCACAACACCAA-3’	52.8

Table 3.3. Primers used for this study for relative quantification.

Gene amplified	Primer name	Primer sequence	Tm (°C)	Ta (°C)
qacJ	qacJ rev	5'-CGTTAAGAAGCACAACACC-3'	51.6	47
qacJ	qacJ forw	5'-GGCCAACATTAGGCACACTTA-3'	60.6	
gyrA	gyrA rev	5'-CTTGACCACTTGTTAAGCG-3'	51.8	47
gyrA	gyrA forw	5'-CAATGATTGCTGTTAAAGACC-3'	51.0	
proC	proC rev	5'-TTTTCTAGACCAAGTTTCGTACC-3'	53.6	47
proC	proC forw	5'-CAAACAACTTTGACCCTAAATCTA-3'	53.3	
Rho	rho rev	5'-GGTCAACGTGGTTTAATAGTG-3'	51.7	47
Rho	rho forw	5'-TCTGTTACCTCTTCAGGACG-3'	51.9	

3.2.6 Screening of cDNA for the presence of reference genes

Conventional PCR was used to screen for the reference genes. The total volume of the reaction mixture was 50 µl containing 1 µl cDNA (undiluted), 0.2 mM dNTP mix, 0.4 mM primer, 5 µl 10 x Thermopol Reaction Buffer (New England Biolabs Inc.) and 1 U of *Taq* DNA polymerase (New England Biolabs Inc.) made up to 50 µl with sterile Milli-Q water. Reactions were thermocycled using a Gstorm Thermocycler (Vacutec) with initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s and extension at 72 °C for 1 min. Final elongation was conducted at 72 °C for 5 min and then the reaction was cooled to 4 °C. Amplified fragments were observed under ultraviolet (UV) illumination on a 1% agarose gel stained with ethidium bromide.

3.2.7 Real-Time PCR

All real-time PCR experiments performed in this study are compliant with the MIQE guidelines (Bustin *et al*., 2009). These guidelines describe the minimum information that is necessary for evaluating qPCR experiments. This is to help ensure the integrity of scientific literature, promote consistency between laboratories, and increase experimental

transparency. Reviewers can assess the validity of the protocol, since all relevant experimental conditions and assay characteristics are provided.

3.2.7.1 Qualitative detection

Qualitative real-time PCR was performed to screen for the presence of *smr, qacJ, qacH* and *qacG* genes in the *S. aureus* strains: VB1_qacG, VB2_qacH, VB3_qacJ, VB4_smr and ATCCT 25923 as well as the avian pathogenic *Escherichia coli* (APEC) strain. The APEC strain represents Gram-negative bacteria. Real-time PCR was performed with the LightCycler® 2.0 instrument (Roche Diagnostics) with the *qac* primers described in Chapter 2 (Table 2.2). The optimised reaction mixture consisted of 1 µl DNA (DNA used in Chapter 2), 0.5 µM primer mix (Table 2.2), 4 µl master mix (LightCycler® FastStart DNA Master^{PLUS} SYBR® Green I, Roche Diagnostics) and water up to 20 µl. Reaction for each gene was done in separate capillaries. The real-time PCR conditions consisted of activating the *Taq* DNA polymerase at 95 °C for 10 min followed by 45 amplification cycles of denaturation at 95 °C for 10 s, annealing at 47 °C (*qacJ* and *qacG*) and 50 °C (*smr* and *qacH*) for 10 s and elongation at 72 °C for 10 s. The amplification of the genes was monitored during the PCR run by measuring the SYBR® Green fluorescence. The SYBR® Green binds to dsDNA and this DNA-dye complex results in fluorescence increase which increases the more dsDNA there are.

The reaction conditions for the melting curves were as follows: denaturation at 95 °C, followed by a decrease in the temperature at a ramp rate of 20 °C s^{-1} to 65 °C and then an increase in the temperature to 95 °C at a ramp rate of 0.1 °C s^{-1}, to form single stranded DNA again. The fluorescence was continuously monitored. The temperature was then decreased to 40 °C at a ramp rate of 20 °C s^{-1} and held at this temperature for 30 s. This allowed the single stranded PCR product to anneal and form double-stranded PCR product.

3.2.7.2 Standard curves for reference genes and qacJ gene

Standard curves were generated for each of the reference genes as well as the *qacJ* gene to obtain the amplification efficiency of the PCR reaction. Since PCRs do not always have an ideal efficiency, the relative quantification is calculated using efficiency correction so as not to overestimate the starting concentration (Wong & Medrano, 2005). Therefore, the amplification efficiency must be determined beforehand.

All the samples were screened for these genes using conventional PCR. Optimal primer concentration for each of the genes was obtained to minimise the formation of primer-dimers using real-time PCR (LightCycler® 2.0, Roche Diagnostics). The standard curves were constructed using the cDNA from the calibrator (untreated) sample. Two-fold dilutions were

made of the undiluted cDNA, with the concentration of the undiluted cDNA being measured using the Nanodrop-1000. The dilution series were made in triplicate for each gene, consisting of a six series dilution range. The PCR reaction was set up for each reference gene as well as the gene of interest (GOI) (*qacJ*). The PCR was performed with the LightCycler® 2.0 instrument (Roche Diagnostics). The optimised reaction mixture consisted of 1 µl cDNA, 0.2 µM primer mix for reference genes and 0.4 µM primer mix for *qacJ* gene, 4 µl master mix (LightCycler® FastStart DNA Master^{PLUS} SYBR® Green I, Roche Diagnostics) and water up to 20 µl. Reaction for each gene was done in separate capillaries. The real-time PCR conditions consisted of activating the *Taq* DNA polymerase at 95 °C for 10 min followed by 45 amplification cycles of denaturation at 95 °C for 10 s, annealing at 47 °C for 10 s and elongation at 72 °C for 10 s. The amplification of the genes was monitored during the PCR run by measuring the SYBR® Green fluorescence.

After the real-time PCR run, absolute quantification analysis was conducted to generate the standard curve of each gene, thereby obtaining the PCR efficiency. Melt curve analysis was conducted as described in section 3.2.7.1. The primer sequences, melting temperatures and annealing temperatures for the primer sets are listed in Table 3.3.

3.2.7.3 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was conducted as described in section 3.2.7.2. The calibrator sample was included in each real-time PCR run, and all the genes were amplified in all the samples. The Cp values were determined for all the samples, for subsequent analysis.

Melting curve analysis was conducted during the PCR process as described in section 3.2.7.1 to help detect primer-dimers, non-specific or mismatched sequences, as these generate a lower melting temperature than the specific product of the reaction (Keer, 2008). Therefore, multiple peaks would show multiple products that were formed during the PCR.

3.2.8 Analysis of the real-time PCR data

The data generated by the real-time PCR was used to determine relative expression of the *qacJ* gene in the presence of different DDAC concentrations. The Cp values obtained were used to calculate the relative expression. The Pfaffl model was used, since gene quantification and normalisation is combined in a single step according to the calculation below:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(\text{control}-\text{sample})}}$$

Equation 3.1. Pfaffl model. *E* = efficiency; ΔCP= difference between control and sample.

3.3 Results

3.3.1 Qualitative detection

In Chapter 2 it was shown that the *qac* resistance genes were detected in the VB1_qacG, VB2_qacH, VB3_qacJ and VB4_smr strains when cultivated in the presence of DDAC. However, when it was cultivated in the absence of DDAC, only the gene corresponding to that strain was detected using conventional PCR. In this study, however, the use of real-time PCR showed that the *qac* resistance genes could be detected in the absence of DDAC. Table 3.4 shows the results obtained from the LightCycler® software. From Table 3.4 it can be seen that the Cp value was the lowest for the gene to the corresponding strain. The different genes are detected in almost all the strains, except the *qacH* gene, which was only detected in the VB1_qacG and VB2_qacH strains.

Table 3.4. Real-time PCR results for the qualitative detection of *qac* resistance genes in the different *Staphylococcus aureus* strains. Crossing point (Cp) as well as melting temperature (Tm) is indicated.

Strain	***Smr***		***qacH***		***qacJ***		***qacG***	
	Cp	**Tm**	**Cp**	**Tm**	**Cp**	**Tm**	**Cp**	**Tm**
VB1_qacG	23.27	78.32	27.89	69.14	22.60	77.40	7.64	77.49
VB2_qacH	30.27	78.27	28.42	76.14	27.00	77.23	31.98	78.70
VB3_qacJ	29.99	78.19	-	-	11.47	77.40	27.58	77.64
VB4_smr	9.22	78.21	-	-	27.84	77.22	28.88	78.07
ATCC	30.54	77.99	-	-	29.87	77.30	32.48	78.15
APEC	25.61	78.07	-	-	27.67	77.21	30.00	77.63

3.3.2 RNA isolation from *S. aureus*

The RNeasy® minikit (Qiagen) was used to extract RNA from the *S. aureus* VB3_qacJ and ATCC 25923 strains. The concentrations of total RNA obtained from each strain are listed in Table 3.5. The RNA was then transcribed to cDNA for real-time PCR use.

The Nanodrop was used to determine the concentration of the RNA and to assess the purity of the RNA. The ratio at 260 and 280 nm is used to assess the purity of the RNA. A ratio of approximately 2.0 is generally accepted as relatively pure for RNA. A ratio much lower than 2.0 would indicate the presence of protein, phenol or other contaminants that absorb at 280nm. The 260/230 ratio is a secondary measure of the nucleic acid purity. Pure nucleic acid usually has a higher 260/230 ratio than 260/280 ratio. The range of the 260/230 ratio is usually 1.8-2.2.

Table 3.5. Results obtained from Nanodrop, indicating the concentration as well as the purity of the RNA.

Sample ID	**Concentration (ng µl^{-1})**		**260/280**		**260/230**	
	Extraction 1	**Extraction 2**	**Extraction 1**	**Extraction 2**	**Extraction 1**	**Extraction 2**
ATCC 0.01	192.11	66.4	2.16	2.01	2.19	1.18
ATCC 0.1	149.64	43	2.14	1.96	1.20	0.14
ATCC 1	187.67	35.3	2.15	2.11	2.44	2.03
J 0	96.53	76.5	2.02	2.15	1.36	2.15
J 0.01	73.23	110.1	1.97	2.10	0.68	1.34
J 0.1	77.73	119.5	2.03	2.02	1.57	1.79
J 1	66.82	121.4	2.00	2.10	0.70	2.35

3.3.3 Screening of cDNA for the presence of reference genes

The extracted RNA was converted to cDNA, which was then screened for the presence of the reference genes. Figure 3.1 shows that the genes were amplified in most of the samples, and with real-time PCR, the genes were amplified in all of the samples.

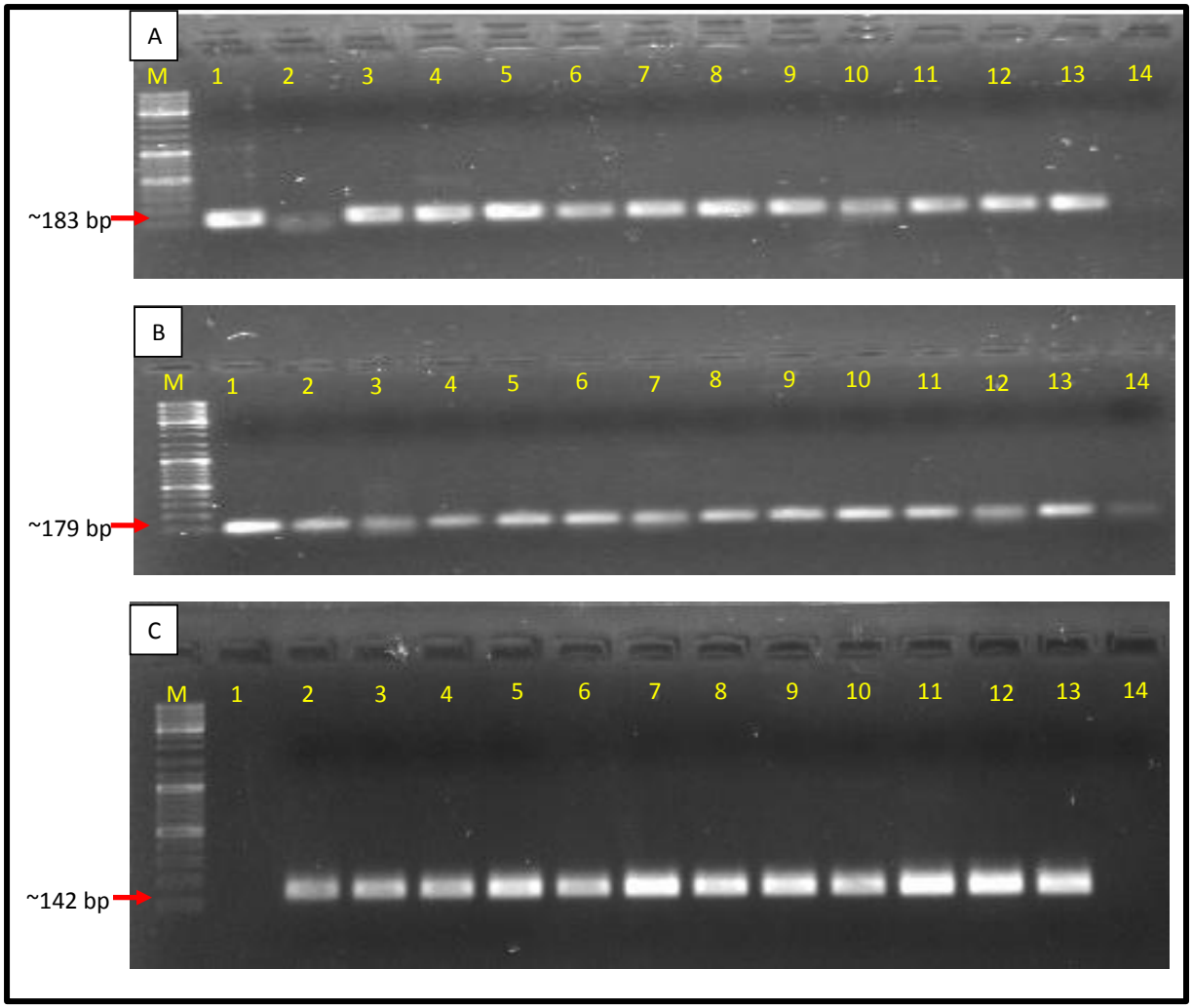

Figure 3.1. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the reference genes A) *proC,* B) *gyrA* and C) *rho* genes. The PCR products are from cDNA synthesised from the RNA extracted from the samples. The expected band sizes of A) 183 bp B) 179 bp C) 142 bp were achieved during amplification. M – marker (Fermentas GeneRuler™ DNA ladder mix); lane 1- J0; lanes 2 & 8 – J0.01; lanes 3 & 9 – J0.1; lanes 4 & 10 – J1; lanes 5 &11 – ATCC0.01; lanes 6 & 12 – ATCC 0.1; lanes 7 & 13 – ATCC1; lane 14 – negative control.

3.3.4 Quantitative Real-Time PCR

The *qacJ* primer set used in Chapter 2 was used for the real-time PCR, however, no amplification was obtained using the LightCycler® 2.0. Since the Mastermix kit already contained the optimal $MgCl_2$ concentration for most reactions, the first optimisation step involved testing of different concentrations of the primers, after which three primer sets were tested in various combinations. The primer set that gave the lowest Cp value was chosen to

be used in the rest of the experiments. The primer pair highlighted in Tables 3.2 and 3.6 was used in the real-time experiments.

Table 3.6. Qualitative detection with different *qacJ* primer sets in different combination. The set in bold gave the lowest Cp value and was used in the rest of the study.

Sample	Target	Cp	Score
A_FA_R	-	-	-5
B_FB_R	+	30.00	5
C_FC_R	+	23.52	5
A_FB_R	-	-	-5
A_FC_R	-	-	-5
B_FA_R	**+**	**23.13**	**5**
B_FC_R	+	23.75	5
C_FA_R	+	23.26	5
C_FB_R	+	30.06	5

Standard curves were generated for the reference genes (Figures 3.2; 3.4; 3.6) as well as for the GOI (Figure 3.8). The calibrator sample was used for all the standard curves and triplicate dilutions were used to generate the standard curves. From the standard curves, the PCR efficiency for each gene was obtained (Figures 3.2; 3.4; 3.6; 3.8). The efficiencies obtained for *proC*, *gyrA*, *rho* and *qacJ* were 1.699, 1.693, 1.609 and 1.725, respectively. These efficiencies are not ideal, since an efficiency of more than 1.8 is more accepted.

From the melt curve analysis (Figures 3.3; 3.5; 3.7; 3.9), the formation of one peak suggests that only one product was formed during the PCR process. This is also confirmed by the peaks for each gene being formed at the same melting temperature (Figures 3.3; 3.5; 3.7; 3.9). The melting temperature is based on the dissociation of dsDNA, therefore a higher GC content would result in a higher Tm. The melting temperatures for *proC*, *gyrA*, *rho* and *qacJ* was approximately 79 °C, 80 °C, 79.5 °C and 78 °C, respectively. In the melting curves there

is a very small peak before the peak of the product. This is probably due to primer-dimers being formed, even though the primer concentration was optimised for this application. It is unlikely to be another product that formed, since the primers were analysed and no other products were detected. However, this peak is negligible. In Figures 3.6 and 3.7 the negative control showed some amplification, therefore there would be a slight increase in the fluorescence in the major peak.

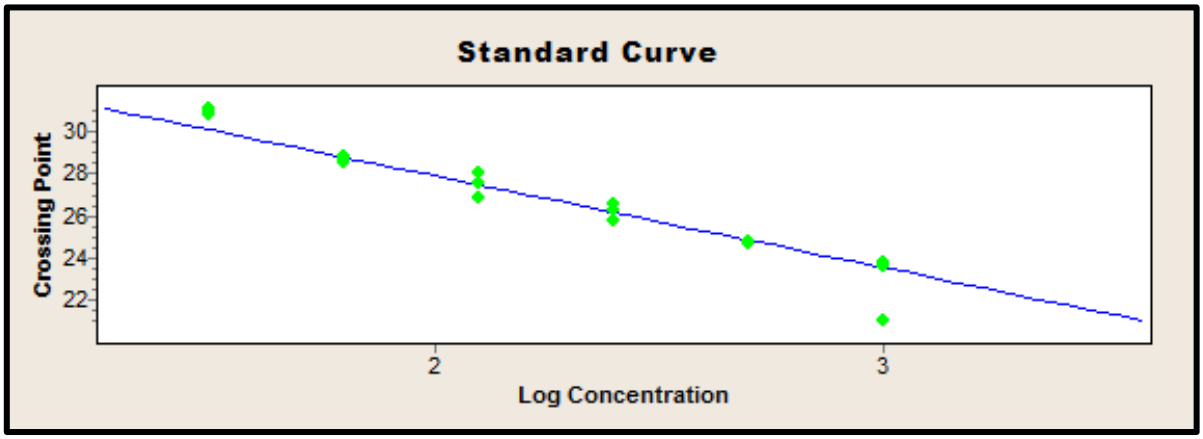

Figure 3.2. Standard curve calculated from the Cp of triplicate dilutions of the calibrator cDNA for the reference gene *proC* with a PCR efficiency of 1.699.

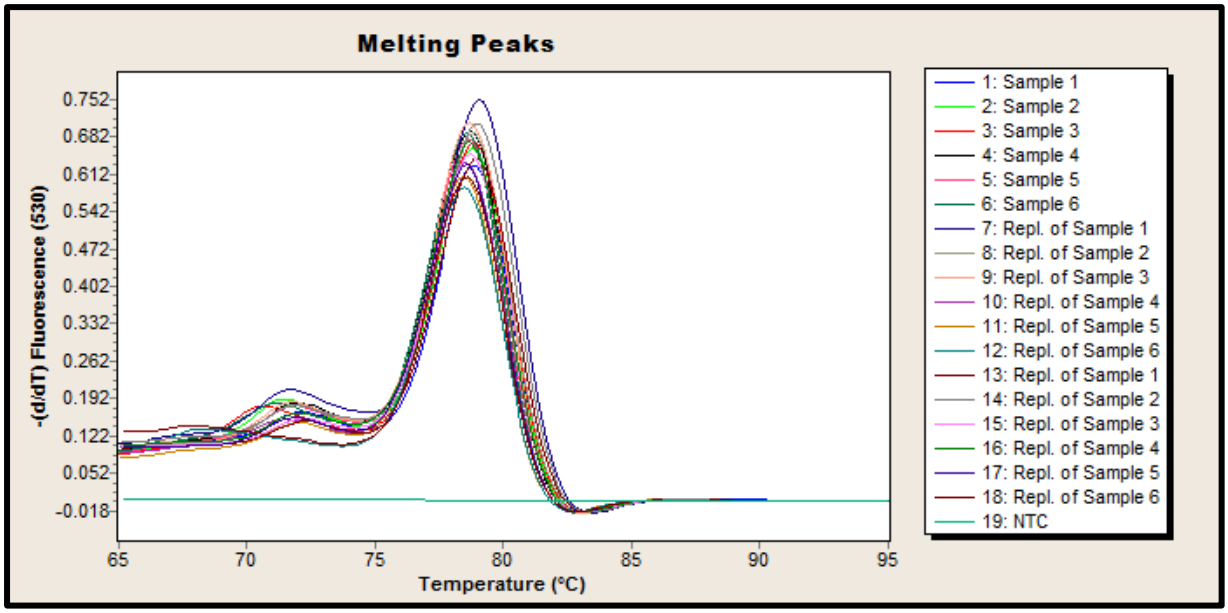

Figure 3.3. Melting peak analysis of *proC* gene after the relative quantification using SYBR® Green indicating that the primers were specific.

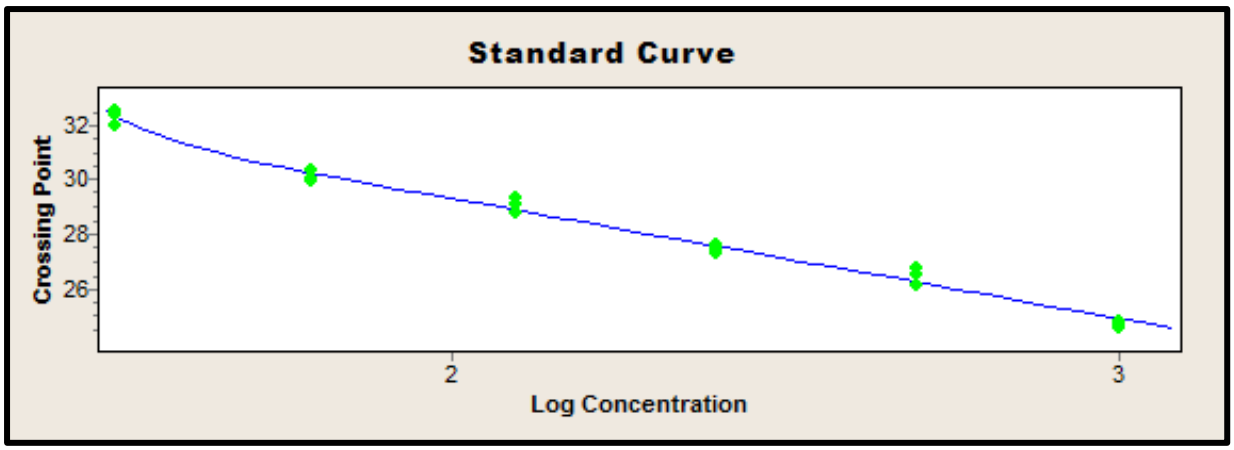

Figure 3.4. Standard curve calculated from the Cp of triplicate dilutions of the calibrator cDNA for the different reference gene *gyrA* with a PCR efficiency of 1.693.

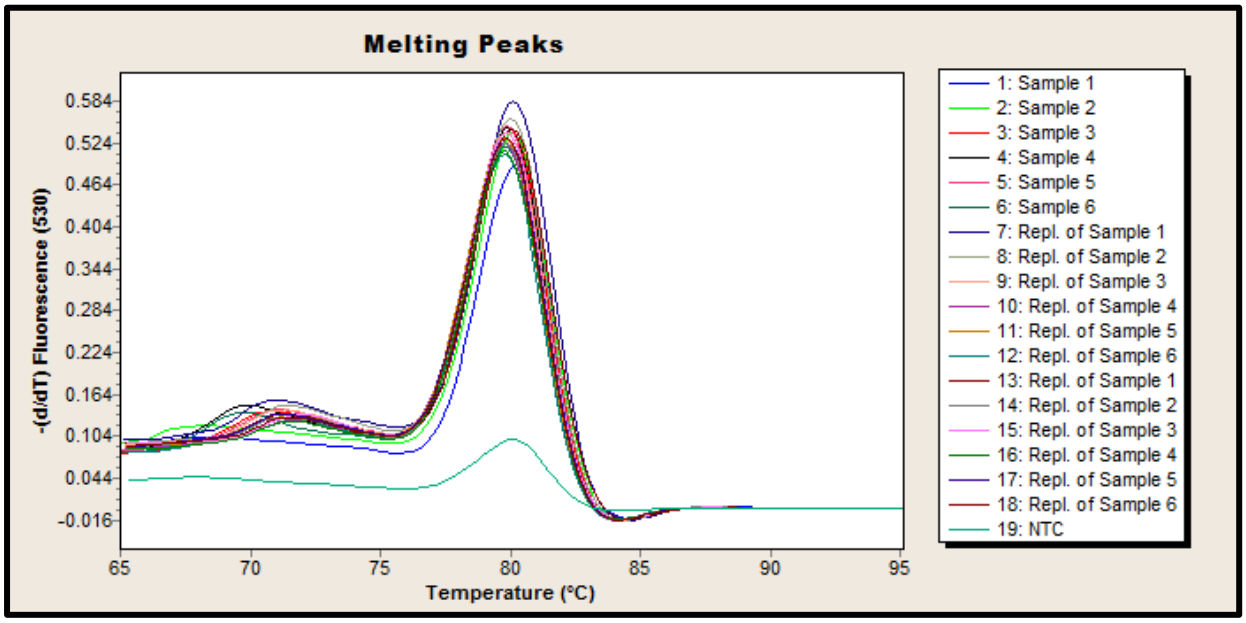

Figure 3.5. Melting peak analysis of *gyrA* gene after the relative quantification using SYBR® Green indicating that the primers were specific.

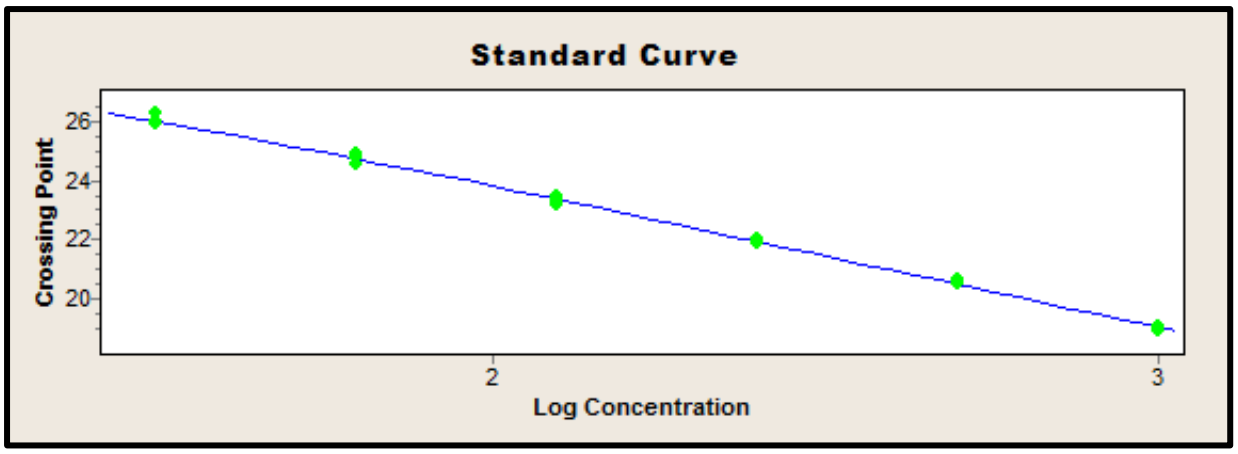

Figure 3.6. Standard curve calculated from the Cp of triplicate dilutions of the calibrator cDNA for the reference gene *rho* with a PCR efficiency of 1.609.

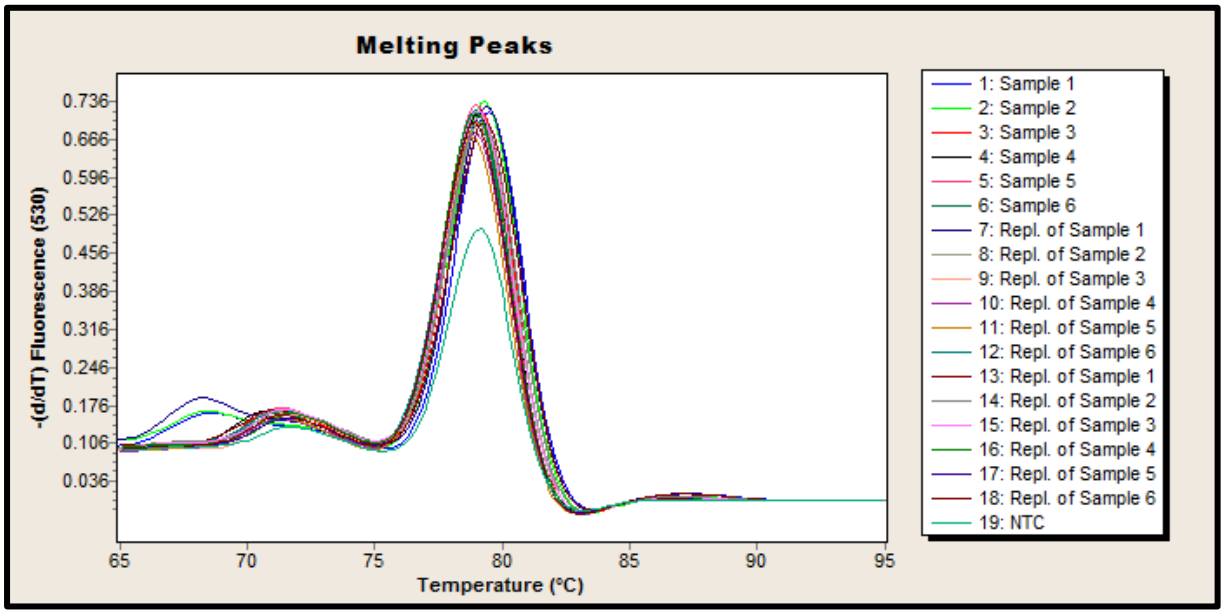

Figure 3.7. Melting peak analysis of *rho* gene after the relative quantification using SYBR® Green indicating that the primers were specific.

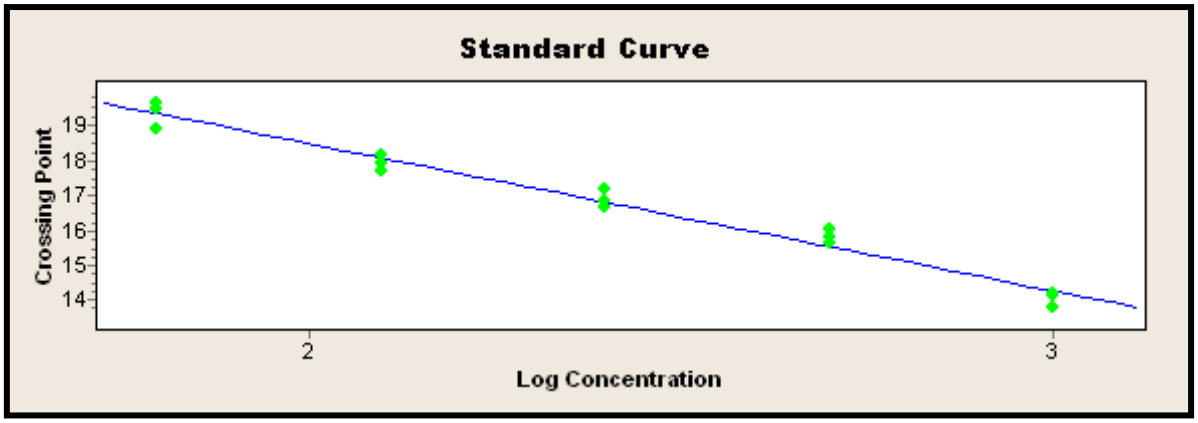

Figure 3.8. Standard curve calculated from the Cp of triplicate dilutions of the calibrator cDNA for the *qacJ* gene with a PCR efficiency of 1.725.

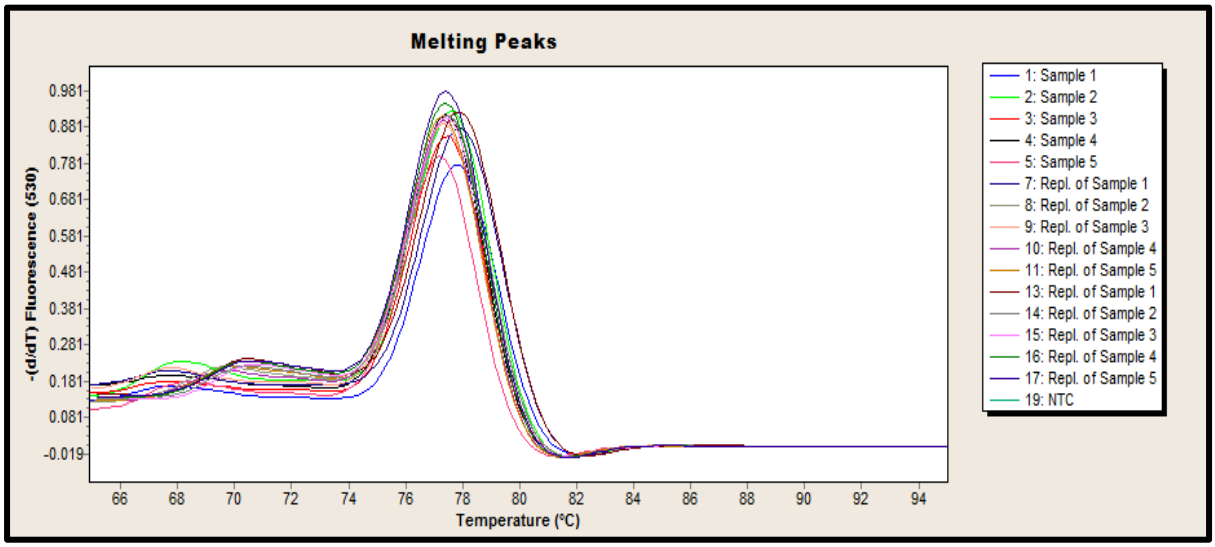

Figure 3.9. Melting peak analysis of *qacJ* gene after relative quantification using SYBR® Green indicating that the primers were specific.

The reference genes as well as *qacJ* were amplified in the samples, and the Cp values were used to calculate the relative expression (Table 3.7). The amplification curves of the genes amplified in the ATCC strain are shown in Figure 3.10. The amplification curves show the Cp value where the fluorescence detected is much higher than the baseline fluorescence; therefore the amplification of dsDNA was detected.

The Pfaffl model was used to calculate the relative expression of the GOI. This model incorporates the gene quantification and normalisation in one step. The quantities of the

reference genes (*proC*, *gyrA* and *rho*) were used in the calculation for normalisation to compensate for differences in starting amount of template, the quality of template of the differences in the efficiency of cDNA synthesis and PCR amplification (Pfaffl, 2001).

The results obtained are shown in a bar graph displaying the relative expression of the GOI, *qacJ* gene (Figure 3.11). The calibrator was cultivated in the absence of DDAC; therefore the expression of the other samples is compared to the calibrator. The *qacJ* gene was expressed in the VB3_qacJ strain, whereas in the ATCC strain the expression of the *qacJ* gene was down-regulated. From the graph, it can be seen that under the different conditions there was not much difference between the expression levels in each strain. There was not even a two-fold increase in the expression levels in the VB3_qacJ strain (Figure 3.11).

Analysis of variance (ANOVA) single factor analysis was used to test for differences in the expression level of the *qacJ* gene in the cells cultivated in different concentrations of DDAC with a level of significance of $P \leq 0.05$. The null hypothesis stated that there is no significant difference in the expression levels of the *qacJ* gene. Since the $P > 0.05$, the null hypothesis was accepted and means that there was no difference in the expression levels of the *qacJ* gene.

Table 3.7. Cp and Tm values for genes during relative quantification real-time PCR. Gene expression study of *qacJ* gene during the cultivation of strains VB3_qacJ and ATCC in the presence of different concentrations of DDAC.

DDAC Treatment (µg ml^{-1})	**Strains**	**Genes**	**First run**		**Second run**	
			Cp	**Tm**	**Cp**	**Tm**
No DDAC	Calibrator	*proC*	20.79	78.51		
		gyrA	19.60	79.81		
		rho	17.73	79.35		
		qacJ	15.85	77.4		
0.01	VB3_qacJ	*proC*	29.02	78.53	21.52	79.24
		gyrA	26.26	79.67	20.54	80.52
		rho	25.05	79.19	18.00	79.72

		qacJ	24.84	77.29	16.28	78.00
	ATCC 25923	*proC*	24.19	79.88	22.18	79.76
		gyrA	19.61	80.51	19.73	80.45
		rho	20.75	79.86	19.90	79.73
		qacJ	37.64	78.08	33.52	78.01
0.1	VB3_qacJ	*proC*	24.79	78.49	23.46	79.44
		gyrA	23.54	79.75	21.60	80.56
		rho	22.78	79.12	20.08	79.80
		qacJ	17.08	77.34	17.15	78.21
	ATCC 25923	*proC*	22.68	79.80	23.46	79.72
		gyrA	20.81	80.56	21.47	80.51
		rho	21.31	79.89	22.26	79.89
		qacJ	35.81	78.09	34.12	78.24
1	VB3_qacJ	*proC*	23.42	78.57	25.15	79.32
		gyrA	24.28	79.89	23.96	80.59
		rho	23.73	79.42	23.59	80.01
		qacJ	16.73	77.46	18.63	78.19
	ATCC 25923	*proC*	23.66	79.76	24.73	79.69
		gyrA	22.01	80.56	23.41	80.42
		rho	22.81	79.80	23.93	79.82
		qacJ	35.58	80.41	35.80	78.11

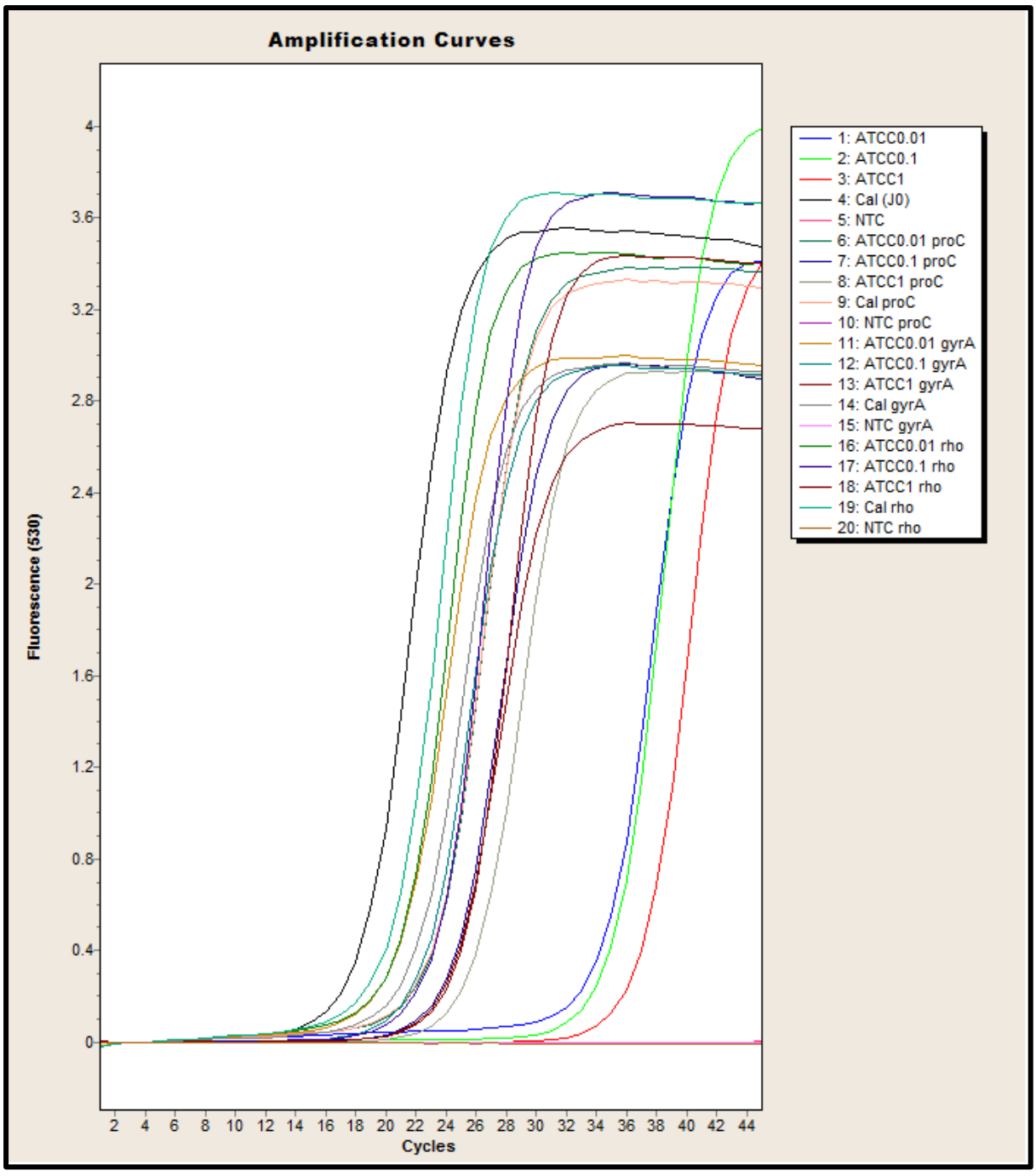

Figure 3.10. Amplification curves of the amplification of the reference genes and the *qacJ* gene in the ATCC strain. 1-5 with *qacJ* primers, 6-10 with *proC* primers, 11-15 with *gyrA* primers and 16-20 with *rho* primers. The *qacJ* gene amplified in the ATCC 0.01, ATCC 0.1 and ATCC 1 samples amplified only much later than the reference genes.

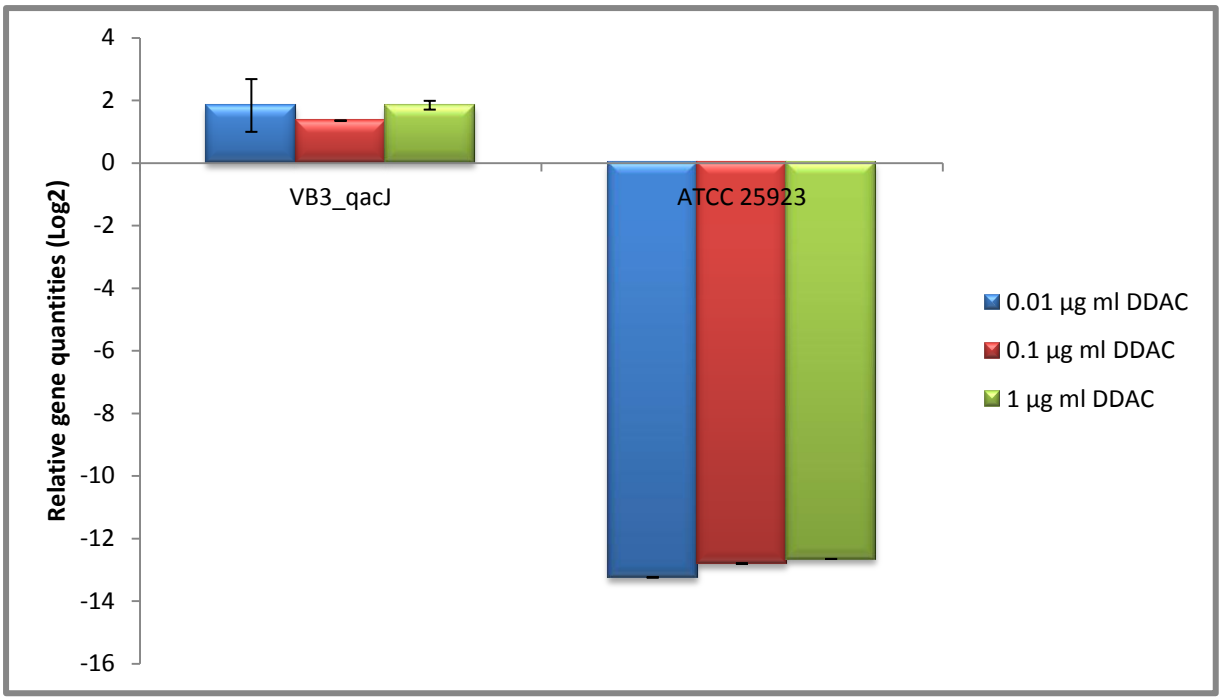

Figure 3.11. Relative amounts of *qacJ* gene expression in *Staphylococcus aureus* cultivated in the absence of DDAC, in the presence of 0.01 µg ml^{-1} DDAC, 0.1 µg ml^{-1} DDAC and 1 µg ml^{-1} DDAC. VB3_qacJ strain containing *qacJ* represented QAC resistant *Staphylococcus aureus* strain, whereas the ATCC 25923 strain is susceptible to QACs.

Table 3.8. ANOVA single factor analysis.

Anova: Single Factor	**α**	**0.05**				
SUMMARY						
Groups	***Count***	***Sum***	***Average***	**Variance**		
0.01 µg ml DDAC	2	3.56558	1.78279	6.355946		
0.1 µg ml DDAC	2	2.547968	1.273984	3.245357		
1 µg ml DDAC	2	3.585834	1.792917	6.427991		
ANOVA						
Source of Variation	***SS***	***Df***	***MS***	***F***	***P-Value***	***F crit***
Between Groups	0.352185	2	0.176093	0.032957	0.968	9.552094
Within Groups	16.02929	3	5.343098			
Total	16.38148	5				

3.4 Discussion

The ability to acquire resistance genes may give bacteria a survival advantage in harsh environments (White & McDermott, 2001). Gillings and co-workers (2009a, b) showed that the *qac* resistance genes are readily found in environmental samples. Furthermore, these genes are commonly found on gene cassettes, located on integrons. Integrons are mobile genetic elements that contain gene cassettes and transposons that contain resistance genes and can be easily transferred between species (Gillings *et al*., 2009a). In Chapter 2, it was shown that several *qac* resistance genes can be found in one strain and in the presence of DDAC, the genes were even detected in susceptible strains such as the *S. aureus* ATCC strain. However, in the absence of DDAC the genes were only detected in their respective strains. Therefore, a more sensitive technique was employed to detect these genes. It was shown with real-time PCR that the genes are present in most of the strains even in the absence of DDAC and the *qac* resistance genes were even detected in the ATCC and APEC strains. These results confirm the findings by Jansen (2012) and Gillings and co-workers (2009a, b).

The *qac* resistance family codes for multidrug efflux pumps. Most of the proteins encoded by the *qac* genes are part of the SMR family (Bjorland *et al*., 2005). The *qacJ* gene codes for a multidrug efflux pump protein that is part of the SMR family (Bjorland *et al*., 2005).

The hypothesis for this study was that the expression of the target gene would increase with increase in concentration of a QAC. Real-time PCR has been shown to be a valuable technique for the quantification of gene expression. Relative quantification can be used to determine the relative expression of the target gene (Pfaffl, 2001). In this study, relative quantification was used to relatively quantify the expression of the target gene – in this case the *qacJ* gene. Calibrator controls and reference genes were used to normalise the gene expression data to correct for sample-to-sample variation. The calibrator that was used was a cDNA sample that contains the GOI of known concentration. The cDNA was synthesised from a RNA sample extracted from the VB3_qacJ strain that was not treated with DDAC, to represent normal gene expression without external influences. The calibrator is needed so that changes in gene expression can be measured. Theis and co-workers (2007) identified a range of genes that could be used as reference genes for gene quantification in *S. aureus*. Reference genes should be present in all the organisms used in the study and should not be influenced by the experimental conditions. The reference genes were amplified in all of the samples and included: Pyrroline-s-carboxylate reductase gene (*proC*) (amino acid biosynthesis), DNA gyrase A gene (*gyrA*) (replication) and the transcription termination factor Rho gene (*rho*) (Theis *et al*., 2007).

The Pfaffl model was used to calculate the relative expression of the GOI, *qacJ*. Efficiency correction is essential, since ideal amplification is not always obtained during PCR, meaning that the amplicon may not be doubled in each PCR cycle. The PCR efficiency is calculated using the slope of the standard curve (Wong & Medrano, 2005). Thus, standard curves were generated for the reference genes and the target gene. The LightCycler® software generated the efficiencies from the standard curves. The calibrator cDNA, obtained from an untreated sample, was used to set up the standard curves and the same calibrator was used for the entire experiment.

Studies determining the expression of a *qac* resistance gene have largely focused on *qacA* gene which codes for a protein that is part of the Major Facilitator Superfamily (MFS) (Smith *et al*., 2008; Theis *et al.,* 2007; Jansen 2012), whereas the *qacJ* gene codes for a protein that is part of the SMR family (Bjorland *et al*., 2005). Smith and co-workers (2008) determined the expression of the *qacA/B* gene by first determining the expression of the *qacR* gene, which is responsible for regulation of the expression of the *qacA/B* genes. This study showed an increase in expression of the *qacA/B* gene in the presence of disinfectants

when assayed with a luciferase reporter. A real-time study by Theis and co-workers (2007) also showed an increase in expression of the *qacA* gene with increasing concentration of different substrates for the protein. However, Jansen (2012) showed that the expression of the *smr* gene did not increase with the increase in disinfectant concentration. The findings of the current study supports previous findings, as a two-fold increase was not obtained when induced with different concentrations of DDAC. The *qacJ* gene also codes for a protein part of the SMR family, therefore similar findings can be expected.

In this study, the induction of the *qacJ* gene was investigated in *S. aureus* strains VB3_qacJ and ATCC 25923. The VB3_qacJ gene contained the *qacJ* gene and the ATCC strain is a susceptible strain, however in Chapter 2 it was shown that this strain also contained the *qacJ* gene in the presence of QAC. Therefore the ATCC strain contains the gene but is not tolerant towards QACs. The gene was induced using different concentrations, 0.01 µg ml^{-1}, 0.1 µg ml^{-1} and 1 µg ml^{-1}, of DDAC. In the VB3_qacJ strain, the relative quantities calculated from the Cp values, showed a less than two-fold increase in expression. Also, the fold increase is approximately the same with all the concentrations; however induction with 0.1 µg ml^{-1} DDAC showed an increase in expression that was slightly less than with other concentrations. The ANOVA analysis showed that there were no differences in the expression of the gene under the different DDAC conditions. Therefore, the expression level of the gene stays the same even in the absence of DDAC. It is possible that the gene may be constitutively expressed, and is transcribed continually compared to a facultative gene which is only transcribed as needed. However, a time study of the expression is needed to conclude this.

Furthermore, an overexpression of the genes was not observed when *S. aureus* was exposed to various QAC agents in a study by DeMarco and co-workers (2007). Therefore, it was suggested that the *qac* resistance genes do not always play a significant role in the resistance against QACs (DeMarco *et al.,* 2007).

In the ATCC strain, the gene was not induced, and it seems that there was a down-regulation of the gene. The gene was detected in the ATCC strain, and this correlates with Chapter 2 where very faint bands were observed for the *qacJ* gene in the presence of DDAC, as well as in the qualitative detection study performed in this study. Since the ATCC strain is susceptible, it is possible that the strain might rather use the energy for survival rather than using energy to produce the pump. However, one would expect that the resistance gene would be up-regulated since it codes for a protein that would lower the concentration of DDAC inside the cell. However, this was not the case and a possible explanation could be that the gene is on a plasmid with a weaker promoter and therefore

cannot be as highly expressed to allow the cell to survive at any concentration of DDAC and therefore other survival mechanisms must be activated.

Kierzek and co-workers (2001) determined that the promoter can have an effect on the expression of genes. Therefore, it is possible that the ATCC strain contains a weak promoter, and therefore the gene expression is down-regulated. A study by Heir and co-workers (1999a) found that the *smr* gene had a stronger promoter than the *qacG* gene. It is therefore important to look at the expression at protein level, as this could be a more accurate reflection on the resistance caused by the proteins coded by these *qac* resistance genes.

Previous research on the resistance of *Listeria monocytogenes* indicated that the presence of certain resistance genes did not appear to impart resistance to disinfectants and antibiotics (Romanova *et al*., 2006). It was also proposed that there might be more than one resistance mechanism present that causes *Listeria monocytogenes* to be resistant to benzalkonium chloride (Romanova *et al*., 2006). Therefore, it is possible that in *S. aureus* these genes are present but are not completely responsible for the resistance seen in the strains used for the study. Since these genes have a wide range of substrates, it is possible that DDAC is not the primary substrate for the qacJ protein. Gillings and co-workers (2009a) showed that these genes can be present in environments where QACs are not used, and it was previously suggested that there might be natural toxins that are substrates for the proteins (Gilbert & McBain, 2003).

Huet and co-workers (2008) found that pump gene expression did not always correlate with the ethidium bromide efflux observed. It was then suggested that only a limited amount of multidrug resistance pump can be tolerated and therefore excess transcripts could be degraded prior to translation. In their study they also showed a MIC increase without change in the expression of the pumps. It was then proposed that the organism probably has increased expression of pumps other than the ones evaluated (Huet *et al.,* 2008). In this study the expression of the *qacJ* strain did not increase, therefore it is possible that another resistance gene has increased expression, which will need to be further studied. The qualitative detection done in this study showed that the VB3_qacJ strain contains more than one of the *qac* resistance genes.

Gillings and co-workers (2009a;b) showed that the *qac* resistance genes form part of a gene cluster, and therefore it is possible that they function as an operon. This suggests that some of the genes can be activated while the others are down-regulated.

From this study it can be seen that the resistance to QAC is a more complex process than initially thought, since one would expect an increase in expression with the increase in DDAC concentration. It is also possible that the gene does not confer resistance alone, and therefore other resistance genes might be induced so that the cell can survive harsh conditions. Future research includes investigating the expression of the known *qac* resistance genes simultaneously. This is in order to determine whether there is a relationship between the *qac* genes in conferring resistance. Also, it would be interesting to determine the full mechanism used by *S. aureus* in surviving harsh environments.

Chapter 4 Optimisation of solid phase extraction of DDAC for liquid chromatography-mass spectrometry

4.1 Introduction

Quaternary ammonium compounds (QAC) are cationic surfactants that target the cytoplasmic membrane of the bacterial cells where they cause damage to the cell membranes and influence membrane permeability (Gilbert & Moore, 2005). However, bacteria have the ability to develop mechanisms that can circumvent the action of disinfectants (Hegstad *et al*., 2010). Bacterial resistance against disinfectants can be obtained either through mutations or by the acquisition of genetic material (White & McDermott, 2001; Hegstad *et al*., 2010).

The primary bacterial resistance mechanism against disinfectants is efflux (Hassan *et al*., 2010). Bacteria have the ability to extrude the disinfectant out of the cell using efflux pumps, reducing the concentration of the disinfectant inside the cell to a non-lethal level (Piddock, 2006). The efflux pumps responsible for the reported resistance against QACs are coded by quaternary ammonium compound (*qac*) resistance genes (Bjorland *et al*., 2005). These resistance genes are acquired from plasmids or transposons (Hegstad *et al*., 2010). The proteins encoded by these genes are part of the multidrug transporter family (Bjorland *et al*., 2005).

Jansen and colleagues (2013) studied the effects that QACs have on the morphology of *Staphylococcus aureus*. Semi-quantitative elemental analysis revealed the presence of trace amounts of chlorine within cells treated with didecyldimethylammonium chloride (DDAC) (Jansen *et al*., 2013). However, the chlorine concentration was too low to definitely conclude its presence. Therefore, it was hypothesised that the bacteria changed the structure of the disinfectant before being pumped out of the cell (Jansen *et al*., 2013). In that particular study, the chlorine was used for the detection of DDAC since that is the only element that differed from normal biomass. Figure 4.1 shows the structure of DDAC, with the positively charged quaternary nitrogen as well as the chlorine.

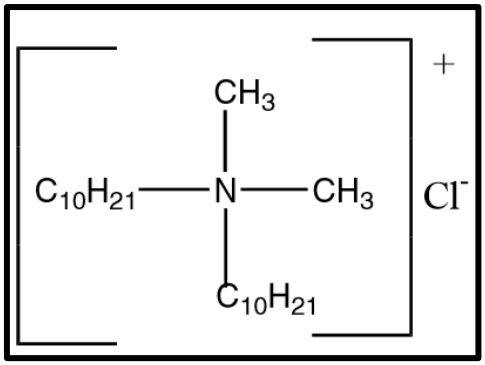

Figure 4.1. Structure of didecyldimethylammonium chloride (Lonza Int, 2013).

Solid phase extraction (SPE) was reported to successfully extract QACs from water samples (Martínez Vidal *et al*., 2004). Solid phase extraction is frequently employed as it is economical and due to its ease of use (Castro *et al*., 2000). Liquid-liquid extraction is also used for extraction of QACs, however this method is more costly and more labour intensive (Castro *et al*., 2000). Solid phase extraction is used to extract the analyte from the sample and prepare it for the use in LC-MS (Żwir-Ferenc & Biziuk, 2006). It separates a mixture by using the affinity of the solutes dissolved in a liquid for a solid (sorbent) phase through which the sample is passed (Żwir-Ferenc & Biziuk, 2006). The general procedure involves the loading of the sample onto the SPE sorbent, which retains the analyte, washing away contaminants and then washing off the desired analyte with another solvent (Żwir-Ferenc & Biziuk, 2006; Camel, 2003).

Liquid-chromatography-mass spectrometry (LC-MS) is a widely used technique to analyse QACs present in environmental samples (Bassarab *et al*., 2011). This is because LC-MS has greater detection sensitivity than alternative types of liquid chromatography (LC) (Castro *et al*., 2000). Liquid chromatography separates a sample into individual components, whereas a mass spectrometer detects the analyte (Agilent technologies, 2001). Mass spectrometer ionises the molecules to a charged state and the ions are analysed and identified based on their mass-to-charge ratio (*m/z*) (Agilent technologies, 2001; Pitt, 2009). Using the *m/z* ratio, it is possible to detect and identify the QAC in a sample and therefore possible to determine the structure of the QAC as well as any structural changes.

The use of LC-MS allows the determination of the structure of DDAC. The aim of the study was to quantify the uptake of DDAC by *S. aureus* and possibly determine structural changes of DDAC*,* by a series of optimisation steps for SPE to prepare samples for the use in LC-MS..

4.2 Materials and Methods

4.2.1 Chemicals

Didecyldimethylammonium chloride was obtained in liquid form (Lonza Int. (80%)). This was diluted to the concentration needed; acetonitrile HPLC grade (LabScan Analyical Sciences); Isopropanol (Merck); methanol (Merck); formic acid (Sigma-Aldrich); acetic acid (BDH AnalaR); Nanopure water from the MilliQ sysem.

4.2.2 Cultivation of *Staphylococcus aureus*

All strains were routinely cultivated in tryptic soy broth (TSB). A growth curve of the QAC tolerant *S. aureus* VB4_smr strain was constructed. An inoculum of *S. aureus* VB4_smr strain was prepared by cultivation in tryptic soy broth (TSB) overnight. The pre-inoculum was then inoculated in 5 ml TSB to an absorbance value of 0.1 measured at a wavelength of 600nm and incubated at 37 °C. The optical density (OD_{600nm}) was measured at 2 hourly intervals for 12 hrs after inoculation and again at 24 hrs to follow growth.

The VB4_smr strain was cultivated in TSB for sampling before LC-MS. A pre-inoculum was prepared, after which, it was inoculated into TSB containing 0.05 µg ml^{-1} DDAC incubated at 37 °C. Samples of 2 ml each were taken after 18 hrs inoculation, and centrifuged at 5000 x *g* for 5 min at room temperature. The supernatant was used further in solid phase extraction, since DDAC would be pumped out of the cell. Therefore the DDAC uptake and expulsion can be measured. Controls samples were TSB with the same concentration DDAC to determine if DDAC concentration in the media decreased.

4.2.3 Solid Phase Extraction

Solid phase extraction was performed to prepare the sample for liquid chromatography. Therefore, DDAC was isolated, purified, and concentrated from TSB. A Strata-X 33µ (200 mg/6 ml) Polymeric reversed phase (Phenomenex) column was used according to the manufacturer's instructions. The sample pH was adjusted to pH 8 using K_2HPO_4. The column was conditioned to prepare the sorbent for effective interaction with the analyte namely DDAC. Following conditioning, the column was equilibrated using a solvent similar to the sample matrix. The sample was loaded on the column that was subsequently washed to remove any impurities bound to the sorbent. These impurities were less strongly bound to the sorbent and could therefore be washed away without washing away the analyte. The analyte was removed using a solvent that disrupted the analyte-sorbent interaction. An organic solvent such as acetonitrile disrupts the hydrophobic interactions between the

analyte and the sorbent. Various protocols were tested to determine which protocol would elute the maximum amount of DDAC.

4.2.3.1 Phenomenex protocol

The manufacturer's protocol was followed to extract DDAC from the growth medium. The sample was pre-treated by adjusting the pH of the sample to 8 with K_2HPO_4. The column was conditioned with 6 ml acetonitrile and then equilibrated with 6 ml water. The sample was loaded, which was TSB spiked with various concentrations (0.8 µg ml^{-1}, 0.2 µg ml^{-1} and 0.05 µg ml^{-1}) of DDAC to represent high, intermediate and low concentrations, respectively. The column was washed with 6 ml methanol:water in a 40:60 ratio. The column was then dried under vacuum for 10 min, and then the DDAC was eluted with 6 ml acetonitrile.

The protocol was optimised for extraction of DDAC from growth medium and modified for this application. Several optimisation steps were performed. The analyte was eluted into microcentrifuge tubes (Eppendorf) or conical tubes (Falcon) since DDAC absorbs to glass (Fogh *et al*., 1954). The concentrations of DDAC in TSB were decreased to improve the results obtained from MS. The DDAC concentrations used was decreased to 0.08 µg ml^{-1} and 0.01 µg ml^{-1}. Controls used in the study were TSB without DDAC as well as MilliQ water samples. Two wash steps were incorporated as well as a longer drying step of the cartridge. The volumes were adjusted so that 1 ml was eluted in the final step and 10 elution steps were incorporated. The volumes were adjusted so that 5 ml was eluted in the final step and 4 elution steps were incorporated. The elution solvents were also changed using 5 ml acetonitrile acidified with 5% formic acid followed by incubating the column with the elution solvent at room temperature for 15 min or 30 min before eluting. Also 1 column was incubated for 15 min with elution solvent after each elution step. Another elution solvent was tested which was a combination of acetonitrile and isopropanol. The DDAC was eluted with 5 ml acetonitrile and isopropanol in different ratios (85:15; 75:25; 50:50 acetonitrile:isopropanol).

The final protocol used of this method was as follows: the sample pH was adjusted to 8. The column was conditioned with 5 ml acetonitrile and equilibrated with 5 ml water. The sample was loaded and the column was washed 2 x with 5 ml methanol:water (40:60). The column was dried for 60 min under full vacuum. Didecyldimethylammonium chloride was eluted with 5 ml acetonitrile:isopropanol (75:25).

4.2.3.2 Protocol of Bassarab and co-workers (2011)

An additional method of Bassarab and co-workers (2011) was also followed. This method involved conditioning with 5 ml acetonitrile, equilibrating with 10 ml distilled water, after

which 2 ml of the sample was loaded. The column was then washed with 20 ml distilled water with 10% (v/v) acetic acid. The column was dried under full vacuum. The analyte was eluted with 8.5 ml 90(A):10(B) with A being acetonitrile acidified with 10% acetic acid and B was distilled water with 10 % acetic acid. This method was modified by incorporating the pre-treatment of the sample, therefore adjusting the pH of the sample to 8. Also the volumes were adjusted to elute DDAC in 1 ml.

4.2.3.3 Protocol of Van de Voorde and co-workers (2012)

The method followed by Van de Voorde and co-workers (2012) was as follows: The column was conditioned with 10 ml methanol and equilibrated with 10 ml deionised water. The sample was loaded and the column washed with 10 ml methanol. The column was dried under vacuum for 30 min and DDAC was eluted in 10 ml methanol acidified with 5% formic acid. This method was modified by adjusting the pH of the sample to 8. The volumes were adjusted to elute DDAC in 1 ml of acidified methanol.

After elution, the samples were dried in a Speedy Vac (Thermo Scientific) for 1 hr, to evaporate the acetonitrile or other elution solvents used. The sample was re-suspended in MilliQ water containing 0.1% formic acid. Formic acid favours ion-pair formation by increasing the retention with the ammonium cation, and forming of an ion pair with the cationic analyte. All samples obtained with the various procedures were subjected to high performance liquid chromatography (HPLC).

Samples were submitted in batches to the Genomics and Proteomics laboratory situated in the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein. Every batch of samples included solvent blank that was run between each sample analysed.

4.2.4 Sample Analysis

The samples were analysed using liquid chromatography-mass spectrometry (LC-MS). Liquid chromatography separates the sample through a stationary phase containing the chromatographic packing material and MS identifies the compound in the samples. The compound that has a higher affinity for the packing material will have a longer retention time, since it would move slower through the column.

Standards of the DDAC were prepared to determine the retention time of the DDAC as well as the upper and lower detection limits of DDAC. Serial dilutions of the concentrated DDAC were prepared, and were directly injected into LC-MS.

Samples were analysed using AB SCIEX 3200 QTRAP hybrid triple quadrupole ion trap mass spectrometer with an Agilent 1200 SL HPLC stack as a front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

4.2.4.1 High Performance Liquid Chromatography (HPLC)

Twenty microliter (20 µl) of each extracted sample was separated on a C18 (150 mm x 4.6 mm, Gemini NX, Phenomenex) column at a flow rate of 400 µl min^{-1} using a 1 min gradient from 80% solvent A (H_2O/0.1% formic acid) to 95% solvent B (MeOH/0.1% formic acid) with a total run time of 9 min to allow for column re-equilibration. The eluates were analysed using positive ionization mode in the TurboV ion source. The following settings were used: ion spray voltage of 5500 V, 500 °C heater temperature to evaporate excess solvent, 50 psi nebuliser gas, 50 psi heater gas and 25 psi curtain gas.

A second set of conditions was also used to analyse the extracted samples. Twenty microliter of each extracted sample was separated on a C18 (150 mm x 3 mm, Luna 3µ, Phenomenex) column at a flow rate of 500 µl min^{-1}, starting at 60% mobile phase B (MeOH/0.1% formic acid), at 1 min gradient increasing to 90% B and staying there for 4 minutes, before re-equilibrating the column at 60% B for 5 minutes. Eluting analytes were analysed in positive ionization mode in the TurboV ion source using an ion spray voltage setting of 5500 V, 500 ^{o}C heater temperature to evaporate excess solvent, 50 psi nebuliser gas, 50 psi heater gas and 25 psi curtain gas.

4.2.4.2 Mass spectrometry

Target analyses of DDAC were performed using 5 multiple reaction monitoring (MRM) transitions per analyte. During the MRM scan, the instrument was used in triple quadrupole mode, where every ionised analyte (the precursor) eluting from the column was fragmented in the collision cell to produce fragment masses. A set of masses consists of the precursor mass and one fragment mass, in turn, this set constitutes a transition.

The instrument changed between different transitions in the MRM transition list during the analysis cycle, with each cycle lasting a few seconds. When a transition was detected, the instrument's response was registered and a chromatogram was generated. The peak area on the chromatogram, generated from the first and most sensitive transition, was used as the quantifier while the rest of the transitions were used as a qualifier.

The qualifier served as an additional level of confirmation for the presence of the analyte. It was essential that the retention time for the 5 transitions stayed the same, as this indicates

the same compound. The transitions used were MRM1: 326.4>186.4; MRM2: 326.4>43.2; MRM3: 326.4>57.1; MRM4: 326.4>58.2; MRM5: 326.4>46.1.

4.3 Results

4.3.1 Growth curve

A growth curve of *S. aureus* VB4_smr strain was constructed. Figure 4.2 shows the growth of the *S. aureus* VB4_SMR strain. From the plot, it can be observed that after 2 hrs the growth reached the early exponential phase. Between 2 and 5 hrs the growth was in the exponential phase. Approximately 5 hrs after inoculation, the growth phase reached early stationary phase.

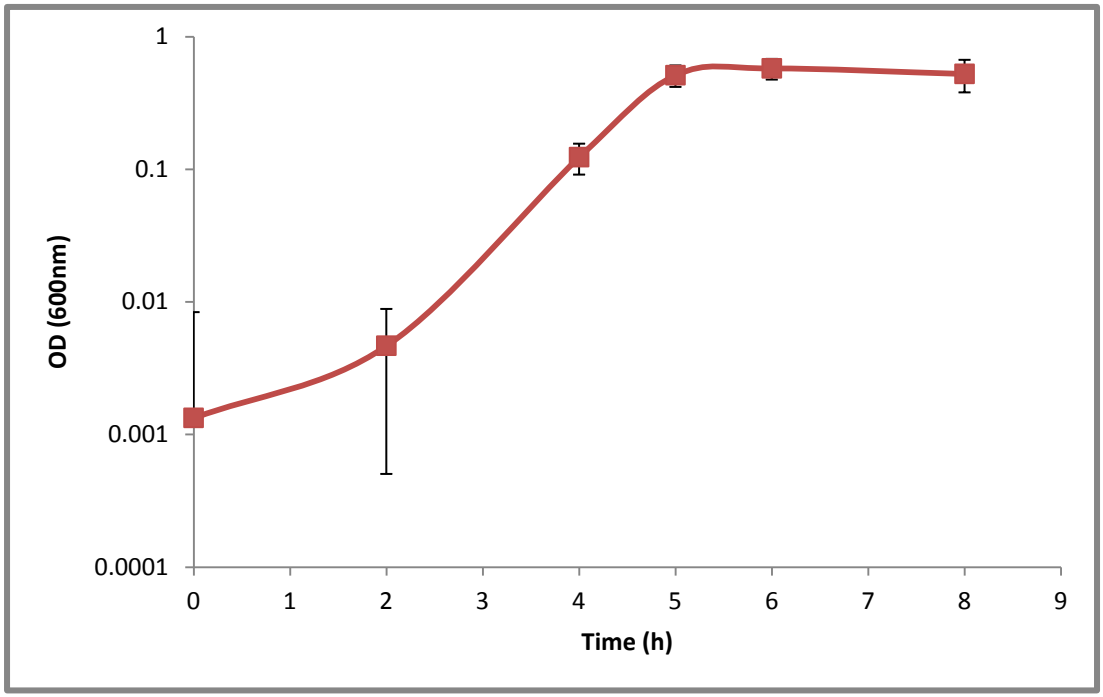

Figure 4.2. Growth curve of *Staphylococcus aureus* VB4_SMR strain. The early exponential phase was reached after 2 hrs. Exponential growth phase was between 2 and 5 hrs after inoculation and the stationary phase is reached after 5 hrs.

4.3.2 Solid phase extraction

Solid phase extraction is frequently used to extract QACs from water samples to prepare the sample for analysis with liquid chromatography-mass spectrometry. The retention time as well as the lower limit of detection was determined, from the prepared dilution series of DDAC. Figure 4.3 shows the chromatogram of one standard. The retention time for DDAC

was determined at approximately 5.5 min. The lower limit was determined to be 0.001 µg ml^{-1}. The peaks all have the same retention time, thus it can be deduced that the fragments were from the same compound.

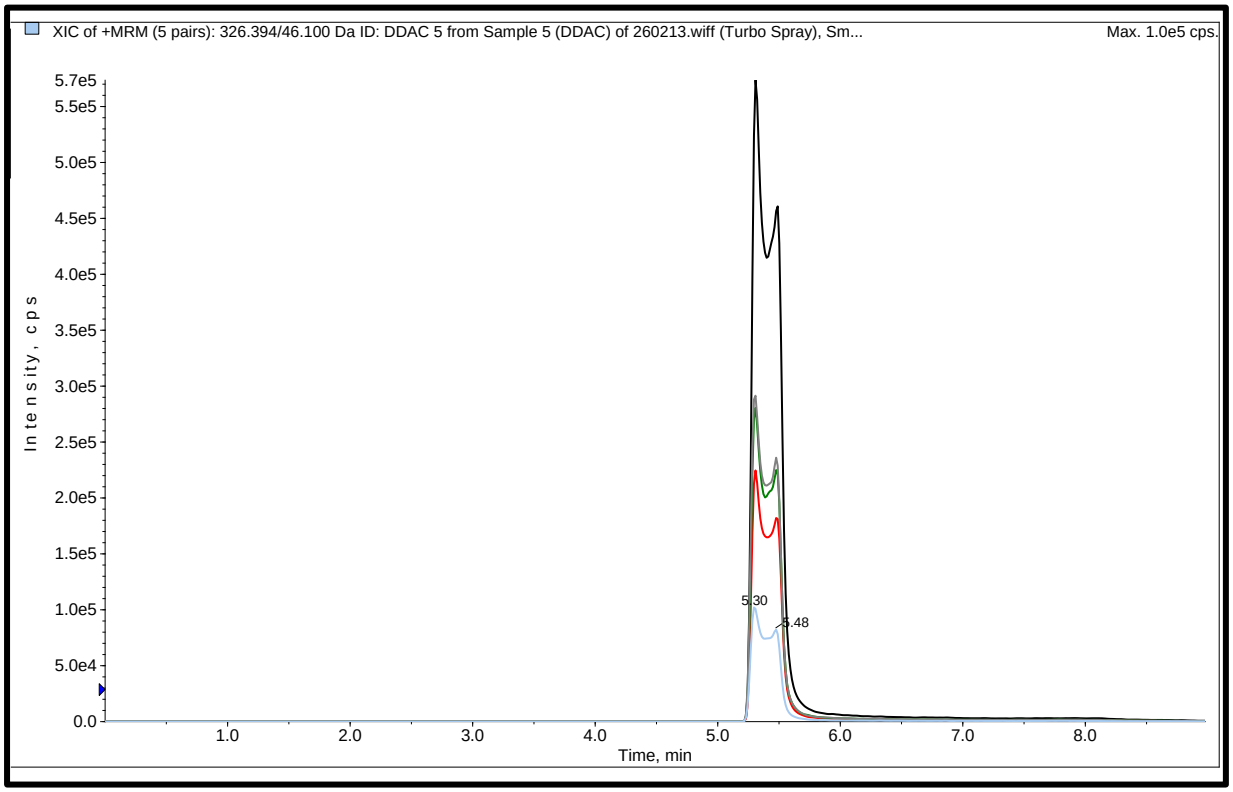

Figure 4.3. Chromatogram of a DDAC standard as obtained from the MS. The retention time for DDAC is at approximately 5.5 min. The different colours indicated in the chromatogram represent different fragments obtained.

Several optimization steps were performed to determine the most efficient method of DDAC extraction from TSB. Several methods were tested, that differed in the steps used in SPE. TSB was spiked with various concentrations of DDAC to determine the efficiency of the SPE method. When TSB was spiked with 0.8 µg ml^{-1}, 0.2 µg ml^{-1} and 0.05 µg ml^{-1} DDAC, the same retention time as the standard, which was 5.5 min indicating that DDAC was extracted from the media. However, the highest and lowest concentrations showed similar amounts of DDAC extracted.

The DDAC concentration used in the growth medium was decreased to obtain more accurate results from the MS. Therefore; the concentrations were decreased to 0.08 µg ml^{-1} and 0.01 µg ml^{-1}. DDAC can adsorb to glass which could influence the detection thereof (Fogh *et al*., 1954) and thus the analytes were eluted in either microcentrifuge tubes

(Eppendorf) or conical tubes (Falcon). The volumes of the procedure were decreased from 6 ml to 1 ml so that only 1 ml was eluted in the elution step. The sample with 0.08 µg ml^{-1} DDAC was eluted twice and was found that there is still DDAC present in the second elution. After incorporating additional elution steps, it was established that DDAC was present even after eluting 10 ml (10 x 1 ml) (Figure 4.4). The first two elution samples showed the highest concentration of DDAC; however the amount of DDAC decreased, forming a plateau where all samples contained the same amount of DDAC. Further adjustment to the volumes of the SPE protocols was done so that 5 ml was eluted at a time; DDAC was still detected in all the samples.

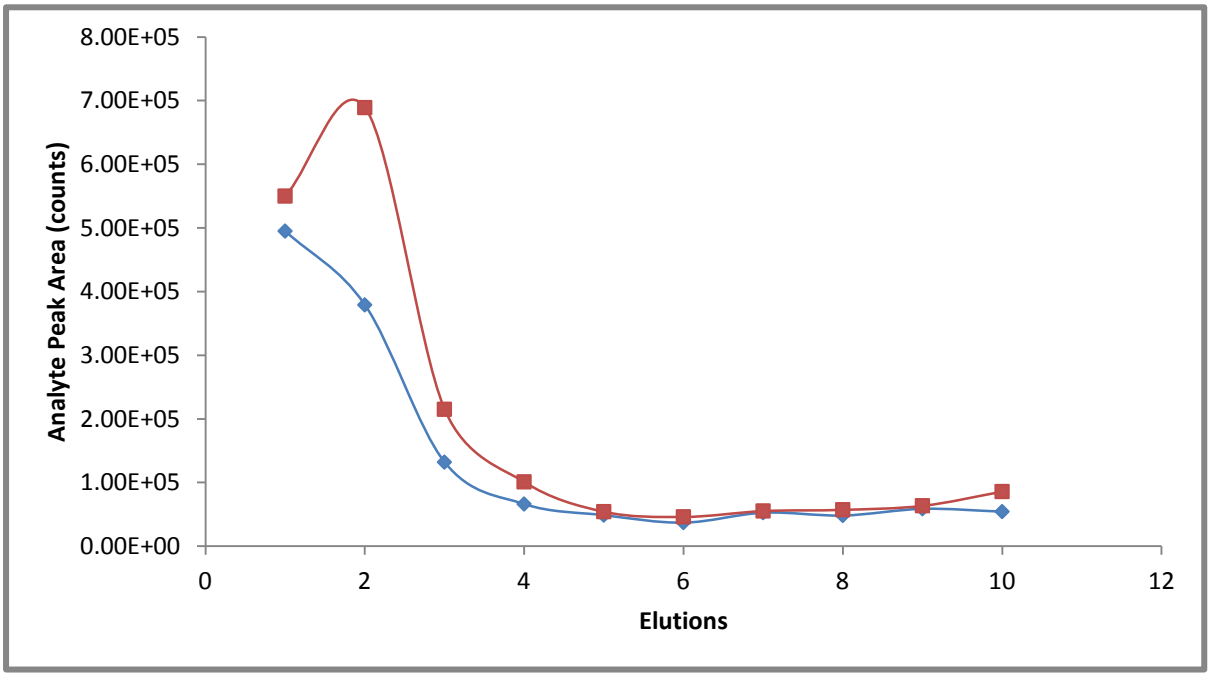

Figure 4.4. Graph showing DDAC eluted with acetonitrile. DDAC eluted after one wash step ◆ and DDAC eluted after two wash steps ■.

It was also observed that TSB was co-eluted with DDAC. Background noise was observed in the chromatogram, indicating an impure sample (Figure 4.5). Therefore, the second wash step as well as a longer drying under vacuum step after washing was incorporated to wash away all contaminants.

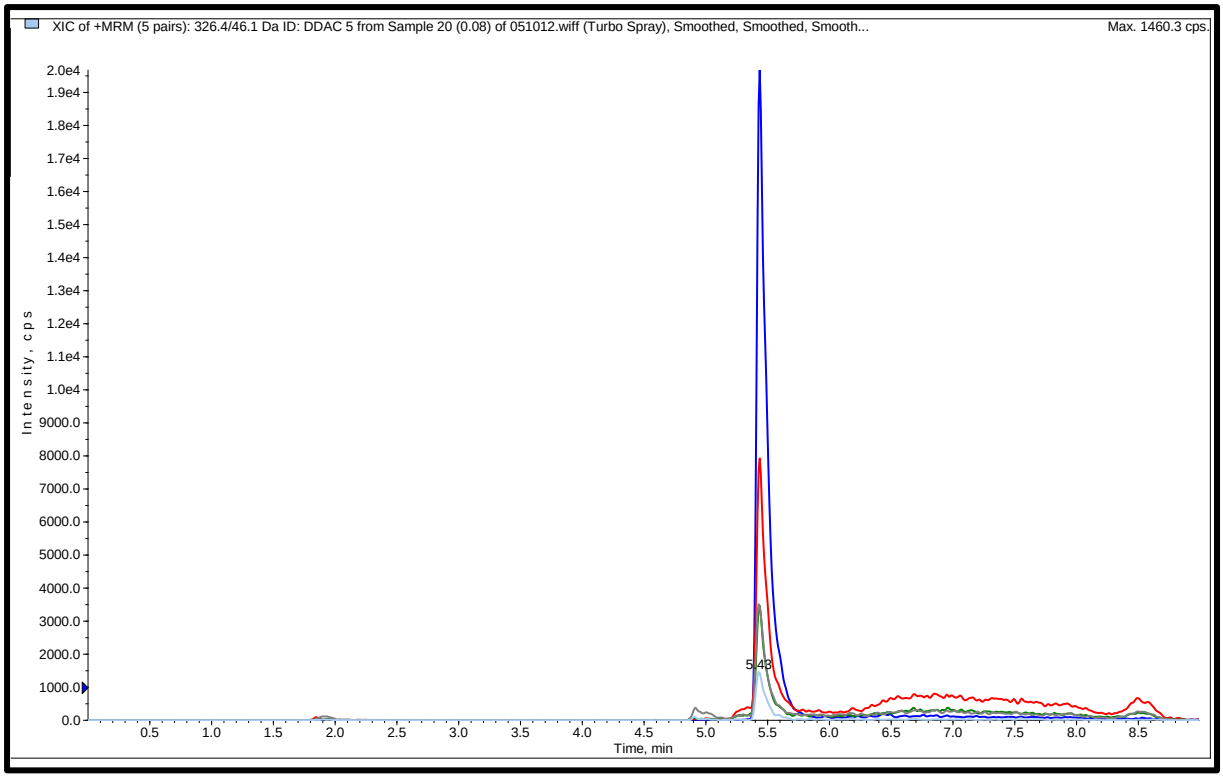

Figure 4.5. Chromatogram of TSB sample spike with DDAC eluted with 1 ml acetonitrile. The retention time of the DDAC is at approximately 5.5 min. The different colours indicated in the chromatogram represent the fragments obtained.

Since DDAC was detected in all elution samples, different SPE methods were explored to increase the efficiency of DDAC extraction. Two different SPE methods from literature were tested (Bassarab *et al*., 2011; Van de Voorde *et al*., 2012). However, DDAC was detected again in all of the elution samples with both of these methods as seen in Figure 4.6. It can be seen in Figure 4.7 that the method from Van de Voorde and co-workers (2012) was the more efficient method of the two with extracting DDAC; since more DDAC was extracted with this method. However, elution with acetonitrile in the previous experiments still seemed to be the most efficient.

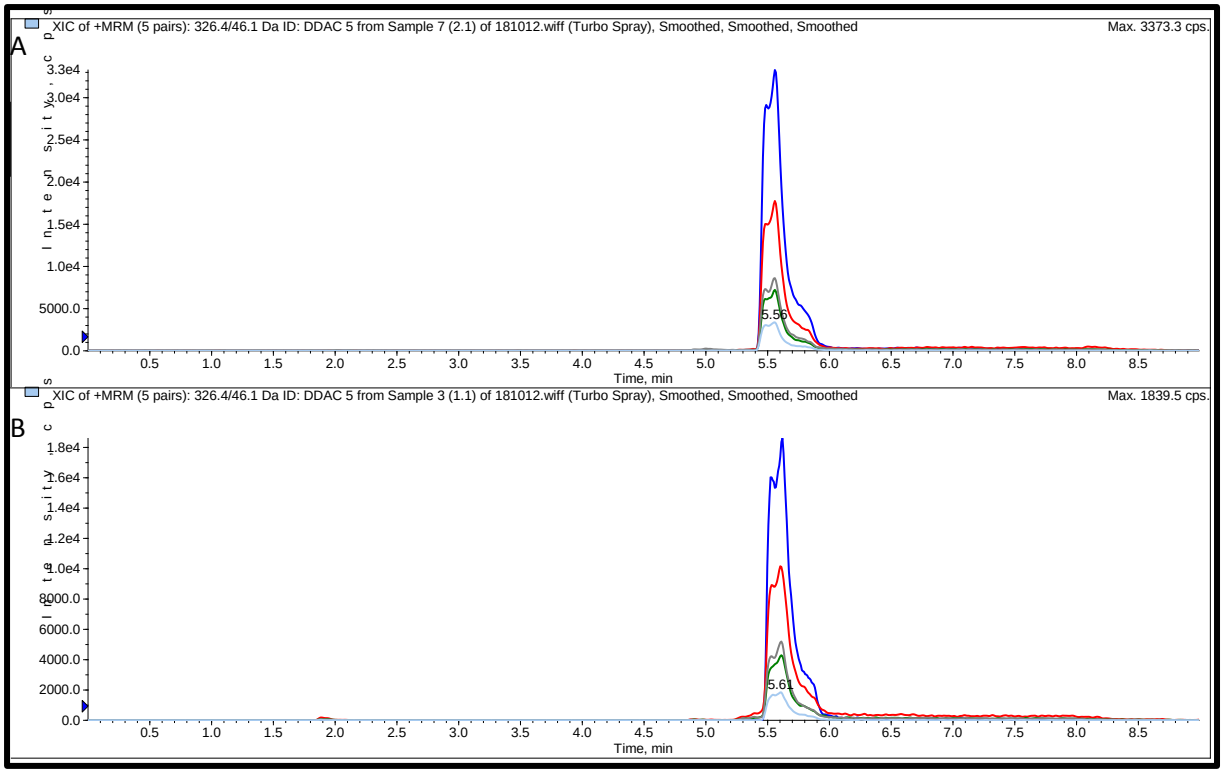

Figure 4.6. Chromatogram of 2 spiked TSB samples eluted with A) acidified methanol (Van de Voorde *et al*., 2012) and B) acetonitrile, water, and acetic acid (Bassarab *et al*., 2011). The retention time is at approximately 5.5 min. Fragments obtained are represented by the different colours indicated in the chromatogram.

Eluting DDAC with acidified acetonitrile (5% formic acid) was tested. Figure 4.8 shows that the acidified acetonitrile was more efficient than eluting with acetonitrile. However, DDAC was still detected in all of the eluted samples. Figure 4.9 show three different ratios of acetonitrile with isopropanol used. The 75:25 ratio of acetonitrile:isopropanol showed extraction of the most DDAC. However, more DDAC was eluted in the second elution step than the first (Figure 4.9). This was still not sufficient, since DDAC was still detected in all the eluted samples. It would be preferable to elute all the DDAC in the first elution step. The results of the 85:15 and 50:50 ratios were the same (Figure 4.9). Higher extraction was achieved with acetonitrile diluted with isopropanol than with acidified acetonitrile.

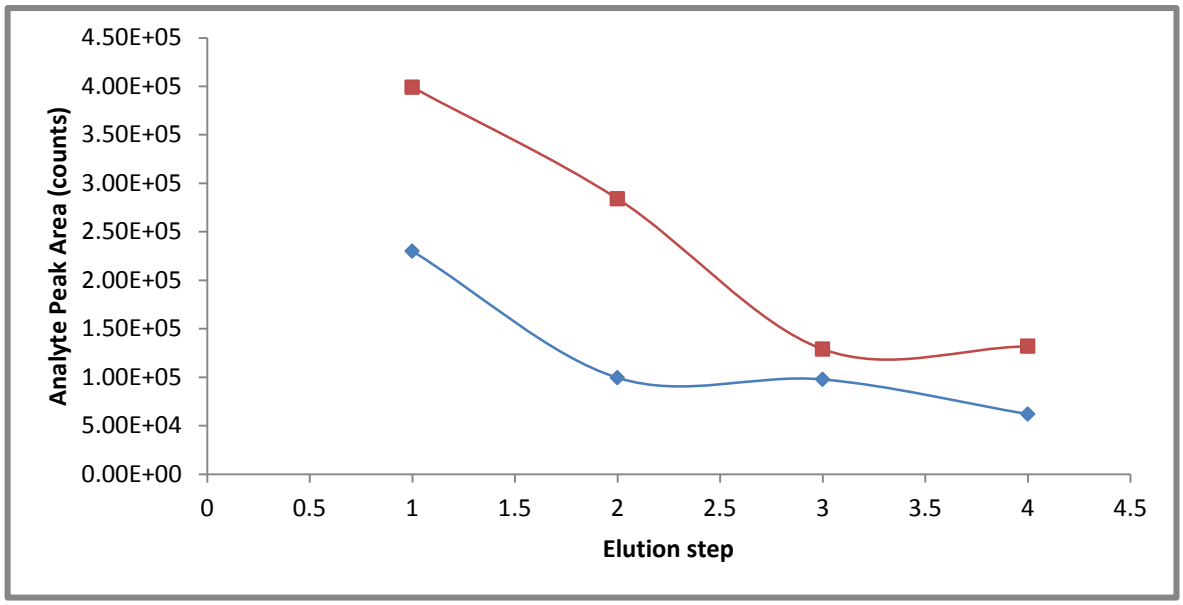

Figure 4.7. Graph showing different amounts of DDAC eluted using various elution solvents. DDAC eluted using acidified methanol (5% formic acid) (Bassarab *et al*., 2011) ◆. DDAC eluted with the method using acetonitrile, water and acetic acid by Van de Voorde and co-workers, 2012 ■.

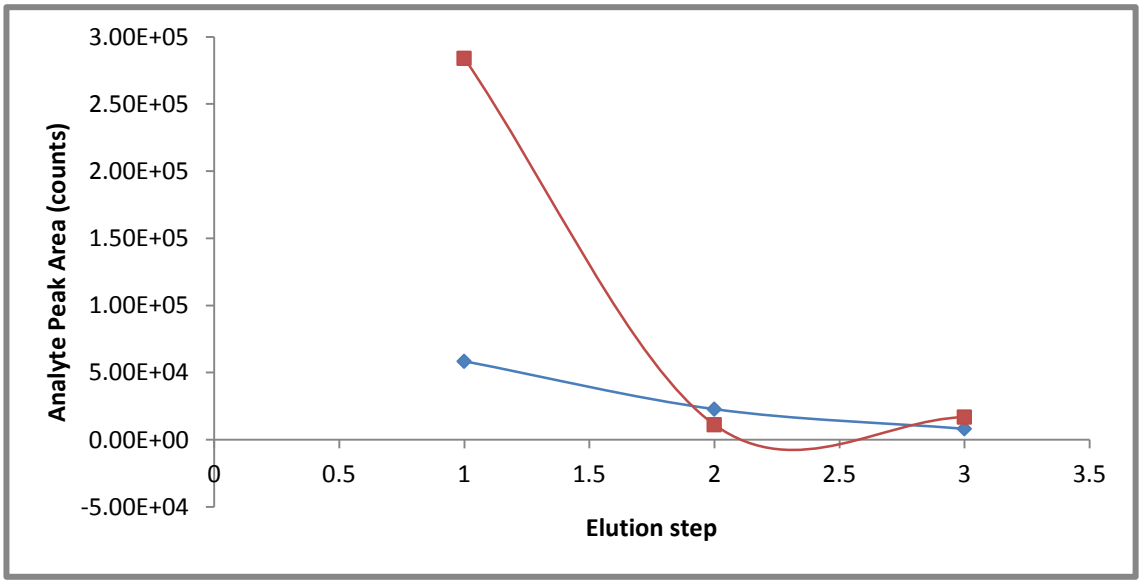

Figure 4.8. Graph showing various amounts of DDAC eluted using different elution solvents. DDAC was eluted using only acetonitrile ◆, as well as the DDAC eluted using acetonitrile acidified with 5% formic acid ■.

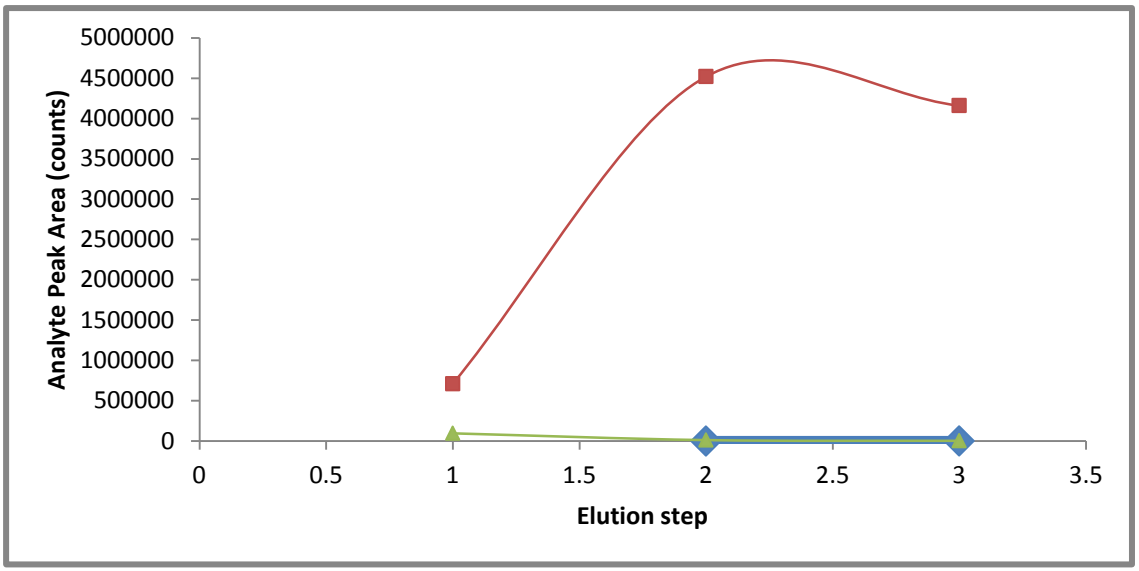

Figure 4.9. Graph showing different amounts of DDAC eluted with various elution solvents. Isopropanol was added to the acetonitrile. DDAC eluted using 85:15 acetonitrile ◆. DDAC eluted using a ratio of 75:25 acetonitrile:isopropanol ■. DDAC eluted using 50:50 ratio of acetonitrile:isopropanol ▲.

Control samples containing no DDAC were tested, since DDAC was detected in all of the eluted samples. A sample consisting of only TSB was extracted. Figure 4.10 shows that DDAC was detected in both the control sample as well as the spiked TSB sample. This indicated that there might be contamination of the TSB with DDAC. Nanopure water from the MilliQ system was extracted to determine if a compound was co-extracted from TSB. Figure 4.11 shows the chromatogram from the sample, with a high amount of DDAC present. The retention time was the same as the retention time for DDAC as seen with the standards of DDAC, which was approximately 5.5 min. Therefore, it can be assumed that the water was either contaminated with DDAC or that another compound with the same retention time as DDAC was detected. Solvent blank runs were included for LC-MS in order to eliminate possible DDAC contamination.

A freeze-dried water sample was directly injected into the LC to determine if the water was contaminated or if the compound was eluted from the SPE cartridge. A compound was still detected in the freeze dried water sample but at very low concentrations. It was decided to dismiss this negligible amount (Figure 4.12).

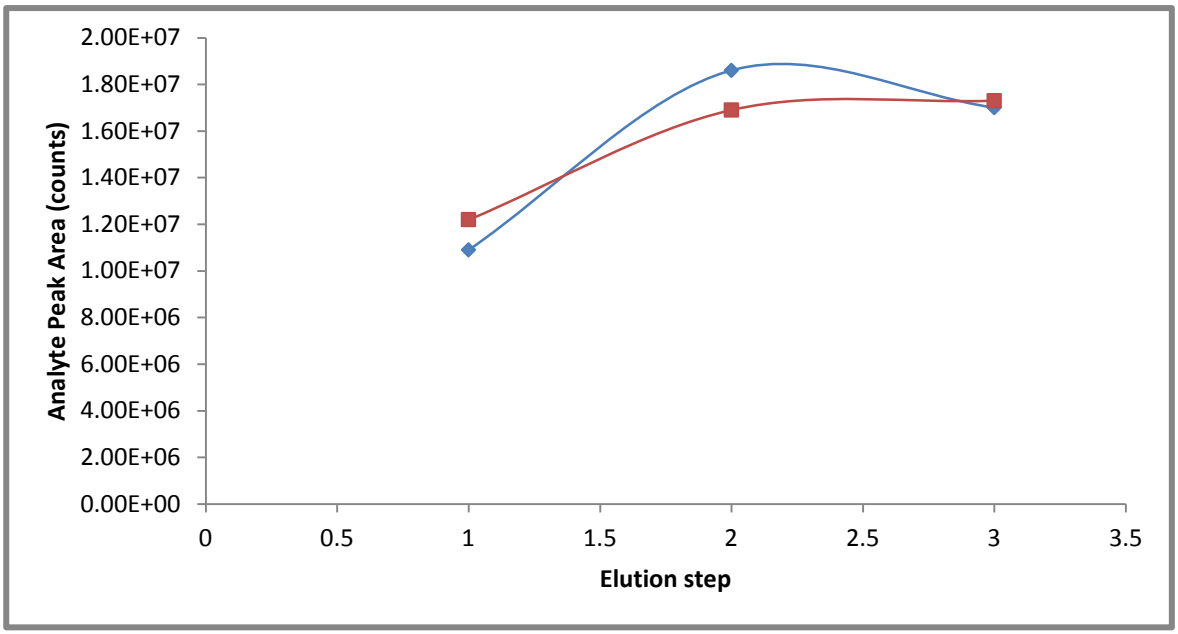

Figure 4.10. Graph showing DDAC eluted from a sample containing 0.01µg ml^{-1} ◆ and a control sample not containing DDAC ■.

Different LC conditions were used, the retention time for DDAC was determined to be 4.24 min. All media was prepared in Nanopure water and sterile plastic containers. DDAC was extracted from an 18 hrs TSB culture containing 0.05 µg ml^{-1} DDAC as well as from control samples, TSB containing DDAC and a water sample. DDAC was detected in the supernatant of the growth sample as well as the TSB control sample, with much more DDAC extracted from the control sample (Figure 4.13) than the sample from the 18 hrs culture (Figure 4.14). Very low levels of non-specific compounds were detected in the water sample (Figure 4.15), which was negligible. Unknown compounds were also detected in the blank sample that was run between samples (Figure 4.16), however this was also negligible.

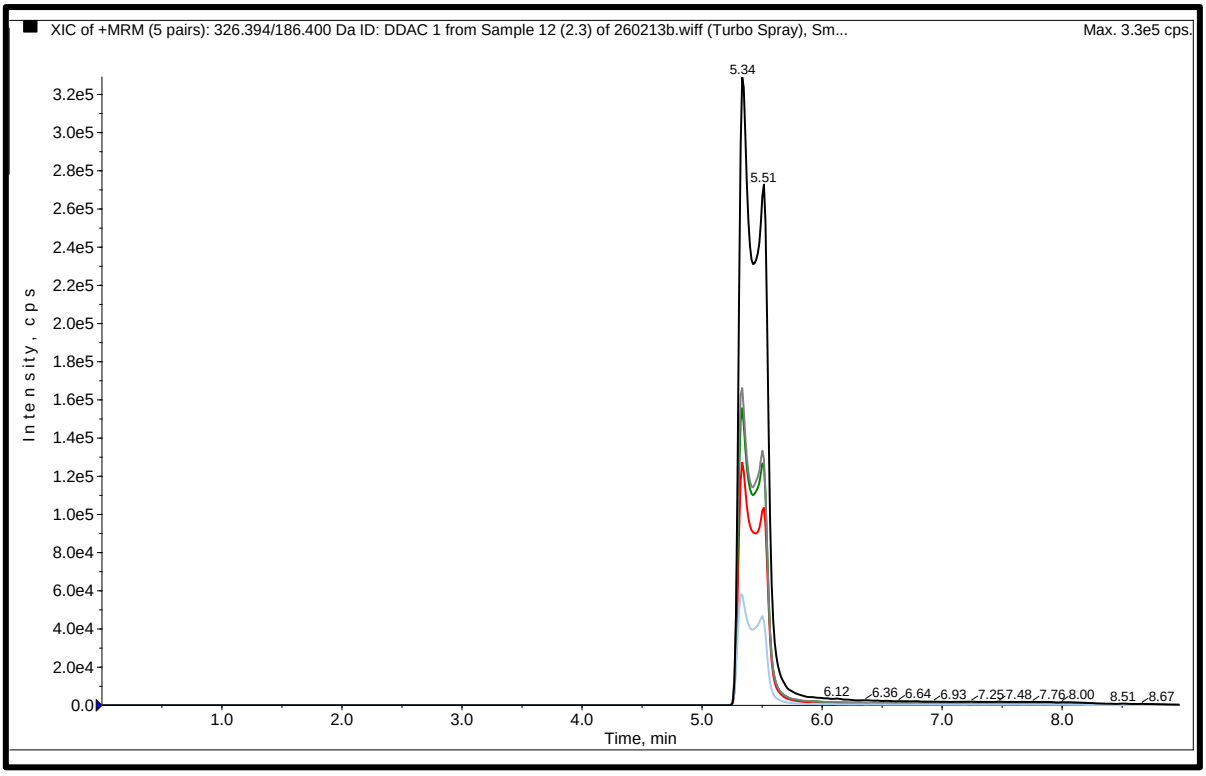

Figure 4.11. Chromatogram of a water sample extracted with SPE. The same retention time for the water sample was obtained as the DDAC standard. The retention time is at approximately 5.5 min. The different colours indicated in the chromatogram represent the various fragments obtained.

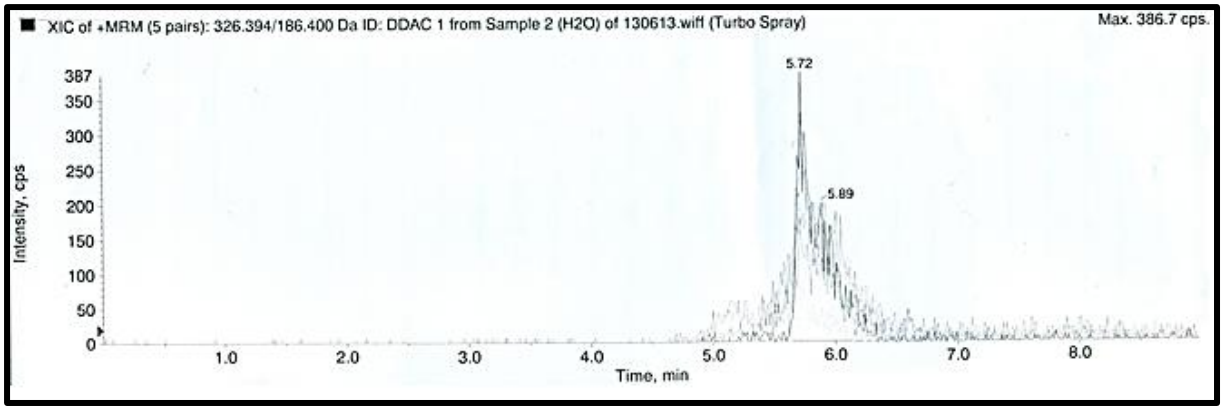

Figure 4.12. Chromatogram of freeze dried water sample. The retention time is 5.72 min.

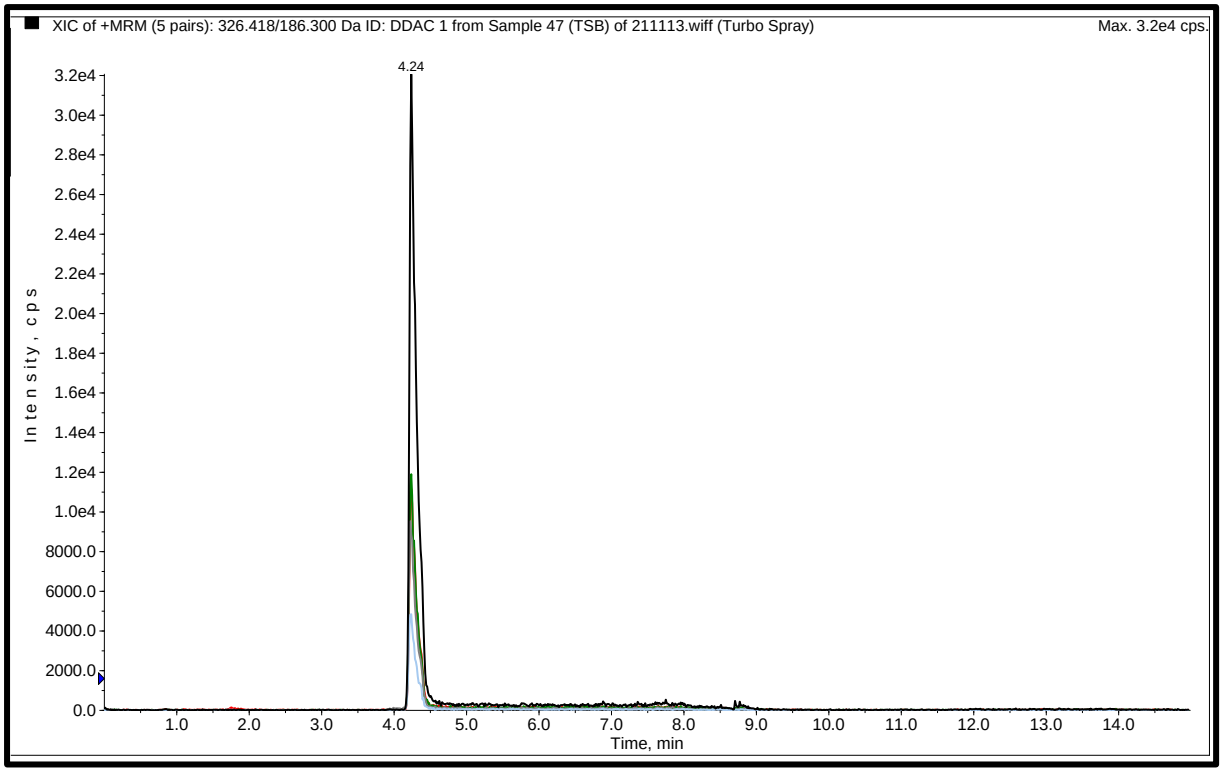

Figure 4.13. Chromatogram of a spiked TSB sample extracted with SPE – elution solvent is 75:25 acetonitrile:isopropanol. The retention time is 4.24 min, under the following LC conditions: a flow rate of 500 uL/min, starting at 60% mobile phase B (MeOH/0.1% formic acid), at 1 min increasing to 90% B and staying there for 4 minutes, before re-equilibrating the column at 60% B for 5 minutes. The different colours represent the various fragments generated.

In Figure 4.13 it can be seen that much more DDAC was extracted than from the sample cultivated for 18 hrs (Figure 4.14), as the height of the former peak is greater than that obtained with the 18 hrs culture. In Figure 4.14 it was seen that the extract was not pure, and that other compounds were also detected. After 4.26 min more peaks are detected in lower amounts, as compared to the DDAC peak. Before 4.26 min, some small peaks can also be observed, however this is very low compared to the DDAC peaks, therefore it may represent background noise.

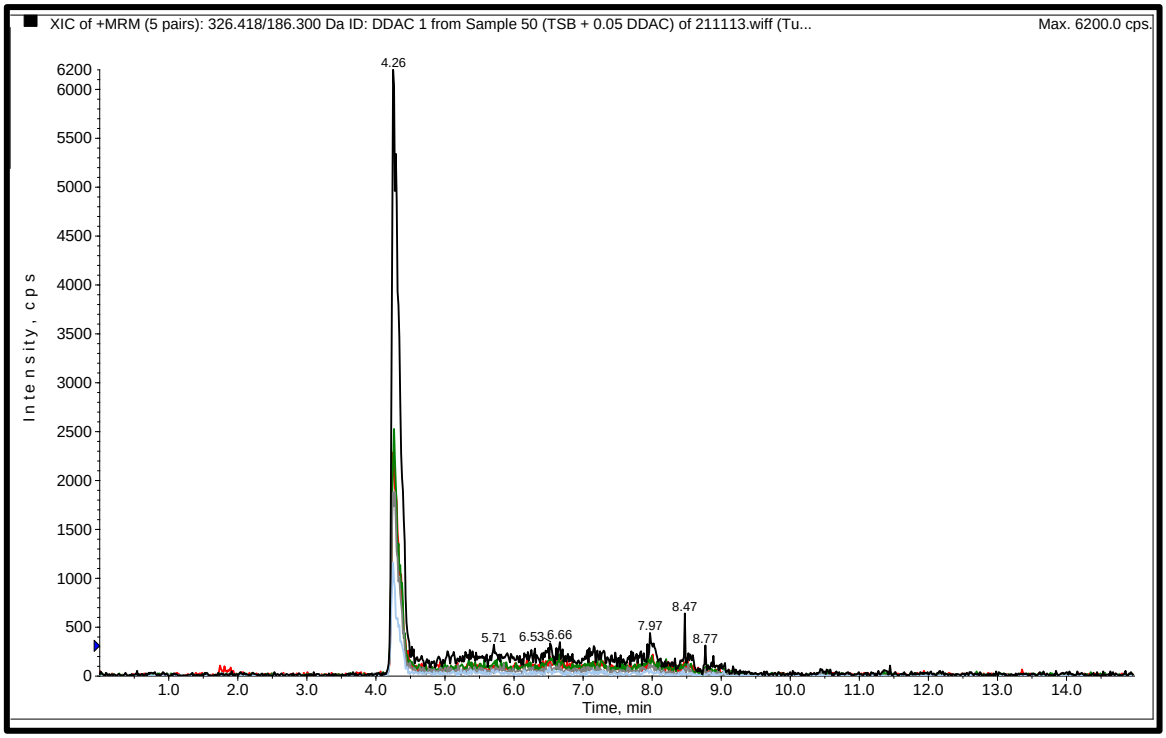

Figure 4.14. Chromatogram of DDAC extracted from TSB containing DDAC inoculated with *Staphylococcus aureus* and cultivated for 18 hrs before extraction. The retention time is 4.26 min, under the new LC conditions. There are other products also detected.

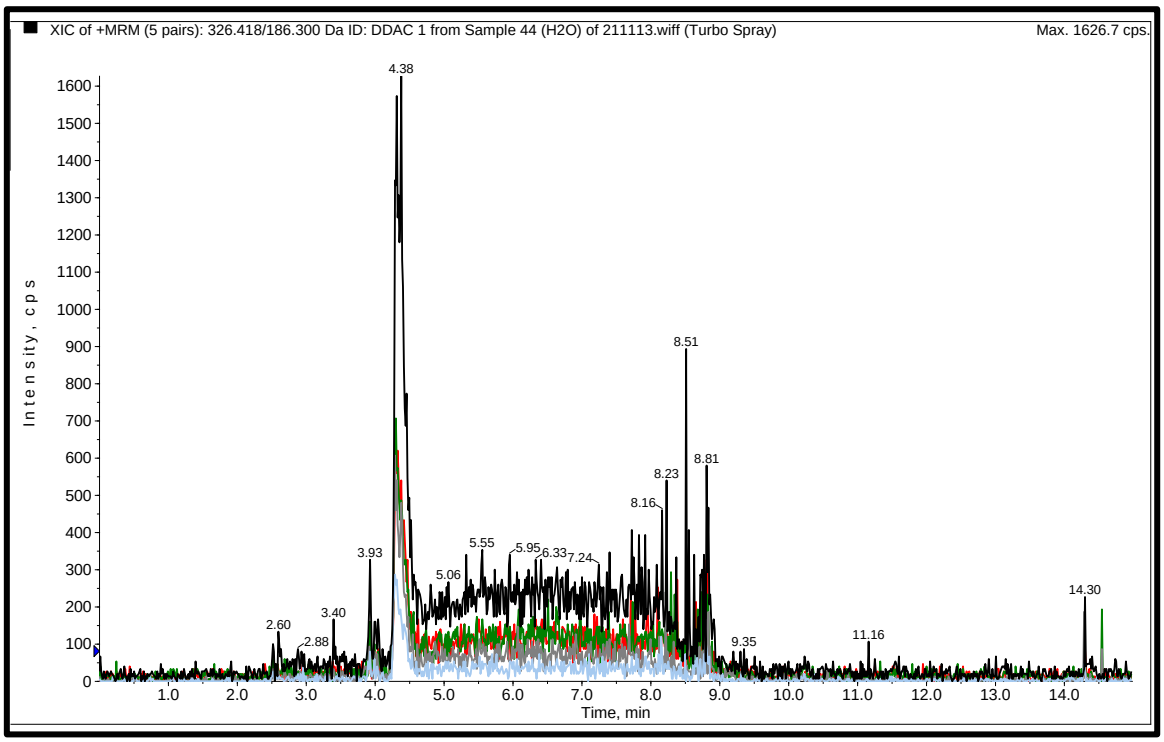

Figure 4.15. Chromatogram of water as control sample with the same SPE procedure being followed. Water sample gives smaller peaks, with much lower amounts.

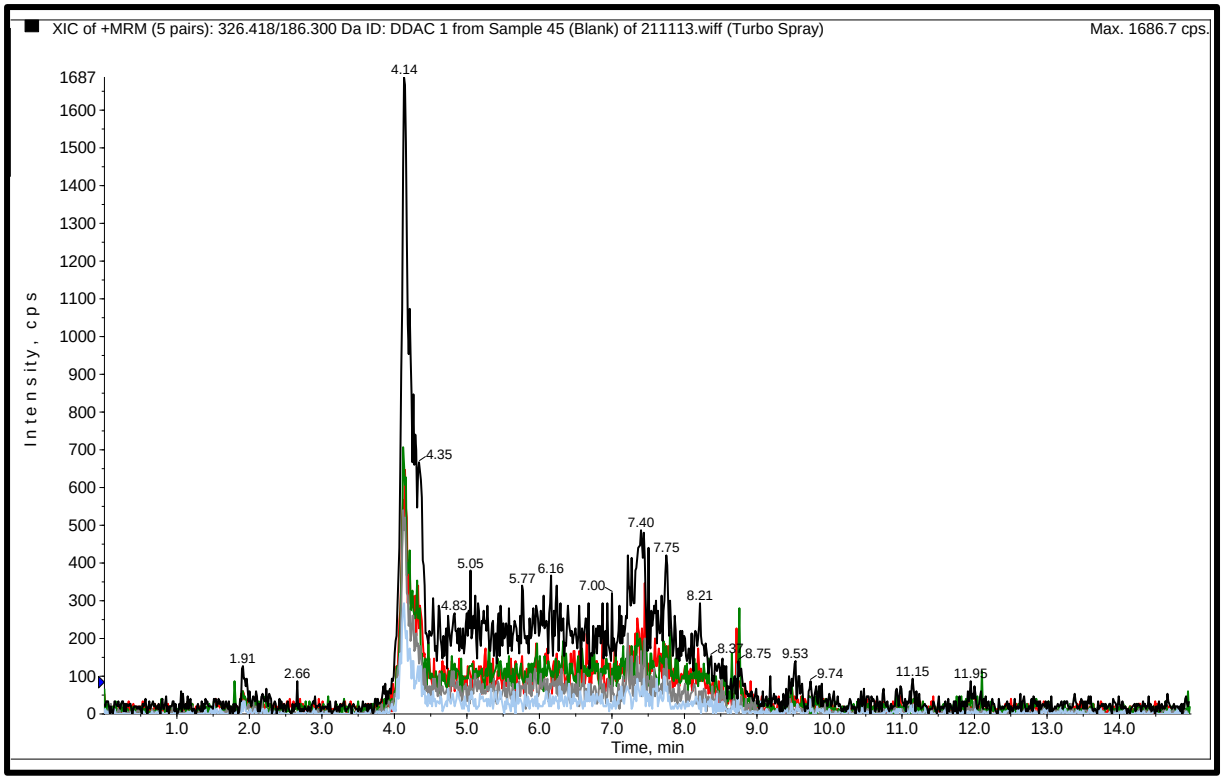

Figure 4.16. Chromatogram of solvent blank used between samples, showing detection of other compounds.

4.4 Discussion

Bacteria have the ability to extrude antimicrobial compounds through the bacterial cell using efflux pumps thus, reducing the concentration of the compound to a non-lethal level inside the cell (Piddock, 2006). In a study by Jansen and co-workers (2013) involving the semi-quantitative elemental analysis on *S. aureus* cells treated with DDAC, trace amounts of chlorine were found. However, the concentration was too low to definitely conclude the presence (Jansen *et al*., 2013). Therefore, the hypothesis for this study was that *S. aureus* modifies the structure of the disinfectant upon influx of the disinfectant before it is pumped out again.

Liquid chromatography-mass spectrometry was used to analyse DDAC extracted from growth samples. LC-MS has been widely used to analyse environmental samples for QACs (Martínez Vidal *et al*., 2004). The LC separates the analytes and the MS detects the analyte (Agilent technologies, 2001). It is also possible to determine the structure of the DDAC using MS and to quantify the concentration or amount of QAC in the growth media by determining the concentration or amount of QAC taken up and expelled by the bacteria.

Solid phase extraction is used to prepare the sample for analysis using LC-MS. Solid phase extraction is a preferred method due to its simplicity and economy in terms of the solvents needed (Castro *et al*., 2000). It is frequently applied to environmental analysis, including extracting QACs from environmental samples (Martínez Vidal *et al*., 2004; Bassarab *et al*., 2011; Van de Voorde *et al*., 2012).

Several modifications were made to the SPE procedure, to optimise the procedure for the extraction of DDAC from growth medium. Extra wash steps and longer drying steps were introduced to reduce elution of contaminants by washing away more of the contaminants and remove all the liquid containing contaminants from the column. Various procedures were used with different elution solvents to find the most efficient SPE method to extract DDAC. However, elution with acetonitrile was more effective. A mixture of acetonitrile and isopropanol was also tested, and seemed to be the most effective in eluting the DDAC. Although DDAC was eluted in all of the elution steps, the obtained results were still not optimum, since not all the DDAC is eluted from the cartridge.

Since DDAC was detected in the control samples it was thought that either the water was contaminated with DDAC or that another compound was eluting from the cartridge. However, the freeze-dried sample showed only little amounts of other compounds, which can be disregarded. The different LC conditions showed that there was no other compound extracted from the SPE cartridge. Therefore, any of the solvents used for the SPE procedure could have been contaminated. This is possible, since even a very small amount of DDAC would ionise and be detected by LC-MS (Kemp, personal communication, 2013).

Solid phase extraction was successfully performed in other studies to prepare samples for LC-MS for the quantification of QACs (Martínez Vidal *et al*., 2004; Bassarab *et al*., 2011; Van de Voorde *et al*., 2012). However, the QACs were extracted mostly from water. In this experiment DDAC was extracted from growth media. Two methods from literature were used to extract DDAC (Bassarab *et al*., 2011; Van de Voorde *et al*., 2012). These methods were selected since some of the QACs they detected were DDAC. These methods were not as efficient as the method obtained from the manufacturer, Phenomenex. It was shown that the 75:25 acetonitrile:isopropanol ratio was the most effective for eluting DDAC. Solid phase extraction did not give optimum results, however, further optimisation of the procedure is needed, or a baseline needs to be determined, so that it can be subtracted from the amount detected. This can be done by disregarding the detected material under a certain amount.

The transition list produced by the MS shows that the parent fragment size of the DDAC is 326.4 g mol^{-1}. This is the size of the DDAC structure without the chlorine. Therefore, the chlorine was also not detected by LC-MS, which is similar to what Jansen and co-workers

(2013) observed. Figure 4.1 shows the structure of DDAC. It is therefore possible that the chlorine cleaved from the QAC molecule, since QACs integrate itself into the outer leaflet of the membrane (Gilbert & Moore, 2005).

Staphylococcus aureus strain VB4_smr was cultivated in TSB in the presence of 0.05 µg ml^{-1} DDAC. The results indicate that the DDAC concentration in the TSB decreased severely after 18 hrs. Thus, it is hypothesized that the DDAC was taken up by the bacterial cell. However, it is uncertain whether the DDAC was pumped out, or the structure modified before being pumped out. The results showed more background noise in the sample than in the control sample as more peaks were detected. Therefore, it is possible that the structure changed and thus the peak of the DDAC would decrease. However, compared to the peak obtained with DDAC this would be very low amounts. It would also be difficult to determine this, since DDAC ionizes very well and would be detected in very low amounts (Kemp, personal communication, 2013). Therefore, any other peak would seem insignificant compared to the DDAC peak. To determine if these other peaks are components of DDAC, the fragment must be isolated and analysed. It is possible that the other peaks detected were due to the extract not being pure enough. The bacteria might also be able to degrade the DDAC. Smaller peaks were detected, however compared to the DDAC peak, it seems insignificant. Degradation of DDAC was shown to occur in fungi (Dubois, 1999), as well as in bacteria such as *Pseudomonas fluorescens* and *Alcaligenes xylosoxidans*, which is Gram-negative bacteria (Nishihara *et al*., 2000). Therefore it is possible that other resistance mechanisms may exist in bacteria in addition to efflux systems including degradation of the QAC.

From the results obtained, it can be seen that determining structural changes of DDAC is not as clear-cut as initially thought. Therefore, other techniques can be used to determine structural changes of DDAC, such as nuclear magnetic resonance. This method is a powerful analytic tool used in the identification of organic compounds (Dubois, 1999). However, different and or optimised extraction methods need to be investigated to obtain better results analysing DDAC.

Chapter 5 General Discussion and Conclusions

Bacteria can obtain resistance against quaternary ammonium compounds (QACs) through the acquisition of plasmids that contain resistance genes (Hegstad *et al*., 2010). These genes code for efflux proteins that can transport the QACs out of the cell (McDonnell & Russell, 1999). In this study the genes *smr, qacJ, qacH* and *qacG,* coding for the small multidrug resistance (SMR) proteins were studied. The aim of this study was to understand bacterial resistance to QACs. By understanding the resistance and how resistance is achieved, it would be possible to prevent similar problems such as antibiotic resistance.

Field isolates identified as *Enterococcus* species were screened for the presence of these four genes, and *qac* resistance genes were present. The results showed that bacteria could contain more than one of these genes, which correlates to studies by Gillings and co-workers (2009a, b). In the studies by Gillings and co-workers, *qac* gene cassettes were found on class 1 integrons from environmental samples, and therefore it was shown that microorganisms can possess the *qac* resistance genes even in the absence of QACs. It was suggested that the efflux pumps help protect microorganisms against toxins in natural ecosystems (Gilbert & McBain, 2003). The role of class 1 integrons is to confer antibiotic resistance (Gaze *et al*., 2005). If antibiotic and *qac* resistance genes are carried on class 1 integrons the selection of QAC resistance may result in co-selection for antibiotic resistance (Gaze *et al*., 2005). Therefore care should be taken so that resistance is not caused by the use of QACs and antibiotics. The *qac* resistance genes are not commonly found in *Enterococcus* species, with only a few reports of *qac* genes being detected, and these include *qac*Δ1, *smr* and *qacA/B* (Bischoff *et al*., 2012).

Using the field isolates the minimum inhibitory concentration (MIC) was calculated for three QACs. This was done in order to correlate the MIC to the number of *qac* resistance genes present in the field isolates. The three QACs tested were benzalkonium chloride (BC), didecyldimethylammonium chloride (DDAC) and Virukill®. However, the MIC did not correlate with the number of resistance genes present in the strain because there were strains that contained more of the *qac* resistance genes than other strains, but did not show a significant increase in resistance against the QACs. It was hypothesised that the strain with the most *qac* resistance genes would be highly tolerant to QAC. However, the current data suggests that the presence of a higher number of resistance genes does not indicate a higher tolerance towards QACs. It is also possible that the *qac* genes present might be

associated with resistance to other cationic biocides, and therefore would have a much higher increase in tolerance if tested with another biocide. This was observed by Smith *et al*., 2008, where there were statistically significant but lower than twofold differences between bactericidal concentrations of QACs between *qac* positive and *qac* negative isolates. However, it was observed that the *qac* resistance genes were associated with at least four times higher resistance to other cationic biocides (Cervinkova *et al.,* 2013).

The *qac* resistance genes code for proteins that are multidrug transporters and they have a wide range of structurally similar substrates (Piddock, 2006). It is possible that another substrate compound was present in the environment which resulted in strains that possessed the *qac* resistance genes. Studies on the resistance to disinfectants in *Listeria monocytogenes* showed that the presence of certain resistance genes do not infer resistance to disinfectants and antibiotics, therefore the presence of resistance genes do not always cause increased tolerance (Romanova *et al*., 2006). Even though resistance against QACs was observed, the MICs are still much lower than the concentrations used in the industry. Concentrations used as disinfectants are usually below 1000 ppm (Tiedink, 2001). However, special care must be taken to ensure that the correct concentrations of disinfectants are used and the proper instructions followed, so that bacteria are not exposed to sub-lethal levels and subsequently produce resistant clones. Therefore, it would be advisable to rotate the usage of QACs with other disinfectants (actives other than QACs) since bacteria can lose their resistance towards a specific compound (Jansen, 2012).

Staphylococcus aureus strains with known *qac* resistance genes as well as an avian pathogenic *Escherichia coli* strain were screened for these resistance genes in the presence and absence of DDAC at different time points. It was found that in the absence of DDAC only the *smr* gene was expressed in all of the strains, whereas the other (*qacJ, qacH* and *qacG*) genes were only detected in their respective reference strains. However, in the presence of DDAC, the four *qac* genes were detected in all the strains that were screened. The *qac* resistance genes were also detected in the QAC susceptible *S. aureus* ATCC 25923 strain. This was unexpected since it was anticipated that the genes would be amplified regardless of whether DDAC was present. It is however possible that the plasmid containing the resistance genes was lost when there was no selective pressure from DDAC. It can be assumed that the respective gene from the strains is integrated into the genome or is inherent to the strain. In the presence of DDAC, the plasmids of the other strains are retained by the bacteria and the different genes were detected. It is possible that conventional PCR is not sufficiently sensitive to detect these genes when the strain is cultivated in the absence of DDAC. This was observed in Chapter 3 where qualitative detection with real-time PCR was employed to detect the genes when the strains were

cultivated in the absence of DDAC. It was found that the *S. aureus* strains contained most of the genes. However, the *qacH* gene was only detected in the VB2_qacH and VB1_qacG strains. Therefore, conventional PCR is not as sensitive as real-time PCR to detect the genes when selective pressure is not present.

In the absence of DDAC, the *smr* gene was detected in the *E. coli* strain, which could be expected since there are similar *qac* resistance genes in Gram-negative bacteria. The *smr* gene was detected in the *E. coli* strain by Jansen (2012). An additional, much larger gene product was amplified with the *smr* primers from *E. coli* in the absence of DDAC. Since Gram-negative organisms have a more complex cell membrane, additional larger proteins might be involved in the efflux since there are two membranes that need to be crossed (Kumar & Schweizer, 2005). Therefore, this larger band detected might be another resistance gene. A larger gene was also observed in a study conducted by Jansen (2012). In that study the two products formed were identified as *smr* and *qacG.* It was suggested that since it is a Gram-negative organism the genes might have different sizes. However, no additional tests were done to identify this gene in this study. The gene was not detected when *E. coli* was cultivated in the presence of DDAC therefore, it is possible that the gene was on another plasmid and that the plasmid was lost. According to Gaze and co-workers (2005), *qacG* is unusual since it is reported to be carried by staphylococcal multiresistance plasmids and class 1 integrons in gram-negative bacteria. However, in this study it was shown that the Gram-negative bacteria can also have the other *qac* resistance genes.

It was shown that the *qac* resistance genes can be found in diverse bacterial species, as seen from the results of this study and what is seen in literature. The major problem with these genes is that they are found on mobile elements such as plasmids and transposons, and can therefore spread easily (Hegstad *et al*., 2010). Some of these elements can even be transferred to different bacterial species (Hegstad *et al*., 2010). Therefore, correct use of disinfectants is crucial to control the bacterial population to prevent an even greater rise in bacterial resistance.

Real-time PCR was used to determine the relative expression of the *qacJ* gene in the QAC tolerant *S. aureus* VB3_qacJ strain and the susceptible strain ATCC 25923. The hypothesis was that the expression of the target gene would increase with increasing concentration of QAC. The gene was induced with 0.01 µg ml^{-1}, 0.1 µg ml^{-1} and 1 µg ml^{-1} DDAC. The relative quantities for the gene were determined using Pfaffl model with efficiency correction. The gene was induced in VB3_qacJ however; there was less than a two-fold increase in expression, indicating no significant increase in expression. The induction with 0.1 µg ml^{-1} was lower than with the 0.01 µg ml^{-1} and 1 µg ml^{-1} concentrations in the VB3_qacJ strain.

The *qacJ* gene was severely down-regulated in the ATCC strain. However, ANOVA analysis showed that there was no significant difference in the expression levels when induced with the different concentrations of DDAC. Thus, the expression level stays consistent in the different concentrations of DDAC. It is possible that the *qacJ* gene is constitutively expressed in the VB3_qacJ strain; however more research on the expression needs to be undertaken before it can be concluded that the gene was constitutively expressed. The findings of the current study are consistent with those of DeMarco and co-workers (2007) where no over-expression of the resistance genes was observed when *S. aureus* was exposed to various QAC agents. DeMarco and co-workers (2007) suggested that the *qac* resistance genes do not always play a significant role in resistance against QACs. The number of copies per cell might also increase the expression of QAC resistance (Bischoff *et al*., 2012; Sasatsu *et al*., 1995). Gilbert and McBain (2003) proposed that there might be natural toxins that are substrates for these genes since these genes were also found in environments devoid of QACs (Gillings *et al*., 2009a). Since different *qac* resistance genes were detected in the VB3_qacJ strain, it is possible that another gene might also be up-regulated to confer resistance. Various studies focused on the expression of the *qacA* gene that code for a protein that is part of the Major Facilitator Superfamily (MFS). The studies show an increase in expression of the *qacA* gene with an increase in substrate concentration (Smith *et al*., 2008; Theis *et al*., 2007). A recent study by Cervinkova and co-workers (2013) has shown significant differences in the expression of *qacA* under certain conditions. The expression was found to be relatively low when QAC concentration was gradually increased. It suggested a dose-dependent induction of the transcription of *qacA*. Furthermore, when a high concentration was added to the bacteria during the exponential growth phase, there was much higher expression. This suggests that the growth phase is important for expression, therefore resistance might be growth-phase dependent (Cervinkova *et al*., 2013). Similar results to the current study were obtained by Jansen (2012) which is contradictory to the studies of Smith *et al*. (2008) and Theis *et al*. (2007), but is similar to some of the results obtained by Cervinkova *et al*., 2013. Results obtained by Jansen (2012) indicated that there was no significant increase in the expression of the *smr* gene with increasing DDAC concentration. This was anticipated, as the *smr* and *qacJ* genes code for proteins within the SMR family. As these genes are part of the SMR family, it is likely that the expression differs to that of the MFS family.

The *qacJ* gene was also detected in the susceptible ATCC strain. However, the gene expression was significantly down-regulated in this strain. It would have been expected that the gene would be up-regulated to survive the harsh conditions through lowering the intracellular concentration of DDAC. Since the strain is more susceptible to QACs, it might

be more energetically favourable for the strain to use other survival mechanisms rather than producing the protein. It is probable that the gene is located on a plasmid with a weak promoter and therefore the gene cannot be over-expressed to allow the cell to survive the concentrations of DDAC. It was shown by Kierzek and co-workers (2001) that the promoter can have an effect on the expression of the gene. Differences between promoter strength were shown for the *smr* and *qacG* gene by Heir and co-workers (1999a). Gillings and co-workers (2009a, b) also showed that the *qac* resistance genes form part of a gene cluster. Thus, it is possible that they function as an operon suggesting that some of the genes can be activated while the others are repressed.

A study by Jansen and co-workers (2013) confirmed that QACs cause leakage of intracellular content. From the study it was hypothesised that *S. aureus* modified the structure of the disinfectant upon the influx of the disinfectant before it is pumped out again. Semi-quantitative elemental analysis was done on *S. aureus* cells treated with DDAC and only trace amounts of chlorine were detected. Chlorine is associated with the DDAC structure as seen in Figure 4.1. Since chlorine was the only marker different from normal biomass it was used to detect DDAC. In this study liquid chromatography-mass spectrometry (LC-MS) was used to determine the DDAC structure as well as to quantify the amount of DDAC in the growth medium after cultivation of VB4_smr in the presence of DDAC.

Solid phase extraction (SPE) is frequently applied in extracting QACs from environmental samples (Martínez Vidal *et al*., 2004; Bassarab *et al*., 2011; Van de Voorde *et al*., 2012). Solid phase extraction was optimised for extraction of DDAC from tryptic soy broth (TSB) and preparation of the sample for LC-MS. Although the SPE method was not optimal, the most effective method was used to determine if there was a difference in the DDAC concentration and structure, before and after 18 hrs of inoculation. Figures 4.13 and 4.14 showed that the amount of DDAC detected was much lower in the sample taken after 18 hrs, as compared to the control sample. Therefore, it was concluded that the *S. aureus* VB4_smr strain assimilated the DDAC. However, it was not determined whether DDAC was pumped out of the cell or if any structural changes occurred. Figure 4.14 showed that the DDAC peak was much smaller with more background noise detected compared to the control sample that contained the same concentration of DDAC. There were also more peaks detected at a later retention time, suggesting that other products were formed by the bacteria. It was possible that small peaks could be obtained in the background noise, which might be unknown compounds being detected. However, observing the compounds was complicated by the fact that DDAC could be detected in trace amounts as it ionises well. The fragment therefore needs to be isolated and identified. A technique that can be used to determine if

there are structural changes to DDAC is nuclear magnetic resonance, since it is a powerful tool to identify organic compounds.

S. aureus may potentially degrade DDAC, as demonstrated in certain Gram-negative bacteria and fungi (Nishihara *et al*., 2000; Dubois, 1999; Gaze *et al*., 2005). From the LC-MS results it can be seen that the molecular size of the DDAC detected was without the chlorine. The size detected was approximately 326 g mol^{-1}. The molecular weight of chlorine is 35 g mol^{-1}, and the complete molecular weight of DDAC is 362 g mol^{-1}. The findings of the current study were consistent with those of Jansen and co-workers (2013) showing that chlorine cannot be detected. Since QACs integrate into the outer leaflet of the membrane, it is possible that the chlorine is cleaved from the QAC molecule upon entering the cell membrane (Jansen *et al*., 2013).

Bacteria have the ability to develop resistance towards QACs, to ensure survival in unfavourable environments (Hegstad *et al*., 2010; Ioannou *et al*., 2007). This is done by acquiring genetic material, such as plasmids, transposons or integrons, which contains resistance genes that code for proteins that confer resistance (Hegstad *et al*., 2010). However, the MICs determined were lower than the concentrations normally used in the industry. Nonetheless, exposing bacteria to sub-lethal levels could produce more clones that are resistant to QACs. Therefore, it is important to use correct disinfectant programmes and to rotate the disinfectants used, so that resistance can be reduced. It was seen that the expression of a *qac* resistance gene did not increase with increasing QAC concentration, as previously hypothesised. This result may be explained by the fact that the gene does not act alone in conferring resistance, and therefore other genes might also be induced. It is thus necessary to investigate the expression of known *qac* resistance genes simultaneously, to determine whether there is a relationship between the different *qac* resistance genes. It is possible that there might be other resistance mechanisms involved including the efflux system.

This dissertation has investigated the resistance towards QACs, as well as the mechanism of resistance. It was shown that disinfectant resistance is much more complex than initially thought and further research is required to understand the full mechanism of resistance against QACs. This would include investigating the *qac* resistance genes simultaneously and other resistance genes that might be involved in the resistance against QACs, as well as expression at protein level. Investigating the efflux mechanism would clarify resistance profiles and help curb the spread of resistance genes.

Summary

Bacterial infections, a major problem in the poultry industry, are controlled through the use of antibiotics. Due to the increase in antibiotic resistance and the restrictions placed on the use of antibiotics in animals, the poultry industry is slowly heading for a post-antibiotic era. The use of disinfectants, like quaternary ammonium compounds (QACs), could possibly be the last resort in the fight against bacterial infections. Resistance against QACs has been observed but needs to be investigated in order to prevent similar resistance problems.

The overall aim of this study was to understand bacterial resistance to QACs, using the following objectives:

- To examine the presence of *qac* resistance genes in field isolates and determining if the number of genes present confer higher resistance.
- To study the expression of one of these *qac* resistance genes in the presence of increasing QAC concentration.
- To study the efflux system, to determine the uptake and efflux of disinfectant, and to determine if the bacteria causes structural changes of the disinfectant.

Bacterial resistance is conferred through acquisition of resistance genes; therefore *qac* resistance genes were studied to understand resistance to QACs. Screening of field isolates using conventional PCR for *qac* resistance genes (*smr, qacJ, qacH* and *qacG*) showed that one strain could contain more than one resistance gene. This could not be correlated with the minimum inhibitory concentration (MIC) of the three QACs tested and possession of more genes did not necessarily make the strain more resistant. *Staphylococcus aureus* strains known to contain at least one of the resistance genes were also screened for these *qac* genes. Conventional PCR showed that all the genes could only be detected in the strain when it was exposed to QAC. Conversely, real-time PCR showed that the genes could be detected even in the absence of QAC, and was detected in susceptible strains as well. Therefore containing the genes does not necessarily confer resistance.

Relative quantitative real-time PCR was used to determine the expression of the *qacJ* gene in the *S. aureus* strains VB3_qacJ and ATCC 25923. It was hypothesised that the expression of the *qacJ* gene would increase with increasing didecyldimethylammonium chloride (DDAC) concentration. However, in the VB3_qacJ strain there was no significant difference in expression when induced with different DDAC concentrations. Expression of

known *qac* resistance genes needs to be investigated simultaneously to determine whether a relationship exists between the *qac* genes conferring resistance.

The mechanism of resistance is mainly efflux of the disinfectants; thereby reducing the disinfectant concentration inside the cell. The efflux mechanism of *S. aureus* was also studied using liquid chromatography-mass spectrometry (LC-MS). The hypothesis was that the bacteria are able to alter the structure of QAC before extruding it from the cell. Samples were prepared for LC-MS using solid phase extraction (SPE). The SPE protocol was optimised for extraction DDAC from growth media. It was shown that the DDAC concentration in the growth media decreased after 18 hrs of growth. It was not determined if structural changes of DDAC occurred.

Resistance to disinfectants is much more complex than originally thought, and therefore resistance is still not fully understood. Further research is required to understand the full mechanism of resistance against QACs, so that similar problems associated with antibiotic resistance can be prevented.

Keywords: Quaternary ammonium compounds, PCR, quantitative real-time PCR, *Staphylococcus aureus*, liquid chromatography-mass spectrometry, solid phase extraction

Opsomming

Bakteriese infeksies, wat 'n groot probleem in die pluimvee bedryf is, word beheer deur die gebruik van antibiotika. As gevolg van die toename in weerstandigheid teen antibiotika en die beperkings wat geplaas is op die gebruik van antibiotika in diere, is die pluimvee bedryf stadig oppad na ‘n post-antibiotiese era. Die gebruik van ontsmettingsmiddels, soos kwaternêre ammonium verbindings (KAVs), kan moontlik die laaste uitweg in die stryd teen bakteriese infeksies wees. Weerstand teen KAVs is al waargeneem, maar dit moet ondersoek word in orde om soortgelyke weestandigheid te voorkom.

Die oorhoofse doel van hierdie studie was om bakteries weerstand teen KAVs te verstaan, met behulp van die volgende doelwitte:

- Die teenwoordigheid van die *kav* weerstands gene in veld isolate te ondersoek en te bepaal of die aantal gene teenwoordig hoër weerstand verleen.
- Die uitdrukking van een van hierdie *kav* weerstands gene te bestudeer in die teenwoordigheid van toenemende KAV konsentrasie.
- Die uitpomp stelsel te bestudeer, deur die opname en uitvloei van die ontsmettingsmiddel te bepaal, en om te bepaal of die bakterieë strukturele veranderinge van die ontsmettingsmiddel veroorsaak.

Bakteriese weerstand word toegeken aan die verkryging van weerstands gene, dus was *kav* weerstands gene bestudeer om weestand teen KAVs te verstaan. Sifting vir die *kav* gene (*smr, qacJ, qacH,* en *qacG*) in veld isolate is gedoen met behulp van konvensionele PKR en het getoon dat een stam meer as een weerstands geen kan bevat. Dit het nie gekorreleer met die minimum inhiberende konsentrasie (MIK) van die drie KAVs was getoets is nie, aangesien die besit van meer gene nie noodwendig die stam meer bestand maak nie. *Staphylococcus aureus* stamme wat bekend was om ten minste een van die weerstands gene te bevat is ook gesif vir hierdie *kav* gene. Konvensionele PKR het getoon dat al die gene net opgespoor kon word as die stamme blootgestel was aan KAV. Aan die ander kant het werklike tyd PKR getoon dat die gene opgespoor kon word selfs in die afwesigheid van KAV, en dit was ook gesien in die vatbare stam. Daarom verleen die teenwoordigheid van die gene nie noodwendig weerstand.

Relatiewe kwantitatiewe werklike tyd PKR is gebruik om die uitdrukking van die *qacJ* geen in die *Staphylococcus aureus* stamme VB3qacJ en ATCC 25923 te bepaal. Die hipotese was

gestel dat die uitdrukking van die *qacJ* geen sal toeneem met toenemende didecyldimethylammonium chloried (DDAC) konsentrasie. In die VB3_qacJ stam was daar geen beduidende verskil in die uitdrukking wanneer geïnduseer is met verskillende DDAC konsentrasies nie. Dus moet uitdrukking van bekende *kav* weerstands gene gelyktydig ondersoek word om te bepaal of daar ‘n verband is tussen die gene.

Die meganisme van weerstand teen ontsmettingsmiddels is hoofsaaklik die uitpomp van die ontsmettingsmiddel. Dus word die konsentrasie van die ontsmettingsmiddel in die sel verlaag. Die uitpomp meganisme van *S. aureus* is ook bestudeer met behulp van vloeistofchromatografie-massa spektrometrie (VC-MS). Die hipotese was dat die bakterieë in staat is om die struktuur van KAV te verander voordat dit uit die sel gepomp word. Monsters was voorberei vir VC-MS met behulp van soliede fase onttrekking (SFO). Die SFO protokol is aangepas vir die onttrekking van DDAC uit groei media. Daar was gewys dat die DDAC konsentrasie in die groei media afgeneem het na 18 uur groei. Dit is egter nie bepaal of strukturele veranderinge van DDAC plaasgevind het nie.

Weerstand teen ontsmettingsmiddels is veel meer kompleks as wat aanvanklik gedink is, en daarom word weerstand nog nie ten volle verstaan nie. Verdere navorsing is nodig om die volle meganisme van weerstand teen KAVs te verstaan, sodat soortgelyke probleme wat verband hou met antibiotiese weerstand verhoed kan word.

Sleutel woorde: Kwaternêre ammonium verbindings, PKR, kwantitatiewe werklike-tyd PKR, *Staphylococcus aureus*, vloeistofchromatografie-massa spektrometrie, soliede fase onttrekking

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