

**Evaluation of the role of ATP-binding cassette (ABC) transporters on
chlorhexidine digluconate biocide activity in nosocomial pathogens**

By

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**Thesis submitted in partial fulfilment of the degree of Masters of Science in
Biotechnology**



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November 2015

DECLARATION

I Molly Mombeshora declare that this thesis is the result of my own independent experimental work, carried out from March 2015 to November 2015 at the University of Zimbabwe except where otherwise stated. Other sources used are acknowledged in the thesis by explicit references. The work has at no time been submitted to meet the requirements for any other award.

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ABSTRACT

The use of biocides against bacterial infections over the years has brought numerous benefits to the health sector, but a disturbing prospect of biocide resistance to nosocomial pathogens is emergent. The evolution of biocide resistant microorganisms increases the frequency of nosocomial infections. Nosocomial infections are a cause of elevated morbidity and mortality and impose extra expenses upon the health service. Nosocomial pathogens have been reported to show signs of resistance towards the commonly used biocide chlorhexidine digluconate. Efflux is a common mechanism liable for bacterial resistance to biocides. The aim of the study was to evaluate the role of ATP-binding cassette (ABC) transporters on accumulation of chlorhexidine in bacterial cells. In this study, it was hypothesized that the effectiveness of chlorhexidine against bacteria could be reduced by ABC transporters. Understanding the role of the ABC transporter in the effluxing of chlorhexidine digluconate from bacterial cells would be a basis for rationale development of efflux pump inhibitor (EPI) to counteract the pumping out of the biocide. The inhibition of efflux pump activity by an EPI would maintain or increase the effectiveness of the biocide. A clinical strain each of *P. aeruginosa* and *S. aureus* and their respective laboratory strains ATCC 27853 and ATCC 9144 were used in the study. All four isolates were tested by the broth microdilution method to compare the changes in the minimum inhibitory concentration (MIC) values in the absence and presence of an EPI. The comparison served to indicate if the isolates exhibited any efflux pump activity. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine were the EPIs utilised. A spectrophotometric method to quantify the amount of chlorhexidine digluconate accumulated within bacterial cells was developed. The suitability of using the UV/VIS spectrophotometer in quantifying chlorhexidine at a wavelength of 255 nm was validated based on the linear regression value obtained from the calibration curve for the absorbance of a series of chlorhexidine concentrations (12 μ M to 0.75 μ M). The spectrophotometric method developed was used to measure the amount of chlorhexidine accumulated in the presence of EPIs: reserpine or CCCP. Sub-inhibitory concentrations of CCCP and reserpine pre-determined in the study using the MICs of the respective EPIs were used in the accumulation assays. The MIC values of chlorhexidine digluconate against the clinical strains of *P. aeruginosa* and *S. aureus* showed a two-fold reduction (from 6.3 μ g/ml to 3.2 μ g/ml) in the presence of reserpine. The presence of reserpine failed to show any change on the MIC value of chlorhexidine digluconate against ATCC isolates of both *P. aeruginosa* and *S. aureus*. The MIC values of chlorhexidine digluconate in the presence of CCCP for the clinical and ATCC strain of *P. aeruginosa* remained unchanged at 6.3 μ g/ml and 3.2 μ g/ml respectively. In the presence of CCCP, the two isolates of *S. aureus* showed a magnified reduction in their MIC value of chlorhexidine digluconate. A linear regression value of 0.99 was obtained from the calibration curve for the absorbance of a series of chlorhexidine concentrations. The linear regression value validating the suitability of using the spectrophotometric method developed to quantify the amount of chlorhexidine accumulated in cells. The bioassay may be used to find novel inhibitors of efflux of chlorhexidine. At $P < 0.05$, only the clinical strain of *P. aeruginosa* showed a significant active efflux of chlorhexidine, while the ATCC strain of *P. aeruginosa*, the clinical and ATCC strains of *S. aureus* exhibited no signifying active efflux. The findings of this study suggest the participation of ABC transporters in pumping out chlorhexidine digluconate from clinical strain cells of *P. aeruginosa*.

ACKNOWLEDGEMENTS

I would like to sincerely thank the following people:

1. Prof S. Mukanganyama for being a great mentor as I was carrying out this research. His unlimited academic guidance and exemplary maintenance of integrity cannot be fully expressed but is greatly appreciated.
2. The International Program in the Chemical Sciences (IPICS), Uppsala University, Sweden and the International Foundation for Science (IFS), Stockholm, Sweden who's funding through the efforts of my supervisor made adequate equipment for the study available.
3. Members of the Biomolecular Interactions Analyses (BIA) for the constructive criticism and sharing of knowledge.
4. Colleagues whose suggestions and remarks have been of significant value to my final work.
5. Members of the Biochemistry staff who played their respective roles to make the study accomplishable.
6. My family for understanding and supporting me.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ANOVA	Analysis of Variance
ATCC	American Type Control Culture
ATP	Adenosine Triphosphate
BIA	Biomolecular Interactions Analyses
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standards Institute
DMSO	Dimethyl Sulfoxide
EPI	Efflux Pump Inhibitor
HPA	Health Protection Agency
ICC	Infection Control Committees
MATE	Multidrug and Toxic Compound Extrusion
MBC	Minimum Bactericidal Concentration
MDR	Multiple Drug Resistance
MFS	Multidrug Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>

OD	Optical Density
PBS	Phosphate Buffered Saline
RND	Resistance Nodule Division
rpm	Revolutions per minute
SMR	Small Multidrug Resistance
spp	Species
SSI	Surgical site infection
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

1 Introduction

1.1 Background

The institutional epidemics of infectious diseases experienced in the 1960s led to the appearance of Infection Control Committees (ICCs) in the United States (Smith and Rusnak, 1997). The ICCs managed to establish hospital acquired infection control programs which are now a requirement in healthcare facilities. Hospital-acquired infections alternatively termed nosocomial infections can be defined as, any infection picked up in hospital by a patient who was admitted for a different reason from the acquired infection (Ducel *et al.*, 2002).

Nosocomial microorganisms are the major infectious agents of hospital acquired infections, resulting in significant morbidity and mortality within the healthcare facilities (Sun *et al.*, 2013). *Staphylococcus aureus*, *Enterococcus*, *Klebsiella* spp, *Acinetobacter* spp and *Pseudomonas aeruginosa* are some of the major nosocomial pathogens (Todorova-Christova *et al.*, 2014) with methicillin-resistant *Staphylococcus aureus* (MRSA) and multiple drug-resistant (MDR) *M. tuberculosis* being among the most problematic. Most pathogens are found on hospital equipment such as catheters, stethoscopes, endotracheal tubes and soap dispensers (Joshi *et al.*, 2013). In the event of inadequate disinfection, the adulterated equipment may end up being a means of transmission of the nosocomial pathogen to the susceptible host. In order to eliminate nosocomial pathogens from causing infections, control programs have to be diligently implemented to break the vicious triangle of the infectious agent, means of transmission and the susceptible host-the patient (Ashraf *et al.*, 2014).

Recommendations for infection control programs are developed centred on the understanding of currently available evidence. The recommendations put in place cover the structure as well as the role of the infection control program. Infection control programs include

surveillance, employee health, isolation precautions, patient care and outbreak control (Smith and Hunter, 2008). The use of disinfectants and antiseptics is the main mode of action used to try and combat the proliferation of nosocomial pathogens.

Commonly used disinfectants include triclosan, benzalkonium chloride, sodium hypochlorite, peracetic acid and chlorhexidine gluconate (Morrissey *et al.*, 2014). Chlorhexidine gluconate is one of the notably effective broad spectrum biocide used, both as a disinfectant and antiseptic (Sun *et al.*, 2014). Chlorhexidine is effective against Gram-positive and negative bacteria and exhibits bactericidal as well as bacteriostatic activity depending on antimicrobial concentration (Behiry *et al.*, 2012). When biocides are correctly used, bacteria generally show susceptibility to biocides. However, their indiscriminate use in personal care products, food production, human medicine and agriculture has raised concerns about the development of bacterial biocide resistance (Horner *et al.*, 2012; Arderbili *et al.*, 2014).

According to literature, due to resistance, occasionally the presence of nosocomial pathogens may fail to be completely eradicated, even after employing antimicrobial agents in cleaning hospital floors and equipment, (Smith and Hunter, 2008; Ashraf *et al.*, 2013). Changes in cell permeability, biofilm formation, metabolism of the biocide and efflux pumping are among some possible reasons for the continued survival of these pathogens (Saleh *et al.*, 2012).

Both prokaryotic and eukaryotic organisms have efflux pumps. The exporting pumps are protein in nature and they span the bacterial cell membrane, playing the role of transporting a particular substrate or an array of structurally similar compounds (Gestin *et al.*, 2013). The major classes of efflux pumps are: MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily), SMR (small multidrug resistance), RND (resistance nodule division) and ABC (ATP-binding cassette) (Piddock, 2006). Efflux pumps may be broadly classified according to the energy source used for the export of substrates. ATP-binding

cassette (ABC) transporters utilize the energy of ATP binding and hydrolysis to export a wide range substrates across cellular membranes (Holmes *et al.*, 2012).

The activity of efflux pumps can be repressed by efflux pump inhibitors (EPIs). Verapamil, reserpine, chlorpromazine and carbonyl cyanide m-chlorophenylhydrazone (CCCP) are some of the most common efflux pump inhibitors which can increase absorption of some compounds (Askoura *et al.*, 2011; Sun *et al.*, 2014). The inhibitory effect of EPIs can be applied to conduct trials in drug development, which can be used in conjunction with biocides to act as inhibitors for the efflux-mediated extrusion in order to improve efficacy. In this study the role of ABC transporters in the efflux of the biocide chlorhexidine digluconate was evaluated using CCCP and reserpine as efflux pump inhibitors.

1.2 Nosocomial infections

The key elements primary to a nosocomial infection are; the infectious agent, a predisposed host and a transmission vector. In a hospital set up, all three elements are almost permanently found together. The most frequent nosocomial infections include infections of the respiratory tract, surgical wounds, urinary tract and gastrointestinal tract (Smith *et al.*, 2008). According to a WHO study (WHO/CDS/CSR/EPH/2002.12) carried out in 2002, nosocomial infections are more prevalent in orthopaedic wards, acute surgical and intensive care units. It has been assessed in England, that the development of a surgical site infection (SSI) results in a patient sustaining an estimated extra hospital stay of one week and twice the hospital charges (Plowman *et al.*, 2001).

1.2.1 Nosocomial pathogens

There are numerous microorganisms associated with nosocomial infections, which include both Gram-positive and Gram-negative bacteria such as *P. aeruginosa*, *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella* spp, *Enterococcus* spp., *Streptococcus* spp, *Escherichia coli* as well as yeasts such as *Candida albicans* and fungi (Weigelt *et al.*, 2010). According to studies carried out by the health protection agency in 2010, *S. aureus* was the causative microorganism in thirty-one percent of cases of infections in the UK (Health Protection Agency, September, 2010). *Pseudomonas* spp., *Klebsiella* spp and *E. coli*, were associated with antimicrobial resistant bacterial septicaemia in Ile-Ife, Nigeria (Komolafe and Adegoke, 2008).

The activity of a biocide against nosocomial pathogens depends on a range of factors, some intrinsic to the biocide and others to the microorganisms. Figure 1.1 shows the general scale of resistance to biocidal exposure among infective agents. Prions show the greatest resistance to antiseptics and most routine methods of decontamination (McDonnell and Burke, 2003) while enveloped viruses show the least resistance. The lack of resistance to biocides in viruses has been attributed to the absence of a varied range of resistance mechanisms. Only a few viral resistance mechanisms have been identified; the formation of viral aggregates during biocide exposure being the most important (Chen *et al.*, 2014).

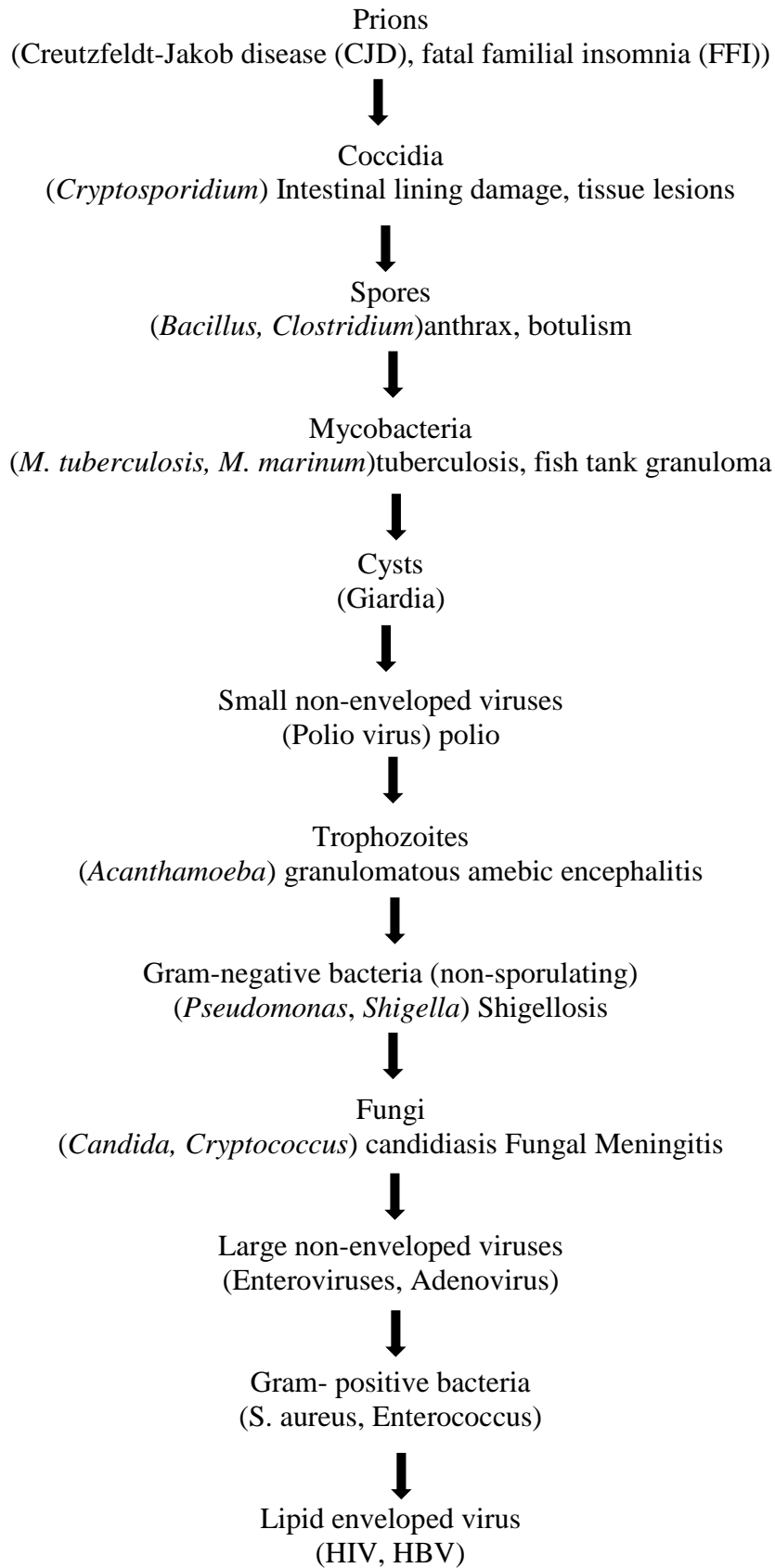


Figure 1.1: Descending order of resistance to antiseptics and disinfectants among infective agents adapted from McDonnell and Russell (1999).

The physiological adaptation of the microorganism to its environment also affects susceptibility to biocides, with planktonic cells showing greater susceptibility when compared to adherent biofilms (Pidcock, 2006). Assessment of the change in resistance of *Burkholderia cepacia* to ceftazidime and cefrofloxacin by Desai *et al.*, (1998), revealed that bacteria forming a biofilm were 15 times more resistant to the antibiotics than the planktonically grown bacteria.

The structure of a microorganism plays a distinctive role in the microbes' susceptibility to biocides. Maillard, (2001) highlighted that enveloped viruses display greater sensitivity to disinfection when compared to unenveloped viruses. The Gram-negative bacteria are largely considered to be less sensitive to many antimicrobials than Gram-positive bacteria (Pidcock, 2006). Traditionally, this has been accredited to the "permeability barrier" in Gram-negatives conferred by the bacterial cell envelope, which limits uptake into the cell (Lambert, 2002). A simplified diagram (Figure 1.2) shows the differences in the structure of Gram-negative and Gram-positive and potential targets of biocides. A typical Gram-positive bacteria used in this study is *S. aureus* and *P. aeruginosa* represents a typical Gram-negative bacteria.

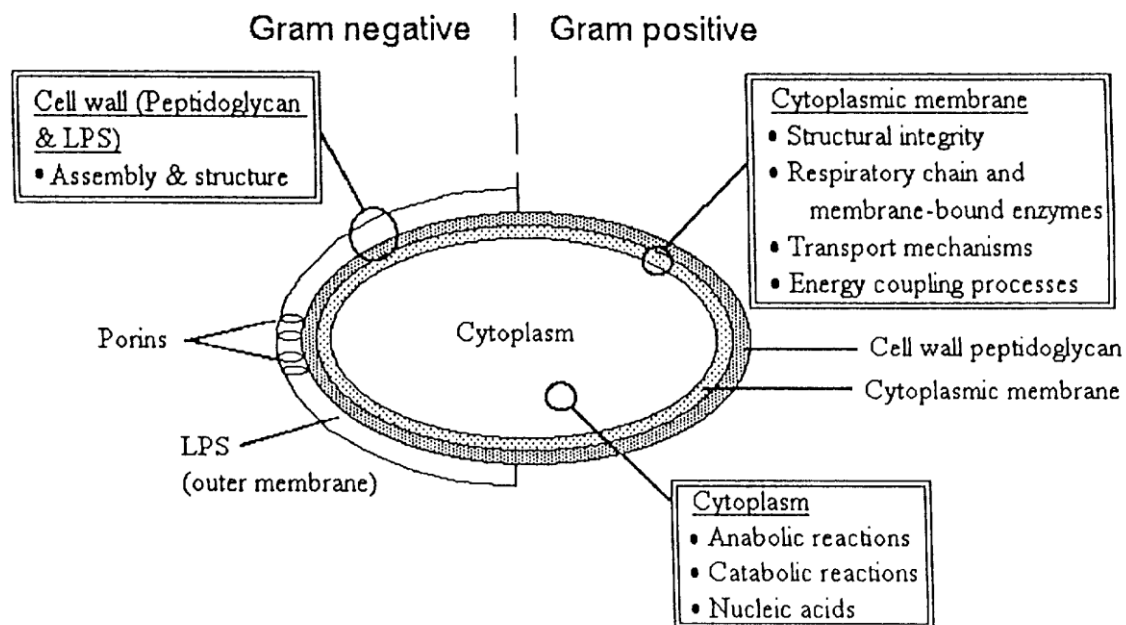


Figure 1.2: Differences in the structure of Gram-negative and Gram-positive and potential targets of biocides; cytoplasm, cytoplasmic membrane and cell wall (Denyer and Stewart, 1998).

1.2.1.1 *Staphylococcus aureus*

Staphylococcus is both a commensal and a nosocomial pathogen commonly playing an opportunistic role when associated with foreign body implants (Prag *et al.*, 2013).

Staphylococcus is responsible for more alien body and device- associated infections than any other microorganism, making it of particular research interest (Rohde *et al.*, 2010). This is where pre-operative sterile preparation becomes an important approach for reducing the threat of complications like surgical site infection (SSI). The virulence of *Staphylococcus* is chiefly due to the capacity of particular strains to produce biofilms. Biofilms facilitate the microbes to irreversibly attach to each other and onto animate or inanimate surfaces (Curtin and Donlan, 2006). The Gram-positive *S. aureus* has a history of increasing reports of antibiotic and biocide resistance (Paniagua-Contreras *et al.*, 2014). Energy-dependent efflux has been reported in *S. aureus* (Paulsen, 1996; Chitemerere and Mukanganyama, 2011). Efflux is one reason thought to be responsible for biocide resistance in *S. aureus*.

1.2.1.2 *Pseudomonas aeruginosa*

P. aeruginosa is among some gram-negative pathogens found in hospitals, known to cause hospital-acquired infections (Alaghebandan *et al.*, 2012). The Gram-negative rod usually has extra simple nutritional requirements and can utilise a varied range of organic substrates. The simple nutritional requirements of *P. aeruginosa* allow the species to proliferate in environments other microorganisms may find difficult to thrive in due to inadequate nutrients (Madigan *et al.*, 2001). *P. aeruginosa* commonly infect damaged tissues or individuals with compromised immunity. Problems that may result from skin incision during surgery or insertion of indwelling medical devices are widely documented, and are frequently caused by resident skin microorganisms such as *Staphylococcus epidermidis*, *C. albicans* as well as *P. aeruginosa* (Todorova-Christova *et al.*, 2014). The ability of these microorganisms to form biofilms further complicates infection as biofilm formation lowers permeation of antimicrobials. In addition to biofilm formation, Soto, (2013) suggested that efflux systems for the intrinsic resistance observed in *P. aeruginosa*. Sun and co-workers recently referred to an operon coding for an efflux system, *MexAB–OprM*, in wild-type *P. aeruginosa* (Sun *et al.*, 2014). The presence of the *MexAB–OprM* operon in *P. aeruginosa* was confirmed by deleting the genes encoding components of the efflux system conferring hypersusceptibility to a variety of antimicrobial agents (Aparna *et al.*, 2014). Dreier and Ruggerone, (2015) also confirmed the frequent detection of an elevated expression of chromosomally encoded multidrug efflux pumps in clinical isolates of *P. aeruginosa*. Efflux pump activity in *P. aeruginosa* is, therefore, a great contributor to the worrying emergence of multi-drug resistance phenotypes.

1.2.2 Determining the susceptibility of nosocomial pathogens to biocides

Validating the effectiveness of disinfection is an important but often challenging task. In order to compare or study susceptibility of selected microorganisms, minimal inhibitory concentrations (MICs) are commonly used (Mazzola *et al.*, 2009). The determination of MICs involves using serial dilutions of the biocide to determine the concentration intervals, which reduce microbial growth for starting inocula of 1×10^6 C.F.U/ml. A low MIC value shows that a low concentration of a given antimicrobial is needed to inhibit microbial growth therefore the tested microorganism is highly susceptible to the antimicrobial (Sun *et al.*, 2014).

1.3 Biocides

Biocides are important compounds that provide the world with numerous benefits. A biocide is defined as any substance or mixture capable of generating one or more active substances with the intention of destroying or inactivating the action of any harmful microorganism (McDonnell and Russell, 1999). Biocides generally possess a greater spectrum of activity when compared to antibiotics. The major difference between biocides and antibiotics is that, biocides tend to have less specific intracellular targets or multiple targets (Ashraf *et al.*, 2013). According to the European Commission's Biocidal Product Directive 98/8/EC (1998), biocides can be categorised into four main groups – disinfectant, pest control, preservative, and other biocidal products. A disinfectant is highly similar to an antiseptic; their major differentiating aspect is that, a disinfectant acts on inanimate objects or surfaces while an antiseptic is used in or on living tissue (Mckenry and Salerno, 2001). A summary table of commonly used biocides is shown in Table 1.1. A single biocide may have a more than one use, for example chlorhexidine may be used as an antiseptic, an antiplaque agent, a disinfectant and a preservative agent (Maillard, 2005).

Table 1.1: A summary of classes of biocides and their use

Biocide class	Typical examples	Use
Alcohols	Ethanol, isopropanol	Antisepsis Disinfection Preservation
Aldehydes	Glutaraldehyde Formaldehyde Formaldehyde-releasing agents o-Phthalaldehyde	Disinfection Preservation Sterilisation
Biguanides	Chlorhexidine, Alexidine, Polymeric biguanides	Antisepsis Antiplaque agents Disinfection Preservation
Bisphenol	Triclosan, Hexachlorophene	Antisepsis Antiplaque agents Deodorants Preservation
Halogen-Releasing Agents	Chlorine compounds, iodine compounds	Antisepsis Cleaning Disinfection
Quaternary Ammonium Compounds	Benzalkonium chloride, Cetrimide	Antisepsis Cleaning Disinfection Preservation

Adapted from McDonnell and Russell (1999)

In an attempt to reduce the number of infections caused by nosocomial pathogens and raise levels of infection control within hospitals, there is extensive use of biocides as disinfectants and antiseptics.

1.3.1 Biocide use in healthcare settings

The innovative usage of biocides in healthcare institutes started in the 19th century with the advent of antiseptics and the use of chlorinated water (Rotter, 2001). Most biocides may be used singly or in combination with other products with varying antimicrobial activity in healthcare settings, as disinfectants and/or antiseptics. The biocides are of great importance in the disinfection of surfaces and water; the sterilisation of medical devices; skin antiseptics and preservation of medicinal formulations (Russell, 1999). Biocides are used extensively in health establishments in order to reduce the potential risk of transmission of infection to patients (Maillard, 2005). Invasive catheters, implantation materials, endotracheal cannula, gynaecological speculum, respiratory equipment are some examples of materials that may be sterilised using biocides (Saginur *et al.*, 2006). Maillard, (2005) showed that a single chemical agent can be used for diverse applications, targeting a wide range of microbes and as illustrated in Figure 1.3. For example chlorhexidine is shown to target the plasma membrane of fungus and bacterium. Quaternary ammonium compounds (QACs) are shown to target all four microorganisms; bacterial spores, bacterium, viruses as well as fungus. The wide spectrum of biocidal activity makes QACs good antiseptic and disinfectant agents as they can be used in a variety of clinical applications.

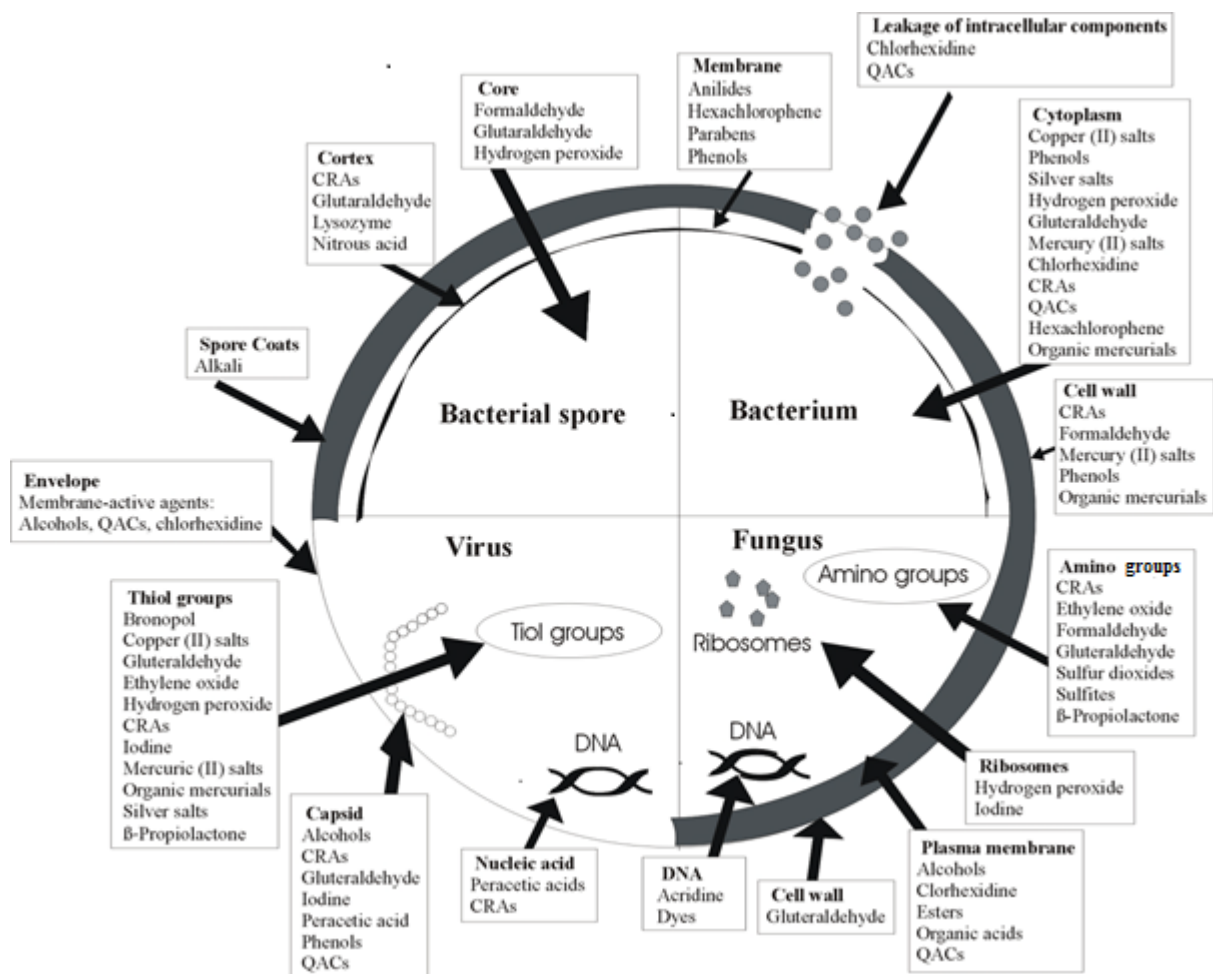


Figure 1.3: Mechanisms of biocide actions on microorganisms. CRAs, chlorine-releasing agents; QAC, quaternary ammonium compounds (Ashraf *et al.*, 2013).

The major variance in biocide application is the concentration at which the biocide is employed, which is dependent on the intended purpose. A typical example is the use of chlorhexidine for preservation at a concentration of 0.0025 %–0.01 % volume/volume (v/v) for preservation, for antiseptics at 0.02 %–4 % v/v and for surface disinfection at 0.5 %–4 % (v/v) (Maillard, 2005). Results from a number of research studies on biocides, in the health sector, domestic applications and industrial settings, have raised concern regarding the overuse of biocides (Saleh *et al.*, 2012; Ashraf *et al.*, 2013). Over usage has been cited as a possible contributing factor to the development of antimicrobial-resistant strains

(Pidcock, 2006). One example of a biocide reported to be showing reduced effectiveness against nosocomial pathogens is the cationic biguanide chlorhexidine.

1.3.1.1 Chlorhexidine

Chlorhexidine took centre stage in the 20th century because of its broad spectrum activity, ability to bind to skin and mucous membrane as well as low toxicity in mammals (Paulson, 2003). Because of the many favourable properties associated with chlorhexidine, it is widely used for both domestic and clinical purposes. Chlorhexidine is found in association with a number of compounds. Few of these associated form are highly soluble and the bulk slightly soluble in water and most organic solvents (Hendry *et al.*, 2009). Chlorhexidine itself is hardly soluble in water. The digluconate form; with the structure shown in figure 1.4 is one of the soluble formulations. The soluble formula is the one commonly found in aqueous disinfectants and antiseptics.

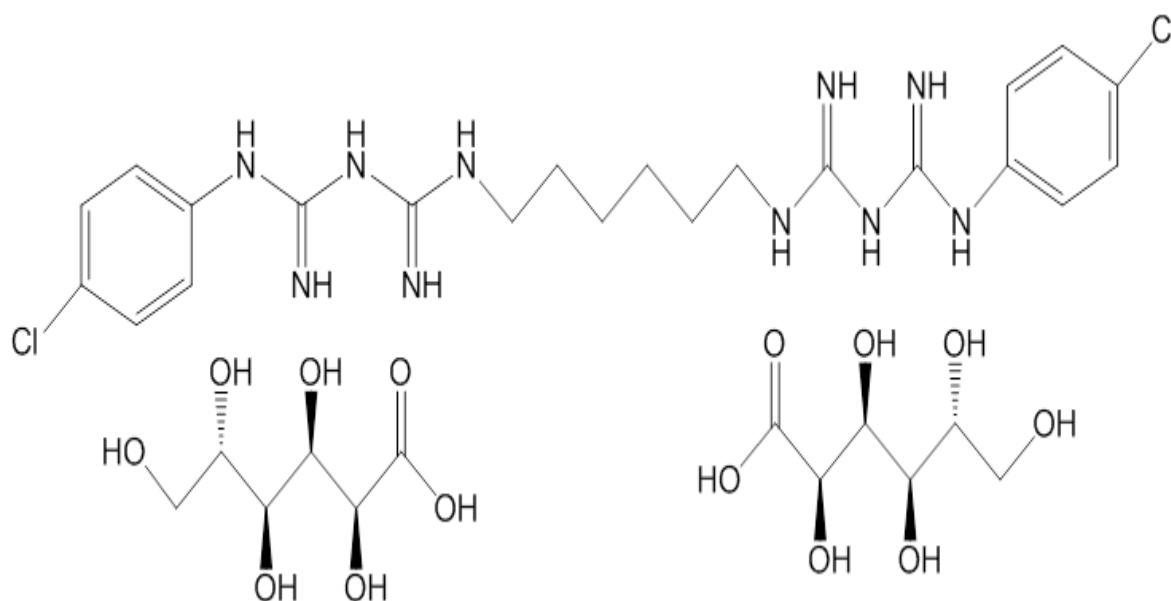


Figure 1.4: The structure of chlorhexidine digluconate

Aqueous solutions of chlorhexidine show stability from pH five to eight, beyond pH eight, chlorhexidine base is precipitated and at acidic pH, gradual degradation, and lowered antibacterial activity can be observed (Block, 1991). Chlorhexidine is a bactericidal agent effective against a number of Gram-negative and Gram-positive bacteria (Denyer, 1995) and is also effective against yeasts like *C. albicans*. The action of chlorhexidine against bacteria is by the disruption of the phospholipids making up the bacterial cell membrane resulting in the loss of membrane integrity and consequent leakage of intracellular components (Hemani and Lepor, 2009). Precipitation of proteins and nucleic acids in addition to coagulation of cytoplasmic components occurs at greater concentrations of chlorhexidine (Sheppard *et al.*, 1997). However, reports of the emergence of organisms resistant to chlorhexidine, such as *B. cepacia* and *P. aeruginosa* has been cited (Maillard, 2005).

1.3.2 Factors affecting biocide efficacy

The successful prevention of nosocomial infections in hospitals is highly dependent on the effective disinfection of these healthcare facilities. The efficacy of biocides is immensely affected by the destructive interactions between the nosocomial pathogen cell and biocide (Ashraf *et al.*, 2013). In general, antimicrobial activity can be described as either “static” if it inhibits growth or “cidal” if it kills the targeted microorganism (Saleh *et al.*, 2012). Factors affecting antimicrobial activity are not limited to, but include concentration, synergy, presence of organic load and pre-exposure experience (Stickler and King, 1999).

1.3.2.1 Concentration

The concentration of a biocide contained in a product is of key significance in its antimicrobial activity. Studies on the uptake and interaction of chlorhexidine with bacteria have shown that the uptake of the biocides by bacteria was highly dependent on concentration

Lambert, 2004). Biocide activity was reported to increase with increasing biocide concentration. The activity of chlorhexidine is also pH-dependent (Prag *et al.*, 2014), with the optimum pH at five to seven. This range corresponds with the pH typically found on human tissue where the antiseptic property of chlorhexidine gluconate is commonly applied (Paulson, 2003). However, in practical applications high concentration may be impractical as a balance has to be struck between efficacy against microorganisms and toxicity in humans. Lack of understanding of the concentration factor can lead to failure to remove microbial survival on surfaces, and/or in products, leading to infection or spoilage. The use of low concentrations in therapeutic devices such as catheters has been linked to bacterial survival attributed to inappropriate or sub-lethal concentration usage (Maillard, 2005).

1.3.2.2 Synergy

Synergy between chlorhexidine and cationic compounds as well as quaternary ammonium compounds has been widely reported. The penetration of chlorhexidine into human skin and bacterial biofilms was found to be poor when used on its own (Hendry *et al.*, 2009).

However, it was demonstrated that increased penetration of chlorhexidine could be achieved by using the biocide in combination with crude eucalyptus oil or 1,8-cineole against suspensions of *P. aeruginosa*, *S. aureus*, MRSA, *C. albicans*, *E. coli* and biofilm cultures of MRSA (Hendry *et al.*, 2009). In another study, it was noted that cationic peptides in combination with chlorhexidine or xylitol may be more effective in growth inhibition of oral streptococci (Kim *et al.*, 2003).

1.3.2.3 Presence of organic load

The presence of organic load such as pus, serum or blood showed significantly lowered activity of chlorhexidine (Russell, 1993). The effect of organic material remaining on prostheses disinfection was evaluated and found to impede the effectiveness of chlorhexidine digluconate solution (Puranik and Kumar, 2010).

1.3.2.4 Pre-exposure to biocide

Increases in post-exposure MICs of chlorhexidine against MRSA noted by Vila *et al.*, (2007) showed that biocide efficacy can be reduced by pre-exposure of microorganism to antimicrobials such as antibiotics or biocides. A similar study carried out recently by Prag *et al.*, (2014) using *Staphylococcus epidermidis* isolated from post-operative infections, showed decreased susceptibility to chlorhexidine to a significantly higher extent when compared to commensals which lacked antimicrobial predisposition. Optimum disinfection can be eluded by the use of incorrect disinfectants for inadequate periods of time and may result in bacterial resistance (Gilbert and McBain, 2003).

1.3.3 Mechanisms of bacterial resistance in biocides

“Biocide resistance” refers to reduced susceptibility of microorganism after exposure to the antimicrobial agent, over a defined period of time and conditions (Gilbert *et al.*, 2002). For *in vitro* studies using antibacterial agents, susceptibility is usually quantified using minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) to gauge concentration required to exert growth inhibition. To date, bacterial resistance against diverse forms of biocides has been documented and characterised to a lesser extent when equated to work carried out of antibiotic resistance (Piddock, 2006). Bacteriological, genetic and biochemical data has provided evidence that the utilisation of active molecules in biocidal products may possibly play a role in the increased emergence of bacteria which is resistant to biocide. *In vitro* studies using pure cultures of *P. aeruginosa* provided evidence that supports the link between predominance of multispecies biofilms and sites of biocidal products deployment, confirming the wide speculation that biocide misuse may be the causative factor contributing to bacterial resistance to antimicrobials (Gilbert *et al.*, 2002).

Resistance of bacteria can be:

- a. Intrinsic- a natural biochemical or physiological property of the microorganism found in all members of the species like *tet* genes found in MRSA coding for tetracycline resistance (Sun *et al.*, 2013)
- b. Acquired- brought about by mutation and/or plasmids or the acquisition of new genes in only certain isolates of a species an example being the acquisition of *qac*-genes coding for quaternary ammonium compounds (QACs) resistance (Poole, 2002)
- c. A change in susceptibility

Intrinsic resistance mechanisms to chlorhexidine are predominantly characteristic of Gram-negative bacteria. This mechanism has also been displayed in mycobacteria, bacterial spores and under special conditions, staphylococci (McDonnell and Russell, 1999). Published data on acquired chlorhexidine resistance in bacteria is limited. However, from reviewed literature; acquired resistance to chlorhexidine has been prominent from members of the *Enterobacteriaceae*, *Streptococcus* spp. and *Staphylococcus* spp. (McDonnell and Russell, 1999). Possible causes of the acquired resistance in these members may include: attainment of plasmid-encoded efflux pumps, amplified expression of chromosomally situated efflux pumps, or alterations in susceptibility due to additional mechanisms presently unknown (Maillard, 2005). An ideal disinfectant to overcome the antimicrobial resistant pathogens should have broad spectrum of antimicrobial activity (Mandell *et al.*, 1995).

1.4 Efflux pumps and EPIs

1.4.1 Efflux pumps

An efflux pump is essentially a protein channel that transports antimicrobials and other compounds out of the cell. Both eukaryotic and prokaryotic cells are able to decrease the

intracellular concentration of lethal compounds by using a variety of efflux pumps (Nikaido, 1998). In bacteria, mobile genetic elements such as plasmids can chromosomally encode efflux pump genes (Pidcock, 2006). The efflux pumps involved in pumping out of compound can be divided into five main classes diagrammatically presented in Figure 1.5.

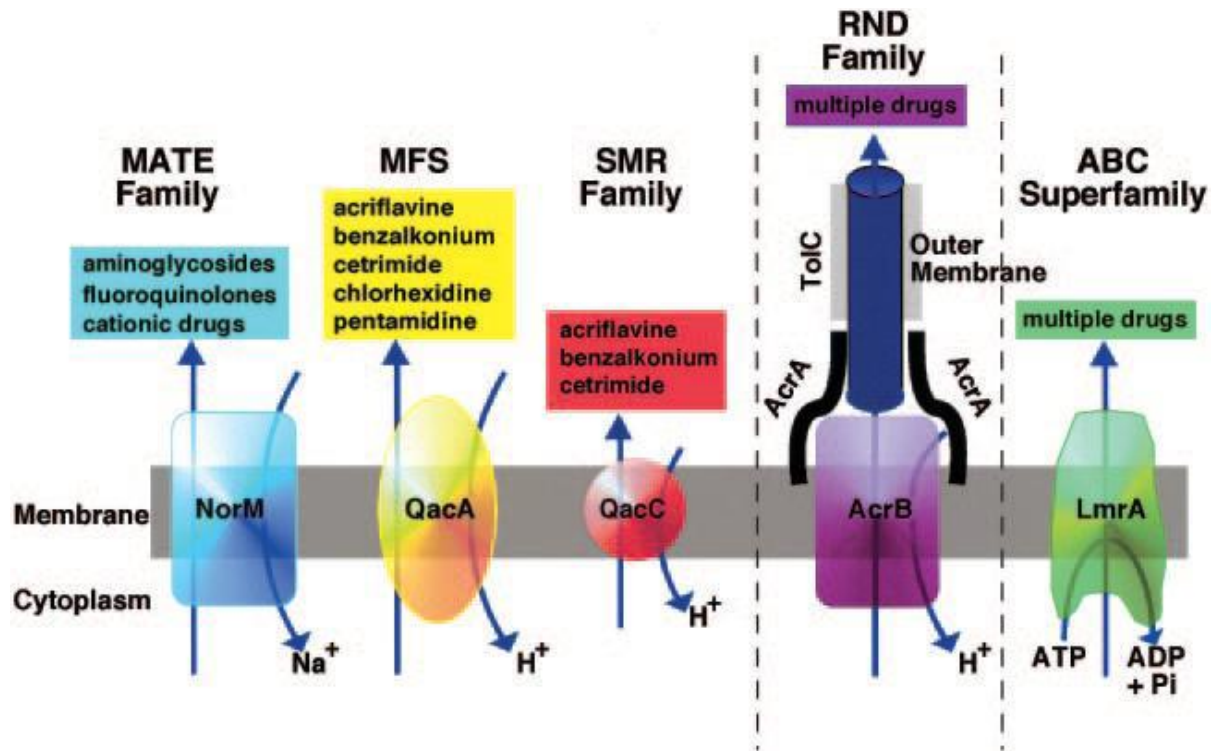


Figure 1.5: Classes of bacterial efflux pumps. MATE = multidrug and toxic compound extrusion. MFS = major facilitator superfamily. SMR = small multidrug resistance. RND = resistance nodule division. ABC = ATP-binding cassette (Pidcock, 2006).

ABC transporters are unique from the other four families of efflux pumps in that they are the only class of pumps that uses ATP as a primary source of energy to pump out compounds from cells (Gestin *et al.*, 2013). The other four classes utilise secondary sources of energy to pump out compounds from cells (Van Bambeke *et al.*, 2010). Efflux pumps can transport a precise substrate or a range of structurally unrelated compounds. The involvement of multidrug efflux pumps in bacterial resistance to various compounds including QACs, phenolics, biguanides and intercalating agents has been widely reported (Lomovskaya *et al.*, 2001; Horner *et al.*, 2012), with particular reference to *S. aureus* and *P. aeruginosa*. The

identified pumps in *S. aureus* include *QacA* to *QacD* (Mitchell *et al.*, 1998) which are involved in the uniport, antiport and symport of a variety of compounds across the cell membrane. In Gram-negative *P. aeruginosa* polyspecific efflux such as, *MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, and *MexJK* (Poole, 2004) were also identified. The polyspecific efflux pumps are essential defence mechanisms for *P. aeruginosa* against biocides, inhibitors and antibiotics. The heavy use of biocides has been blamed for the dissemination of efflux pump genes and the spread of efflux pumps (Paulsen *et al.*, 1996; Poole, 2002), however, additional evidence is needed to confirm the link between the overuse of biocides and the emergence of resistance in bacteria (Piddock, 2006). Additionally, it has been shown that efflux pumps are involved in virulence (Ramon-Garcia *et al.*, 2009) as well as oxidative stress responses (Van Bambeke *et al.*, 2006). The efflux pumps of importance to this study are ABC transporters which have ATP as their primary source of energy.

1.4.2 ATP binding cassette (ABC) transporters

According to their telling name, ABC transporters are the only class of MDR protein pumps that use ATP as their primary source of energy to carry out translocation processes of various molecules across the membrane (Goffeau *et al.*, 2013). The ABC family can be further divided into seven subfamilies designated A to G based on structural homology (Tegos *et al.*, 2011). The transmembrane protein consists of four domains illustrated in Figure 1.6. The four domains consist of; two transmembrane spanning domains and the other two are ATP-binding domains. The ATP-binding domains are called ATP-binding cassettes (ABCs). The pump starts in a closed state when not bound to substrate or ATP molecule (Piddock, 2006).

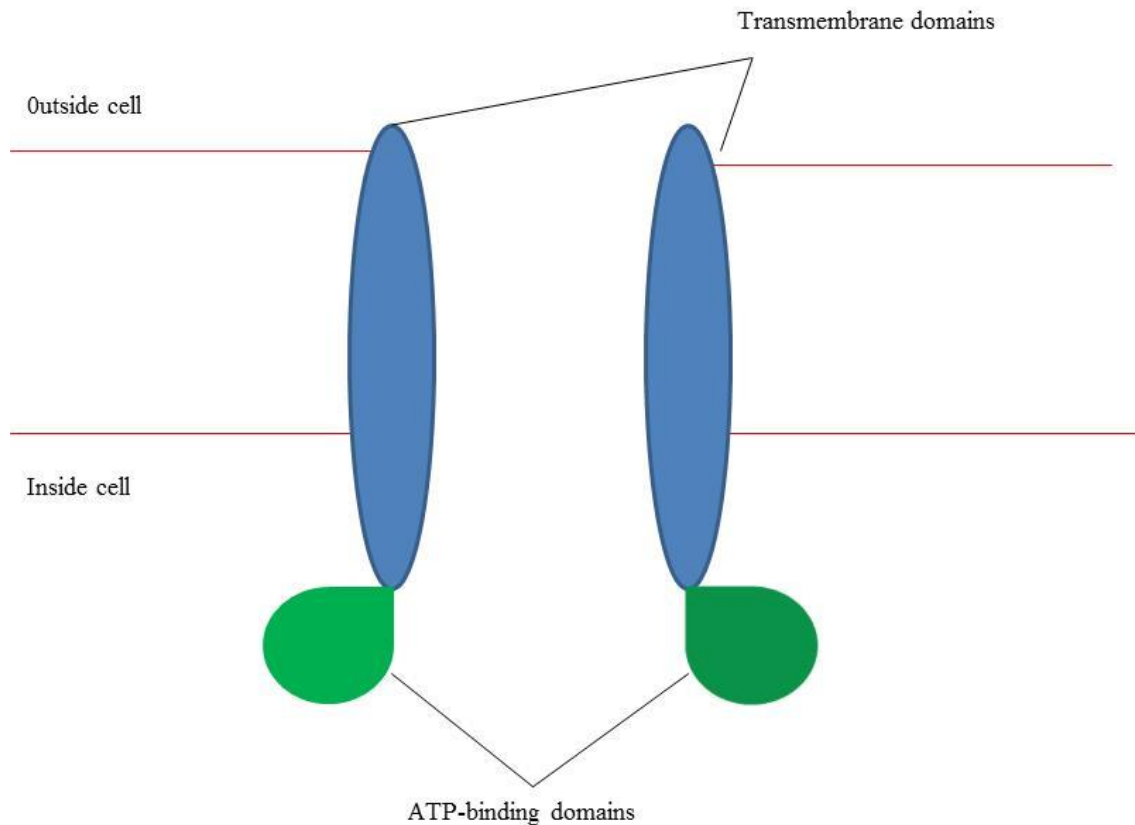


Figure 1.6: Illustration of an ABC transporter showing the transmembrane domains and the ATP-binding domains.

The four domains consist of; two transmembrane spanning domains and the other two are ATP-binding domains. The ATP-binding domains are called ATP-binding cassettes (ABCs). The pump starts in a closed state when not bound to substrate or ATP molecule (Piddock, 2006). Once a substrate binds to the transporter, it converts to its open state. The substrate then enters the transmembrane domain space. Substrate binding to the transmembrane space closes the transmembrane domain, and induces a conformational change in the ATP domains, that increases their affinity for ATP (Jones and George, 2002). Two ATP molecules then bind to the ABCs causing a conformational change in the transmembrane domains, which results in the release of the substrate to the opposite side. Hydrolysis of both ATP molecules resets the ABC transporter (Chang, 2003).

ABC transporters were first discovered in studies involving chemotherapy resistance of tumour cells. In the 1970s, it was realised that initially cancer cells were dying after addition of antimicrobials, but later continued to grow (Chang, 2003). It was later discovered that antimicrobials were being actively pumped out of the cancer cells (De Rossi *et al.*, 2006) and ABC transporters were implicated for the drug resistance in both eukaryotes and prokaryotes. Bacterial ABC transporters play a vital role in cell viability, pathogenicity as well as virulence (Kumar *et al.*, 2013). There is great correlate between the physiological roles of a bacteria and the total quantity of ABC transporters encoded in the bacterial genetic makeup (Gestin *et al.*, 2013). Bacterial pathogenesis can be enhanced by ABC transporters by the extrusion of toxins, disinfectants and drugs from the cell, rendering them ineffective against the bacterium (Davidson and Chen, 2004). However, the extrusion of antimicrobials by efflux pumps can be reversed or limited by the use of efflux pump inhibitors (EPIs).

1.4.3 Efflux pump inhibitors

Efflux pump inhibitors (EPIs) are compounds that can be co-administered with antimicrobials to perform as inhibitors of the efflux-mediated extrusion of antimicrobials (Askoura *et al.*, 2011). EPIs are a promising approach to counteract MDR efflux, which may fix the performance of antimicrobials (Kumar and Varela, 2012). Examples of EPIs include reserpine, biricodar (VX-710), timcodar (VX-853), Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil (Tegos *et al.*, 2011).

Reserpine is a plant alkaloid that inhibits both bacterial and mammalian ABC-system P-glycoprotein and is widely used in in vitro studies of efflux pumps (Ahmed *et al.*, 1993). The use of reserpine in vivo is very rare due to toxicity problems (Van Bambeke *et al.*, 2010).

According to Kumar *et al.*, (2013) the inhibitory activity of reserpine was first demonstrated on the multidrug transporter *Bmr* of *Bacillus subtilis*.

CCCP is the other commonly used EPI in *in vitro* studies of efflux pump activity. CCCP is an energy-dependent hydrogen ion ionophore which disperses the Hydrogen ion gradient resulting in the uncoupling of the electron transport from ATP synthesis (Aparna *et al.*, 2014). The ionophore has been reported to greatly reduce biofilm formation using its efflux pump inhibitory properties (Baugh *et al.*, 2012)

The activity of EPIs can be evaluated by combining an EPI compound with a multi-resistant drug (MDR) substrate at concentrations below the MIC. It has been established that the lowering of the MICs of most antimicrobials when used in combination with reserpine indicates the presence of an active efflux structure (Garvey and Piddock 2008; Ardebili *et al.*, 2014).

The major benefit of presently available EPIs is the difficulty to build bacterial resistance against them, but their limitation is their toxic nature which is a great deterrent in their clinical use (Askoura *et al.*, 2011). However, alternatives from natural sources such as plants are being explored (Mangoyi and Mukanganyama, 2011; Chitemerere and Mukanganyama, 2014). Extracts of *Combretum zeyheri* and *Combretum molle* plants were found to possess efflux pump inhibitory activity against *C. albicans* and *Candida krusei* (Mangoyi and Mukanganyama, 2011). *Callistemon citrinus* and *Vernonia adoensis* plant extracts were also shown to have inhibitory activity against *S. aureus* (Chitemerere and Mukanganyama, 2014).

2 Rationale, Hypothesis and Objectives

2.1 Rationale

Chlorhexidine gluconate is believed to be the ‘gold’ disinfectant and, thus, it is widely used in disinfecting healthcare facilities. Chlorhexidine is mainly used for the disinfection of surfaces, equipment, antisepsis, and also for the sterilization of medical devices. However, there have been concerns of nosocomial pathogens becoming resistant to chlorhexidine. Determining the possible mode of action of microorganisms resistance to this biocide may help in coming up with alternative combinations that can evade these modes of resistance. Resistance to biocides occurs mainly as a result of target alteration, reduced accumulation due to decreased permeability inactivation/modification and increased efflux. The role played by increased efflux due to ABC transporter pumping out chlorhexidine gluconate was evaluated in this study. Information on specific efflux mechanisms utilised by bacteria to confer reduced susceptibility to biocide, may be used to develop ways of preventing the efflux of the biocide from nosocomial pathogens resulting in higher disinfection activity.

2.2 Research question

The research question was, “are ABC transporters involved in reducing the activity of chlorhexidine digluconate against some nosocomial pathogens?”

2.3 Hypothesis

It was hypothesised that ABC transporters pump out chlorhexidine out of *P. aeruginosa* and *S. aureus* resulting in reduced accumulation of the biocide within the cells after exposure to a defined quantity of the biocide.

2.4 Objectives

The main objective of the study was to evaluate the role of ABC transporters on the accumulation of chlorhexidine in bacterial cells of *P. aeruginosa* and *S. aureus* .

The specific objectives of this study were to:

1. Develop a method for quantifying amount of chlorhexidine accumulated in *P. aeruginosa* and *S. aureus* cells.
2. Determine the MICs of:
 - i. Chlorhexidine only
 - ii. CCCP and reserpine
 - iii. Chlorhexidine in the presence of CCCP
 - iv. Chlorhexidine in the presence of reserpine against *P. aeruginosa* and *S. aureus*.
3. Determine and compare amount of chlorhexidine accumulated in bacterial cells using the method developed in the study.

3 Materials and methods

3.1 Chemicals and materials

The study was carried out in the Biomolecular Interactions Analyses (BIA) Laboratory, Department of Biochemistry, University of Zimbabwe, Harare, Zimbabwe. Chemicals used in the study included; chlorhexidine digluconate aqueous standard solution (purity 20.0%, batch number: 07/1306), reserpine, ampicillin, dimethyl sulphoxide (DMSO), Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide [MTT, thiazolyl blue] from Sigma Aldrich (Germany). Tryptic soy broth (TSB) and Tryptic soy agar (TSA) were also from Sigma Aldrich (Germany).

3.2 Bacterial strains.

A total of four bacterial isolates drawn from the list of common nosocomial pathogens (Diekema *et al.*, 2001) were employed in the study. The test isolates included a single clinical strain each of *P. aeruginosa* and *S. aureus* as well as their respective reference strains; *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 9144. Both clinical strains were isolated from patients at Parirenyatwa Hospital (Department of Medical Microbiology, College of Health Sciences, Harare, Zimbabwe). The reference strains were obtained from the Microbiological Section in the Department of Biological Sciences at the University of Botswana (Gaborone, Botswana). All bacteria were kept as 750 µl TSB overnight culture: 250 µl 50 % glycerol stocks at -35°C. Prior to each assay, bacteria were resuscitated by sub culturing twice in TSB for 24 hours at 37°C followed by streaking on TSA agar plates. For each assay, an overnight culture of the test isolate was prepared by obtaining a single colony of the strain from TSA plates, transferring it to 20 ml of TSB and incubating at 37°C overnight (15 hours).

3.3 Development of a chlorhexidine accumulation assay protocol

The basics of an accumulation assay technique outlined by Mortimer and Piddock (1993) were adapted in the development of a chlorhexidine accumulation assay protocol. A spectrophotometric method to quantify the amount of chlorhexidine digluconate accumulated within bacterial cells was developed. A 2800 UNICO UV/VIS spectrophotometer (UNICO United Products and Instruments Inc., Dayton, United States) was used for quantifying the chlorhexidine digluconate. Chlorhexidine absorbs maximally at 255 nm (Manisha *et al.*, 2012). Therefore the quantification of chlorhexidine using a UV/VIS spectrophotometer was carried at a wavelength of 255 nm. The method was validated, by determining the OD of a series of standards of chlorhexidine prepared on the day. Standards of chlorhexidine (12 μM to 0.75 μM) were prepared in distilled water and the standards analysed in duplicate. A calibration curve was generated from absorbances obtained as a function of concentration using GraphpadTM version 5 for Windows (GraphpadTM Software Inc., San Diego, California, USA). The linear regression value obtained was used to determine the suitability of using a UV/VIS spectrophotometer for quantifying chlorhexidine in the chlorhexidine accumulation assay. The calibration curve was used to interpolate concentrations of chlorhexidine in samples in the accumulation assay. Sub-inhibitory concentrations of chlorhexidine, CCCP and reserpine used in the accumulation assay were determined using half the MIC value (MIC_{50}) values calculated from MIC values obtained using the microdilution method.

3.4 Preparation of serial dilutions of chlorhexidine, CCCP and reserpine used in MIC determinations

Aqueous 20 % chlorhexidine digluconate was first diluted in sterile distilled water to give a 5 % solution which was further diluted with TSB to obtain a 100 $\mu\text{g}/\text{ml}$ working solution. A

1.0 mg/ml ampicillin stock solution was prepared using sterile distilled water. Reserpine and CCCP were dissolved in DMSO to give 1.0 mg/ml solutions. All double dilutions for the microdilution assays were prepared using TSB.

3.5 Determination of MIC and MBC of chlorhexidine

The plate count method was used to determine the viable cell count of serially diluted overnight culture and appropriate dilutions made to reach 2×10^6 C.F.U/ml. Microdilution tests, as recommended by the Clinical and Laboratory Standards Institute (CLSI M7-A7 2006) were carried out in quadruplicate using 96-well microtitre plates as shown in Figure 3.1.

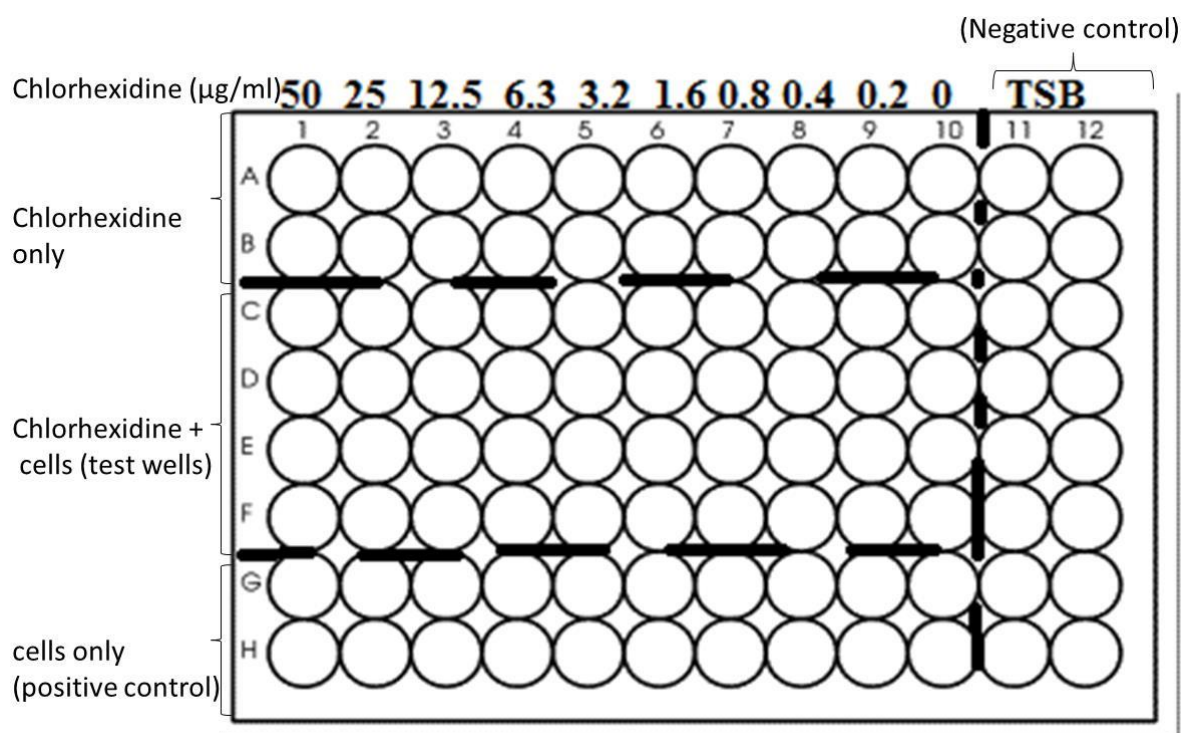


Figure 3.1: Example of a 96-well plate set up for determination of the MIC of chlorhexidine using the broth microdilution method. TSB (Tryptic Soy Broth) is the media used in the microdilution assay. Each test well contain 100 µl of defined chlorhexidine dilution and 100 µl of bacterial suspension. Negative control wells contain 200 µl media only to provide a turbidity control for reading end points. The positive control contained 100 µl of TSB and 100 µl of 2×10^6 C.F.U/ml of the test isolate to give a final concentration of 10^6 C.F.U/ml.

A separate ampicillin microtitre plate was set up following the same procedure used for chlorhexidine. The assay plate with ampicillin served as a reference plate for antimicrobial susceptibility of the test bacteria. All microtitre plates were incubated overnight at 37°C in a Lab Companion incubator (Korea) under a closed humidified atmosphere. After incubation, bacterial growth was determined with and without use of an instrument for panel readings. Two methods were utilised so that in the event of microplate readings failing to clearly distinguish the point of inhibition the MTT assay would act as a confirmatory test. The instrument-based method measured optical density (OD) read as absorbance at 590 nm using a Genios Pro microplate reader (Tecan Group Ltd, Austria) prior to and after incubation. The mean absorbance difference of varying concentrations of each bacterium in the presence chlorhexidine was compared to that of TSB only (negative control). The MIC was read as the minimum concentration of chlorhexidine that showed higher absorbance than the negative control. For non-instrument detection of bacterial viability, the MTT assay as outlined by Hansen *et al*, (1989) with minor modifications was utilised to visually observe bacterial viability. The MTT assay involved the addition of 30 µl of 1 mg/ml MTT to each well and reincubated for 1 hour at 37°C. The yellow colour of the tetrazolium salt would persist if cells were non-viable or be reduced by dehydrogenases and reductases in viable cells to a blue or purple colour (Arun and Rabeeth, 2010). The reduced salt would form an insoluble precipitate, which was dissolved by the addition of 30 µl DMSO at the end of the incubation period. The MIC was read as the minimum concentration of chlorhexidine in which no colour change in MTT was observed.

In order to determine the minimum bactericidal concentration (MBC), samples from the MIC microtiter plate were used. A sterile inoculating loop was used to collect a loopful sample

from the well before the well read as the MIC (Figure 3.2). The samples were plated onto TSA in duplicate and bacterial survival was evaluated after overnight incubation at 37°C.

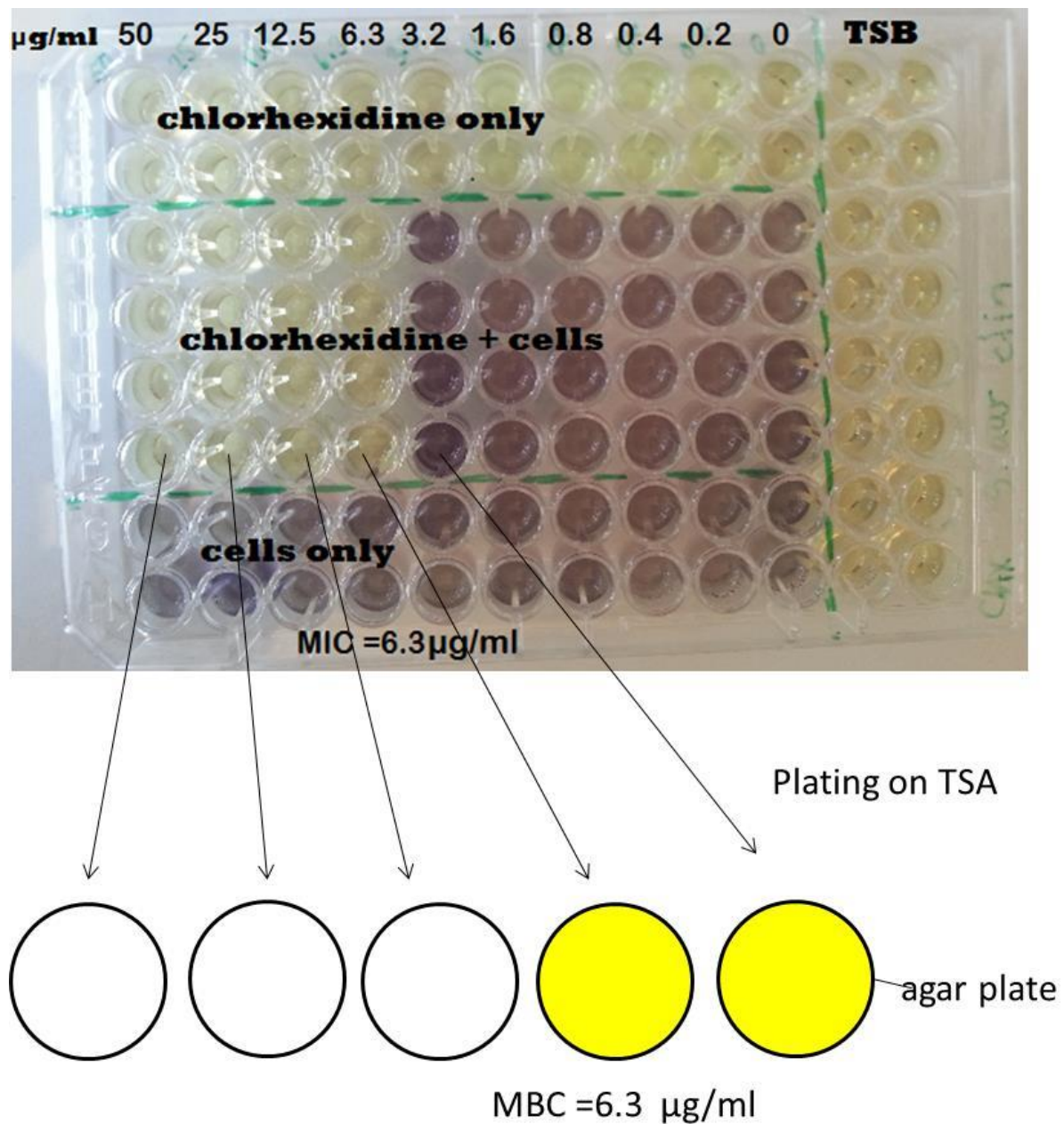


Figure 3.2: A picture of a typical microplate showing how the MBC was determined. Yellow plates show bacterial survival and white plates show no bacterial growth.

3.6 Determination of MIC of CCCP and reserpine

The maximum concentration of EPI that could be used for efflux pump inhibitory studies without bactericidal effects was attained by determining the MICs of CCCP and reserpine

against all four isolates. MIC determination of the EPIs was carried out using CCCP concentration of 0.4 µg/ml to 100 µg/ml and reserpine concentration of 1 µg/ml to 250 µg/ml. The MIC determination of CCCP and reserpine against all four isolates was carried out using the same microplate set up as for chlorhexidine (Figure 3.1). From the MIC obtained, half the MIC value of CCCP or reserpine was calculated. MIC₅₀ was used as the final concentration in the determination of the MIC of chlorhexidine in the presence of CCCP or reserpine as well as in the accumulation assay.

3.7 Determination of MIC of chlorhexidine in the presence of an inhibitor CCCP or reserpine

The extent of efflux pump mediated pumping out of chlorhexidine in the test bacterial isolates was determined by evaluating the change in MIC levels for chlorhexidine in the presence of an EPI; CCCP or reserpine. MICs of chlorhexidine in the presence of the EPIs were determined using the broth Microdilution assay. Using a microplate set up similar to that in Figure 3.1, half the MIC value for each EPI was used as the final concentration in combination with chlorhexidine concentrations used in the absence of an EPI.

The accumulation of chlorhexidine in the presence of an EPI, either CCCP or reserpine into intact cells of *P. aeruginosa* and *S. aureus* was investigated using the developed method. All tests were performed in duplicate. Cultures used in the accumulation assay involved large masses of cells, achieved by transferring 200 µL of an overnight culture into 200 ml TSB and incubated overnight at 37°C with shaking (120 r.p.m). Cells were harvested by centrifuging at 3000 r.p.m for 15 minutes using a Rotafix Centrifuge (Germany), washed twice using phosphate buffer solution (PBS), pH 7.4. The washed cells were resuspended in PBS and

centrifuged for 15 minutes. The pellet was resuspended in 10 Mm sodium azide to give a final cell concentration of 40 mg/ml. A final concentration of half the MIC of chlorhexidine digluconate, calculated from the value MIC of chlorhexidine in the absence of an EPI value was added to the cells. The mixture was incubated at 37°C for 30 minutes with shaking (120 r.p.m). Cells were collected by centrifuging at 4000 r.p.m for 15 minutes and distributed into 5 ml aliquots treated as illustrated in Figure 3.3.

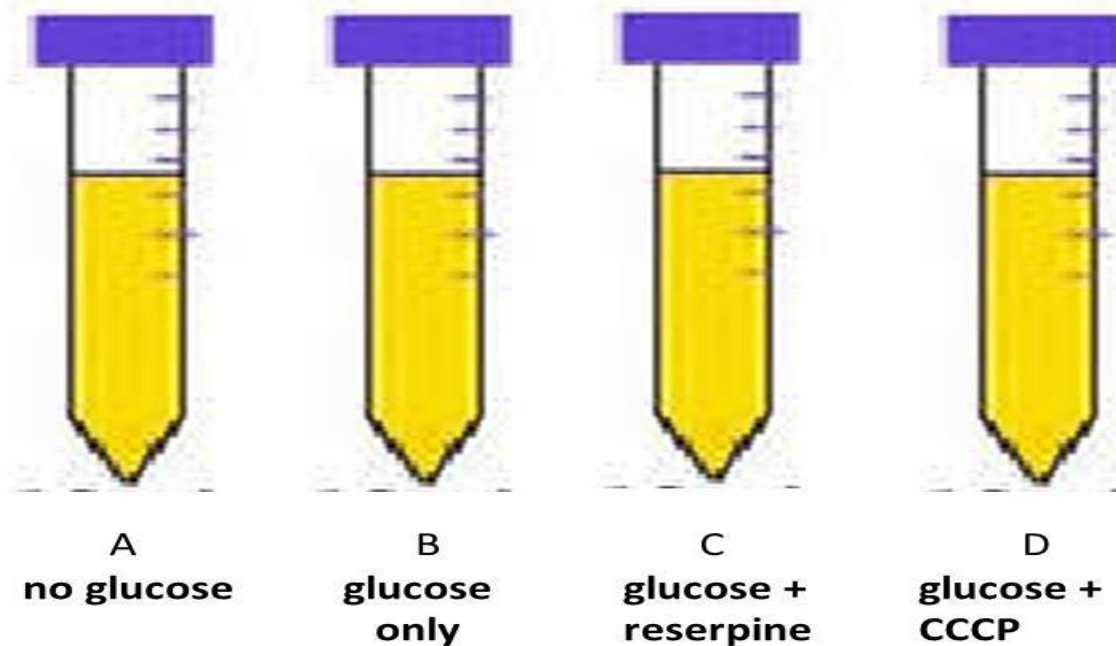


Figure 3.3: Set up of reaction test tubes for chlorhexidine accumulation assay. Tube A was the negative control containing no glucose thus no source of energy. Tube B was the positive control with glucose being the source of ATP energy. Tubes C and D contained a source of energy as well as an EPI.

All tubes were incubated at 37°C with shaking (120 r.p.m) for one hour. Cells were collected by centrifuging at 4000 r.p.m for 15 minutes and the supernatant stored for chlorhexidine efflux quantification. The pellet obtained from each tube was resuspended in 0.1 M glycine HCl (Sigma, Germany) pH 3, vortexed and incubated overnight to allow cell lysis. The tubes with lysed cells were centrifuged at 4000 r.p.m for 15 minutes and the supernatant retained

for chlorhexidine accumulation quantification. The quantification of chlorhexidine of the supernatants was by means of using a 2800 UNICO UV/VIS spectrophotometer at a wavelength of 255 nm. Sample concentrations were extrapolated from a standard curve initially plotted using known standards of chlorhexidine. Sample quantities were expressed as percentages of maximum possible accumulation or efflux in the presence of energy in the form of ATP from glucose.

3.8 Statistical Analyses

Graphpad™ version 5 for Windows (Graphpad™ Software Inc., San Diego, California, USA) was used for statistical analysis of the results obtained from MIC determinations and accumulation assays. The means, standard deviations and standard errors obtained for the minimum inhibitory concentrations of chlorhexidine, CCCP and reserpine were used to obtain graphs. One Way ANOVA and a post-test using Dunnet's test was used to compare results obtained for the positive control against that of test samples from the accumulation assay to determine level of significant difference from the control with $P < 0.05$.

4 Results

4.1 Validation of the quantification of chlorhexidine using a UV/VIS spectrophotometer

The calibration curve generated from absorbances obtained as a function of the concentration of chlorhexidine is as shown in Figure 4.1.

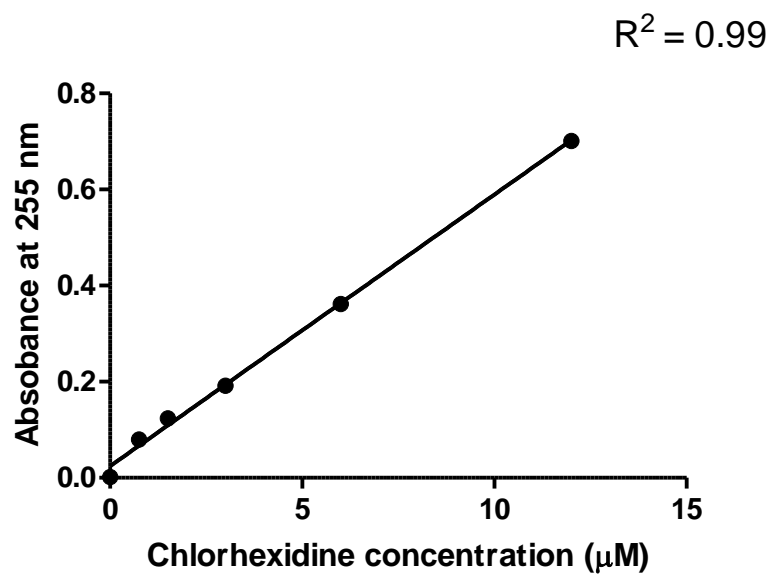


Figure 4.1: Absorbance of chlorhexidine at 255 nm as a function of concentration.

There was linear correlation between absorbance and concentration of chlorhexidine at 255 nm.

4.2 MICs and MBCs of chlorhexidine for *P. aeruginosa*

A typical microplate after carrying out the MMT assay is as shown in Figure 4.2. The MTT assay aided visual determination of the MIC value.

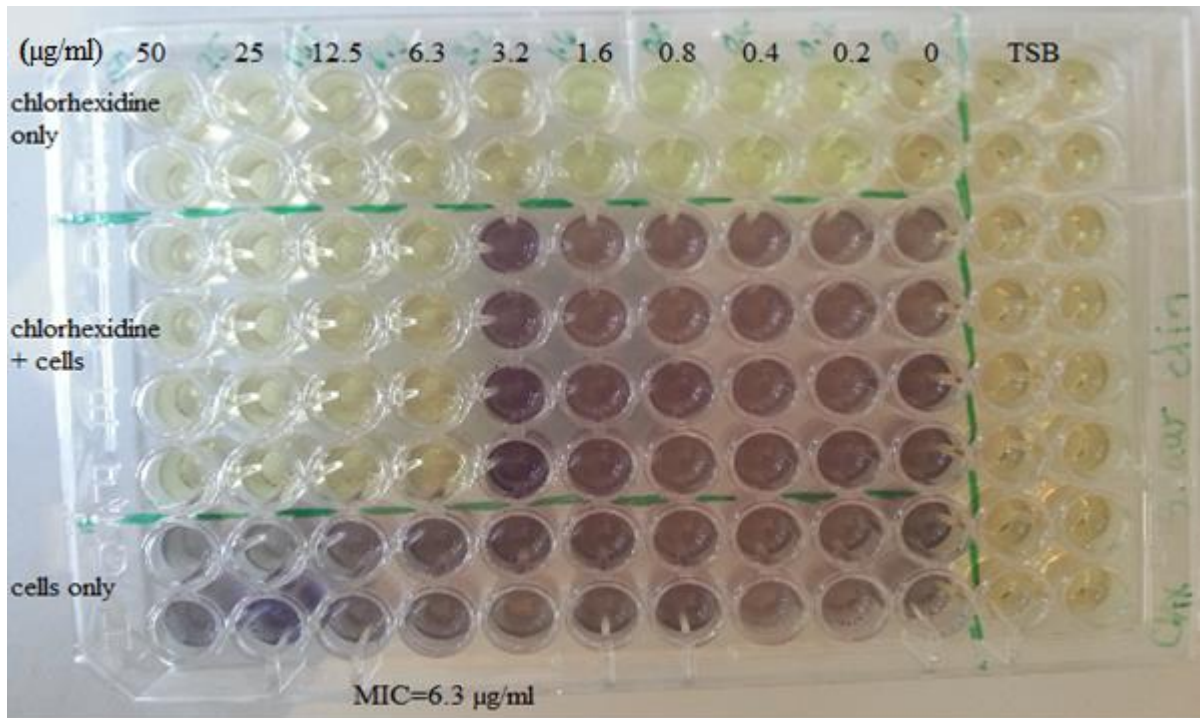
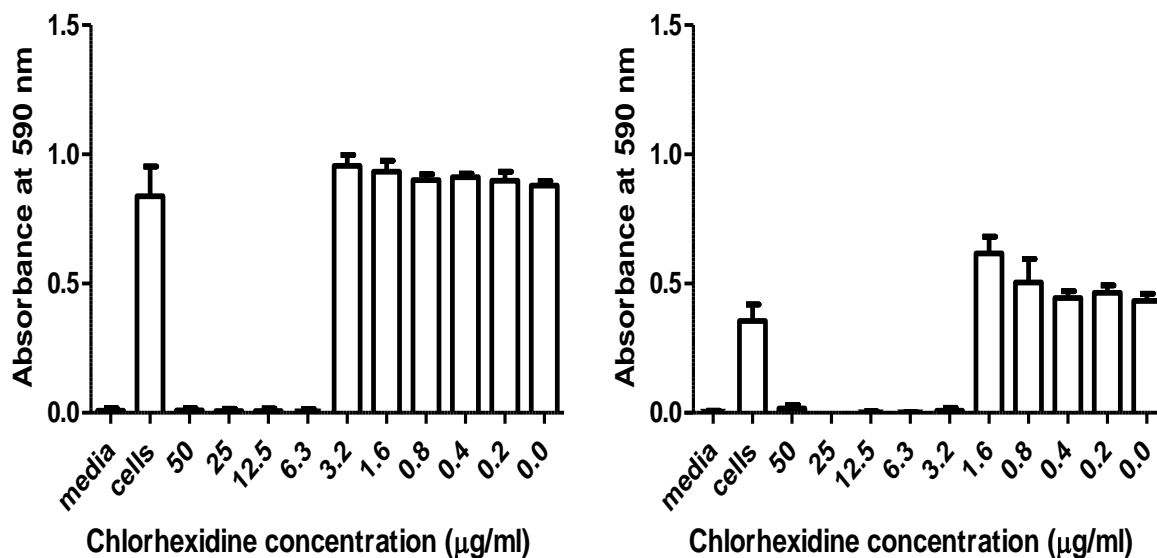


Figure 4.2: Typical appearance of microplate after carrying out the MTT assay. Wells with a purple colour show bacterial growth. Wells with a yellow colour show no bacterial growth. Wells with TSB is the media tryptic soy broth and was the negative control showing no growth. Wells with cells only was the positive control showing bacterial growth.

The MIC in the plate in Figure 4.2 is 6.3 µg/ml since it is the minimum concentration of chlorhexidine that shows no cell growth. The MTT assay was only used as a visual confirmatory assay for all MIC determinations obtained using the microplate reader which are the results presented in this study.

The absorbances corresponding to different cell densities of *P. aeruginosa* after incubation with different concentrations of chlorhexidine is as shown in Figure 4.3.



(a) *P. aeruginosa* clinical strain

(b) *P. aeruginosa* ATCC strain

Figure 4.3: Growth inhibition by varying concentration of chlorhexidine graphs of *P. aeruginosa* clinical and laboratory strains. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars shows the standard deviation from the mean of quadruplicate samples.

The clinical strain of *P. aeruginosa* cell inhibition by chlorhexidine digluconate was first noted at 6.3 µg/ml. Therefore the MIC of chlorhexidine digluconate against the clinical strain of *P. aeruginosa* was 6.3 µg/ml. The MIC for the same biocide against the laboratory strain of *P. aeruginosa* (3.2 µg/ml) was lower than that of the clinical strain. The laboratory strain of *P. aeruginosa* exhibited greater susceptibility to chlorhexidine in comparison to the clinical strain. Both strains were susceptible to the reference antibiotic ampicillin with MIC values of 25 µg/ml.

A typical plate of the determination of MBC for chlorhexidine digluconate against *P. aeruginosa* obtained by subculturing from the MIC wells is as shown in Figure 4.4.

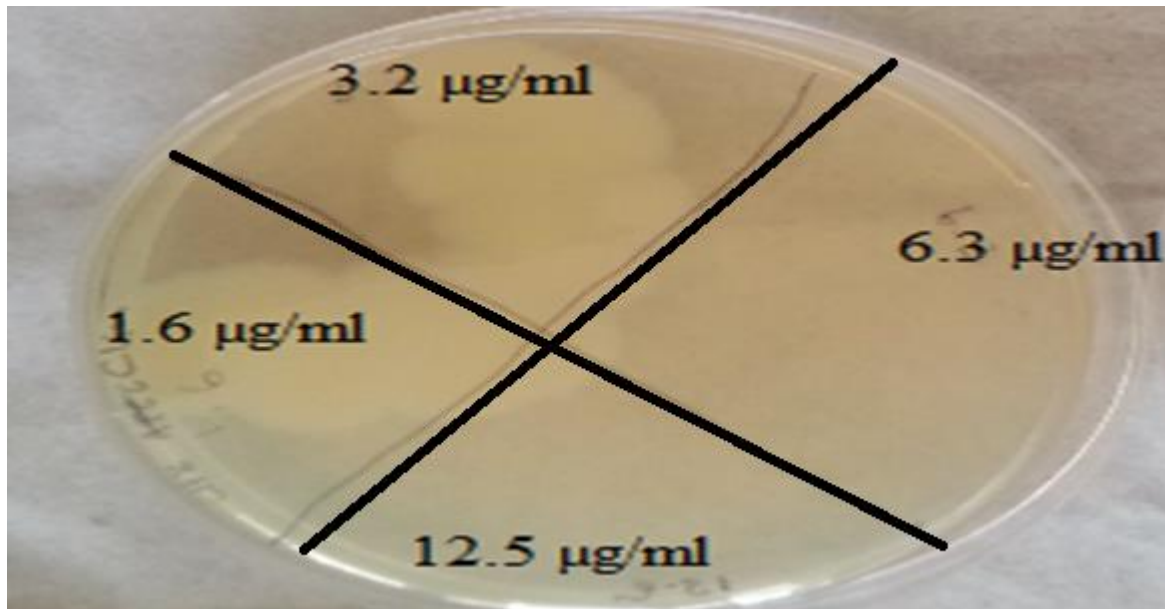


Figure 4.4: A typical plate for the determination of the MBC of chlorhexidine against *P. aeruginosa*. The MBC is 6.3 µg/ml as it is the minimum concentration of chlorhexidine digluconate that does not allow bacterial growth.

The minimum concentrations of chlorhexidine that were lethal to the clinical and ATCC strains of *P. aeruginosa* are tabulated in Table 4.1.

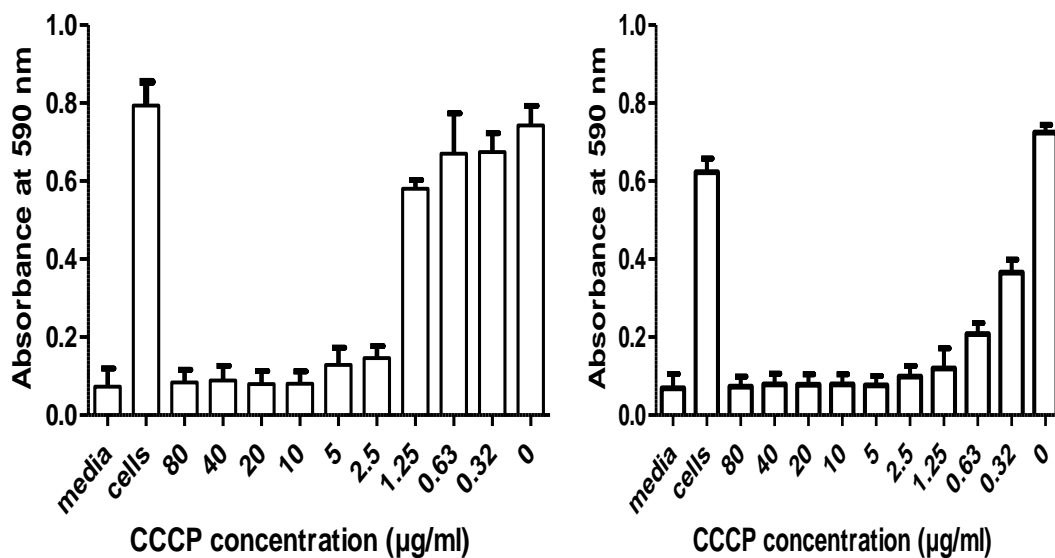
Table 4.1: MBC of chlorhexidine against *P. aeruginosa*.

<i>P. aeruginosa</i> strain	chlorhexidine digluconate MBC
Clinical	6.3 µg/ml.
ATCC	6.3 µg/ml.

The concentration of chlorhexidine for which both the clinical and laboratory strains of *P. aeruginosa* were killed was 6.3 µg/ml.

4.3 MICs of CCCP and reserpine against *P. aeruginosa*

The absorbances equivalent to different cell turbidity of *P. aeruginosa* after incubation with different concentrations of CCCP is shown in Figures 4.5.



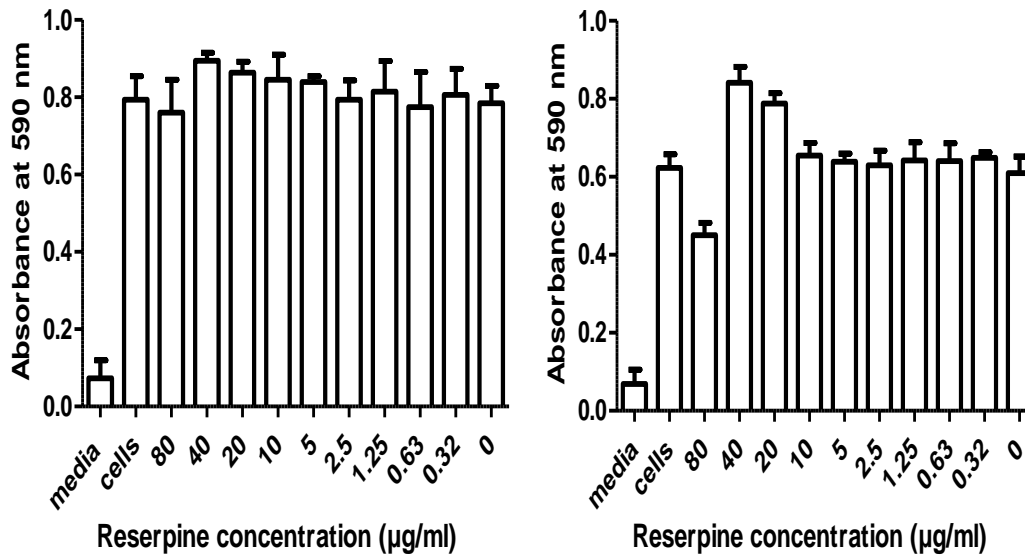
(a) *P. aeruginosa* clinical strain

(b) *P. aeruginosa* ATCC strain

Figure 4.5: *P. aeruginosa* clinical and ATCC strains antibacterial susceptibility to CCCP. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars show the standard deviation from the mean of quadruplicate samples.

The graph in Figure 4.5 showed no growth of *P. aeruginosa* clinical strain from a minimum CCCP concentration of 10 µg/ml. The MIC of CCCP against the clinical strain of *P. aeruginosa* was 10 µg/ml. The MIC against the laboratory strain was 5 µg/ml as shown by growth inhibition from a CCCP concentration of 5 µg/ml (Figure 4.5b). The laboratory strain showed greater susceptibility to CCCP when compared to the clinical strain.

The absorbances equivalent to different cell turbidity of *P. aeruginosa* after incubation with different concentrations of reserpine is shown in Figures 4.6.



(a) *P. aeruginosa* clinical strain

(b) *P. aeruginosa* ATCC strain

Figure 4.6: *P. aeruginosa* clinical and ATCC strains antibacterial susceptibility to reserpine. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars shows the standard deviation from the mean of quadruplicate samples.

There was growth of *P. aeruginosa* for both the clinical and laboratory strains at all concentrations of reserpine used. However, the clinical strain in Figure 4.6a showed greater microbial growth with an average absorbance of 0.8 compared to the laboratory strain which had an average absorbance of 0.6 in Figure 4.6b. The MIC of reserpine against *P. aeruginosa* was greater than 80 µg/ml for both the clinical and laboratory strains, as all concentrations used showed microbial growth.

4.4 MICs of chlorhexidine in the absence and presence of reserpine or CCCP for *P. aeruginosa*

MICs of chlorhexidine against the clinical and laboratory strains of *P. aeruginosa* obtained in the absence of efflux pump inhibitors were compared with those obtained in the presence of efflux inhibitors (Table 4.2).

Table 4.2: MICs of chlorhexidine against the clinical and ATCC strains of *P. aeruginosa* obtained in the absence and presence of CCCP or reserpine.

Determined MIC	<i>P. aeruginosa</i> clinical strain	<i>P. aeruginosa</i> ATCC strain
chlorhexidine only	6.3 µg/ml	3.2 µg/ml
chlorhexidine + 2.5 µg/ml CCCP	6.3 µg/ml	3.2 µg/ml
chlorhexidine + 80 µg/ml reserpine	3.2 µg/ml	3.2 µg/ml

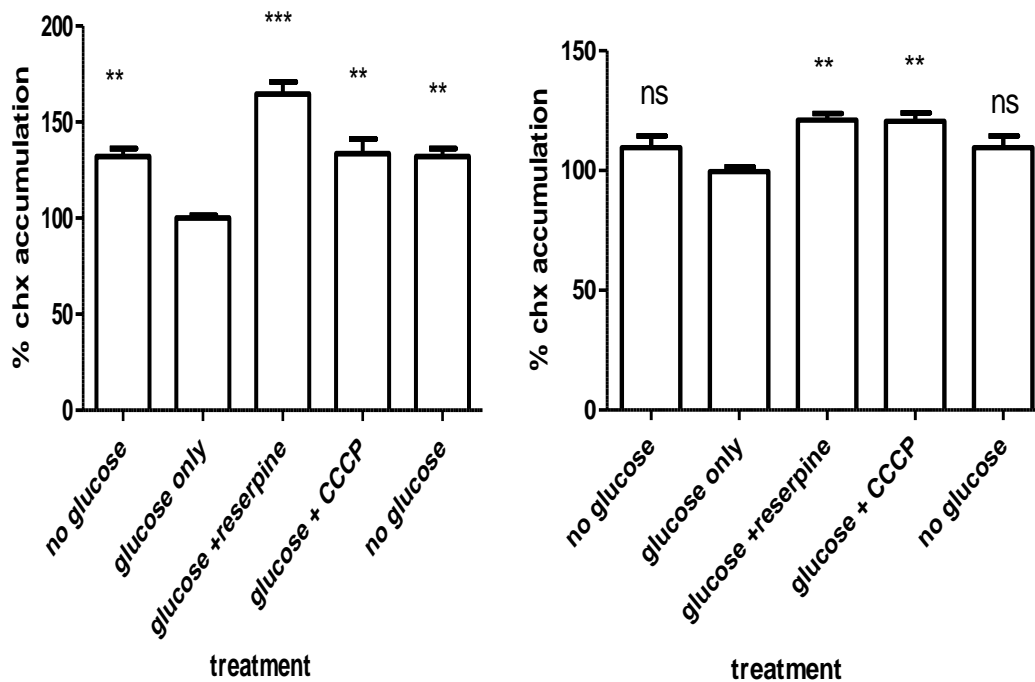
Boldface indicates a decrease in the MIC of chlorhexidine in the presence of an EPI.

A reduction in MIC levels of chlorhexidine was considered as an indication of presence of efflux activity in the bacterial isolates. It was observed that the MIC levels of chlorhexidine remained unchanged for both the clinical and laboratory isolates (6.3 µg/ml and 3.2 µg/ml respectively) in the presence of CCCP. A twofold reduction in MIC was observed when chlorhexidine was used together with 63 µg/ml reserpine against the clinical strain of *P. aeruginosa*. Reserpine exhibited higher inhibition potential compared to CCCP when used in conjunction with chlorhexidine against the clinical isolate. No difference in CCCP and reserpine activity was noted against the laboratory isolate as the MIC of chlorhexidine remained unchanged even in the presence of the EPIs.

4.5 Chlorhexidine accumulation and efflux for *P. aeruginosa*

Accumulation assay

The amount of chlorhexidine accumulated in the clinical and ATCC strain cells of *P. aeruginosa* is as shown in Figure 4.7.



(a) *P. aeruginosa* clinical strain

(b) *P. aeruginosa* ATCC strain

Figure 4.7: Chlorhexidine accumulation assays for the clinical and ATCC strains of *P. aeruginosa*. The tube with glucose is the positive control. The error bars show the standard deviation from the mean of two samples. The asterisk (*) indicates a significant difference from the control with $P < 0.05$ and ns shows no significant difference from the control.

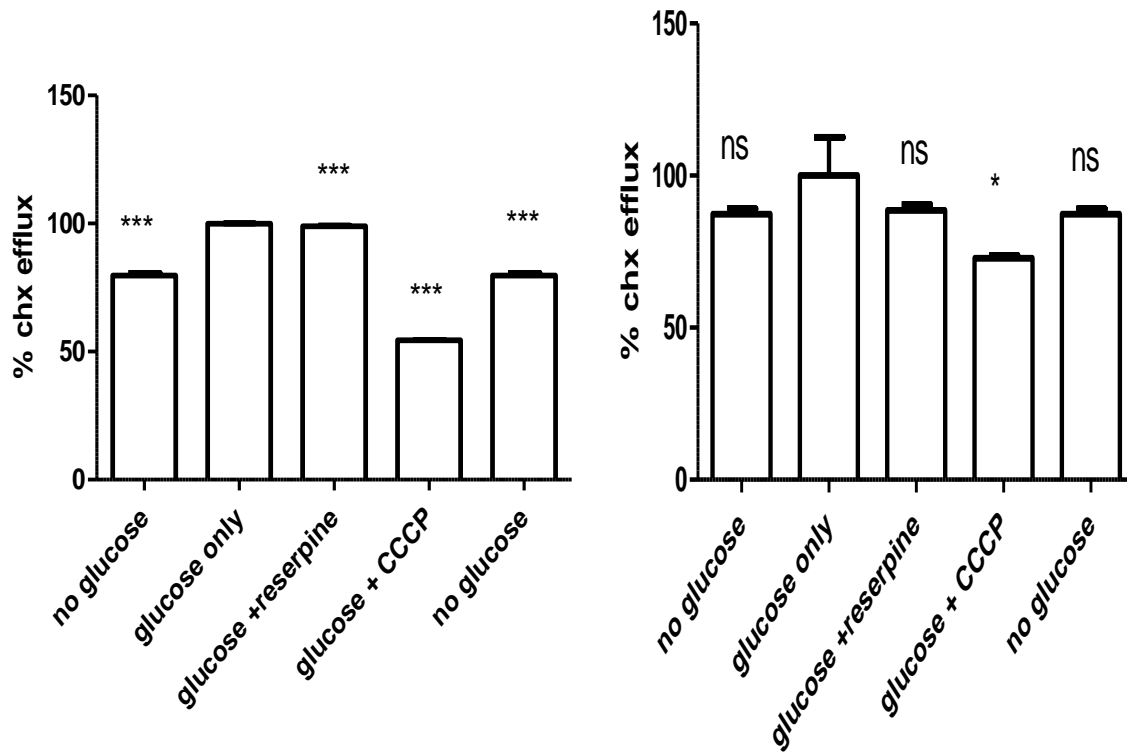
The glucose tube had the lowest amount of accumulated chlorhexidine showing that glucose was actively pumped out resulting in the net low chlorhexidine accumulated in both the clinical and laboratory strains. When compared to the glucose tube, the no glucose tube had moderately significant higher chlorhexidine accumulated within the clinical strain cells. This could be evidence of lack of active pumping out of chlorhexidine from the clinical strain

cells, as the tube contained no glucose for the provision of ATP. There was no signifying difference between the no glucose and glucose tubes for the ATCC strain showing lack of active pumping out of chlorhexidine. Chlorhexidine accumulation in the presence of both CCCP and reserpine was significantly higher for both the clinical and ATCC strains.

Reserpine resulted in a much higher accumulation of chlorhexidine than CCCP within the clinical strain cells. However, the accumulation of chlorhexidine in the presence of reserpine or CCCP was almost equivalent. The clinical strain showed more efflux activity than the laboratory strain as the tubes with no glucose and the ones with the inhibitors had significantly higher amounts of accumulated chlorhexidine.

4.6 Efflux assay for *P. aeruginosa*

The efflux activity for the clinical and ATCC strains of *P. aeruginosa* is as shown in Figure 4.8.



(a) *P. aeruginosa* clinical strain

(b) *P. aeruginosa* ATCC strain

Figure 4.8: Chlorhexidine efflux assays for the clinical and ATCC strains of *P. aeruginosa*. The tube with glucose is the positive control. The error bars show the standard deviation from the mean of two samples. The asterisk (*) indicates a significant difference from the control with $P < 0.05$ and ns shows no significant difference from the control.

The glucose tube showed the highest amount of pumped out chlorhexidine for both the clinical and ATCC strains, because of the presence of energy supplied by glucose in the form of ATP. For the clinical strain cells, the amount of chlorhexidine effluxed out for the no glucose tube and the tube containing CCCP was significantly lower than that of the no glucose tube. However, the reserpine containing tube had no significantly different

chlorhexidine pumped out. For the ATCC strain only CCCP showed significantly low inhibition of efflux.

4.7 MICs and MBCs of chlorhexidine against *S. aureus*

The absorbances resulting from the growth of the clinical and laboratory strains of *S. aureus* after incubation with different concentrations of chlorhexidine digluconate is as shown in Figures 4.9.

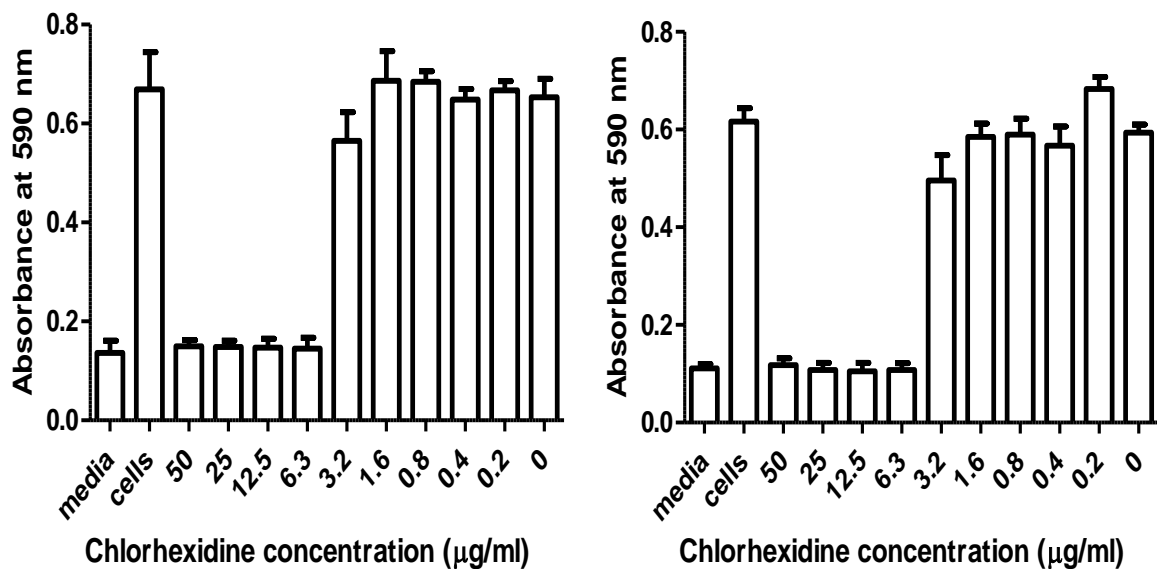


Figure 4.9: *S. aureus* clinical and ATCC strain antibacterial susceptibility to chlorhexidine. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars show the standard deviation from the mean of quadruplicate samples.

The clinical and laboratory strains of *S. aureus* displayed no difference in their susceptibility to chlorhexidine as both strains had an MIC value of 6.3 µg/ml. However, the clinical strain of *S. aureus* exhibited lower susceptibility to the reference antibiotic ampicillin giving an MIC of 3.2 µg/ml whereas the laboratory strain had a fourfold lower value of 0.8 µg/ml.

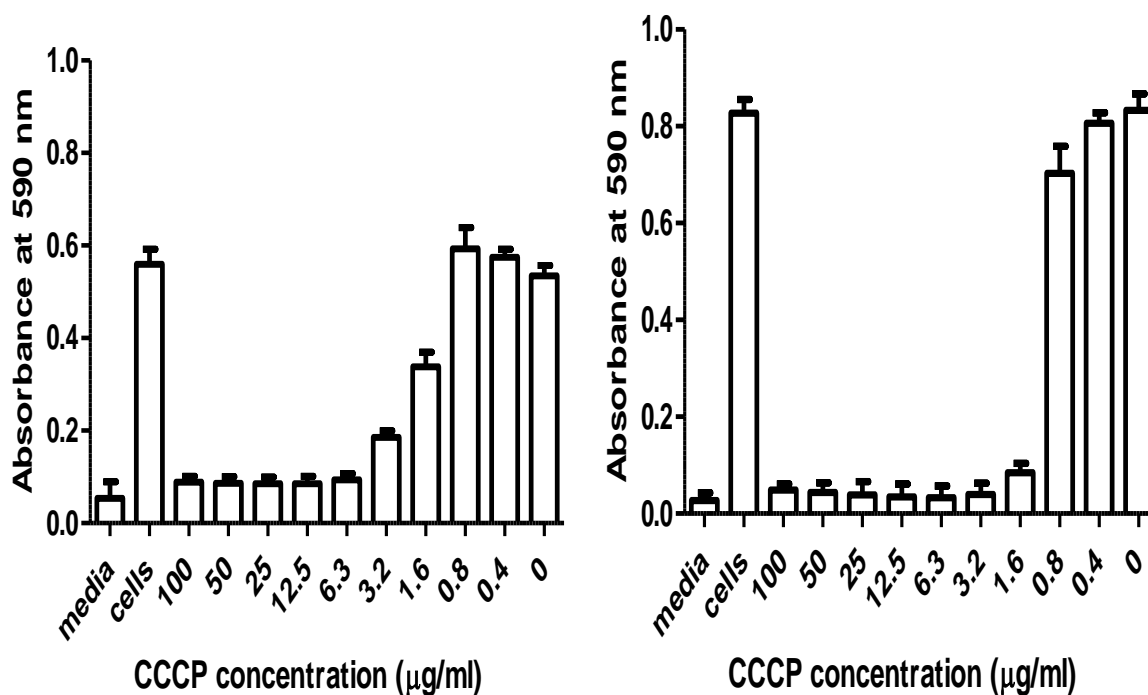
The minimum bactericidal concentration (MBC) for chlorhexidine digluconate is as shown in Table 4.3.

Table 4.3: MBC of chlorhexidine digluconate against *S. aureus*.

<i>S. aureus</i> strain	chlorhexidine digluconate MBC
Clinical	6.3 µg/ml.
ATCC	6.3 µg/ml.

4.8 MIC of CCCP or reserpine against *S. aureus*

The absorbances obtained for the clinical and ATCC strains of *S. aureus* after incubation with different concentrations of CCCP is as shown in Figures 4.10.



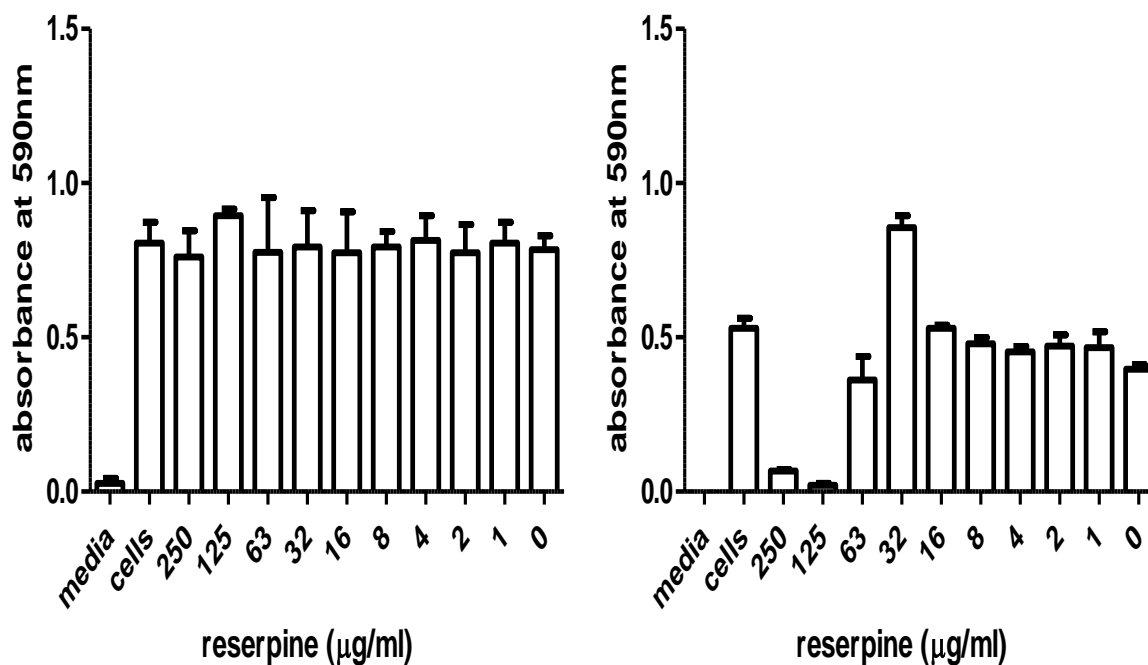
(a) *S. aureus* clinical

b. *S. aureus* ATCC

Figure 4.10: *S. aureus* clinical and ATCC strain antibacterial susceptibility to CCCP. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars show the standard deviation from the mean of the quadruplicate samples.

The MIC of CCCP against the clinical strain of *S. aureus* was double that of the laboratory strain with the clinical and laboratory strains having MIC values of 6.3 µg/ml and 3.2 µg/ml respectively.

The absorbances obtained for the clinical and ATCC strains of *S. aureus* after incubation with different concentrations of reserpine is as shown in Figures 4.11.



(a) *S. aureus* clinical

b. *S. aureus* ATCC

Figure 4.11: *S. aureus* clinical and ATCC strain antibacterial susceptibility to reserpine. The media is the negative control showing no microbial growth. The positive control is the cells showing microbial growth. The error bars shows the standard deviation from the mean of quadruplicate samples.

All concentrations of reserpine used against the clinical strain of *S. aureus* were unable to inhibit growth (Figure 4.11a), showing that the MIC of reserpine was greater than 250 $\mu\text{g/ml}$. However, growth inhibition was noted for the laboratory strain at a concentration of 125 $\mu\text{g/ml}$ of reserpine as depicted in Figure 4.11b. Therefore the MIC for the clinical strain is greater than 250 $\mu\text{g/ml}$ while that for the laboratory strains is 125 $\mu\text{g/ml}$.

4.9 MIC of chlorhexidine in the absence and presence of an inhibitor CCCP or reserpine for *S. aureus*

The MIC of chlorhexidine in the absence and presence of inhibitors of efflux pumps CCCP and reserpine against the clinical and laboratory strains of *S. aureus* are summarized in Tables 4.4.

Table 4.4: MIC of chlorhexidine in the absence and presence of inhibitors of efflux pumps CCCP and reserpine against the clinical and ATCC strain of *S. aureus*.

Determined MIC	<i>S. aureus</i> clinical strain	<i>S. aureus</i> ATCC strain
chlorhexidine only	6.3 µg/ml	6.3 µg/ml
chlorhexidine + 1.6 µg/ml CCCP	< 0.1 µg/ml	< 0.1 µg/ml
chlorhexidine + 63 µg/ml reserpine	3.2 µg/ml	6.3 µg/ml

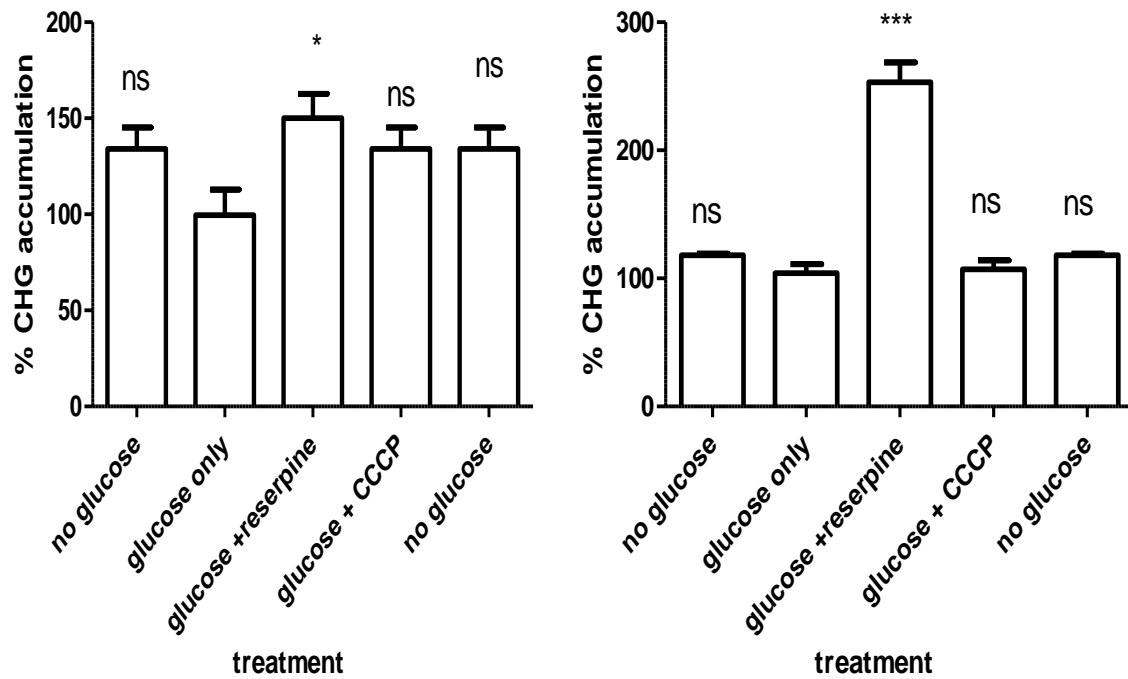
Boldface indicates a decrease in the MIC of chlorhexidine in the presence of an EPI.

Decreased susceptibilities of both isolates to chlorhexidine used in combination with CCCP were observed. The MIC of chlorhexidine for the two bacterial strains lie below 0.1 µg/ml, which is many times lower than 6.3 µg/ml the value obtained when chlorhexidine is used on its own for both strains. The MIC of chlorhexidine with reserpine against the clinical strain of *S. aureus* was reduced twofold from 6.3 µg/ml to 3.2 µg/ml. However, the MIC of chlorhexidine against the laboratory strain remained at the same level of 6.3 µg/ml despite the presence of reserpine. CCCP lowered the MICs of chlorhexidine more than those of reserpine, with same level reductions in MICs of chlorhexidine for the clinical and laboratory strains.

4.10 Chlorhexidine accumulation and efflux for *S. aureus*

Accumulation assay

The amount of chlorhexidine accumulated in the clinical and ATCC strains of *S. aureus* is as shown in Figure 4.12.



(a) *S. aureus* clinical strain

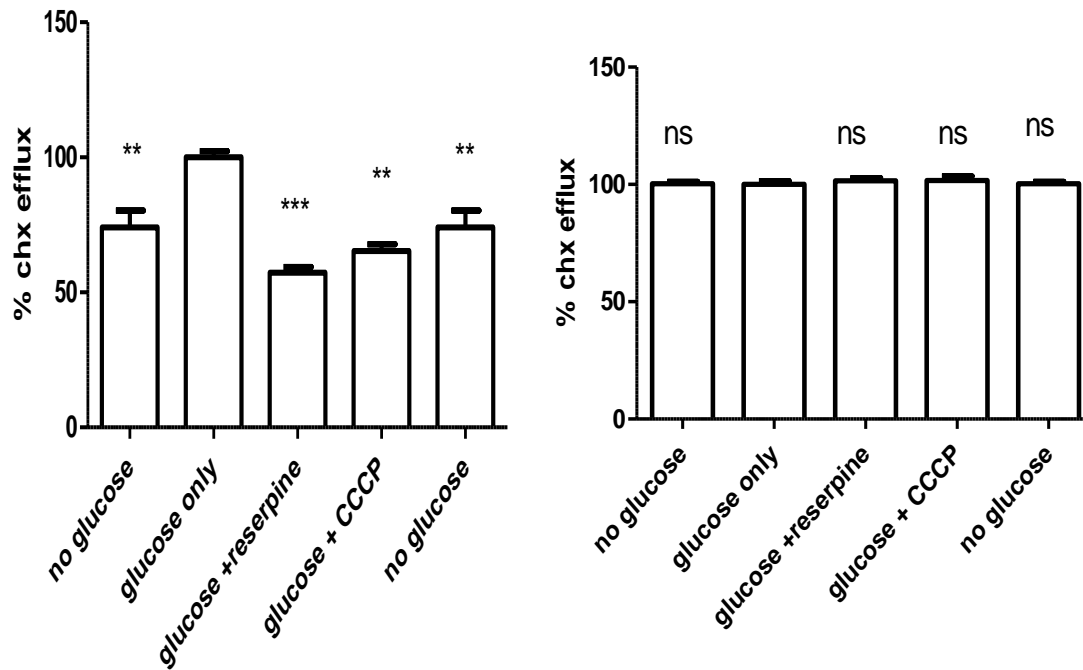
(b) *S. aureus* ATCC strain

Figure 4.12: Chlorhexidine accumulation assays for the clinical and ATCC strains of *S. aureus*. The tube with glucose is the positive control. The error bars show the standard deviation from the mean of two samples. The asterisk (*) indicates a significant difference from the control with $P < 0.05$ and ns shows no significant difference from the control.

Efflux activity for both the clinical and ATCC strain was not significant. Inhibition by reserpine was the only signifying difference from the positive control.

4.11 Efflux assay

The amount of chlorhexidine pumped out by the clinical and ATCC strains of *S. aureus* is as shown in Figure 4.13.



(a) *S. aureus* clinical strain

(b) *S. aureus* ATCC strain

Figure 4.13: Chlorhexidine efflux assays for the clinical and ATCC strains of *S. aureus*. The tube with glucose is the positive control. The error bars show the standard deviation from the mean of two samples. The asterisk (*) indicates a significant difference from the control with $P < 0.05$ and ns shows no significant difference from the control.

The efflux activity of the clinical strain of *S. aureus* was significantly inhibited by CCCP and reserpine. However, there was no significant inhibition of efflux activity for the ATCC strain of *S. aureus*.

5 Discussion

5.1 Development of a chlorhexidine accumulation assay protocol

There is a lot of documented work on the antimicrobial properties; efficacy and disinfecting efficiency of chlorhexidine (Coenye *et al*, 2011; Solmaz and Korachi, 2012) but very limited information is available for the quantification of chlorhexidine accumulated within bacterial cells. The available methods makes use High Performance liquid chromatography HPLC (Hebert *et al*, 2003) to quantify the amount of chlorhexidine in samples such as milk and saliva. Due to the unavailability of an HPLC in the Department, an alternative method had to be developed using the available machinery. Chlorhexidine absorbance was to be measured at 255 nm because the biocide absorbs maximally at 255 nm (Manisha *et al.*, 2012). The use of a UV/VIS spectrophotometer was decided on as it was the only machine available that could detect an absorbance of 255 nm. The use of the UV/VIS spectrophotometer was validated by generating a calibration curve using a series of chlorhexidine concentrations. The linear regression (R^2) value of 0.99 (Figure 4.1) obtained confirmed that at 255 nm there was a significant linear relationship between absorbance and the concentration of chlorhexidine. Therefore, the UV/VIS spectrophotometer could be used to quantify the concentration of chlorhexidine in the accumulation assay. To ensure that growth of cells used in the accumulation assay was not inhibited sub-inhibitory (MIC_{50}) concentrations of chlorhexidine, CCCP and reserpine were calculated from MIC obtained using the broth microdilution.

5.2 Determination of MIC for chlorhexidine and ampicillin against clinical and ATCC strains of *P. aeruginosa*

The MIC of chlorhexidine digluconate against the clinical strain of *P. aeruginosa* was 6.3 $\mu\text{g/ml}$ (Fig) and the MIC of the ATCC strain of *P. aeruginosa* was lower with a value of 3.2 $\mu\text{g/ml}$. In general, clinical strains of microorganisms show higher MICs of biocides,

evidence of reduced susceptibility. The reduced susceptibility commonly observed in clinical strains, has been attributed to the clinical strains having higher chances of being extensively exposed to biocides in the hospital set up where they live (Sun *et al.*, 2014). The high exposures to biocides increases their chances of developing mechanisms that may make the microorganisms more adaptable to the harsh environment, potentially by a reduced susceptible to antimicrobials (Odore *et al.*, 2000). MIC values of chlorhexidine against *P. aeruginosa* of 3.2 µg/ml and 6.3 µg/ml obtained in this study, correlate with previous studies by Amorim and colleagues (Amorim *et al.*, 2004). Amorim *et al.*, (2004) reported MIC values of chlorhexidine against *P. aeruginosa* ranging from 2.67 to 80.00 µg/ml. There was no difference between the MBC of chlorhexidine against the ATCC and clinical strains of *P. aeruginosa* pegged at 6.3 µg/ml. The equivalence in the MBC values observed, may be explained by the fact that chlorhexidine is known to be bacteriostatic at low concentrations and bactericidal at high concentrations. It is well documented that at higher concentrations chlorhexidine results in the rapturing of membranes (McDonnell and Russell, 1999; Maillard, 2005) hence, the cidal effect. In order to confirm that the *P. aeruginosa* strains used were susceptible to antimicrobials, their susceptibility to a standard antibiotic (ampicillin) was tested. The MIC of ampicillin against both strains was 25 µg/ml; this is evidence that both strains used were susceptible to a standard antibiotic as the values obtained lay within the range reported by other authors. Khan *et al.*, (2008) reported that ampicillin MIC against *P. aeruginosa* may be as low as 1 µg/ml or as high as 2048 µg/ml.

Using *P. aeruginosa* allowed the investigation of the susceptibility of typical Gram-negative bacteria against chlorhexidine. To study the susceptibility of Gram-positive bacteria to chlorhexidine *S. aureus* was used in the determination of MICs of the biocide.

5.3 Determination of MIC for chlorhexidine and ampicillin against clinical and ATCC strains *S. aureus*

The MICs of chlorhexidine against the clinical and ATCC strains of *S. aureus* (6.3 µg/ml) were not different from one another which was not the expected outcome. Clinical strains should generally show reduced susceptibility to biocides when compared to their laboratory counterparts since they are highly exposed to biocides in the clinical set up (Maillard, 2005; Piddock, 2006). However, the results of this study were similar to the ones obtained by Hogan and Smith, (1989) who found that the response of *S. aureus* to chlorhexidine was not affected by prolonged exposure to the biocide after testing eight strains of *S. aureus* to determine if repeated 15 *in vitro* exposures to sub-lethal concentrations of chlorhexidine could increase bacterial tolerance (Hogan and Smith, 1989). Behiry *et al.*, (2012) also reported the lack of reduced susceptibility of *S. aureus* to chlorhexidine digluconate after ten rounds of exposures (Behiry *et al.*, 2012). With the aim of confirming that the *S. aureus* used in the study were susceptible to antimicrobials; antibiotic (ampicillin) susceptibility tests were carried out for the clinical and ATCC strains.

The clinical strain of *S. aureus* exhibited lower susceptibility to the reference antibiotic ampicillin giving an MIC of 3.2 µg/ml whereas the laboratory strain had a fourfold lower value of 0.8 µg/ml, as clinical strains are generally supposed to show reduced susceptibility to antibiotics when compared to laboratory strains. Susceptibility shown by *S. aureus* could be an indication that the pre-exposure may not affect susceptibility. The difference in susceptibility to chlorhexidine of a Gram-negative (*P. aeruginosa*) and a Gram-positive (*S. aureus*) were then compared.

5.4 Determination of MIC for chlorhexidine against *P. aeruginosa* compared to *S. aureus*

The MIC of chlorhexidine against the clinical strain of *P. aeruginosa*, the clinical strain of *S. aureus* and the ATCC strain of *S. aureus* were all equivalent with a value of 6.3 µg/ml. The expectation was that *P. aeruginosa* being Gram-negative bacteria would have a higher MIC value than *S. aureus* which is a Gram-positive. Gram-negatives are basically known to show reduced susceptibility to antimicrobials due to lack of membrane permeability rendered by their thick outer membrane which limits uptake into the cell (Breidenstein *et al.*, 2011). The equivalence in MIC values obtained in the present study may be explained by the mechanism of attacking the cell membrane by which the cationic chlorhexidine works against microorganisms (Horner *et al.*, 2012). It may be speculated that, both *P. aeruginosa* and *S. aureus* had their membranes damaged promoting the uptake of the chlorhexidine hence the difference in their membrane structure falls out, which may be one of the reasons in the same susceptibility to chlorhexidine observed. After observing the similarities and differences in the susceptibility to chlorhexidine of the four bacterial isolates, their susceptibility to chlorhexidine in the presence of an EPI was to be investigated determine the presence of an EPI would increase susceptibility. Increased susceptibility would imply that the EPI had managed to keep the biocide inside the cell resulting in a lower concentration of the biocide being needed to inhibit growth. The EPIs used (CCCP and reserpine), were expected to exclusively act as efflux pump inhibitors and not as antimicrobial agents in the determination of the MIC of chlorhexidine in the presence of either EPIs. In an attempt to rule out the antimicrobial effect of CCCP and reserpine in subsequent assays, the MICs of CCCP and reserpine against the four test organisms were carried out so that sub-inhibitory concentrations of either EPIs could be determined and used in the subsequent assays.

5.5 Determination of MIC for CCCP and reserpine

The MIC of CCCP against the clinical and laboratory strain of *P. aeruginosa* was 10 µg/ml and 5 µg/ml respectively. The higher MIC value obtained for the clinical strain suggests reduced susceptibility which was also highlighted by Piddock (2006). Concentrations of reserpine of up to 80 µg/ml used against both the clinical and laboratory strains of *P. aeruginosa* showed microbial growth suggesting that the MIC of reserpine lies beyond 80 µg/ml. Jin *et al.*, (2010) reported an MIC of 256 µg/ml for reserpine against *Mycobacterium tuberculosis* which is greater than 80 µg/ml, thus the value obtained in the study is within range of other studies of reserpine.

The ATCC strain of *S. aureus* showed greater susceptibility to CCCP and reserpine when compared to the clinical strain were the expected results. The clinical strain of *S. aureus* had an MIC of reserpine greater than 250 µg/ml and while that for the ATCC strains was 125 µg/ml, both values were quite high but in line with Rodrigues *et al.*, (2008) findings. Rodrigues and colleagues obtained an MIC of 256 µg/ml for reserpine.

5.6 Determination of MIC for chlorhexidine in the presence of EPIs against *P. aeruginosa* and *S. aureus*

The MICs of chlorhexidine in the absence and presence of EPIs against the clinical and ATCC strains of *P. aeruginosa* and *S. aureus* were compared, in order to see if the presence of an EPI reduces the MIC value of chlorhexidine. No difference in MIC values was noted for chlorhexidine only or in combination of with CCCP against the clinical and ATCC strains of *P. aeruginosa*. However, there was a reduction in the MIC of chlorhexidine in the presence of reserpine, which was evidence of inhibitory activity according to Omoregie *et al.*,

(2007). The reduction in the MIC value in the presence of the efflux pump inhibitor was found to give an inhibitory effect on *P. aeruginosa* efflux pumps (Omoregie *et al.*, 2007).

The reduction in MIC values of chlorhexidine in the presence of an EPI (Table 4.2 and Table 4.4) obtained for the clinical strain of *P. aeruginosa*, the clinical and ATCC strains of *S. aureus* could only show the presence of efflux activity without bringing out the type of efflux pump involved. An accumulation assay in the presence and absence of an energy source (glucose) was also carried out for all isolates in order to evaluate the role of ABC transporters.

5.7 Chlorhexidine accumulation assay

Since ABC transporters are the only type of efflux pumps that use ATP as their primary source of energy (Gestin *et al.*, 2013) any differences observed in the amount of chlorhexidine accumulated in the presence of glucose could be attributed to the activity of ABC transporters. The clinical strain of *P. aeruginosa* showed a significant reduction in levels of chlorhexidine accumulated in presence of glucose than in the absence of glucose (Figure 4.7), this evidence may suggest active efflux of chlorhexidine by ABC pumps. Cells of the clinical strain of *P. aeruginosa* incubated in the presence of glucose plus reserpine showed significantly higher levels of chlorhexidine accumulated (Figure 4.7) than cells incubated in the presence of glucose only. This showed that reserpine was inhibiting efflux pump activity of the ABC transporters. Concentrations of up to 100 µg/ml reserpine have been reported to give an inhibitory effect on *P. aeruginosa* efflux pumps (Omoregie *et al.*, 2007). Both strains of *S. aureus* showed no significant reduction in the levels of chlorhexidine accumulated in presence of glucose, evidence that there was no active efflux of chlorhexidine by ABC transporters. Efflux activity in *S. aureus* suggested by the reduction in MIC values of chlorhexidine in the presence of an EPI (Table 4.4) could be attributed to other types of efflux pumps. An example of an efflux pump type that can pump out chlorhexidine

without the use ATP as a primary source of energy is the major facilitator superfamily (MFS) (Piddock, 2006). MFS efflux pumps involved in the uniport, antiport and symport of a variety of compounds across the cell have been identified in *S. aureus* (Mitchell *et al.*, 1998).

6 Conclusion

The development and validation of an assay to quantify the amount of chlorhexidine accumulated in bacterial cells was achieved. ABC transporters play a role in the efflux of chlorhexidine from the cells of the clinical strain of *P. aeruginosa*. The role of ABC transporters in the efflux of chlorhexidine from clinical and ATCC strains of *S. aureus* as well as ATCC strains of *P. aeruginosa* was not evident.

7 Future studies

Possible inhibition of ABC transporter activity of the clinical strain of *P. aeruginosa* can be evaluated using the accumulation assay in future studies. Using the chlorhexidine accumulation assay developed in this study, the role of ABC transporters in the efflux of chlorhexidine may also be evaluated for fungi and mycobacteria in future studies.

8 References

- Ahmed M., Borsch C.M., Alexander A. , Neyfakh S.B. and Schuldinern S.** (1993). Mutants of the *Bacillus subtilis* multidrug transporter *Bmr* with altered sensitivity to the antihypertensive alkaloid reserpine. *The Journal of Biological Chemistry*, **268(15)**:11086-11089.
- Alaghebandan R., Azimi L., Rastegar L.A.** (2012). Nosocomial infections among burn patients in Teheran, Iran: a decade later. *Annual Burns Fire Disasters*, **25(1)**: 3–7.
- Amorim C.V.G, Aun C.E. and Mayer M.P.A.** (2004). Susceptibility of some oral microorganisms to chlorhexidine and paramonochlorophenol. *Brazilian Oral Research*, **18(3)**:242-6.
- Aparna V.,Dineshkumar K., Mohanalakshmi N., Velmurugan D. and Hopper W.** (2014). Identification of natural compound inhibitors for multidrug efflux pumps of *Escherichia coli* and *Pseudomonas aeruginosa* using *in silico* high-throughput virtual screening and *in vitro* validation. *PLOS ONE* **9(7)**:e101840.
- Arderbili A., Talebi M., Azimi L. and Lari R.A.** (2014). Effect of efflux pump inhibitor Carbonyl Cyanide 3-Chlorophenylhydrazone on the minimum inhibitory concentration of ciprofloxacin in *Acinetobacter baumannii* clinical isolates. *Jundishapur Journal of Microbiology*, **7(1)**:e8681.
- Arun T., Rabeeth M.** (2010). Genotoxic effect of paracetamol containing tablets in cultured human lymphocytes. *International Journal of Biomedical Research*, **1**:21-30.
- Ashraf M.A., Ullah S., Ahmad I., Qureshi A.K., Balkhairf K.S. and Rehmang M.A.** (2013). Green biocides, a promising technology:current and future applications to industry and industrial processes. *Journal of the Science of Food and Agriculture*, pp1-17.
- Askoura M., Mottawea W., Abujamel T. and Taher I.** (2011). Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. *Libyan Journal of Medicine*, **6**:5870-5877.
- Baugh S., Ekanayaka A.S., Piddock L.J. and Webber M.A.** (2012). Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *Journal of Antimicrobial Chemotherapy*, **67**:2409-17.
- Behiry A.E., Schlenker G., Szabo I, Roesler U.** (2012). *In vitro* susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different antimicrobial agents. *Journal of Veterinary Science*, **13(2)**:153-161.
- Block S.S.** 1991. Disinfection, sterilisation and preservation., 4th edition, Lea and Febiger, PA, p. 274.
- Breidenstein E.B., de la Fuente-Nunez C. and Hancock R.E.** (2011). *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends in Microbiology*, **19**:419–426.
- Chang G.** (2003). Multidrug resistance ABC transporters. *FEBS Letters*, **555**:102-105.
- Chen J., Wang F., Liu Q., and Du J.** (2014). Antibacterial polymeric nanostructures for biomedical applications. *Chemical Communications*, **50**:14482-14493.

Chitemerere T.A. and Mukanganyama S. (2011). *In vitro* antibacterial activity of selected medicinal plants from Zimbabwe. *The African Journal of Plant Science and Biotechnology*, **5(1)**:1-7.

Chitemerere T.A. and Mukanganyama S. (2014). Evaluation of cell membrane integrity as a potential antimicrobial target for plant products. *BMC Complementary and Alternative Medicine*, **14**:278.

CLSI: Methods for dilutions antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard-seventh edition CLSI document M7-A7 2006.

Coenye T., Van Acker H., Peeters E., Sass A., Buroni S., Riccardi G. and Mahenthiralingam E. (2011). Molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms. *Antimicrobial Agents and Chemotherapy*, **55(5)**:1912–1919.

Curtin J. J. and Donlan R. M. (2006). Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. *Antimicrobial Agents Chemotherapy*, **50**:1268-75.

Davidson A.L. and Chen J. (2004). ATP-binding cassette transporters in bacteria. *Annual Reviews of Biochemistry*, **73**:241-268.

Denyer S. P. (1995). Mechanisms of action of antibacterial biocides. *International Biodeterioration and Biodegradation*, **36**:227–245.

Denyer S. P. and Stewart G.S.A.B. (1998). Mechanisms of action of disinfectants. *International Biodegradation and Biodegradation*, **41**:261-268.

Desai M., Buhlar T., Weller P.H. and Brown M.R.W. (1998). Increasing resistance of planktonic and biofilm culture of *Burkholderia cepacia* to ciprofloxacin and ceftzidime during exponential growth. *Journal of Antimicrobial Chemotherapy*, **42**:153–160.

De Rossi E., A´insa J.A. and Riccardi G. (2006). Role of mycobacterial efflux transporters in drug resistance:an unresolved question. *FEMS Microbiology Reviews*, **(30)**:36–52.

Diekema D. J., Pfaller M. A., Schmitz F. J., Smayevsky J., Bell J., Jones R. N. and Beach M. (1997). SENTRY participants group. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY antimicrobial surveillance program, 1997–1999. *Clinical Infectious Diseases*, **32**:114–132.

Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. *Official Journal of the European Union*, **123**:0001 – 0063.

Dreier J. and Ruggerone P. (2015). Interaction of antibacterial compounds with RND efflux pumps in *Pseudomonas aeruginosa*. *Frontiers of Microbiology*, **6**:660.

Ducel G., Fabry J. and Nicolle L. Prevention of hospital-acquired infections: A practical guide. 2nd edition — WHO/CDS/CSR/EPH/2002.12.

Garvey M.I. and Piddock L.J.V. (2008). The efflux pump inhibitor reserpine selects multidrug-resistant *Streptococcus pneumoniae* strains that overexpress the ABC transporters *patA* and *patB*. *Antimicrobial Agents and Chemotherapy*, **52(5)**:1677–1685.

Getsin I., Nalbandian G.H., Yee D.C., Vastermark A., Paparoditis P.C.G., Reddy V.S. and Saier M.H. (2013). Comparative genomics of transport proteins in developmental bacteria: *Myxococcus xanthus* and *Streptomyces coelicolor*. *BMC Microbiology*, **13**:279.

Gilbert P., Allison D.G. and McBain A.J. (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance. *Journal of Applied Microbiology Symposium Supplement*, **92**:98–110.

Hansen M. B., Nielsen S. E. and Berg K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*, **119**:203-10.

Health Protection Agency. (September) 2010c. Healthcare-associated infections and antimicrobial resistance: 2009/10 (annual report). [http://www.hpa.org.uk/web/HPAweb &HPAwebStandard/HPAweb_C/1281954478156](http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1281954478156).

Hebert V.R, Middleton J. R., Tomaszewska E. and Fox L.K. (2003). “Methodology for quantifying residues of chlorhexidine in raw dairy milk,” *Journal of Agricultural and Food Chemistry*, **51(3)**:567-570.

Hemani M. L. and Lepor H. (2009). Skin preparation for the prevention of surgical site infection: which agent is best? *Reviews in Urology*, **11** (4): 190-5.

Hendry E. R., Worthington T., Conway B.R. and Lambert P.A. (2009). Antimicrobial efficacy of eucalyptus oil and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures. *Journal of Antimicrobial Chemotherapy*, **64**:1219–1225.

Hogan J.S., Smith K.L. (1989). Prolonged *in vitro* exposure of *Staphylococcus aureus* to germicidal teat dips. *Journal of Dairy Sciences*, **72**:1052-1056.

Holmesa A.N., Keniyaa M., Ivnitiski-Steeleb I., Monka C., Lampinga E., Sklarb L.A. and Cannona R.D. (2012). The monoamine oxidase A inhibitor clorgyline is a broad-spectrum inhibitor of fungal ABC and MFS transporter efflux pump activities which reverses the azole resistance of *Candida albicans* and *Candida glabrata* clinical isolates. *Antimicrobial Agents and Chemotherapy*, **56(3)**:1508-1515.

Horner C., Mawer D. and Wilcox M. (2012). Reduced susceptibility to chlorhexidine in staphylococci: is it increasing and does it matter? *Journal of Antimicrobial Chemotherapy*, **67(11)**:2543-2546.

Jin J., Zhang J., Guo N., Sheng H., Li L., Liang J., Wang X., Li Y., Liu M., Wu X. and Yu L. (2010). Farnesol, a potential efflux pump inhibitor in *Mycobacterium smegmatis*. *Molecules*, **15**: 7750-7762.

- Jones P.M. and George A.M.** (2002). Mechanism of ABC transporters: A molecular dynamics simulation of a well characterized nucleotide-binding subunit. *PNAS* **99(20)**:12639–12644.
- Joshi S.G. and Litake G.M.** (2013). *Acinetobacter baumannii* : An emerging pathogenic threat to public health. *World Journal of Clinical Infectious Diseases*, **3(3)**:25-36.
- Kim S.S., Kim S., Kim E., Hyuna B., Kimb K. and Lee B.J.** (2003). Synergistic inhibitory effect of cationic peptides and antimicrobial agents on the growth of oral *Streptococci*. *Caries Research*, **37**:425–430.
- Khan J.A., Iqbal Z., Rahman S.U., Farzana K. and Khan A.** (2008). Prevalence and resistance pattern of *Pseudomonas aeruginosa* against various antibiotics. *Pakistan Journal of Pharmaceutical Science*, **21(3)**:311-5.
- Komolafe A. O. and Adegoke, A. A.** (2008). Incidence of bacterial septicaemia in Ile-Ife Metropolis, Nigeria. *Malaysian Journal of Microbiology*, **4(2)**:51- 61.
- Kumar S. and Varela M.F.** (2012). Biochemistry of bacterial multidrug efflux pumps. *International Journal of Molecular Sciences*, **13**:4484–4495.
- Kumar S., Mukherjee M. and Varela M.F.** (2013). Modulation of Bacterial Multidrug Resistance Efflux Pumps of the Major Facilitator Superfamily. *International Journal of Bacteriology*, pp1-15.
- Lambert P.A.** (2002). Cellular impermeability and uptake of biocides and antibiotic in Gram-positive bacteria and mycobacteria. *Journal of Applied Microbiology Symposium*, **92**:46-54.
- Lambert R.J.W.** (2004). Comparative analysis of antibiotic and antimicrobial biocide susceptibility data in clinical isolates of methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* between 1989 and 2000. *Journal of Applied Microbiology*, **97**:699–711.
- Li X.Z. and Nikaido H.** (2004). Efflux-mediated drug resistance in bacteria. *Drugs*, **64**: 159-204.
- Lomovskaya O. and Watkins W.** (2001). Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. *Journal of Molecular Microbiological Biotechnology*, **3**:225–36.
- Madigan M.T., Martinko J.M. and Parker J.** (2001). Antimicrobial Drug Resistance. *Brock Biology of Microorganisms*. 10th ed., Pearson Education International.
- Maillard J.** (2001). Virus susceptibility to biocides: an understanding. *Reviews in Medical Microbiology*, **12(2)**:63-74.

- Maillard J.** (2005). Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Therapeutics and Clinical Risk Management*, **1(4)**:307–320.
- Mandell Z., Nikaido H. and Pool K.** (1995). Role of *MexA-Mex B-Opr Min* antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **39**:1984-1953.
- Mangoyi R. and Mukanganyama S.** (2011). *In Vitro* Antifungal activities of selected medicinal plants from Zimbabwe against *Candida albicans* and *Candida krusei*. *The African Journal of Plant Science and Biotechnology*, **5(1)**:1-7.
- Manisha P.** (2012). Development and evaluation of troches containing chlorhexidine HCL and triamcinolone for treatment of dental plaque, gingivitis and mouth ulcers. *International Journal of Pharmaceutical Research and Bio-Science*, **1(5)**: 211-237.
- Mazzola P.G., Jozala A.F., Novaes L.C., Moriel P. and Penna T.C.V.** (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents. *Brazilian Journal of Pharmaceutical Sciences*, **45(2)**: 241-248.
- McDonnell G., and Burke P.** (2003). The challenge of prion decontamination. *Clinical Infectious Diseases*, **36(9)**:1152-1154.
- McDonnell G. and Russell D.** (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews*, **12(1)**:147–179.
- Mckenry L.M. and Salerno E.** 2001. Mosby's pharmacology in nursing. 21st ed., Mosby, Inc., USA. pp:1198-1208.
- Mitchell B.A., Brown M.H. and Skurray R.A.** (1998). *QacA* multidrug efflux pump from *Staphylococcus aureus*: Comparative analysis of resistance to diamidines, biguanidines, and guanilylhydrazones. *Antimicrobial Agents and Chemotherapy*, **42(2)**:475–477.
- Morrissey I., Oggioni M.R., Knight D., Curiao T., Coque T., Kalkanci A. and Martinez J.L.** (2014). Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms. *PLOS ONE*, **9(1)**:e86669.
- Mortimer P. G. S. and Piddock L. J. V.** (1991). A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, **28**:639–653.
- Nikaido H., Basina M. and Nguyen V.** (1998). Multidrug efflux pump *AcrAB* of *Salmonella typhimurium* excretes only those b-lactam antibiotics containing lipophilic side chains. *Journal of Bacteriology*, **180**:4686–92.
- Odore R., Colombatti Vale V. and Re G.** (2000). Efficacy of chlorhexidine against some strains of cultured and clinically isolated microorganisms. *Veterinary Research Communications*, **24**:229-238.

Omorieg R., Airueghionmon D.J.U., Okonkwo J.O., Airueghionmon U.E., Ibeh I.N. and Ogefere H.O. (2007). Prevalence of multidrug efflux pump requiring ciprofloxacin, ofloxacin and pefloxacin as substrates, among clinical isolates of *Pseudomonas aeruginosa*. *Malaysian Journal of Microbiology*, **3(2)**:37-40.

Paniagua-Contreras G.L., Monroy-Pérez E., Vaca-Paniagua F., Rodríguez-Moctezuma J.R., Negrete-Abascal E. and Vaca S. (2014). Implementation of a novel *in vitro* model of infection of reconstituted human epithelium for expression of virulence genes in methicillin-resistant *Staphylococcus aureus* strains isolated from catheter-related infections in Mexico. *Annals of Clinical Microbiology and Antimicrobials*, **13**:6.

Paulsen I. T., Brown M.H., and Skurray R.A. (1996). Proton-dependent multidrug efflux systems. *Microbiological Reviews*, **60**:575–608.

Paulson D. S. 2003. "Chlorhexidine gluconate," in Handbook of topical antimicrobials: industrial applications in consumer products and pharmaceuticals, Marcel Dekker Inc., New York, USA, pp. 117-122.

Piddock L. J. (2006). Multidrug-resistance efflux pumps: not just for resistance. *Nature Reviews Microbiology*, **4**:629–636.

Piddock L.J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Review*, **19(2)**:382–402.

Plowman R., Graves N., Griffin M.A.S., Roberts J.A., Swan A.V., Cookson B. and Taylor L. (2001). The rate and cost of hospital-acquired infections occurring in patients admitted to selected specialities of a district hospital in England and the national burden imposed. *Journal of Hospital Infections*, **47**:198-202.

Poole K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. *Journal of Applied Microbiology Symposium Supplement*, **92**:55–64.

Poole K. (2004). Resistance to beta-lactam antibiotics. *Cellular and Molecular Life Sciences*, **61(17)**:2200-23.

Prag G., Falk-Brynhildsen K., Jacobsson S. and Hellmark B. (2014). Decreased susceptibility to chlorhexidine and prevalence of disinfectant resistance genes among clinical isolates of *Staphylococcus epidermidis*, *APMIS* pp 2-7.

Puranik S.N. and Kumar P. (2010). A comparative study to evaluate the effectiveness of two commercially available disinfectants on denture base acrylic resin with an organic load. *International Journal of dental Clinics*, **2(2)**:1-6.

Ramon-Garcia S., C. Martin, C. J. Thompson, and J. A. Ainsa. (2009). Role of the *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrobial Agents of Chemotherapy*, **53**:3675–3682.

Rodrigues L., Wagner D., Viveiros M., Sampaio D., Couto I., Vavra M., Kern W.V. and Amaral L. (2008). Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*. *Journal of Antimicrobiological Chemotherapy*, **61**:1076-1082.

Rohde H., Frankenberger S., Zahringer U. and Mack D. (2010). Structure, function and contribution of polysaccharide intercellular adhesion (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. *European Journal of Cell Biology*, **89(1)**:103-11.

Rotter M.L. (2001). Argument for alcoholic hand disinfection. *Journal of Hospital Infections*, **48**:4–8.

Saginur R., St. Denis M., Ferris W., Aaron S.D., Chan F., Lee C. and Ramotar K. (2006). Multiple combination bactericidal testing of staphylococcal biofilms from implant associated infections. *Antimicrobial Agents of Chemotherapy*, **50(1)**:55-61.

Saleh R.H., Naher H.S. and Al-Jubory S.A. (2012). A study of efficacy of disinfectants and bacterial contamination in Al-Hilla Teaching Hospital. *Medical Journal of Babylon*, **9(4)**:890-900.

Sheppard F. C., Mason D. J., Bloomfield S. F. and Gant V. A. (1997). Flow cytometric analysis of chlorhexidine action. *FEMS Microbiological Letters*, **154**:283–288.

Smith P.W. and Rusnak P.G. (1997). Infection prevention and control in Long-Term-Care facility. *Infection Control and Hospital Epidemiology*, **18(12)**: 831-849.

Smith K. and Hunter I. S. (2008). Efficacy of common hospital biocides with biofilms of multidrug resistant clinical isolates. *Journal of Medical Microbiology*, **57**:966-73.

Solmaz G. and Korachi M. (2012). Inhibition and disruption properties of chlorhexidine gluconate on single and multispecies oral biofilms. *Jundishapur Journal of Microbiology* **6(1)**:61-66.

Soto M. (2013). Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence*, **4(3)**:223–229.

Stickler D.J. and King J.B. 1999. Bacterial sensitivity and resistance. A. Intrinsic resistance. In *Principles and practice of disinfection, preservation and sterilization*. 3rd ed. pp284-296. Russell AD., Hugo WB and Ayliffe GAJ. Oxford: Blackwell Scientific Publications.

Sun D., Maa X.X., Hua J., Tiana Y., Panga L., Shang H. and Cui L.Z. (2013) Epidemiological and molecular characterization of community and hospital acquired *Staphylococcus aureus* strains prevailing in Shenyang, North-eastern China. *Brazilian Journal of infectious diseases*, **17(6)**:682–690.

Sun J.1., Deng Z. and Yan A. (2014). Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications*, **453(2)**:254-267.

Tegos G.P., Haynes M., Strouse J.J., Khan M.T., Bologa C.G., Oprea T.I. and Sklar L.A. (2011). Microbial efflux pump inhibition: tactics and strategies. *Current Pharmaceutical Design*, **17(13)**:1291–1302.

Todorova-Christova M., Vatcheva R. , Filipova R., Kamenova T., Arnaudov Y., Radulova Y., Ivanov I. and Dobрева E. (2014). Frequency distribution of the microbial isolates in major nosocomial infections groups. *Journal of Bacteriology and Parasitology*, **6(2)**:1-9.

Van Bambeke F., Barcia-Macay M., Lemaire S. and Tulkens P.M. (2006). Cellular pharmacodynamics of antibiotics: current views and perspectives. *Current Opinions on Drug Discovery and Development*, **9(2)**:218-30.

Van Bambeke F., Pagès J. and Lee V.G. (2010). Inhibitors of Bacterial Efflux Pumps as Adjuvants in Antibacterial Therapy and Diagnostic Tools for Detection of Resistance by Efflux. *Frontiers in Anti-Infective Drug Discovery*, **1(2)**:138-175.

Vila J., Martí, S. and Sánchez-Céspedes J. (2007). Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*, **59**:1210-1215.

Weigelt J. A., Lipsky B. A., Tabak Y. P., Derby K. G., Kim M. and Gupta V. (2010). Surgical site infections: causative pathogens and associated outcomes. *American Journal of Infection Control*, **38(2)**:112-20.

Appendices

Absorbances for the determination of MIC of chlorhexidine for clinical *S. aureus*

Second reading

0.1778	0.1616	0.1622	0.1628	0.1612	0.6175	0.6838	0.6712	0.6328	0.1671	0.1681	0.1777
0.2147	0.1993	0.1971	0.1953	0.1951	0.6508	0.6930	0.6539	0.6107	0.1689	0.1714	0.1754
0.2108	0.1850	0.1724	0.1655	0.6000	0.6801	0.7103	0.7132	0.7427	0.6790	0.2029	0.2068
0.2291	0.2135	0.2154	0.2153	0.6465	0.7243	0.7552	0.6859	0.6996	0.6879	0.2044	0.2071
0.2257	0.2097	0.2085	0.2072	0.6795	0.8227	0.7539	0.6816	0.7263	0.7616	0.2149	0.2180
0.2089	0.2005	0.2043	0.2048	0.5465	0.7327	0.7339	0.7266	0.7119	0.7083	0.1764	0.2198
0.7015	0.7492	0.7311	0.7940	0.8216	0.6931	0.7983	0.6748	0.5630	0.6864	0.1620	0.1618
0.6337	0.7294	0.6292	0.6740	0.7281	0.6594	0.6090	0.5938	0.6069	0.4963	0.1628	0.1591

First reading

0.0645	0.0537	0.0527	0.0530	0.0535	0.0531	0.0527	0.0532	0.0518	0.0527	0.0528	0.0541
0.0652	0.0536	0.0530	0.0534	0.0540	0.0533	0.0533	0.0540	0.0522	0.0534	0.0539	0.0545
0.0714	0.0539	0.0512	0.0523	0.0520	0.0544	0.0545	0.0539	0.0537	0.0551	0.0537	0.0549
0.0675	0.0543	0.0533	0.0531	0.0544	0.0538	0.0546	0.0538	0.0535	0.0547	0.0541	0.0548
0.0664	0.0537	0.0530	0.0539	0.0527	0.0529	0.0529	0.0532	0.0520	0.0550	0.0542	0.0538
0.0693	0.0545	0.0540	0.0531	0.0546	0.0542	0.0536	0.0538	0.0538	0.0593	0.0487	0.0540
0.0528	0.0520	0.0527	0.0535	0.0529	0.0526	0.0529	0.0512	0.0523	0.0515	0.0528	0.0535
0.0530	0.0524	0.0532	0.0518	0.0533	0.0532	0.0523	0.0518	0.0529	0.0497	0.0533	0.0528

Second reading - First reading

0.1133	0.1079	0.1095	0.1098	0.1077	0.5644	0.6311	0.6180	0.5810	0.1144	0.1153	0.1236
0.1495	0.1457	0.1441	0.1419	0.1411	0.5975	0.6397	0.5999	0.5585	0.1155	0.1175	0.1209
0.1394	0.1311	0.1212	0.1132	0.5480	0.6257	0.6558	0.6593	0.6890	0.6239	0.1492	0.1519
0.1616	0.1592	0.1621	0.1622	0.5921	0.6705	0.7006	0.6321	0.6461	0.6332	0.1503	0.1523
0.1593	0.1560	0.1555	0.1533	0.6268	0.7698	0.7010	0.6284	0.6743	0.7066	0.1607	0.1642
0.1396	0.1460	0.1503	0.1517	0.4919	0.6785	0.6803	0.6728	0.6581	0.6490	0.1277	0.1658
0.6487	0.6972	0.6784	0.7405	0.7687	0.6405	0.7454	0.6236	0.5107	0.6349	0.1092	0.1083
0.5807	0.6770	0.5760	0.6222	0.6748	0.6062	0.5567	0.5420	0.5540	0.4466	0.1095	0.1063

Absorbances for the determination of MIC of chlorhexidine for *ATCC S. aureus*

Second reading

0.1735	0.1556	0.1585	0.1558	0.1579	0.5956	0.6595	0.6432	0.6356	0.1647	0.1623	0.1747
0.2079	0.1903	0.1891	0.1896	0.1847	0.5941	0.5887	0.6430	0.6299	0.1581	0.1608	0.1499
0.1953	0.1582	0.1557	0.1563	0.5775	0.6084	0.6040	0.5627	0.7160	0.6331	0.1659	0.1754
0.2031	0.1847	0.1841	0.1837	0.4739	0.6597	0.6850	0.6589	0.7662	0.6422	0.1615	0.1749
0.1857	0.1532	0.1534	0.1546	0.5858	0.6233	0.6337	0.6296	0.7243	0.6713	0.6095	0.1605
0.1686	0.1564	0.1548	0.1566	0.5666	0.6665	0.6553	0.6327	0.7560	0.6499	0.1588	0.1618
0.6977	0.6393	0.7069	0.6913	0.6876	0.6653	0.6667	0.6450	0.6246	0.6833	0.1594	0.1610
0.7097	0.6615	0.6776	0.6646	0.6097	0.6345	0.6277	0.6189	0.7170	0.6419	0.1606	0.1593

First reading

0.0679	0.0531	0.0529	0.0526	0.0533	0.0530	0.0529	0.0529	0.0534	0.0534	0.0531	0.0533
0.0694	0.0539	0.0528	0.0533	0.0531	0.0530	0.0533	0.0535	0.0532	0.0531	0.0535	0.0517
0.0729	0.0551	0.0549	0.0545	0.0549	0.0536	0.0532	0.0517	0.0583	0.0567	0.0539	0.0542
0.0694	0.0555	0.0538	0.0547	0.0550	0.0553	0.0560	0.0548	0.0577	0.0548	0.0540	0.0543
0.0709	0.0551	0.0621	0.0553	0.0552	0.0544	0.0551	0.0546	0.0567	0.0550	0.0549	0.0537
0.0691	0.0558	0.0577	0.0572	0.0549	0.0549	0.0555	0.0554	0.0574	0.0561	0.0538	0.0545
0.0533	0.0530	0.0536	0.0529	0.0551	0.0574	0.0549	0.0546	0.0536	0.0545	0.0532	0.0536
0.0548	0.0530	0.0535	0.0543	0.0543	0.0534	0.0532	0.0526	0.0530	0.0546	0.0539	0.0524

Second reading - First reading

0.1056	0.1025	0.1056	0.1032	0.1046	0.5426	0.6066	0.5903	0.5822	0.1113	0.1092	0.1214
0.1385	0.1364	0.1363	0.1363	0.1316	0.5411	0.5354	0.5895	0.5767	0.1050	0.1073	0.0982
0.1224	0.1031	0.1008	0.1018	0.5226	0.5548	0.5508	0.5110	0.6577	0.5764	0.1120	0.1212
0.1337	0.1292	0.1303	0.1290	0.4189	0.6044	0.6290	0.6041	0.7085	0.5874	0.1075	0.1206
0.1148	0.0981	0.0913	0.0993	0.5306	0.5689	0.5786	0.5750	0.6676	0.6163	0.5546	0.1068
0.0995	0.1006	0.0971	0.0994	0.5117	0.6116	0.5998	0.5773	0.6986	0.5938	0.1050	0.1073
0.6444	0.5863	0.6533	0.6384	0.6325	0.6079	0.6118	0.5904	0.5710	0.6288	0.1062	0.1074
0.6549	0.6085	0.6241	0.6103	0.5554	0.5811	0.5745	0.5663	0.6640	0.5873	0.1067	0.1069

Absorbances for the determination of MIC of ampicillin for clinical *S. aureus*

Second reading

0.0557	0.0520	0.0516	0.0529	0.0568	0.5552	0.5105	0.4648	0.0575	0.0566	0.0559	0.0552
0.0860	0.0860	0.0850	0.0813	0.0762	0.4301	0.0676	0.4406	0.0609	0.4035	0.0527	0.0534
0.0826	0.0696	0.0563	0.0550	0.5980	0.6406	0.5962	0.6464	0.1006	0.6085	0.0647	0.0697
0.0922	0.0939	0.0902	0.0873	0.0844	0.6104	0.5459	0.0741	0.5163	0.1805	0.0672	0.0688
0.0861	0.0755	0.0660	0.0554	0.4513	0.5891	0.0567	0.0557	0.3241	0.5334	0.0581	0.0581
0.9277	0.0563	0.0567	0.0550	0.4031	0.6222	0.5098	0.5044	0.4348	0.6609	0.3654	0.0579
0.4058	0.4337	0.1629	0.4424	0.4405	0.4655	0.4375	0.4557	0.4941	0.4956	0.0538	0.0522
0.4202	0.4572	0.3862	0.4509	0.4447	0.4703	0.4268	0.4669	0.5105	0.4875	0.0519	0.3954

First reading

0.0519	0.0504	0.0508	0.0515	0.0549	0.0559	0.0518	0.0516	0.0521	0.0521	0.0517	0.0524
0.0512	0.0525	0.0525	0.0521	0.0512	0.0526	0.0534	0.0520	0.0541	0.0548	0.0520	0.0519
0.0530	0.0534	0.0538	0.0551	0.0546	0.0561	0.0567	0.0560	0.0492	0.0609	0.0555	0.0532
0.0537	0.0541	0.0547	0.0552	0.0555	0.0553	0.0557	0.0548	0.0480	0.0610	0.0546	0.0529
0.0533	0.0535	0.0576	0.0534	0.0559	0.0610	0.0544	0.0543	0.0469	0.0621	0.0520	0.0524
0.0534	0.0542	0.0551	0.0545	0.0549	0.0552	0.0548	0.0551	0.0507	0.0609	0.0523	0.0523
0.0566	0.0545	0.0418	0.0555	0.0564	0.0566	0.0505	0.0571	0.0582	0.0572	0.0593	0.0514
0.0547	0.0555	0.0463	0.0547	0.0552	0.0560	0.0522	0.0542	0.0558	0.0556	0.0511	0.0515

Second reading - First reading

0.0038	0.0016	0.0008	0.0014	0.0019	0.4993	0.4587	0.4132	0.0054	0.0045	0.0042	0.0028
0.0348	0.0335	0.0325	0.0292	0.0250	0.3775	0.0142	0.3886	0.0068	0.3487	0.0007	0.0015
0.0296	0.0162	0.0025	-0.0001	0.5434	0.5845	0.5395	0.5904	0.0514	0.5476	0.0092	0.0165
0.0385	0.0398	0.0355	0.0321	0.0289	0.5551	0.4902	0.0193	0.4683	0.1195	0.0126	0.0159
0.0328	0.0220	0.0084	0.0020	0.3954	0.5281	0.0023	0.0014	0.2772	0.4713	0.0061	0.0057
0.8743	0.0021	0.0016	0.0005	0.3482	0.5670	0.4550	0.4493	0.3841	0.6000	0.3131	0.0056
0.3492	0.3792	0.1211	0.3869	0.3841	0.4089	0.3870	0.3986	0.4359	0.4384	-0.0055	0.0008
0.3655	0.4017	0.3399	0.3962	0.3895	0.4143	0.3746	0.4127	0.4547	0.4319	0.0008	0.3439

Absorbances for the determination of MIC of CCCP for ATCC *S. aureus*

Second reading

0.0596	0.0630	0.0669	0.0646	0.0661	0.0672	0.0651	0.0679	0.0723	0.8774	0.9410	0.8880
0.1029	0.1064	0.1037	0.1003	0.0950	0.0888	0.0861	0.0755	0.7073	0.9043	0.0655	0.8784
0.0951	0.0788	0.0656	0.0633	0.0649	0.0772	0.1270	0.8506	0.8889	0.9396	0.0892	0.0904
0.1191	0.1241	0.1252	0.1215	0.1168	0.1216	0.1616	0.7520	0.8802	0.8912	0.8583	0.0979
0.1123	0.1043	0.0972	0.0938	0.0893	0.0984	0.1528	0.7460	0.8765	0.8972	0.8603	0.1043
0.1193	0.1229	0.1222	0.1184	0.1158	0.1278	0.1746	0.7266	0.8401	0.8630	0.4300	0.0956
0.9335	0.8978	0.8976	0.9352	0.8447	0.9554	0.9664	0.9804	0.9656	0.9497	0.0746	0.0708
0.8879	0.9325	0.9179	0.8782	0.9102	0.8439	0.8687	0.9035	0.9075	0.9217	0.0637	0.0729

First reading

0.0596	0.0642	0.0695	0.0653	0.0651	0.0648	0.0662	0.0636	0.0664	0.0714	0.0714	0.0626
0.0632	0.0609	0.0634	0.0647	0.0659	0.0661	0.0662	0.0626	0.0647	0.0689	0.0634	0.0609
0.0654	0.0629	0.0627	0.0635	0.0624	0.0662	0.0667	0.0647	0.0652	0.0612	0.0612	0.0575
0.0620	0.0632	0.0633	0.0644	0.0652	0.0662	0.0660	0.0696	0.0659	0.0658	0.0621	0.0639
0.0614	0.0643	0.0635	0.0662	0.0640	0.0680	0.0730	0.0644	0.0639	0.0652	0.0640	0.0631
0.0630	0.0661	0.0647	0.0638	0.0637	0.0668	0.0708	0.0639	0.0643	0.0681	0.0606	0.0555
0.0659	0.0667	0.0734	0.0735	0.0722	0.0716	0.0722	0.0706	0.0726	0.0749	0.0674	0.0627
0.0656	0.0652	0.0716	0.0718	0.0708	0.0695	0.0750	0.0699	0.0684	0.0722	0.0643	0.0679

Second reading - First reading

0.0000	-0.0012	-0.0026	-0.0007	0.0010	0.0024	-0.0011	0.0043	0.0059	0.8060	0.8696	0.8254
0.0397	0.0455	0.0403	0.0356	0.0291	0.0227	0.0199	0.0129	0.6426	0.8354	0.0021	0.8175
0.0297	0.0159	0.0029	-0.0002	0.0025	0.0110	0.0603	0.7859	0.8237	0.8784	0.0280	0.0329
0.0571	0.0609	0.0619	0.0571	0.0516	0.0554	0.0956	0.6824	0.8143	0.8254	0.7962	0.0340
0.0509	0.0400	0.0337	0.0276	0.0253	0.0304	0.0798	0.6816	0.8126	0.8320	0.7963	0.0412
0.0563	0.0568	0.0575	0.0546	0.0521	0.0610	0.1038	0.6627	0.7758	0.7949	0.3694	0.0401
0.8676	0.8311	0.8242	0.8617	0.7725	0.8838	0.8942	0.9098	0.8930	0.8748	0.0072	0.0081
0.8223	0.8673	0.8463	0.8064	0.8394	0.7744	0.7937	0.8336	0.8391	0.8495	-0.0006	0.0050

Absorbances for the determination of MIC of CCCP for ATCC *P. aeruginosa*

Second reading

0.0738	0.0501	0.0502	0.8071	0.7588	0.6936	0.6522	0.7848	0.8111	0.8675	0.0576	0.4581
0.0882	0.0912	0.0897	1.0091	0.8024	0.6889	0.6978	0.9129	0.9563	0.7745	0.4056	0.1685
0.0668	0.0494	0.0877	0.9985	0.7665	0.6913	0.7143	0.8828	1.0158	0.8045	0.0464	0.0466
0.0643	0.0501	0.4643	0.9285	0.8027	0.7539	0.7093	0.9004	0.8757	0.8163	0.3527	0.0460
1.1023	1.0539	1.0401	1.0276	0.9601	0.7899	0.9580	0.8852	0.8604	0.9781	0.3815	0.0450
1.0734	1.0431	1.0326	0.9994	1.0464	0.9491	1.0120	0.9940	1.0322	1.0418	0.0452	0.3583

First reading

0.0479	0.0463	0.0455	0.0456	0.0457	0.0454	0.0458	0.0458	0.0456	0.0464	0.0462	0.0460
0.0455	0.0456	0.0453	0.0450	0.0460	0.0457	0.0452	0.0471	0.0469	0.0468	0.0460	0.0460
0.0498	0.0498	0.0497	0.0497	0.0506	0.0507	0.0530	0.0512	0.0500	0.0510	0.0463	0.0456
0.0498	0.0487	0.0495	0.0504	0.0509	0.0505	0.0512	0.0506	0.0507	0.0512	0.0461	0.0466
0.0504	0.0494	0.0495	0.0494	0.0505	0.0504	0.0513	0.0512	0.0514	0.0508	0.0456	0.0457
0.0503	0.0489	0.0502	0.0497	0.0500	0.0504	0.0501	0.0550	0.0500	0.0513	0.0470	0.0466
0.0507	0.0507	0.0502	0.0506	0.0505	0.0501	0.0505	0.0495	0.0513	0.0504	0.0460	0.0461
0.0507	0.0497	0.0497	0.0501	0.0494	0.0497	0.0513	0.0498	0.0500	0.0498	0.0455	0.0465

Second reading - First reading

-0.0020	-0.0012	-0.0009	-0.0008	-0.0006	0.1138	0.3049	0.3330	0.3840	0.0056	0.0028	0.0016
0.0420	0.0433	0.0405	0.0347	0.0286	0.1233	0.3162	0.3222	0.3278	-0.0008	-0.0006	0.3491
0.0240	0.0003	0.0005	0.7574	0.7082	0.6429	0.5992	0.7336	0.7611	0.8165	0.0113	0.4125
0.0384	0.0425	0.0402	0.9587	0.7515	0.6384	0.6466	0.8623	0.9056	0.7233	0.3595	0.1219
0.0164	0.0000	0.0382	0.9491	0.7160	0.6409	0.6630	0.8316	0.9644	0.7537	0.0008	0.0009
0.0140	0.0012	0.4141	0.8788	0.7527	0.7035	0.6592	0.8454	0.8257	0.7650	0.3057	-0.0006
1.0516	1.0032	0.9899	0.9770	0.9096	0.7398	0.9075	0.8357	0.8091	0.9277	0.3355	-0.0011
1.0227	0.9934	0.9829	0.9493	0.9970	0.8994	0.9607	0.9442	0.9822	0.9920	-0.0003	0.3118

**Absorbances for the determination of MIC of chlorhexidine in the presence of reserpine
for clinical *P. aeruginosa***

Second reading

0.0814	0.0598	0.0585	0.0581	0.0581	0.0590	0.0583	0.0585	0.0580	0.0586	0.0595	0.0593
0.0965	0.0828	0.0808	0.0760	0.0719	0.0718	0.0613	0.0577	0.0580	0.1984	0.0597	0.0594
0.1241	0.1078	0.0757	0.0751	0.1242	0.5432	0.6334	0.6427	0.6876	0.6023	0.0667	0.0668
0.1055	0.0979	0.0972	0.0912	0.1285	0.5098	0.6439	0.6606	0.7320	0.6734	0.0598	0.1883
0.0970	0.1197	0.0765	0.1162	0.1158	0.5249	0.6522	0.6853	0.6598	0.6416	0.0611	0.0606
0.0963	0.0779	0.0749	0.0741	0.1935	0.5743	0.6324	0.5947	0.5611	0.5595	0.0592	0.0596
0.5711	0.5978	0.6608	0.6436	0.5674	0.5642	0.6731	0.7042	0.5523	0.5496	0.0592	0.0596
0.5727	0.5785	0.6112	0.6591	0.5839	0.5728	0.6423	0.6371	0.6335	0.6081	0.0592	0.0588

First reading

0.0876	0.0624	0.0586	0.0582	0.0576	0.0570	0.0573	0.0578	0.0569	0.0650	0.0587	0.0585
0.0936	0.0631	0.0591	0.0579	0.0588	0.0577	0.0586	0.0582	0.0575	0.0581	0.0588	0.0591
0.1122	0.0852	0.0803	0.0787	0.0791	0.0802	0.0824	0.0796	0.0797	0.0798	0.0590	0.0586
0.1117	0.0837	0.0808	0.0796	0.0801	0.0789	0.0802	0.0805	0.0797	0.0805	0.0593	0.0584
0.1129	0.0841	0.0787	0.0806	0.0786	0.0801	0.0783	0.0788	0.0794	0.0788	0.0595	0.0627
0.1110	0.0827	0.0790	0.0795	0.0795	0.0796	0.0795	0.0782	0.0777	0.0783	0.0583	0.0588
0.0715	0.0774	0.0782	0.0773	0.0777	0.0793	0.0782	0.0782	0.0772	0.0773	0.0582	0.0577
0.0719	0.0777	0.0779	0.0764	0.0776	0.0787	0.0846	0.0842	0.0831	0.0822	0.0578	0.0578

Second reading - First reading

-0.0062	-0.0026	-0.0001	-0.0001	0.0005	0.0020	0.0010	0.0007	0.0011	-0.0064	0.0008	0.0008
0.0029	0.0197	0.0217	0.0181	0.0131	0.0141	0.0027	-0.0005	0.0005	0.1403	0.0009	0.0003
0.0119	0.0226	-0.0046	-0.0036	0.0451	0.4630	0.5510	0.5631	0.6079	0.5225	0.0077	0.0082
-0.0062	0.0142	0.0164	0.0116	0.0484	0.4309	0.5637	0.5801	0.6523	0.5929	0.0005	0.1299
-0.0159	0.0356	-0.0022	0.0356	0.0372	0.4448	0.5739	0.6065	0.5804	0.5628	0.0016	-0.0021
-0.0147	-0.0048	-0.0041	-0.0054	0.1140	0.4947	0.5529	0.5165	0.4834	0.4812	0.0009	0.0008
0.4996	0.5204	0.5826	0.5663	0.4897	0.4849	0.5949	0.6260	0.4751	0.4723	0.0010	0.0019
0.5008	0.5008	0.5333	0.5827	0.5063	0.4941	0.5577	0.5529	0.5504	0.5259	0.0014	0.0010

Absorbances for the determination of MIC of CCCP for clinical *P. aeruginosa*

Second reading

0.0220	0.0171	0.0153	0.0204	0.0279	0.0286	0.0374	0.0455	0.0519	0.0476	0.0617	0.0625
0.1016	0.1034	0.1018	0.0972	0.0919	0.0772	0.0762	0.0733	0.2743	0.0574	0.0642	0.0639
0.0975	0.0941	0.0941	0.0945	0.1233	0.1682	0.6476	0.6502	0.6711	0.7681	0.1156	0.1170
0.1442	0.1693	0.1440	0.1409	0.1983	0.2006	0.6616	0.8280	0.7146	0.7586	0.1186	0.1172
0.1417	0.1401	0.1393	0.1453	0.1931	0.2003	0.6234	0.8015	0.7735	0.8706	0.1532	0.1573
0.1782	0.1769	0.1764	0.1724	0.2315	0.2418	0.6123	0.6258	0.7629	0.7958	0.1562	0.1677
0.9396	0.7802	0.8823	0.8852	0.8839	0.7416	0.8634	0.8210	0.9131	0.8099	0.1746	0.1762
0.8538	0.7906	0.8684	0.8688	0.8736	0.6793	0.8772	0.7963	0.8145	0.6814	0.1851	0.1765

First reading

0.0546	0.0559	0.0557	0.0595	0.0581	0.0551	0.0559	0.0562	0.0546	0.0458	0.0553	0.0536
0.0549	0.0562	0.0562	0.0563	0.0544	0.0477	0.0512	0.0538	0.0626	0.0454	0.0544	0.0535
0.0555	0.0561	0.0583	0.0570	0.0569	0.0568	0.0559	0.0553	0.0568	0.0552	0.0564	0.0550
0.0560	0.0577	0.0573	0.0571	0.0567	0.0564	0.0549	0.0558	0.0551	0.0561	0.0558	0.0537
0.0561	0.0566	0.0613	0.0616	0.0567	0.0568	0.0561	0.0559	0.0560	0.0563	0.0561	0.0568
0.0577	0.0557	0.0594	0.0565	0.0615	0.0556	0.0554	0.0553	0.0543	0.0549	0.0553	0.0549
0.0570	0.0565	0.0565	0.0593	0.0564	0.0536	0.0626	0.0574	0.0638	0.0557	0.0561	0.0557
0.0558	0.0547	0.0544	0.0563	0.0561	0.0532	0.0568	0.0548	0.0552	0.0536	0.0552	0.0553

Second reading - First reading

-0.0326	-0.0388	-0.0404	-0.0391	-0.0302	-0.0265	-0.0185	-0.0107	-0.0027	0.0018	0.0064	0.0089
0.0467	0.0472	0.0456	0.0409	0.0375	0.0295	0.0250	0.0195	0.2117	0.0120	0.0098	0.0104
0.0420	0.0380	0.0358	0.0375	0.0664	0.1114	0.5917	0.5949	0.6143	0.7129	0.0592	0.0620
0.0882	0.1116	0.0867	0.0838	0.1416	0.1442	0.6067	0.7722	0.6595	0.7025	0.0628	0.0635
0.0856	0.0835	0.0780	0.0837	0.1364	0.1435	0.5673	0.7456	0.7175	0.8143	0.0971	0.1005
0.1205	0.1212	0.1170	0.1159	0.1700	0.1862	0.5569	0.5705	0.7086	0.7409	0.1009	0.1128
0.8826	0.7237	0.8258	0.8259	0.8275	0.6880	0.8008	0.7636	0.8493	0.7542	0.1185	0.1205
0.7980	0.7359	0.8140	0.8125	0.8175	0.6261	0.8204	0.7415	0.7593	0.6278	0.1299	0.1212

**Absorbances for the determination of MIC of chlorhexidine in the presence of reserpine
for ATCC *P. aeruginosa***

Second reading

0.0769	0.0579	0.0574	0.0573	0.0567	0.0580	0.0569	0.0566	0.0565	0.2361	0.0587	0.0584
0.1012	0.0895	0.0852	0.0799	0.0751	0.0746	0.0674	0.0624	0.0579	0.0690	0.0588	0.0586
0.0760	0.0625	0.0602	0.0603	0.0594	0.6788	0.9517	1.0083	0.9959	0.9638	0.0660	0.0707
0.1078	0.0981	0.0973	0.0925	0.1177	0.7200	1.0595	1.0508	1.0503	1.0847	0.0592	0.0660
0.0923	0.1358	0.0617	0.0611	0.0619	0.7160	1.0327	1.0460	1.0164	0.9430	0.0582	0.0595
0.0768	0.0619	0.0605	0.0620	0.0619	0.6926	1.0234	1.0370	0.9200	1.0547	0.0581	0.0586
1.0724	1.0433	1.0598	1.0797	1.0527	1.0845	1.0647	1.0642	1.0723	1.0689	0.0578	0.0580
1.0588	1.1061	1.0858	1.0807	1.0789	0.9615	1.0469	1.0549	1.0577	1.0642	0.0574	0.0577

First reading

0.0886	0.0607	0.0580	0.0577	0.0567	0.0571	0.0568	0.0564	0.0565	0.0849	0.0587	0.0580
0.0909	0.0611	0.0542	0.0535	0.0544	0.0586	0.0583	0.0580	0.0571	0.0686	0.0589	0.0586
0.0971	0.0695	0.0617	0.0620	0.0615	0.0626	0.0585	0.0588	0.0580	0.0687	0.0582	0.0582
0.0978	0.0671	0.0630	0.0623	0.0630	0.0616	0.0619	0.0619	0.0627	0.0628	0.0589	0.0581
0.1121	0.0681	0.0614	0.0634	0.0624	0.0625	0.0619	0.0709	0.0620	0.0616	0.0578	0.0585
0.0973	0.0670	0.0615	0.0623	0.0621	0.0616	0.0619	0.0620	0.0614	0.0616	0.0580	0.0580
0.0616	0.0607	0.0616	0.0615	0.0630	0.0621	0.0620	0.0626	0.0626	0.0607	0.0574	0.0576
0.0602	0.0625	0.0613	0.0721	0.0618	0.0623	0.0613	0.0613	0.0604	0.0611	0.0577	0.0574

Second reading - First reading

-0.0117	-0.0028	-0.0006	-0.0004	0.0000	0.0009	0.0001	0.0002	0.0000	0.1512	0.0000	0.0004
0.0103	0.0284	0.0310	0.0264	0.0207	0.0160	0.0091	0.0044	0.0008	0.0004	-0.0001	0.0000
-0.0211	-0.0070	-0.0015	-0.0017	-0.0021	0.6162	0.8932	0.9495	0.9379	0.8951	0.0078	0.0125
0.0100	0.0310	0.0343	0.0302	0.0547	0.6584	0.9976	0.9889	0.9876	1.0219	0.0003	0.0079
-0.0198	0.0677	0.0003	-0.0023	-0.0005	0.6535	0.9708	0.9751	0.9544	0.8814	0.0004	0.0010
-0.0205	-0.0051	-0.0010	-0.0003	-0.0002	0.6310	0.9615	0.9750	0.8586	0.9931	0.0001	0.0006
1.0108	0.9826	0.9982	1.0182	0.9897	1.0224	1.0027	1.0016	1.0097	1.0082	0.0004	0.0004
0.9986	1.0436	1.0245	1.0086	1.0171	0.8992	0.9856	0.9936	0.9973	1.0031	-0.0003	0.0003

**Absorbances for the determination of MIC of chlorhexidine in the presence of CCCP
for ATCC *P. aeruginosa***

Second reading

0.0796	0.0582	0.0600	0.0560	0.0567	0.0573	0.0571	0.0575	0.0565	0.3754	0.0582	0.0584
0.0830	0.0596	0.0601	0.0574	0.0595	0.0591	0.0589	0.0725	0.0570	0.0576	0.0582	0.0586
0.0794	0.0618	0.1050	0.0594	0.0610	0.7063	0.9083	0.9494	1.0438	0.9596	0.0578	0.0589
0.0800	0.0632	0.0625	0.1125	0.0630	0.7270	0.8983	1.0651	1.0658	1.0565	0.0593	0.0587
0.0810	0.0615	0.0608	0.0608	0.0605	0.7240	0.9895	1.0601	1.0475	1.0321	0.0575	0.0585
0.0797	0.0978	0.0605	0.0608	0.0591	0.7042	0.8962	1.0328	1.0111	1.0426	0.0582	0.3274
0.9483	1.0436	0.9140	0.9132	0.9388	1.0692	0.9267	1.0754	1.0594	0.8997	0.0581	0.0580
1.0775	1.0817	1.0723	0.9454	0.9307	1.0646	1.0646	1.0638	0.9050	1.0095	0.0582	0.0593

First reading

0.0890	0.0614	0.0594	0.0568	0.0572	0.0572	0.0572	0.0570	0.0567	0.0579	0.0585	0.0581
0.0915	0.0634	0.0586	0.0574	0.0587	0.0586	0.0590	0.0737	0.0578	0.0581	0.0588	0.0585
0.0954	0.0694	0.0618	0.0625	0.0628	0.0631	0.0630	0.0623	0.0620	0.0665	0.0590	0.0611
0.0973	0.0695	0.0631	0.0618	0.0643	0.0624	0.0633	0.0625	0.0630	0.0629	0.0613	0.0589
0.0978	0.0686	0.0622	0.0652	0.0627	0.0628	0.0620	0.0618	0.0626	0.0622	0.0589	0.0587
0.0970	0.0682	0.0623	0.0632	0.0613	0.0619	0.0622	0.0628	0.0611	0.0625	0.0592	0.0580
0.0609	0.0617	0.0619	0.0614	0.0619	0.0618	0.0614	0.0627	0.0620	0.0621	0.0583	0.0589
0.0605	0.0616	0.0616	0.0608	0.0617	0.0620	0.0619	0.0613	0.0622	0.0617	0.0590	0.0595

Second reading - First reading

-0.0094	-0.0032	0.0006	-0.0008	-0.0005	0.0001	-0.0001	0.0005	-0.0002	0.3175	-0.0003	0.0003
-0.0085	-0.0038	0.0015	0.0000	0.0008	0.0005	-0.0001	-0.0012	-0.0008	-0.0005	-0.0006	0.0001
-0.0160	-0.0076	0.0432	-0.0031	-0.0018	0.6432	0.8453	0.8871	0.9818	0.8931	-0.0012	-0.0022
-0.0173	-0.0063	-0.0006	0.0507	-0.0013	0.6646	0.8350	1.0026	1.0028	0.9936	-0.0020	-0.0002
-0.0168	-0.0071	-0.0014	-0.0044	-0.0022	0.6612	0.9275	0.9983	0.9849	0.9699	-0.0014	-0.0002
-0.0173	0.0296	-0.0018	-0.0024	-0.0022	0.6423	0.8340	0.9700	0.9500	0.9801	-0.0010	0.2694
0.8874	0.9819	0.8521	0.8518	0.8769	1.0074	0.8653	1.0127	0.9974	0.8376	-0.0002	-0.0009
1.0170	1.0201	1.0107	0.8846	0.8690	1.0026	1.0027	1.0025	0.8428	0.9478	-0.0008	-0.0002

**Absorbances for the determination of MIC of chlorhexidine in the presence of CCCP
for clinical *P. aeruginosa***

Second reading

0.0814	0.0598	0.0585	0.0581	0.0581	0.0590	0.0583	0.0585	0.0580	0.0586	0.0595	0.0593
0.0965	0.0828	0.0808	0.0760	0.0719	0.0718	0.0613	0.0577	0.0580	0.1984	0.0597	0.0594
0.1241	0.1078	0.0757	0.0751	0.1242	0.5432	0.6334	0.6427	0.6876	0.6023	0.0667	0.0668
0.1055	0.0979	0.0972	0.0912	0.1285	0.5098	0.6439	0.6606	0.7320	0.6734	0.0598	0.1883
0.0970	0.1197	0.0765	0.1162	0.1158	0.5249	0.6522	0.6853	0.6598	0.6416	0.0611	0.0606
0.0963	0.0779	0.0749	0.0741	0.1935	0.5743	0.6324	0.5947	0.5611	0.5595	0.0592	0.0596
0.5711	0.5978	0.6608	0.6436	0.5674	0.5642	0.6731	0.7042	0.5523	0.5496	0.0592	0.0596
0.5727	0.5785	0.6112	0.6591	0.5839	0.5728	0.6423	0.6371	0.6335	0.6081	0.0592	0.0588

First reading

0.0876	0.0624	0.0586	0.0582	0.0576	0.0570	0.0573	0.0578	0.0569	0.0650	0.0587	0.0585
0.0936	0.0631	0.0591	0.0579	0.0588	0.0577	0.0586	0.0582	0.0575	0.0581	0.0588	0.0591
0.1122	0.0852	0.0803	0.0787	0.0791	0.0802	0.0824	0.0796	0.0797	0.0798	0.0590	0.0586
0.1117	0.0837	0.0808	0.0796	0.0801	0.0789	0.0802	0.0805	0.0797	0.0805	0.0593	0.0584
0.1129	0.0841	0.0787	0.0806	0.0786	0.0801	0.0783	0.0788	0.0794	0.0788	0.0595	0.0627
0.1110	0.0827	0.0790	0.0795	0.0795	0.0796	0.0795	0.0782	0.0777	0.0783	0.0583	0.0588
0.0715	0.0774	0.0782	0.0773	0.0777	0.0793	0.0782	0.0782	0.0772	0.0773	0.0582	0.0577
0.0719	0.0777	0.0779	0.0764	0.0776	0.0787	0.0846	0.0842	0.0831	0.0822	0.0578	0.0578

Second reading - First reading

-0.0062	-0.0026	-0.0001	-0.0001	0.0005	0.0020	0.0010	0.0007	0.0011	-0.0064	0.0008	0.0008
0.0029	0.0197	0.0217	0.0181	0.0131	0.0141	0.0027	-0.0005	0.0005	0.1403	0.0009	0.0003
0.0119	0.0226	-0.0046	-0.0036	0.0451	0.4630	0.5510	0.5631	0.6079	0.5225	0.0077	0.0082
-0.0062	0.0142	0.0164	0.0116	0.0484	0.4309	0.5637	0.5801	0.6523	0.5929	0.0005	0.1299
-0.0159	0.0356	-0.0022	0.0356	0.0372	0.4448	0.5739	0.6065	0.5804	0.5628	0.0016	-0.0021
-0.0147	-0.0048	-0.0041	-0.0054	0.1140	0.4947	0.5529	0.5165	0.4834	0.4812	0.0009	0.0008
0.4996	0.5204	0.5826	0.5663	0.4897	0.4849	0.5949	0.6260	0.4751	0.4723	0.0010	0.0019
0.5008	0.5008	0.5333	0.5827	0.5063	0.4941	0.5577	0.5529	0.5504	0.5259	0.0014	0.0010

