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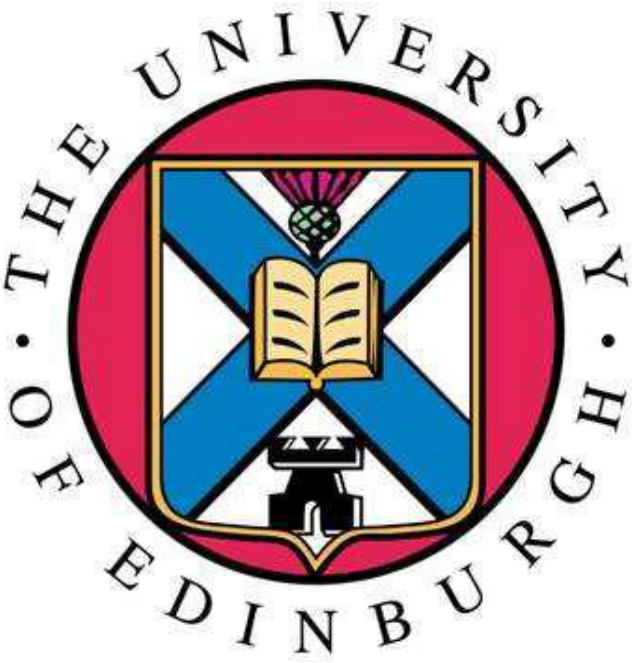
Susceptibility and bactericidal activity of five biocides on *Klebsiella pneumoniae* and its association with efflux pump genes and antibiotic resistance

Abdulmonem Ali Abuzaid



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ABSTRACT

Klebsiella pneumoniae is one of the top eight pathogens in hospitals, causing around 10% of hospital-acquired infections (nosocomial infections). It often produces extended-spectrum β-lactamase enzymes (ESBLs). This has led to numerous outbreaks, especially in intensive care, neonatal and surgical wards, associated with increases in morbidity and mortality. In order to reduce the number of infections caused by multi-resistant *K. pneumoniae* and improve standards of infection control within hospitals, there is extensive use of biocides as disinfectants and antiseptics. However this raises concerns that intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not just to themselves but also to antibiotics.

The reduced susceptibility to biocides and their relationship with resistance to antibiotics was assessed in this thesis. The susceptibility of 64 isolates of *K. pneumoniae* to five biocides preparations, Chlorhexidine (CHX), Benzalkonium chloride (BZK), Trigene, MediHex-4 (MH-4), Mediscrub (MS) and 17 antibiotics, were tested. The isolates of *K. pneumoniae* were collected from Royal Infirmary Hospital in Edinburgh (RIE) between 2006 and 2008 from different sites of infection. Antimicrobial susceptibility was tested by the agar double dilution method (DDM) and disc diffusion methods following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines.

A few isolates of *K. pneumoniae* showed insusceptibility to cephalosporins, colistin, rifampicin, trimethoprim and penicillin but not to carbapenems. Biocide susceptibility testing showed that 57, 55 and 61 strains had reduced susceptibility to Chlorhexidine, Trigene and Benzalkonium chloride, respectively, but not to MediHex-4 and Mediscrub. The effect of efflux pumps were determined by carbonyl cyanide m-chlorophenylhydrazone (CCCP) (10mg/L), which decreased the MICs of Chlorhexidine and Medihex-4 by 2 – 128 fold but had no impact on the MICs of Benzalkonium chloride, Trigene and Mediscrub.

Six isolates of *K. pneumoniae* were chosen for their varying sensitivity to Chlorhexidine (CHX), and were tested for their minimum bactericidal concentration (MBC) to biocides. The high MBCs of Mediscrub and Trigene, over 500-fold greater than the minimum inhibitory concentration (MICs), indicates that these compounds are mainly bacteriostatic. Conversely, the MBCs of Chlorhexidine and MediHex-4, which contains chlorhexidine, were less than 10-times the MIC value indicating they are effective in killing the organism. However, this thesis showed how the killing capability of Chlorhexidine was hindered by the presence of organic matter, which compromised its effect.

The relationship between reduced susceptibility to biocides and the carriage of antiseptic resistance genes, *cep*A, *qac*ΔE1 and *qac*E was determined by polymerase chain reaction. The antiseptic resistance genes *cep*A, *qac*ΔE1 and *qac*E were found in 56, 34 and 1 isolates respectively, and the levels of gene

expression were detected by the reverse transcription polymerase chain reaction (RT-PCR).

These results have shown that there was a close link between carriage of efflux pump genes, *cep*A, *qac*ΔE1 and *qac*E genes and reduced susceptibility to biocides. Most strains showed decreased susceptibility to Chlorhexidine, Trigene and Benzalkonium chloride and this correlated with the carriage of the *cep*A, *qac*ΔE1 and *qac*E genes encoding efflux. There was no correlation between the reduced susceptibility to biocides and antibiotic resistance in clinical isolates of *K. pneumoniae*.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Dedication

I dedicate this thesis to:

My parents, the source of love, kindness and support.

My wife, Amal, for her care, love and encouragement.

My children, Abdullah and Sara who have enriched my life.

Acknowledgement

First, I would like to express my sincerest gratitude to my university supervisors Professor Sebastian Amyes and Dr. Cathy Doherty, A very special thanks goes out to Professor Amyes, thank you for your help in improving my work, constructive comments and guiding me in my research. I appreciate his continuing support of me throughout my thesis with his patience, knowledge and kindness, as well as his academic experience. This study would not have been possible without his motivation and encouragement.

I am extremely grateful to Mr Malcolm Baldock for the processing of orders, I would also like to thank all the colleagues in Molecular Chemotherapy, they offered me guidance, support, encouragement and feedback on my work, and they created a great working atmosphere.

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Finally, I would like to warmly thank my parents for the support they provided me through the years. I must acknowledge my wonderful family. My wife, Amal and my children, Abdullah and Sara, thank you for the care, encouragement and unconditional support without you surrounded me throughout my study, I would not have finished this thesis.

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Publications and presentations

I. Abuzaid, A. Hamouda, S.G.B. Amyes. Assessment of *Klebsiella pneumoniae* susceptibility to biocides and its association with *cep*A, *qac*ΔE1 and *qac*E efflux pump genes and antibiotic resistance. Presented at the Federation of Infection Societies Conference 2011, Manchester (16th – 18th November) Abstract no. 17

II. Abuzaid, A. Hamouda, S.G.B. Amyes. Assessment of *Klebsiella pneumoniae* susceptibility to biocides and its association with *cep*A, *qac*ΔE1 and *qac*E efflux pump genes and antibiotic resistance. Presented at 6th workshop in Antibiotic Research Mechanisms 2011, Birmingham (24th – 25th November) Abstract no. 1

III. Abuzaid A, Hamouda A, Amyes S.G.B. *Klebsiella pneumoniae* susceptibility to biocides and its association with *cep*A, *qac*ΔE1 and *qac*E efflux pump genes and antibiotic resistance. Journal of Hospital Infection 2012; 81; 87 – 91.

IV. Abuzaid A, Hamouda A, Amyes S.G.B. Bactericidal activity of five antiseptics on *Klebsiella pneumoniae* and its relationship to the presence of efflux pump genes and influence of organic matter. Journal of Chemotherapy 2012; 24; 297 – 299.

Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Bloodstream infection
BZK	Benzalkonium chloride disinfectant
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CDC	Center for Disease Control
cep	cation efflux pump
cfu	Colony forming units
CHX	Chlorhexidine disinfectant
CLSI	Clinical and Laboratory Standards Institute
cm	Centi-metres
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPI	Efflux pump inhibitor
ESBL	Extended-spectrum β-lactamase
FIC	Fractional inhibitory concentration
g	Gram
G+C	Percentage of DNA consisting of guanine and cytosine bases
HAI	Hospital acquired infection
HPA	Health Protection Agency
ICU	Intensive care unit
IST	Iso-sensitest
K1	*Klebsiella pneumoniae* serotype *K1*

K2	*Klebsiella pneumoniae* serotype *K2*
kb	Kilo-base pairs
kDa	Kilo-Dalton
KPC	*Klebsiella pneumoniae* carbapenemase
L	Litre
LPS	Lipopolysaccharide
M	Molar
mA	Milli-amps
MATE	Multidrug and toxic compound extrusion
MBC	Minimum Bactericidal Concentrations
MDR	Multidrug resistant
MFS	Major facilitator superfamily
mg	Milligram
MH-4	MediHex-4 disinfectant
MIC	Minimum inhibitory concentration
MIC50	Minimum inhibitory concentration for 50% of strains
MIC90	Minimum inhibitory concentration for 90% of strains
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	Messenger ribonucleic acid
MRSA	*Methicillin-resistant Staphylococcus aureus*
MS	Mediscrub disinfectant
NaCl	Sodium chloride
NCBI	National Center of Biotechnology Information
ng	Nanogram
NHS	National Health Service
nm	Nanometre
NSS	Normal Saline Solution
OD	Optical density

ORF	Open reading frame
PABN	Phenyl alanine arginyl β-naphtylamide
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	potential Hydrogen, measure of acidity or basicity of a solution
pmol	Pico-moles
psi	pounds per square inch
qac	quaternary ammonium compounds
RIE	Royal Infirmary Edinburgh
RNA	Ribonucleic acid
RND	Resistance nodulation division
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SEM	Scanning Electron Micrograph
SMR	Small multi-drug resistance
Spp.	Species
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBE	Tris/borate/ethylenediaminetetraacetic acid
TE	Tris-EDTA
tRNA	Transfer-ribonucleic acid
TSA	Tryptone Soy Agar
U	Units
V	Volts
W	Watts
w/v	Weight by volume
µg	Micro-gram
µL	Micro-litre
µM	Micro-molar

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Introduction

Introduction of *Klebsiella pneumoniae*

The *Klebsiella* genus

The genus *Klebsiella*, a member of the *Enterobacteriaceae* family, *Klebsiella* was named after the German Microbiologist Edwin Klebs, who first described the bacterium in the 19th century (Podschun and Ullmann, 1998, Vincent, 2004, Brisse *et al*., 2006)

The *Klebsiella* genus comprises of the following species: *K. pneumoniae, K. oxytoca, K. rhinoscleromatis, K. ozaenae, K. aerogenes, K. planticola, K. mobilis, K. terrigena, K. trevisanii* and *K. ornithinolytica. Klebsiella* spp. are ubiquitous in our environment, commonly found in water, soil, sewage, drinking water, industrial waste and plants as commensal bacteria (Casewell and Phillips, 1977, Bagley, 1985, Vincent, 2004, Brisse *et al*., 2009). They are identified and differentiated according to their biochemical reactions (Bagley, 1985, Vincent, 2004, Dworkin *et al*., 2006, Otman *et al*., 2007).

General characteristics

Klebsiella spp. are Gram-negative, rod-shaped, lactose fermenting, oxidase negative and non-spore forming bacteria (Figure 1). They are facultative anaerobic bacteria and non-motile (no flagella), although they possess pili/fimbriae for adhesion of the bacteria to host cell (Figure 2)(Podschun and Ullmann, 1998).

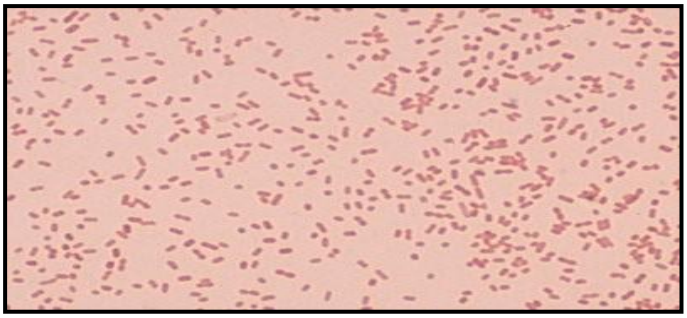


Figure 1. Microscopy, *K. pneumoniae* is Gram-negative, non-motile, encapsulated and rod-shaped. (adapted from http://first6weeks.blogspot.co.uk/) (Last accessed 25 January 2008).

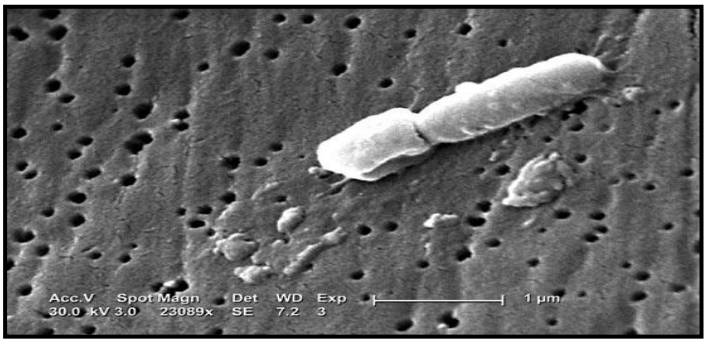


Figure 2. Scanning electron micrograph of *K. pneumoniae*. (adapted from Centers for Disease Control and Prevention (CDC)/Janice Carr http://www.cdc.gov/) (Last accessed 31 August 2011).

K. pneumoniae is lactose-fermenting, it appears as pink colonies on MacConkey agar, forming large moist mucoid colonies (Figure 3) (Bruce *et al*., 1981, Vincent, 2004).

Figure 3. *K. pneumoniae* on MacConkey agar plate is mucous and lactose positive colonies. (adapted from Wikimedia Commons http://commons.wikimedia.org/wiki/File:Klebsiella_pneumoniae_mucoid.jpg) (Last accessed 25 December 2011).

Pathogenicity factors

Klebsiella spp. has many characteristics that promote their virulence. *Klebsiella* spp. express two types of antigens on their cell surface, capsular antigen and lipopolysaccharide antigen. Both antigens play an important role in classification, epidemiology and pathogenicity of *Klebsiella* spp. (Figure 4) (Podschun and Ullmann, 1998).

I. Capsular Antigen

The capsular polysaccharide antigen (CPS) is one of the most important virulence factors. CPS antigen is a polysaccharide with 77 serotypes that promotes adherence and protects *K. pneumoniae* from phagocytosis and bacterial serum factors (Podschun and Ullmann, 1998, Chuang *et al*., 2006).

II. Lipopolysaccharide Antigen

The Lipopolysaccharide antigen (LPS) consists of three components; the O antigen, the core oligosaccharide and lipid A. LPS has 8 serogroups and serves to protect *K. pneumoniae* from complement mediated killing (Podschun and Ullmann, 1998).

III. Adhesins

K. pneumoniae attaches and adheres to host cell by pili (fimbriae) in the early stages of infection. *K. pneumoniae* express two types of pili; type 1 pili which are the most common whilst type 3 pili are less common. There are three new types of *Klebsiella* adhesins that have been recently reported, CF29K adhesin, Aggregative adhesin and KPF-28 (Podschun and Ullmann, 1998, Brisse *et al*., 2006).

IV. Siderophores, Iron is an essential factor for bacterial growth in host and *K. pneumoniae* secure their supply of iron by siderophores (Podschun and Ullmann, 1998).

V. Serum Resistance (Podschun and Ullmann, 1998).

VI. Cytotoxins, enterotoxins and haemolysin proteins (Podschun and Ullmann 1998; Brisse, Grimont *et al*. 2006).

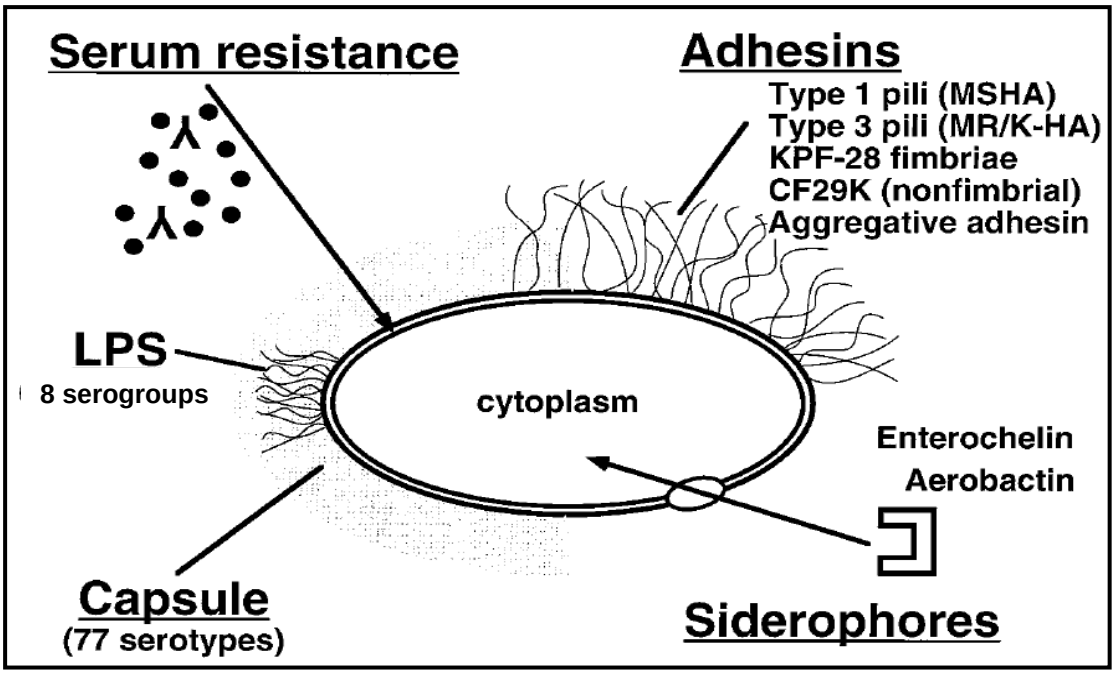


Figure 4. Representative diagram of the pathogenicity factors found in *K. pneumoniae* (Podschun and Ullmann, 1998).

Pathogenesis

K. pneumoniae can colonise the skin, mouth, throat, pharynx and gastrointestinal tract of humans (Bagley, 1985, Vincent, 2004), *K. pneumoniae* is a common opportunistic pathogen for humans and animals; however, under certain circumstances it can cause diarrhoea by enterotoxin release and serious infections, including urinary tract infections, respiratory infections, soft tissue infections, septicaemia, liver abscesses and bacterial meningitis.

Recently about 8% of hospital-acquired infections including hospital-acquired pneumonia are caused by *K. pneumoniae*, this figure indicates that *K. pneumoniae* is a significant nosocomial pathogen. It is generally a real pathogen although it also infects immunocompromised patients due underlying health problems e.g. diabetes mellitus, chronic pulmonary obstruction and malnourished alcoholics or those with invasive medical devices and those hospitalised for long term care (Jarvis *et al*., 1985, Ko *et al*., 2002, Gupta, 2002, Brisse *et al*., 2009).

Epidemiology

During the last decade, the incidence of hospital-acquired infection caused by multidrug resistant strains has increased alarmingly (Reacher *et al*., 2000). As a contribution to this, the spread of multidrug resistant strains of *K. pneumoniae* is a worldwide problem and has impacted on the ability to deal with nosocomial infection. The incidence of hospital acquired infections has escalated and it is estimated that now 10% of all hospital acquired infections (HAI) are caused by *K. pneumoniae* (Vincent, 2004, Struve and Krogfelt, 2004). It has been reported that approximately 7% of nosocomial infections in the United States, Canada and Europe (Sahly and Podschun, 1997) are caused by this organism. Recently it is estimated that 9% of in-patients hospital in England and Wales have a hospital acquired infection (HAI). As result there are 300,000 infected patients, 5000 deaths and cost National Health Service over £1 billion per year (NationalAuditOffice, 2004). *K. pneumoniae* is now among the top eight pathogens in hospitals (Vincent, 2004). The spread of multiresistant *K. pneumoniae* has lead to numerous outbreaks and has increased the morbidity and mortality rate (Jarvis *et al*., 1985, Jacoby and Medeiros, 1991, Gupta, 2002, Vincent, 2004, Marra *et al*., 2006, Otman *et al*., 2007, Khanfar *et al*., 2009).

K. pneumoniae has the ability to adapt to the hospital environment and survives longer than enteric bacteria on hands and on environmental surfaces compared to other. This ability has coincided with inadequate infection control procedures that facilitates cross-infection within hospitals which combined has allowed *K. pneumoniae* to emerge as an important hospital pathogen (Paterson and Bonomo, 2005). As a result, the rate of *Klebsiella* carrier in hospitalized patients have increased markedly, 77% in the stool, 19% in the pharynx, and 42% on the hands of patients (Podschun and Ullmann, 1998).

In the hospital environment HAIs can be transmitted through different routes including medical tools, equipments and contaminated hands. Hand contamination is the most common mode of transmission of *K. pneumoniae* between hospital workers and patients (Casewell and Phillips, 1977). On the other hand, frequent handwash with chlorhexidine and quaternary ammonium compounds (QACs) recorded a significant reduction of 98 – 100% in the number of patients infected with *K. pneumoniae* (Casewell and Phillips, 1977, Girou *et al*., 2002, Paterson and Bonomo, 2005).

Introduction of antibiotics

Antibiotics are defined as natural substances produced by microorganisms that can kill or inhibit growth of other organisms. The accepted view now is that all selective antibacterials are classified as antibiotics and this definition will be used here.

The history of antibiotics can be divided in two periods as shown below:

Early history

Antibiotic use began in ancient times, prior to the 19th century, when people observed how to use natural treatment to control infections based on traditional medicine. Ancient Sumerian used beer soup mixed with snake skins and turtle shells to treat wounds and infections of patients, while Babylonian doctors used frog bile and sour milk as ointment to heal eye infections. In addition, more than 3000 years ago, Egyptians, Chinese, Greeks and Indians used moulds, honey and herbs in medicine as healing agents to treat infected wounds, although they did not understand the connection between natural substances as anti-infection agents and the treatment of diseases (reviewed by Florey *et al*., 1949).

Many books have been written about antibiotics by a wide range of people in the world from different cultures and in several languages, including Roman, Greek, Persian, Turkish, Arabic and Hebrew, which discussed how to control infections caused microorganisms by using natural sources available during the years (reviewed by Florey *et al*., 1949).

Modern history

The beginning of the modern history of antibiotics can be traced from the 19th to early 20th centuries. The modern era of antibiotics began in the late 1800s, when the germ theory of disease came to prominence, which linked the microorganisms as the causation agent to a variety of diseases. As a result, European scientists began to devote their time to searching for an effective medication that would eradicate diseases and their causation agents. Finally, at the beginning of the 20th century, scientists discovered the natural antibiotics produced by microorganisms and made progress in this area, several treatments for infections were introduced (Table1) (reviewed by Neu and Gootz, 1996, Freter and Perry.M.C., 2008).

Table1. Summarized modern history of antibiotics

Years	Name of discoverer	Name of discovery
Early 20th century	Paul Ehrlich	pioneers "chemotherapy"
1929	Alexander Fleming	demonstrates inhibition of bacterial growth with penicillin moulds
Early 1930s	Gerhard Domagk	discovers "prontosil" a prodrug to sulphanilamide
1935	Gerhard Domagk	Sulfanilamide, the first synthetic sulfonamide in human medicine
1942	Florey and Chain	First therapeutic use of penicillin
1944	Selman Waksman	Streptomycin
1947	David Gottlieb	Chloramphenicol, the first broad-spectrum antibiotic
1948	Benjamin Dugger	Chlortetracycline
1960	Giuseppe Brotzu	Cephalosporins
1962	George Lesher	discovers nalidixic acid during chloroquine synthesis
1970s	George Lesher	New 4-quinolones (pipemidic acid, oxolinic acid, cinoxacin)
1980	Kyorin Seiyaku K.K.	Norfloxacin, the first fluoroquinolone
1980	Grohe and Peterson	Enrofloxacin synthesized
1990s	Pharmacia Company	Linezolid

The table adapted from baytril.com.auhttp://baytril.com.au/Product/History.aspx (Last accessed 05 July 2010).

The first pioneer in this area was Paul Ehrlich, a German medical scientist, who first studied the interaction of different substances with microbial infections, and this the treatment of the infectious disease became known as chemotherapy (reviewed by Neu and Gootz, 1996). In 1877 Louis Pasteur, a French chemist and Robert Koch a German physician, observed that bacteria can cause many diseases and bacteria can kill other bacteria. In 1905 Robert Koch was awarded the Nobel Prize in Medicine for his efforts in tuberculosis research (Figure 5) (reviewed by Neu and Gootz, 1996).

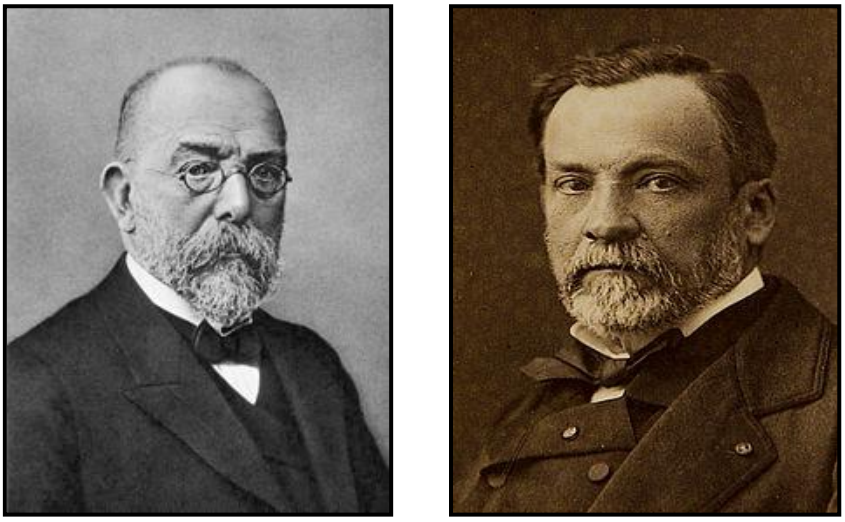


Figure 5. Robert Koch (1843 – 1910) and Louis Pasteur (1822 – 1895). All images are credited to Wikipedia http://en.wikipedia.org (Last accessed 25 February 2013).

In 1935, a German bacteriologist, Gerhard Domagk (1895 – 1964), introduced sulfonamides (Prontosil) as the first *in vivo* treatment against bacterial infections in commercial use. In 1939, Domagk received the Nobel Prize in Physiology and Medicine for his findings (Figure 6) (reviewed by Neu and Gootz, 1996)

Figure 6. Gerhard Domagk (1895 – 1964). The image is credited to official website of Nobel Prize http://www.nobelprize.org (Last accessed 02 March 2013).

The great promise of antibiotics in their modern history was first demonstrated in 1929 when Sir Alexander Fleming (1881 – 1955), a Scottish bacteriologist, while working at St. Mary's Hospital in London, discovered penicillin accidently. Fleming noticed that a mould called *Penicillium notatum* inhibited the growth of the bacterium *Staphylococcus aureus*. He found that a clear zone had been developed around the mould indicating that penicillin moulds had produced a toxic substance that diffused through the agar and inhibited the growth of bacteria *in vitro*.

This lead to the hypothesis that penicillin can produce small molecular weight antibacterial chemicals, which could kill bacteria inside the body (Figure 7) (Rolinson, 1998, Bennett and Chung, 2001, Goldsworthy and McFarlane, 2002, Geddes, 2008).

Figure 7. Fleming's original agar "*Staph*" plate with large mass of mould on left the produced penicillin. While numerous bacterial colonies grew at the edge of plate, a clear zone of inhibition of bacterial growth surrounds the mould colony where penicillin has released into the medium. (adapted from Accelr8.com http://www.accelr8.com/antibiotic_resistance.php) (Last accessed 27 February 2013).

In 1942 Howard Florey (1898 – 1968) and Ernst Chain (1906 – 1979), who headed a team of researchers at the University of Oxford, demonstrated the bactericidal action of penicillin inside the human body. Additionally, they succeeded in purifying a large quantity of penicillin from *Penicillium notatum*. This was the first antibiotic available for commercial and clinical use. This was the first step towards the modern antibiotics. During World War II, penicillin was used widely against many types of bacteria and had saved millions of lives, showing itself to be safe and effective antibiotic (Goldsworthy and McFarlane, 2002). In 1945, Alexander Fleming shared the Nobel Prize in Physiology and Medicine with Howard Florey and Ernst Chain for their work on penicillin (Figure 8)(Goldsworthy and McFarlane, 2002).

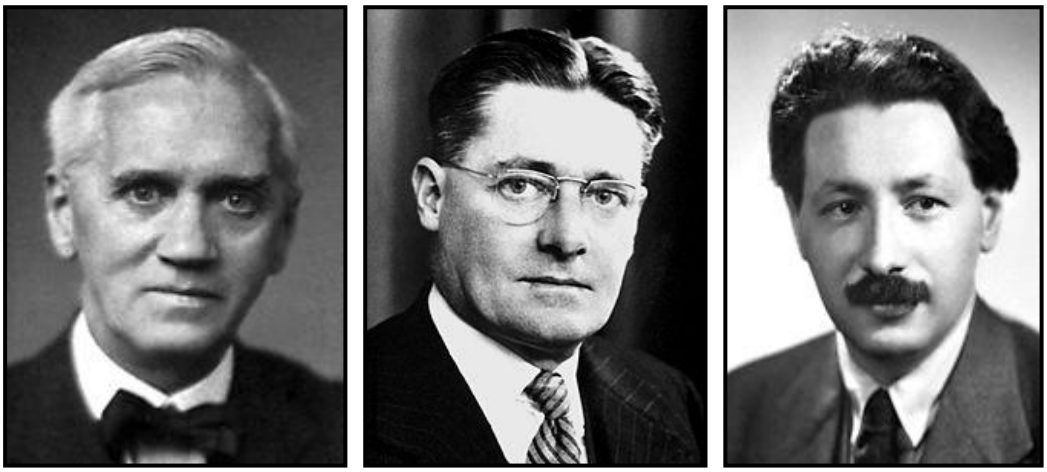


Figure 8.

Sir Alexander Fleming (1881 – 1955) Sir Howard Florey (1898 – 1968) Sir Ernst Chain (1906 – 1979)

All images are credited to official website of Nobel Prize http://www.nobelprize.org (Last accessed 02 March 2013).

Shortly afterwards, the first of the aminoglycoside class of antibiotics was discovered and developed. In 1944, Selman Waksman (1888 – 1973), an American microbiologist, discovered streptomycin from the soil bacteria *Streptomyces griseus*. Streptomycin was the first antibiotic used to treat tuberculosis (Kingston, 2004). Five years later, in 1949, Waksman and his coworkers discovered a new aminoglycoside antibiotic called neomycin, a bactericidal antibiotic used commonly for skin infections. In 1952 he was received the Nobel Prize in Physiology and Medicine for his discovery of streptomycin and several other antibiotics (Figure 9)(Kingston, 2004).

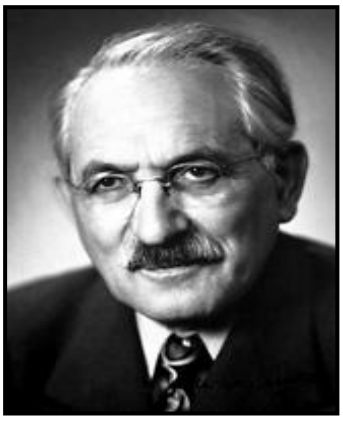


Figure 9. Selman Waksman (1888 – 1973). The image is credited to official website of Nobel Prize http://www.nobelprize.org (Last accessed 02 March 2013).

Since penicillin, the first β-lactam antibiotic, was launched for commercial use in 1942, many new classes of antibiotics have been discovered in the 1950s and 1960s, such as aminoglycoside, chloramphenicol, tetracycline, chlortetracycline, erythromycin, vancomycin, rifampicin and cephalosporines (Table 1) (Rolinson, 1998, Kingston, 2004).

Classification of antibiotics

Fundamentally, antibiotics are classified based on several characteristics; such as

I. Laboratory behaviour or Lethality

   a. Bactericidal antibiotics that have the ability to kill bacteria.
   b. Bacteriostatic antibiotics that preventing bacterial growth.

II. Spectrum of activity

   a. Broad spectrum antibiotics affect a wide range of bacteria.
   b. Narrow-spectrum antibiotics that active against a limited range of bacteria.

III. Mechanism of action

   Antibiotics are commonly classified based on their mechanism of action.

Mechanism of action

There are five main antibiotics targets on the bacteria: (Table 2 and Figure 11) (reviewed by Amyes, 2010).

I. This group antibiotics interfere with the cell wall synthesis

A group of antibiotics contains β-lactam ring, prevents cell wall production by blocking the cross linking of the polysaccharide chains to a new cell wall subunits, causing bacterial cell death.

II. This group antibiotics inhibit the protein synthesis

Disrupts protein synthesis by binding to the subunit of the bacterial ribosomes (30S or 50S subunit).

III. This group antibiotics interfere with the nucleic acid (DNA) synthesis

Interferes with DNA transcription and replication by targeting DNA gyrase and topoisomerase IV.

IV. This group antibiotics interfere the ribonucleic acid (RNA) synthesis

Disrupts RNA transcription by acting on DNA-directed RNA polymerase, which is necessary to make proteins.

V. This group antibiotics inhibit the synthesis of essential metabolites enzymes

Inhibits biosynthesis enzymes by disruption tetrahydrofolate syntheses pathway (tetahydrofolic acid). This prevents folic acid synthesis which is a compound essential for DNA synthesis.

VI. This group antibiotics disrupt of bacterial membrane structure bacterial

Disrupts bacterial membrane structure by binding to lipopolysaccharide (LPS) in the outer membrane of bacteria (polymyxins, daptomycin).

Table 2. The antibiotic classes and their mechanisms of action (reviewed by Amyes, 2010, Findlay, 2011).

Mechanism of Action	Antibiotic Class/Sub-Class	Bacteriostatic/ Bactericidal	Examples
Inhibition of cell wall synthesis	β-lactams-Carbacephems	Bactericidal	Loracarbacef
	β-lactams-Carbapenems		Ertapenem, Meropenem
	β-lactams-Cephalosporins-1stGen		Cefazolin, Cefalexin
	β-lactams-Cephalosporins-2ndGen		Cefoxitin, Cefuroxime
	β-lactams-Cephalosporins-3rdGen		Cefotaxime, Cefpodoxime
	β-lactams-Cephalosporins-4thGen		Cefepime
	β-lactams-Cephalosporins-5thGen		Ceftobiprole
	β-lactams-Monobactams		Aztreonam
	β-lactams-Penicillins		Amoxicillin, Cloxacillin
	Fosfomycin		Fosfomycin
	Glycopeptides		Teicoplanin, Vancomycin
	Lipopeptides		Daptomycin
	Polypeptides		Bacitracin, Colistin

Inhibition of protein synthesis	Aminoglycosides Chloramphenicols Glycylcyclines, Lincomycins Macrolides Oxazolidinones Tetracyclines	Bactericidal Bacteriostatic Bacteriostatic Bacteriostatic Bacteriostatic Bacteriostatic Bacteriostatic	Amikacin, Gentamicin Chloramphenicol, Thiamphenicol Tigecycline Clindamycin, Lincomycin Azithromycin, Erythromycin Linezolid Minocycline, Tetracycline
Inhibition of DNA synthesis	Fluoroquinolones Metronidazole Quinolones	Bactericidal Bactericidal Bactericidal	Ciprofloxacin, Norfloxacin Metronidazole Nalidixic acid
Inhibition of RNA synthesis	Rifampicin	Bactericidal	Rifampicin
Inhibition of tetrahydrofolate synthesis	Diaminopyrim idines Sulphonamides	Bacteriostatic Bacteriostatic	Iclaprim, Trimethoprim Sulphadiazine, Sulphamethoxazole

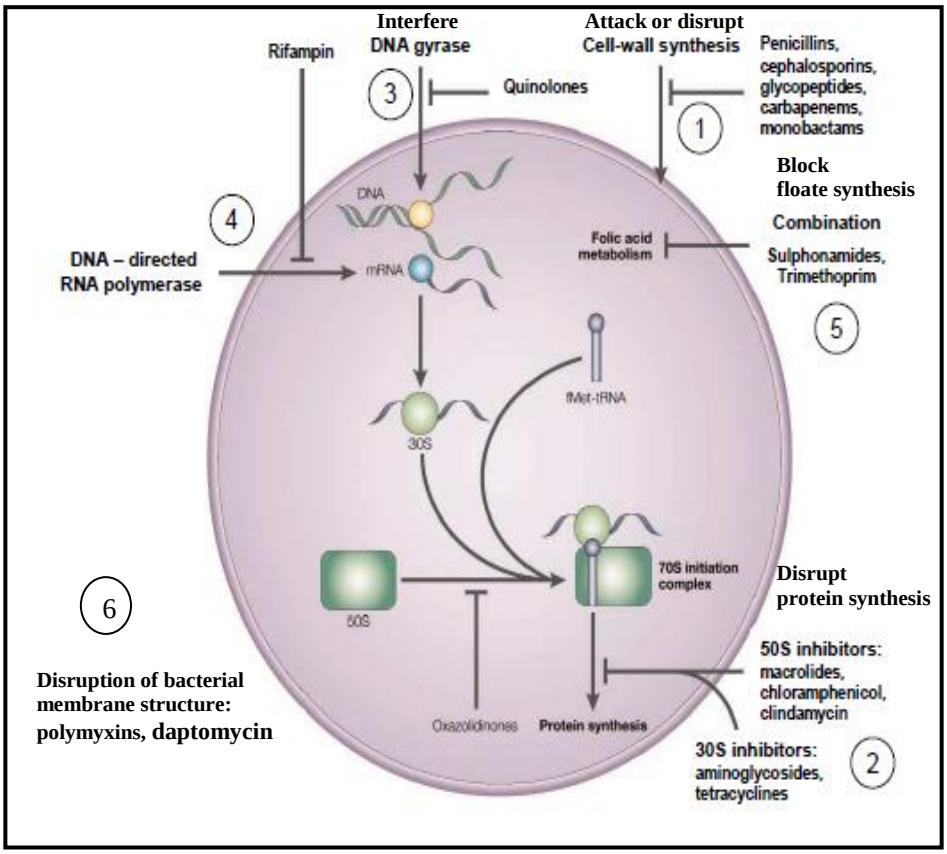


Figure 10. The five main antibiotic targets on the bacteria cell (Coates *et al.*, 2002).

Antibiotic resistance

The term antibiotic resistance usually refers to a change in susceptibility of an organism that no longer responds to treatment (Russell, 1999, Poole, 2002). This is typically noted as an increase in the minimum concentration of antibiotics required to inhibit bacterial growth. Antibiotic resistance can be considered one of the greatest challenge facing hospitals. In the US there are over 2 million nosocomial infections each year and about half of these are caused by resistant strains (Jones, 2001).

Basically, bacteria have developed several ways to defend themselves against antibiotics as a means to survive resulting in the evolution and spread of bacterial resistance. Numerous and various mechanisms of antibiotic resistance have been reported for example, mutation, acquisition of genes via conjugation, reduced permeability, active efflux, inactive of the antibiotic via enzymatic destruction and alternation of antibiotic target (Mazel and Davies, 1999).

Extended spectrum β-lactamases

Originally, β-lactam antibiotics were frequently prescribed to treat these infections caused by *K. pneumonia.* These comprised mainly cephalosporins because this species is usually not considered clinically sensitive to aminopenicillins such as ampicillin and amoxicillin, despite the results of in *vitro* sensitivity tests. Extensive and imprudent use of antibiotics in clinical treatment may have been responsible for an increased prevalence of resistance ultimately leading to development of multidrug resistance. This has been most acutely manifested by the emergence of a group of enzymes called extended spectrum β-lactamases (ESBLs) as well as chromosomal mutation (Podschun and Ullmann, 1998).

The first ESBL was detected in Germany in 1983 (Knothe *et al*., 1983). ESBLs have been detected in a wide variety of Gram-negative bacteria. ESBLs genes are usually encoded on plasmids and classified into TEM, SHV, OXA and CTX-M. All but OXA enzymes are class A β-lactamases, OXAs belong to class D. *K. pneumoniae* continues to be an important ESBLs producer (Livermore, 1995, Philippon *et al*., 2002, Canton *et al*., 2003, Schneiders *et al*., 2003, Poole, 2004, Paterson and Bonomo, 2005).

β-lactamases are able to break and hydrolyze the carbon-nitrogen bond of the β-lactam ring and deactivate these antibiotics as the planar β-lactam ring is no longer functional (Figure 11)(Livermore, 1995, Philippon *et al*., 2002, Poole, 2004, Paterson and Bonomo, 2005).

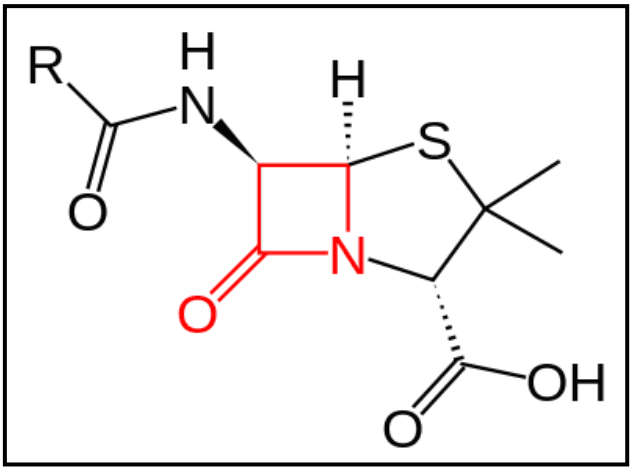


Figure 11. The core chemical structure of penicillin with β-lactam ring in red at the centre.

The incidence of bacterial ESBL varies worldwide with low rates (3 – 8%) reported in Sweden, Japan and Singapore, compared to higher rates reported in Portugal (34%), Italy (37%), New York (44%), Latin American countries (30 – 60%) and Turkey (58%)(Khanfar *et al*., 2009). In the Gulf Cooperation Council (GCC) countries, ESBL rates range from 8.5 – 38.5% in the Kingdom of Saudi Arabia, 31.7% in Kuwait and the highest level in United Arab Emirates with 41%.(Khanfar *et al*., 2009).

Antibiotic resistance mechanisms

The six main mechanisms that bacteria use to resist the action of antibiotics are presented in (Figure12) (reviewed by Russell, 2003, Amyes, 2010).

I. Enzymatic modification or inactivation. Enzymes can destroy or modify antibiotic so the antibiotic will not be able to attack the target site. Resistance occurs by natural, mutational chromosomal genes or acquisition of extrachromosomal genetic elements (plasmids or transposons), for instance, β-lactamase enzymes destroys the β-lactam ring (Livermore, 1995, reviewed by Amyes, 2010).

II. Impermeability features. Bacteria can reduce the susceptibility of the antibiotic agent by reducing the membrane permeability; for example, outer membrane proteins may act as channels that control antibiotic entry into the bacterial cell (reviewed by Russell, 2003, Amyes, 2010).

III. Acquisition of active efflux pump genes is a common mode of resistance. The presence these pumps may be lead to Multiple Drug Resistance (MDR). Multidrug-resistance efflux pumps families have become a great challenge in hospitals (Poole, 2002, Piddock, 2006b). These systems can be classified into five superfamilies based on the energy source used to export their substrates and the sequence of amino acid including (Table 3 and Figure 12).

1. The ATP binding cassette superfamily (ABC).
2. The multidrug and toxic compound extrusion family (MATE).
3. The major facilitator superfamily (MFS).
4. The resistance nodulation cell division superfamily (RND).
5. The small multidrug resistance family (SMR).

The ABC superfamily is primary transporters, it utilize ATP as sources of energy for extrusion of antibiotics and toxic substances from the cell. The other families represent secondary transporters is energized by protons or sodium ions (Zechini and Versace, 2009).

The presence and activity of an efflux pump can be demonstrated by efflux pump inhibitor (EPI) *in vitro* such as cyanide m- chlorophenyl hydrazone (CCCP), phenylalanine arginyl b β-naphthylamide (PAbN) and reserpine (Garvey and Piddock, 2008).

Table 3. A summary of the bacterial efflux pump families (Findlay *et al*., 2012).

Family/ Superfamily	Gram +ve/-ve	Example	Energy Source	TMS	Primary/ Secondary	References
ABC	+ve/-ve	LmrA, *L. lactis*	ATP	12	P	Bambeke *et al*, 2000.
MATE	-ve	YdhE, *E. coli*	PMF	12	S	Borges-Walmsley *et al*, 2003.
MFS	+ve/-ve	NorA, *S. aureus*	PMF	12 or 14	S	Yin *et al*, 2000.
RND	-ve	AcrAB, *E. coli*	PMF	12	S	Yang *et al*, 2003.
SMR	+ve/-ve	QacC, *S. aureus*	PMF	4	S	Leelaporn *et al*, 1994.

PMF – proton motive force, TMS – transmembrane segments

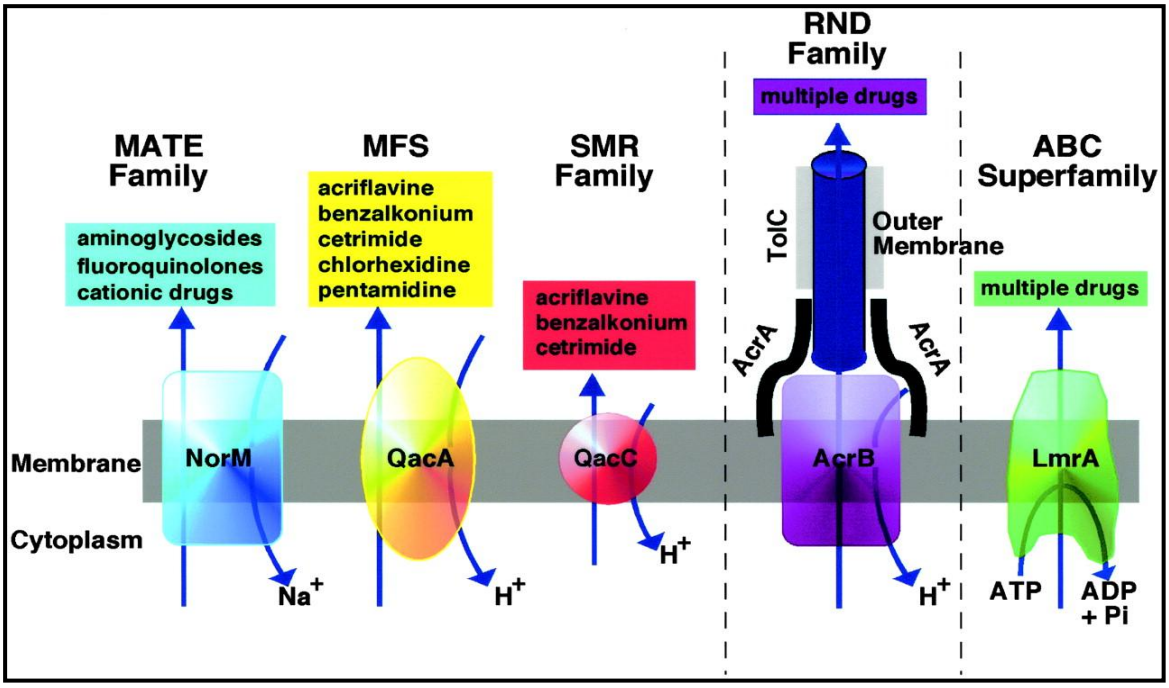


Figure 12. The five families of Multidrug-resistance efflux pumps (Piddock, 2006b).

IV. Alteration of cellular target sites such as enzyme. Ribosome and cell wall is modified by acquiring a plasmid, transposons or mutation so that there is no longer a recognition site for the antibiotic. The consequence of these changes reduce the susceptibility to antibiotics, thus promote resistance (reviewed by Russell, 2003, Amyes, 2010).

V. The inhibited steps can be by-passed. Bacteria can be developed an alternative metabolites pathway that may or may not disable the metabolites target for antibiotic and an additional target makes is less sensitive to the binding of the antibiotic. For example alternative pathway for folic acid synthesis (Amyes and Smith, 1974, reviewed by Russell, 2003, Amyes, 2010).

VI. Overproduction. The target can be produced in larger quantities than normal by the bacteria thus hyperproduction of the target will mop-up the antibiotic (reviewed by Russell, 2003, Amyes, 2010).

VII. Target protection.

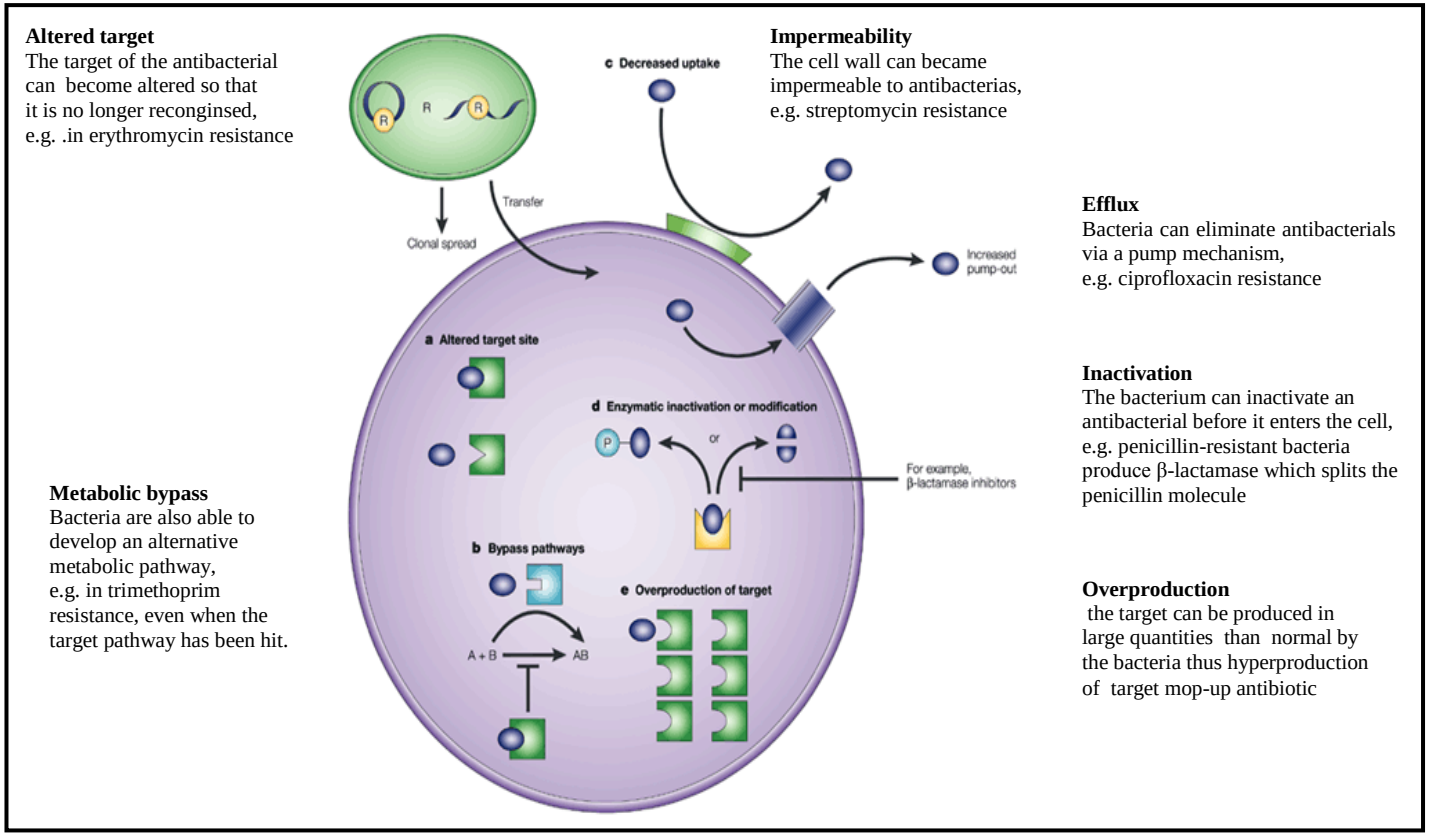


Figure 13. Resistance mechanisms of bacteria to antibiotics (Coates *et al*., 2002) and reproduced with permission from the controller of her Majesty's Stationary office.

Introduction of biocides

Biocide is a general term describing a chemical agent that has a broad spectrum activity, which is capable to inactivative or kill microorganisms (McDonnell and Russell, 1999). Biocides include disinfectants, antiseptics and preservatives (Russell, 1999).

Disinfectant**:** This is a chemical agent that are applied on nonliving objects (e.g., floors, walls, sinks) to destroy microorganisms. Disinfectants are normally too toxic for human use and they are frequently used in food industry, veterinary application and clinical environments such as hospitals, dental surgeries to control the spread of infectious organisms (McDonnell and Russell, 1999).

Antiseptic: This is a chemical agent that is used on skin or living tissue for inhibiting or destroying microorganisms. Antiseptics are less-toxic agents and commonly used for skin cleaning, handwashing, on wound surfaces and as surgical scrubs to eliminate a number of microorganisms. Examples of antiseptics include alcohol, chlorhexidine, chloroxylenol (PCMX), hydrogen peroxide, iodine compounds, triclosan and quaternary ammonium compounds such as benzalkonium chloride, cetrimide (McDonnell and Russell, 1999).

Preservative: This is a compound that is often added to a medicine or food, which has previously been sterilised, to prevent microbial growth once the container is opened (McDonnell and Russell, 1999).

Ideal properties of an antiseptic

1. Broad antimicrobial spectrum.
2. Bactericidal action.
3. Longer antibacterial effects.
4. Easy to use.
5. Least adverse effects.
6. Efficiency in the presence of organic matter (Cecilio *et al*., 1983).

As a part of infection control strategies various types of disinfectants and antiseptics are utilized widely in hospital settings to reduce the incidence of nosocomial infections.

Biocides, usage and mode of action

The modes of action of biocides are less well known compared with antibiotics; however there are similarities between the two. The uses and the mechanisms of action of most common biocides, i.e. list them here, are provided in Table 4. These are the most frequent biocides used in local hospital, although there are many other types of biocides available.

Table 4. Some types of clinical biocide agents with their contents, main targets and use (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Biocides family	Biocides	Main contents	Mode of action	Used
Biguanide compound	Chlorhexidine digluconate 20% in H_2O solution	1% Chlorhexidine	Cationic compound (+)charged interact with outer membrane (-) charged, penetrates into cell, leakages intracellular and cell death	Antiseptic, disinfectant, pharmaceutical preservative
Biguanide compound	MediHex-4 solution	4% Chlorhexidine (manufacture)		Hand cleaning, disinfectant
Biguanide compound	Trigene advance solution	1% Polymeric biguanide (manufacture)		Swimming pool, disinfection, contact lens solutions
Quaternary Ammonium Compounds (QACs)	Benzalkonium chloride	1% Benzalkonium chloride		Skin disinfectant, preoperative antiseptic, disinfectant, pharmaceutical preservative
Bis-phenol	Mediscrub solution	1% Triclosan (manufacture)	Inhibition fatty acid synthesis, increases cell membranes permeability and destroy cell	Body washes, dental hygiene

Chlorhexidine

Chlorhexidine is a biguanide compound, cationic antiseptic and active agent against various types of bacteria. It has been used widely in Europe and United Kingdom hospitals (Fraise, 2002b) .

Chlorhexidine Digluconate 20% in H_2O

Chlorhexidine digluconate 20% in H_2O solution belongs to the biguanide family, it has been used as an antiseptic and disinfectant, It is broad-spectrum antimicrobial, bactericidal, virucidal and fungicidal (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Use

Chlorhexidine is mainly used for handwashing, skin cleansing, surgical scrub, antiplaque (oral rinses and mouthwash) and a preservative agent (Figure14) (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Mechanism of action

Chlorhexidine is cationic and reacts with the anionic microbial cell surface, thus disrupting the cell membrane. Subsequently, chlorhexidine penetrates into the cell and causes leakages of intracellular constituents leading to cell death (Figure15) (McDonnell and Russell, 1999). Chlorhexidine is an effective biocide for Gram-positive bacteria because they are more negatively-charged and have a single membrane; therefore Gram-positive bacteria are more sensitive to this agent (Russell, 1986, Kuyyakanond and Quesnel, 1992).

Figure14. Chlorhexidine Digluconate 20% in H_2O.

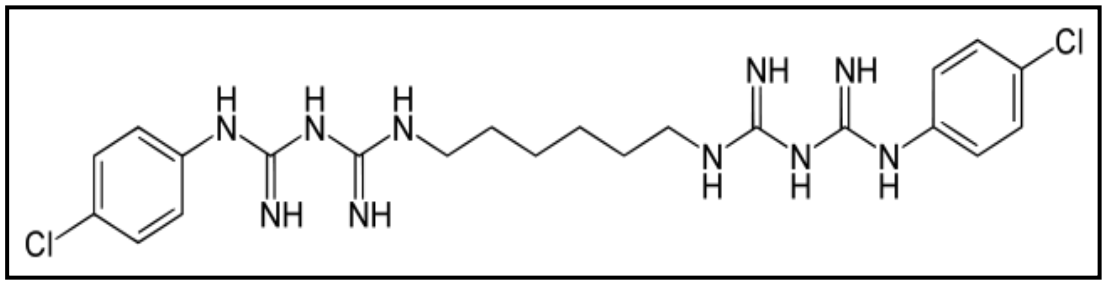


Figure 15. Chemical structure of Chlorhexidine.

MediHex-4 solution

The main ingredient of MediHex-4 solution is 4% of chlorhexidine; it is active against a wide variety of bacteria, fungi and viruses (Medichem International).

Use

MediHex-4 solution applies for hand cleaning and disinfection of the whole skin area, it is semi viscose liquid, red colour (Figure 16) (Medichem International).

Mode of action

MediHex-4 solution has the same mode of action of Chlorhexidine digluconate 20% in H_2O, because it contains 4% chlorhexidine. Positive charged has the ability to interact with the negatively charged of cell walls of bacteria and which increases permeability of membrane thus causing the death of the bacteria (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Figure 16. MediHex-4 solution. (adapted from Medichem International).

Trigene advance solution

Trigene advanced solution, is a biguanide compound containing <1% polymeric biguanide hydrochloride and <1% didecyl dimethyl ammonium chloride and alkyl dimethyl benzyl ammonium chloride. Trigene is broad-spectrum disinfectant, bactericidal, sporicidal, mycobactericidal, virucidal and fungicidal (Medichem International).

Use

Trigene is appropriate for all surfaces , furnitures, equipments and also can be used for all medical areas in hospitals, clinics and dental clinics, Trigene solution is a blue colour liquid (Figure17) (McDonnell and Russell, 1999, reviewed by Russell, 2003) (Medichem International).

Mode of action

Trigene is based on the active ingredients of polymeric biguanide as cationic compounds and it is carried rapidly through the cell walls of bacteria, causing interruption of, and penetration through the bacterial outer membrane, which reduces its permeability and causing death to the cells (McDonnell and Russell, 1999, reviewed by Russell, 2003) (Medichem International).

Figure17. Trigene advance solution. (adapted from Medichem International).

Triclosan

Triclosan is a bis-phenol broad-spectrum disinfectant which is very active against a wide range of pathogenic organism (Fraise, 2002b). It is utilized commonly in hospital as detergents, surgical scrub, soap and hand gels. It is a key agent for disinfecting used against Gram-positive bacteria (Jones *et al*., 2000, Levy, 2001, Russell, 2004).

Triclosan is made more effective by adding ethylenediamine tetraacetic acid (EDTA), which increases the permeability of the outer membrane of bacteria (McDonnell and Russell, 1999, (McDonnell and Russell, 1999, reviewed by Russell, 2003). It should be noted that the EDTA has an antibacterial effect of its own.

Mediscrub solution

Mediscrub solution contains 1% triclosan together with anionic and amphoteric surfactants. It is broad-spectrum antiseptic and effective against a wide variety of bacteria, viruses and fungi (Medichem International).

Use

Mediscrub is suitable for handwashing and cleansing. Mediscrub solution is a high viscosity liquid (sticky) with pink colour (Figure 18) (McDonnell and Russell, 1999, reviewed by Russell, 2003) (Medichem International).

Mode of action

Mediscrub contains triclosan inhibiting fatty acid synthesis by binding to the enoyl-acyl carrier protein reductase enzyme (ENR), which is an essential enzyme for biosynthetic pathway fatty acid in bacteria. Consequently; the bacteria are unable to synthesis fatty acid, which is necessary for building cell membranes and for reproduction. As a consequence the outer membrane of bacteria impairs and increases the permeability. It also has an influence on the cytoplasmic membrane and destroys the cell effectively (figure 19) (Heath *et al*., 1999, McDonnell and Russell, 1999, Jones *et al*., 2000, reviewed by Russell, 2003, Russell, 2004) (Medichem International).

Figure 18. Mediscrub solutions. (adapted from Medichem International).

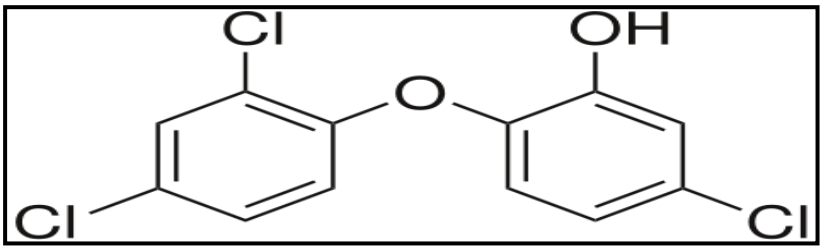


Figure 19. Chemical structures of Triclosan.

Quaternary Ammonium Compounds (QACs)

Quaternary Ammonium Compounds (QACs) are surface-active agents, cationic with very good activity against various types of clinical pathogenic organisms, non corrosive and low toxic properties. Quaternary ammonium compounds (QAC) are widely used as antiseptics and disinfectants in both medical and food environments.

Benzalkonium chloride

Benzalkonium chloride, a cationic surface-acting agent belonging to the quaternary ammonium compound group, is mainly active against Gram-positive bacteria, and it is the most useful disinfectant in hospitals (McDonnell and Russell, 1999, Fraise, 2002b) .

Use

Benzalkonium chloride is usually utilized in wide range of applications because it is an efficient and safe agent. It is used as antiseptics and disinfectants for skin, hand sanitizers, cleansing wounds and hard surfaces (Figure 20) (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Mode of action

Benzalkonium chloride is a cationic compound, which interacts with the inner membrane of bacteria mainly as it is negatively charged; then the agent penetrates into the cell wall, where it reacts with the cytoplasmic membrane and disrupts the integrity of the cytoplasmic membrane causing leakage of the intracellular content of cell and death (Figure 21) (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Figure 20. Benzalkonium chloride.

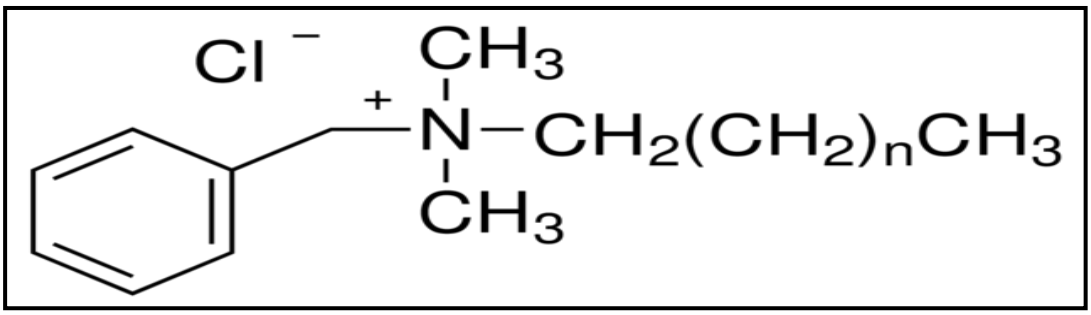


Figure 21. Chemical structure of Benzalkonium chloride.

Bacterial resistance to biocides

The term resistance is used to describe bacterial insusceptibility to antimicrobial agents. According to Russell, (1999) the term “biocide resistance” is usually used to define a bacterial strain surviving exposure to a biocide concentration which kill the rest of the bacterial population.

Biocides are used extensively in modern society as a part of sanitization, they are commonly used in hospitals to decontaminate skin, hands, wounds and surfaces, particularly in operation room as part of infection control to reduce and manage hospital acquired infection. However, there has been increased biocide use such as Chlorhexidine, Triclosan and Benzalkonium chloride for various ranges of applications e.g. skin antiseptics, hand sanitizers and surfaces cleaners. Several of these compounds are available for the general market, outside the direct medical field, and this widespread use had raised some concern on the development of bacterial resistance to biocides.

The first biocide resistance was recognized 70 years ago in *Salmonella typhi* (Fraise, 2002a). Biocides resistance is less common than antibiotics resistance due to most of biocides having multiple modes action against microbes (Poole, 2002, Fraise, 2002b). Recently, there is increasing evidence that indicate the development of biocides resistance has become a significant risk and there are numerous reports concerning the problem (Fraise, 2002b, Maillard, 2007).

Factors affecting biocide activity

Biocide activity is influenced by several factors: concentration, exposure time, pH, temperature, environments, presence of organic matter, interfering compound and the nature, number, location, and condition of the microorganism (Russell, 2002, reviewed by Russell, 2003).

I. **Concentration,** is a crucially important factor in assessing the effect of biocide activity. Concentrations lower than the recommended one have little bactericidal activity, which probably enables organisms to obtain some degree of resistance to an individual biocide (Stickler, 2002, reviewed by Russell, 2003). In hospitals topping up of biocides solution, such as QACs and Chlorhexidine, leads to diluting the concentrated biocide and to incorrect use of biocides, all of which will reduce biocides susceptibility.

II. **Contact time**, exposure of clinical isolates repeatedly and regularly to insufficient periods of time of biocide that bacteria could gradually become less insusceptible to many biocides over a long period of time.

III. **pH**, Many biocides have an optimum pH range. Glutaraldehyde and cationic biocides as chlorhexidine and QACs are active at alkaline pH, whereas hypochlorites and phenolics are most potent at acid pH.

IV. **Interaction with organic matter,** such as blood serum, pus and dirt can adversely affect the efficacy of many biocides.

V. **Environment of biocide residues**, where bacteria could develop low-level of insusceptibility.

VI. **Condition**, phenotypic adaptation is one of the mechanisms of resistance for the bacteria to survive under constantly changing and stressful condition.

It is assumed that biocides insusceptibility may be increasing. Furthermore, it is anticipated that biocides resistance will emerge on a major scale at some point in future (reviewed by Russell, 2003).

Biocides resistance mechanisms

Biocide resistance can occur as a natural resistance of an organism to a biocide agent (Intrinsic) or acquired resistance (reviewed by Russell, 2003).

Intrinsic Resistance mechanisms

Intrinsic resistance is the innate ability of bacteria to resist the activity of a particular biocide agent. Intrinsic resistance is dependent on the natural insusceptibility or natural characteristic of the bacteria, which is demonstrated by a Gram-negative bacteria, spores, mycobacterium and *S. aureus* (reviewed by Neu and Gootz, 1996, Russell, 2003). Some bacteria can display intrinsic resistance through inactivation of biocides, alteration of the target site. Intrinsic resistance is usually chromosomally encoded (Gilbert and McBain, 2003). Intrinsic resistance can be:

I. **Impermeability**, the outer membrane is very important factor in intrinsic resistance (Poole, 2002). The structure of the outer membrane of the cell may act as permeability barrier to reduce biocides entry to the bacterial cell, and change in this structure can include alteration of: surface hydrophobicity, outer membrane ultrastructures, outer membrane protein composition and outer membrane fatty acid structure (Figure 13) (McDonnell and Russell, 1999, reviewed by Russell, 2003). This type of intrinsic resistance is often demonstrated in Gram-negative bacteria.

II. **Inactivation**, some bacteria display resistance to QACs and chlorhexidine at lower concentration. Consistent exposure of sub-inhibitory concentrations of biocides may induce biocides degrading enzymes (McDonnell and Russell, 1999, reviewed by Russell, 2003).

Acquired Resistance mechanisms

Acquired insusceptibility may arise by altering the target sites, mutation, acquisition genetic elements (transposons or plasmid)(Russell, 2001).

I. **Overproduction of target**, this does occur with triclosan where there is overproduction of enoyl-acyl carrier protein reductase enzyme (ENR) (McDonnell and Russell, 1999, reviewed by Russell, 2003).

II. **Alteration or modification of the target sites**, biocides usually affect multiple cellular components (Poole, 2002); for example, triclosan resistance in *Escherichia coli*, is by modification by mutation of the enoyl-acyl carrier protein reductase enzyme. The active target site for triclosan, which leads to failure of triclosan when it is used to treat *E. coli* (McDonnell and Russell, 1999, Levy, 2001, Poole, 2002, reviewed by Russell, 2003, Maillard, 2005).

III. **Acquired plasmid mediated resistance** is mainly associated with mercury compound and metallic salts (McDonnell and Russell, 1999). Resistance plasmids are more associated with antibiotic resistance than biocides resistance (Russell, 1997, Gilbert and McBain, 2003). Plasmid mediated resistances are not common in biocide insusceptibility; however, a direct association between Gram-negative bacteria plasmids and biocide resistance have been linked to efflux mechanisms (Gilbert and McBain, 2003). Plasmids carrying efflux pump genes are the main resistance mechanism to cationic biocides such as chlorhexidine and QACs.

IV. **Acquired resistance** may also occur through efflux pumps, which pump out biocides faster than they can enter (Levy, 2001). The efflux pump is also known as the 'vomit response' allowing bacteria to remove toxic compound from cell (reviewed by Russell, 2003) and respond to stressful changes in the environment (Gilbert and McBain, 2003). Efflux pumps are located in the cell wall of bacteria and remove biocides from the cell, often lowering susceptibility to chlorhexidine and QACs (Suller and Russell, 1999). It has proven to be an important survival strategy for most of bacteria (Gilbert and McBain, 2003).

The efflux system acts as a transport for many antibiotics and biocides. Interestingly, efflux systems are able to accommodate biocides including QACs (Benzalkonium chloride) and Chlorhexidine in Gram-negative and Gram-positive bacteria (Kazama *et al*., 1998, Poole, 2002).

Efflux pump genes can be carried and transmitted via plasmids in both Gram-negative and Gram-positive bacteria (Gilbert and McBain, 2003, Fraise, 2002b). Therefore, there is a possibility of cross linking between biocide resistance genes and antibiotics resistance genes. It is also theoretically possible that the efflux mechanisms involved in the low level resistance to Chlorhexidine (Russell, 1997), could also confer resistance to antibiotics (Fraise, 2002b). QACs resistance genes are classified into two main families; (a) the *qac*A/B genes, which are members of the multifacilitator superfamily, and (b) *qac*C/D, *qac*E, and *qac*EΔ1 genes, which belong to the small multidrug resistance family (Paulsen *et al*., 1996).

The multidrug resistance *qac*E gene was initially identified on the *Klebsiella aerogenes* plasmid R751, where it is located on an integron (Paulsen *et al*., 1996, Kazama *et al*., 1998). The *qac*E and *qac*EΔ1 (disrupted form of *qac*E) are located either on plasmids or on Class I integrons (Paulsen *et al*., 1996).

*qac*EΔ1 gene is distributed widely through Gram-negative bacteria because it is often located on the ubiquitous Class I integron and the spread of this integron may have been facilitated by the use of quaternary ammonium compounds (Kazama *et al*., 1998, Kucken *et al*., 2000, Schneiders *et al*., 2008). The *cep*A efflux gene has been detected on one occasion in a clinical isolate of *K. pneumoniae*, where is believed to contribute to resistance to chlorhexidine (Poole, 2005). No further identification of this gene was made before the start of this thesis.

These efflux genes are of concern because they are active against biocides commonly used in hospitals. They are able to cause resistance to a broad range of biocides so the spread of biocides resistance genes could occur rapidly (Fraise, 2002b). It has been speculated that these genes could well be associated with antibiotic resistance (reviewed by Russell, 2003, Poole, 2005).

Similarity of bacterial biocides and antibiotics resistance

Bacterial biocides and antibiotics may show similarities in their resistance to mechanisms (Russell, 2002, reviewed by Russell, 2003). Nevertheless, there are major differences in their mechanisms of bacterial resistance. Enzymatic degradation (inactivation), efflux pumps and modification of target sites are responsible for the resistance to both antibiotics and biocides (Russell, 2002, reviewed by Russell, 2003). A common mechanism of resistance, particularly as a first response, for biocides and antibiotics is an efflux pump, with enzymatic degradation and target site mutations being less common (Poole, 2002, reviewed by Russell, 2003). As the efflux pumps are a common resistance mechanism to biocides and antibiotics, they may also be responsible for the cross resistance between them. This could result in the inadvertent selection of antibiotic resistant mutants during disinfection, potentially causing an outbreak in hospitals (Figure 22) (Poole, 2002, reviewed by Russell, 2003).

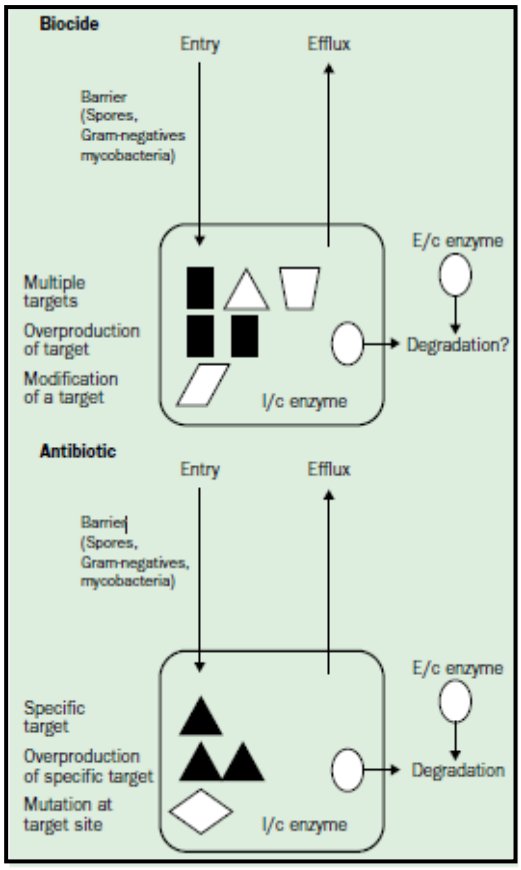


Figure 22. Mechanisms of bacterial insusceptibility to biocides and antibiotics shows similarities (e.g. permeability barrier, efflux) and differences (e.g. single or multiple targets) (reviewed by Russell, 2003).

Aim of this thesis

I. To assess the susceptibility of 64 *K. pneumonia* isolates against five biocides: 1% Chlorhexidine, 1% Benzalkonium chloride, Trigene, MediHex-4, Mediscrub; and to 17 antibiotics against 64 isolates of *K. pneumoniae*.

II. To determine the relationship between the reduced susceptibility to biocides, the carriage of antiseptic resistance genes, *cep*A, *qac*ΔE1, *qac*E and antibiotics resistance in *K. pneumoniae.*

III. To assess the bactericidal activity of five biocides on *K. pneumoniae,* both under ideal conditions and in the presence of organic matter and with the association of efflux pump genes that have been linked to antiseptic resistance (*cep*A, *qac*ΔE1 and *qac*E).

Methods and Materials

Bacterial strains

The standard laboratory strains *E. coli* NCTC 1048, *Pseudomonas aeruginosa* NCTC 10662, *S. aureus* NCTC 6571 and *Acinetobacter baumannii* ATCC 19606 were used as control strains for the determination of MICs. *A. baumannii* ATCC 19606, *K. pneumoniae* ATTC 13883, *K*1, *K*2, *Methicillin-resistant Staphylococcus aureus MRSA*-15 and *MRSA*-16 were used as control strains for *cep*A, *qac*ΔE1 and *qac*E antiseptic resistance genes. Sixty-four isolates of *K. pneumoniae* were obtained from Royal Infirmary Hospital in Edinburgh between 2006 and 2008 from different sites of infection.

All strains were subcultured on MacConkey agar (Oxoid, Basinstoke, UK) and incubated for 18 hours at 37°C. Two to three colonies were inoculated into Iso-Sensitest (IST)(Oxoid, Basinstoke, UK) broth and incubated overnight at 37°C/180rpm in an orbital shaker. The next day 900µL of these IST broths of overnight shaken culture was added to 100µL sterile glycerol and was stored at -70°C in a 2mL eppendorf tube for long-term storage at -80°C. The API 20 E strips were used for the identification of *K. pneumonia* according to the manufacturer's instructions of BioMerieux (Basingstoke, UK), Briefly, colonies (between 1 – 4) taken from an MacConkey agar plate incubated overnight, were suspended into a 2mL ampoule of API NaCl 0.85 % medium, the saline suspension was distributed into the microtubules and about 5mL of distilled water was distributed into the bottom of the tray to create a humid atmosphere. The strip box was incubated at

29°C ± 2°C for 24 hours (± 2 hours). After the incubation period, the strip was interpreted by referring to the Reference Table.

Media, Buffers and Reagents

All media were supplied by Oxoid (Basingstoke, UK) in powdered form. The broths and agars were dissolved with distilled water and autoclaved at 121°C/15 pounds per square inch (psi) for 15 minutes according to the manufacturer's instructions. Agar was cooled to 50ºC before being poured into sterile petri dishes (Sterilin Ltd, Staffordshire, UK) and allowed to solidify into plates to be used immediately or stored at 4ºC.

All chemicals and buffers were purchased from the Sigma-Aldrich (Poole, UK) unless stated otherwise. Normal Saline Solution (NSS), agarose, Tris base and MOPS were obtained from Fisher Scientific (Leicestershire, UK). Normal saline (0.85%) was prepared by dissolving 0.85g of sodium chloride in 100mL of distilled water and sterilized by autoclaving.

Antimicrobial Agents

Antibiotics

The antibiotic agents used in this study were purchased from suppliers as sterile powders as listed below: Polymyxin B, Cefotaxime, Cefoxitin, Colistin, Chloramphenicol, Gentamicin, Rifampicin and Trimethoprim (Sigma, Poole, UK), Ceftazidime (GlaxoSmithKline, Brentford, UK), Imipenem (Merck Sharp and Dohme, Huddleston, UK) and Meropenem (AstraZeneca, Luton UK). The antibiotics were stored in the dark at 4ºC.

All breakpoints values of antibiotic agents were taken from the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for Antimicrobial Susceptibility Testing version 11.1 (Andrews, 2012) with the following exceptions; cefotaxime and trimethoprim (> 2mg/L); colistin, polymyxin-B and gentamicin (> 4mg/L); ceftazidime, cefoxitin, imipenem, meropenem and chloramphenicol (> 8mg/L) and rifampicin (> 16mg/L).

Biocides

The common hospital biocides 1% Chlorhexidine gluconate, a member of biguanide family, 1% Benzalkonium chloride and quaternary ammonium compounds (QAC) were supplied from Sigma (Poole, UK). The commercial biocide preparations Trigene, a mixture of the QAC alkyl dimethyl benzyl ammonium chloride and didecyl ammonium chloride, the cationic biocide MediHex-4, containing 4% chlorhexidine gluconate and Mediscrub, containing 1% triclosan were purchased from Medichem International (Kent, UK) (Smith and Hunter, 2008).

Antibiotic Discs

All antibiotic discs were obtained from Mast Laboratories Ltd (Liverpool, UK) at the contents listed: amikacin (AK) 30µg, ampicillin (AMP) 25µg, aztreonam (ATM) 30µg, carbenicillin (CAR) 100µg, cefotetan (CCT) 30µg, apramycin (APR) 15µg, doxycycline (DO) 30µg, netilmicin (NET) 30µg, piperacillin/ tazobactam (PRL) 75µg, tigecycline (TCG) 15µg, erythromycin (E) 30µg and novobiocin (NV) 30µg. All plates were incubated at 37ºC for 18 hours following inoculation.

Minimum Inhibitory Concentrations (MICs)

A minimum inhibitory concentration is the lowest concentration of the antimicrobial agent that completely inhibits growth of bacteria. MICs were determined by the agar double dilution agar method, and were carried out using Iso-Sensitest (IST) plates containing the particular concentrations of antimicrobial agents following the guidelines of BSAC (Andrews, 2006).

A single colony was suspended in 5mL of IST broth and grown overnight at 37°C in an orbital shaker. Antibiotic powders were weighed and dissolved in an appropriate solvent according to the BSAC (Andrews, 2006). Water was added to ensure that the required concentration of antibiotic stock solution was reached. The rest of the antibiotic concentrations were made from this stock solution. The bacterial inocula were diluted in 0.85% normal saline solution (NSS) to approximately 10^7 cfu/mL, which is equivalent to a 0.5 McFarland standard following BSAC guidelines (Andrews, 2012) by adding 0.5mL of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2$. $2H_2O$) to 99.5mL of 0.18M H_2SO_4. The acceptable absorbance range for the standard is 0.08 – 0.13 at 625 nanometre (nm).

The inocula were always used within the prescribed 30 minute time-frame. An A400 multipoint inoculator (Denley, Surrey, UK), was used to inoculate a 1µL volume of suspension onto the surface of the agar to give a final inoculum of approximately 10^4 cfu/spot. Plates were allowed to dry and were incubated overnight at 35 – 37ºC. The MIC range of antibiotic tested was usually 0.008 – 128mg/L. A plate without antibiotic was used as a positive control. The positive

control plate and the MICs of the standard laboratory strains were used to monitor the quality of the tests.

Disc sensitivity testing

Disc sensitivity testing was complied with the BSAC guidelines (Andrews, 2012). Disc sensitivity testing was performed on IST agar, an overnight IST broth culture was diluted in 10µL sterile 0.85% normal saline solution (NSS) to match 0.5 McFarlane as turbidity standard, a sterile cotton swab was dipped into the suspension and removal of the excess liquid was made by pressing against the side of the test tube, swab the inoculum over the surface of IST agar plate was made in three directions. Antibiotic discs have been applied on the surface agar plate within 15 minutes of inoculation and the plates were incubated in air at 37°C for 18 hours. Disc sensitivity testing was interpreted as sensitive, intermediate or resistant according to the BSAC guidelines (Andrews, 2012).

Minimum Bactericidal Concentrations (MBCs)

The minimum bactericidal concentration (MBC) was defined as the lowest concentration of the biocide that was able to reduce the viable count by 3 $logs_{10}$; in other words, to kill 99.9% of the original bacteria in a given time or complete suppression of bacterial growth (Andrews, 2006, Kawamura-Sato *et al*., 2008, Smith *et al*., 2008, Smith and Hunter, 2008). The evaluation of basic bactericidal activity of five biocides against *K. pneumoniae* was conducted according to the European standard test method EN 1040 guidelines for measuring the reduction in the number of bacteria. MBC testing was carried out on Tryptone Soya Agar (TSA) (Kawamura-Sato *et al*., 2008),

The overnight broth culture was adjusted to an optical density (OD) 0.08 at 660nm (~ $\times 10^8$ cfu/mL) in a spectrophotometer (Camspec M330). An overnight bacterial suspension (100µL of a 10^8 cfu/mL suspension) was added to 900µL biocides and the mixture was left for exactly 3 minutes at 20 – 22°C. After that, 100µL of mixture was transferred to 900µL of neutralizer solution which consisted of 10% Tween 80, 0.1% histidine and 0.5% sodium thiosulphate from Sigma (Poole, UK), 3%lecithin soybean (MP Biomedicals, LLC, Cambridge, UK) and Phosphate Buffer Solution (PBS pH 7.4) (Kawamura-Sato *et al*., 2008), the mixture was kept at 22°C for 3 minutes and diluted 1:10 serially with PBS. A 100µL aliquot of this diluted suspension was spread onto TSA plates and incubated at 37°C for 18 hours (Kawamura-Sato *et al*., 2008).

The toxicity of the neutralizer to the bacterial activity was evaluated by adding 100µL of bacterial suspension to 900µL of neutralizer, the mixture was left for 3 minutes at 20 – 22°C then 100µL of mixture was spread on TSA plate and incubated at 37°C for 18 – 24 hours (Kawamura-Sato *et al*., 2008).

Assessment of the bactericidal activity of disinfectants

Time killing test

Time killing test were performed to assess the bactericidal effects of CHX (360mg/L) following the European standard test method EN 1040 (Kawamura-Sato *et al*., 2008), the disinfection concentration was lower than the MBC value in order to evaluate the bactericidal activity at different exposure times intervals. The bacterial cell suspension (100mL) was added to 900mL of disinfectant and assayed at 1, 3, 5 and 10 minutes. The number of surviving colonies were counted and plotted against with time (Kawamura-Sato *et al*., 2008).

Dirty test

This test assessed bactericidal activity under conditions that simulated the presence of organic matter, in this case by using bovine serum albumin (BSA) (Roche Diagnostics Ltd., Burgess Hill, UK). The test was performed with CHX (360mg/L) using exposure times at 1, 3, 5 and 10 minutes, respectively. However, in addition of BSA 1%, 3% and 10% was added to the tubes complying with the European standard test method EN 1276 (Kawamura-Sato *et al*., 2008). The samples were assayed by following the same method employed in the time killing measurement.

Deoxyribonucleic acid (DNA) extraction

To extract DNA for PCR reaction, a suspension of 1 to 2 fresh colonies, for each isolates, were picked from growth on an overnight MacConkey plate. They were mixed with 100µL sterile distilled water in an Eppendorf tubes and the suspension was boiled in a waterbath to 100ºC for about 10 minutes to release DNA. Eppendorf tubes were centrifuged for 1 minute at 13,000rpm; the supernatant containing the bacterial DNA was transferred to new Eppendorf tube and kept at -20ºC until it was used in PCR reaction as the DNA template.

PCR primers

The primers used in this study were either from previously published papers or were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). They are listed in Tables 5 and 6 including those required to amplify the antiseptic resistance genes *cep*A, *qac*ΔE1 and *qac*E. They were purchased from Life Technologies Ltd (Paisley, UK). The primers were diluted to a concentration of 100pmol/μL according to the manufacturer's guidelines as stock standards. The annealing temperatures are shown in Tables 5 and 6.

Table 5. PCR primers of the antiseptics resistance gene *qac*E, *qac*ΔE1, *sul*1 and *aad*A1 with expecting size of product and the annealing temperatures

Gene	PCR Primers	Product size (bp)	Annealing temperature	References
*qac*ΔE1	F 5'GGGAATTCGCCCTACACAAATTGGGAGA'3 R 5'AACACCGTCACCATGGCGTCGACGTCG'3	370	49°C for 40s	(Kazama *et al*., 1998)
*qac*ΔE1 *sul*1	F 5' AGGCTGGTGGTTATGCACTC 3' R 5' CCGACTTCAGCTTTTGAAGG 3'	238	56°C for 40s	This work
*sul*1 *orf*5	F 5' AGGCTGGTGGTTATGCACTC'3 R 5' CGTATAGGCCACGCAGGTT'3	895	57.5°C for 40s	This work
*sul*1 *chr*A	F 5' ACGAGATTGTGCGGTTCTTC'3 R 5' GGGGTCATGCTCAACAACTT'3	974	56°C for 40s	This work
*aad*A1 *qac*ΔE1	F 5' TGAGGCGCTAAATGAAACCT'3 R 5' AACCAGGCAATGGCTGTAAT'3	1303	56.5°C for 40s	This work
IGR *aad*A1	F 5' ATGCCCGTTCCATACAGAAG'3 R 5'AGGTTTCATTTAGCGCCTCA'3	1836	56.5 for 40s	This work
*dhfr*A1 *aad*A1	F 5' CAATGGGAGCATTACCCAAC '3 R 5' TACTGCGCTGTACCAAATGC'3	844	56°C for 40s	This work
*qac*E	F 5'GCCCTACACAAATTGGGAGA3' R 5'TTAGTGGGCACTTGCTTTGG3'	350	49°C for 40s	(Kazama *et al*., 1998)

bp, base pair; s, seconds; *qac*, quaternary ammonium compounds; IGR, intergenic region

Table 6. PCR and RT-PCR primers of the antiseptics resistance gene *cep*A, *pfk*A, *men*G, *cpx*P and *cpx*P with product size and the annealing temperatures

Gene	PCR Primer	Product size (bp)	Annealing temperature	References
*cep*A	F 5'CAACTCCTTCGCCTATCCCG3' R 5'TCAGGTCAGACCAAACGGCG3'	1051	66°C for 40 s	(Fang *et al*., 2002)
*cep*A *pfk*A	F 5' CGCTGTTCCTGTTTCTCACC3' R 5' CGCGCTTCTTCATGTTTTC 3'	1099	66°C for 40 s	This work
*pfk*A *men*G	F 5' GTTCCTATATGGGGGCGATG 3' R 5' GAATGGCCGTGGTCATATTC 3'	975	63.5°C for 40 s	This work
*cpx*P *cep*A	F 5' CCCCGTTAATGTTAGCGAAA 3' R 5' CTAACGAGGGCGATCAATGT 3'	830	64.5°C for 40 s	This work
*cpx*R *cpx*P	F 5' ATCGAAATTGGCTTCCTGAC 3' R 5' GGCGTAAGCAGGTGGTACAT 3'	830	66°C for 40 s	This work
RT-PCR Primer 16S	F 5' CAGCCACACTGGAACTGAGA3' R 5' GTTAGCCGGTGCTTCTTCTG 3'	220	66°C for 40 s	(Findlay *et al*., 2012)

bp, base pair; s, seconds; *cep*, cation efflux pump.

Polymerase Chain Reaction (PCR) Reagents and Amplification of genes by PCR

PCR reactions were run in total volumes of 50µL. The lists PCR reaction components (Table 7). The PCR mastermix solution (Promega, Southampton, UK) containing (Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers) were added into a sterile 0.5mL PCR tube (Fisher Scientific, Leicestershire, UK), containing DNA template with primers. 37.8µL sterile distilled water was used as the diluent for the reaction and to give a final volume in each tube 50µL (Table 7). A negative control contained all the reaction components minus any bacterial DNA.

The PCR tubes were mixed by pulsing in a centrifuge prior to being placed in a thermal cycler. PCR cycling was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Warrington, UK). Cycling parameters were adjusted according to PCR standard protocol as described below in (Table 8). The annealing temperatures and numbers of cycles were varied depending on length of primer, primer Tm, GC content and target gene.

Table 7. PCR reaction components

Component	Volume (µL)
5×Green or Colorless GoTaq® Flexi Buffer1	5.0
$MgCl_2$ Solution, 25mM	3.0
Go Taq polymerase	0.2
PCR Nucleotide Mix, 10mM	1.0
Upstream Primer ×25pmol/µL	1.0
Downstream Primer ×25pmol/µL	1.0
DNA template	1.0
Nuclease-Free Water	37.8

Table 8. PCR cycling parameters

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	94	5 minutes	1
Denaturation	94	30 seconds	
Annealing	55 – 66	40 seconds	30 – 35
Extension	72	50 seconds	
Final Extension	72	7 minutes	1
Soak	4	Until required	1

10×TAE buffer

The constituents of 10×TAE buffer were made in the following order: 48.44g of Tris Base, 3.72g of ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate 99%, 11.4mL Glacial Acetic Acid (Biokeystone Co LLC, California, USA). The final volume of the buffer was made up to one litre by adding distilled water and mixed well until it completely dissolved. The pH of the buffer was adjusted to 8.0, sterilized and diluted 1:10 in order to give a final concentration of 1×TAE ready for use in gel electrophoresis.

Gel electrophoresis of DNA

The PCR products were separated by agarose gel electrophoresis. 7µL of PCR product containing the 5×Green Buffer was loaded directly into 1.5% agarose gel GenSieve LE agarose (Flowgen Bioscience, UK) in 1×TAE buffer. The amplified PCR containing colorless buffer can be loaded directly into the wells of an agarose gel, combined with a 3µL PCR loading buffer Blue/Green dye from Promega (Southampton, UK) to monitor the progress of electrophoresis.

The agarose was heated in a microwave oven until it dissolved and then left to cool at 50ºC. In order to provide a measure of product size, 7µL of DNA ladder (comprising either 100base pairs (bp) or 1kilo base (kb)), (Promega, Southampton, UK) as molecular weight markers was loaded with 3µL PCR loading buffer Blue/Green dye from Promega (Southampton, UK). The DNA ladder and the negative control were run alongside each PCR amplification; the DNA ladder was run in the first well of the gel and the negative control in second well.

The gel electrophoresis was performed using the Bio-Rad Mini Sub Cell horizontal gel units in 1×TAE buffer at 100 volts in room temperature for 30 – 40 minutes. Gels were stained in 100mL Red Gel from Biotium (Cambridge, UK) according to the manufacturer's instructions, which consisted of 30µL of Red Gel solution diluted with 100mL 0.1M NaCl (11.68g NaCl added to 2 litres of distilled water) for 15 – 20 minutes and Gel Red was stored at room temperature. Finally, the gel was visualised under a UV light on a UV transilluminator and gel image was taken in a Joint Photographic Experts Group (JPEG) with the Diversity Database software image capturing system of Bio-Rad GelDoc system (Hemel Hempstead, UK).

DNA sequencing

The PCR amplifications were purified with the kit QIAquick PCR Purification Kit, which was supplied from QIAGEN Company (West Sussex, UK). The purified DNA was stored at -20°C. A clean 5µL of PCR product and 1µL sequencing primer forward or reverse (3.2 pmol/µL) was in a total volume of 6µL per sample. The nucleic acid sequencing was performed on an ABI 3730 Sanger capillary sequencer instrument supplied by Applied Biosystems (Warrington, UK) by the Gene Pool (Edinburgh University). The sequences were compared to the published sequences of antiseptic resistance genes *cep*A, *qac*ΔE1 and *qac*E using online databases website of nucleotide blast program of the National Centre for Biotechnology Information (http://www.ncbi.nih.gov/BLAST). The Multalin website was used for alignment DNA sequence online to observe for any differences or changes in the sequences (http://multalin.toulouse.inra.fr/multalin/).

Efflux pumps inhibitor and biocides resistance

The main function of an efflux pump is to excrete of various toxic molecules, especially those that are bactericidal, and this mechanism can play an important role in bacterial resistance to antibiotics and biocides (Mahamoud *et al*., 2007). Because of this there are several trials to produce chemicals that can be administered with antibiotics or biocides to act as efflux pump inhibitors. Reserpine, 1-(1-Naphthylmethyl)-piperazine (NMP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma, Poole, UK) have been used. These can inhibit efflux pumps in Gram-negative bacteriaand can be used to determine whether an efflux pump was active.

The efflux pump inhibitors (EPIs) used, such as CCCP, against biocides resistance in *K. pneumoniae*. A 10mg aliquot of the efflux pump inhibitors (EPIs) used, such as CCCP, was dissolved in small volumes of Dimethyl sulfoxide (DMSO) (Sigma, Poole, UK) to1 litre of IST agar to make up final concentration is 10mg/L and 20mL of molten IST agar, was poured in each plate containing increasing concentrations of biocides prepared in a similar manner to that previously described earlier for the MIC experiments. Agar plates were inoculated by multipoint inoculator and incubated within 15 minutes at 37°C for 18 hours. (Piddock, 2006a).

Ribonucleic acid (RNA) extraction

A single colony of each isolate was grown in IST broth to the exponential phase an optical density (O.D) of 0.9 – 1.0 at 600 nanometre (nm) (~1×10^9 cfu). Total RNA was extracted from broth culture according to the manufacturer's instructions using the RiboPure Bacteria kit purchased from Ambion (Warrington, UK). All equipment and the environment including the benches, gloves and the pipettes were decontaminated with TURBO DNase ZAP (Ambion, Warrington, UK).

The eluted RNA was treated with DNA-free DNase (Ambion, Warrington, UK) at 37°C for 30 minutes to eliminate any traces of contaminating genomic DNA**.** The RNA concentration was measured by loading a 2µL sample onto NanoDrop spectrophotometer ND-1000 from Thermo scientific (Cramlington, UK). The NanoDrop spectrophotometer was cleaned and blanked with distilled water. The average of two independent readings was taken. When it fell between 100 and 200nanogram (ng), it was deemed to be suitable to be carried forward for cDNA synthesis. RNA samples were stored at -80°C.

Reverse transcription (RT) PCR

cDNA was synthesized from 100ng of total RNA by reverse transcription according to Access RT-PCR System Kit instructions from Promega. A reverse transcription (RT) reaction consisted of: 25µL Access Quick™ Master Mix, 1.5µL upstream and downstream primer, 5µL RNA and 17µL sterilized distilled water was used to give a final volume in each tube 50µL into a sterile PCR tube (Fisher Scientific, Leicestershire, UK). The RT reaction was mixed thoroughly before adding 1µL (5units) AMV Reverse Transcriptase as the final component and mixed by gentle vortexing or pipetting (Table 9). The reaction tubes were incubated at 45°C for 45 minutes, RT-PCR amplification was run in a 50µL total volume for 25 – 30 cycles.

The RT-PCR amplification product was separated by agarose gel electrophoresis in order to assess gene expression and stained with Gel Red. Visualisation, with the Bio-Rad GelDoc 2000 software, Quantity One, was used to assess gene expression semiquantitatively. The levels of expression RT-PCR products were assessed against the 16S gene as negative control of RT-PCR, which was run for 12 cycles.The intensities of the RT-PCR product bands were normalised and compared to the intensities of the 16S bands in order to compare expression levels between strains.

Table 9. Conditions for RT-PCR

Component	Volume (µL)
Access Quick™ Master Mix, 2X	25
Upstream primer	1.5
Downstream primer	1.5
RNA Template	5
Nuclease-Free Water to a final volume of 50	17
(5u) AMV Reverse Transcriptase	1

1×TBE buffer

This buffer comprised Tris base (0.089M), and of boric acid (0.089M) and 2.5mM disodium EDTA (ICN Biomedical, Aurora, Ohio, USA). The pH was adjusted to 8.0 and the solution buffer was sterilized. 0.5×TBE buffer was prepared from 1×TBE buffer and used for running of the PFGE gels.

Pulsed-Field Gel Electrophoresis (PFGE)

Agarose plugs

PFGE of *K. pneumoniae* isolates were typed on according to modified protocol described by (Miranda *et al*., 1996). A loopful of fresh bacterial colonies from an overnight IST agar plate was inoculated into 3mL of cell suspension buffer (100mM Tris Base and 100mM EDTA pH 8.0) and suspended by vortexing, each bacterial suspension was adjusted to give a cell density of approximately 10^9 cells/mL in the spectrophotometer at 610 nm (Camspec M330) using cell suspension buffer as the blank.

An aliquot of 25µL proteinase K (20mg/mL) was added to a 500µL cell suspension resuspended and incubated at 55°C in a water bath to avoid setting. This was followed by adding equal volume of mixture of 1% Certified Megabase Agarose, (Bio-Rad, Hertfordshire, UK) and 1% sodium dodecyl sulphate (SDS) (Fisher Scientific, Leicestershire, UK) into TE buffer (10mM Tris Base, 1mM EDTA, pH 8.0) for the plugs, The mixture was pipetted immediately into the wells of plug moulds and allowed to solidify at 4°C for 20 –30 minutes.

A single plug was incubated in 5mL of cell lysis buffer, 50mM of EDTA, 50mM Tris Base, 1%N-Lauroyl Sarcosine and 1.0M Sodium chloride at pH 8.0, at 55°C in a water bath for two hours. The plugs were washed five times carefully (15

min/wash) at 55°C, two times with sterile distilled water and three times with TE buffer

Agarose plug digestion

For the restriction endonuclease digestion of the genomic DNA, the plug was gently sliced into 3 pieces of 4 millimetres (mm) into 100µL of the 1x restriction endonuclease buffer (1µL BSA by Promega (Southampton, UK) and 89µL of sterilized distilled water) for at least 15 minutes. The restriction buffer was replaced with100µL fresh buffer containing 3µL of 30U *Xba1* restriction endonuclease by Promega (Southampton, UK), 10µL of restriction buffer, 1µL BSA and 86µL sterilized distilled water, and tubes were incubated in water bath at 25°C for overnight. After that the buffer was removed and the reaction was stopped by adding 100µL of 0.5×TBE buffer (Miranda *et al*., 1996).

Gel preparation and running conditions

The plug slices were loaded into the gel lanes of 1% Pulsed Field Certified Agarose (Bio-Rad, Hertfordshire, UK) prepared in 0.5×TBE buffers. A Lambda ladder PFGE Marker (New England Biolabs Hertfordshire, UK) was used as the size standard marker for PFGE, plugs wells were then overlain with more 1% PFGE-grade agarose to seal.

Electrophoresis was performed with a CHEF-DRII system (Bio-Rad, Hertfordshire, UK). The gel was run for 20 hours at 6.0 volts/cm at 14ºC with 5 seconds initial pulse time and 45 seconds final pulse time in 0.5×TBE running buffer. Gel was stained for 30 minutes in GelRed as described previously, then Gel was visualised by UV transillumination on the Bio-Rad GelDoc system (Miranda *et al*., 1996).

Interpretation of PFGE

All isolates were analysed using BioNumerics software, version 4 (Applied Maths, Sint-Martins-Latem, Belgium). Isolates which clustered together with a similarity of > 85% were considered to belong to the same PFGE type. Results were obtained as phylogenetic trees. A value of 80% was selected as the threshold for the establishment of clonal relatedness of unknown isolates. The banding patterns were also interpreted according to the criteria suggested by Tenover *et al*. (1995).

Results

MICs of biocides and antibiotics against the *Klebsiella* strains

MICs of biocides

The susceptibility of the 64 *Klebsiella* isolates was determined by measuring the MIC of antibiotics and biocides. The ranges of MIC for each biocide are shown in (Table 10 and Figure 23). There was a decrease in susceptibility to the disinfectants 1% Chlorhexidine gluconate (CHX), 1% Benzalkonium chloride (BZK) and the commercial biocide Trigene with MICs ranging from 32 – 128mg/L. This is considered as high level of insusceptibility, it should be noted that 57, 55 and 61 isolates had reduced susceptibility to Chlorhexidine, Trigene and Benzalkonium chloride, respectively, and the high of resistance was verified by the high MIC_{50} as well as MIC_{90} values (Table 10).

The cationic biocide Medihex-4 (MH-4), containing 4% chlorhexidine. Strains showed a moderate level of MICs to MH-4 from 8 – 16mg/L in 58 isolates; these were approximately 4-fold lower than Chlorhexidine reflecting the 4-fold increase in chlorhexidine concentration in this commercial product (Table 10 and Figure 23). On the other hand, 63 isolates were susceptible to Mediscrub (MS), containing 1% triclosan with MICs $\leq$ 4mg/L, though the remaining one was far less susceptible (MICs > 128mg/L) (Table 10 and Figure 23), again was verified by the MIC_{50} and MIC_{90} values (Table 10)

The results show four of the biocides had MIC ranges starting at greater than 1mg/L, with two of them having the lowest MIC at 16mg/L. The exception to this was Mediscrub, which had an MIC range beginning at 0.12mg/L and extending only to 0.5mg/L. Chlorhexidine, Benzalkonium chloride and Trigene had identical MIC_{50} and MIC_{90} values; whereas Medihex was about four-fold lower. In stark contrast Mediscrub had much lower MIC_{50} and MIC_{90} values (Table 10). This means that the MIC_{50} of Chlorhexidine, Benzalkonium chloride and Trigene was at least 256-fold higher than the MIC_{50} of Mediscrub. The MIC details of all biocides are shown in appendix A.

Table 10. The MIC summary of biocide and the ranges of MIC_{50} and MIC_{90} of biocides against 64 *K. pneumoniae* isolates

Biocides	Number of strains at each MIC (mg/L) of biocide											MIC Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
	0.12	0.25	0.5	1	2	4	8	16	32	64	128			
CHX	-	-	-	-	-	1	1	5	34	20	3	4 – 128	32	64
Trigene	-	-	-	-	-	-	-	9	41	14	-	16 – 64	32	64
MH-4	-	-	-	-	1	5	45	13	-	-	-	2 – 16	8	16
MS	45	6	12	-	-	-	-	-	-	-	1	0.12 – 128	0.12	0.5
BZK	-	-	-	-	-	-	-	3	48	13	-	16 – 64	32	64

CHX, Chlorhexidine; MH-4, MediHex-4; MS, Mediscrub; BZK, Benzalkonium chloride.

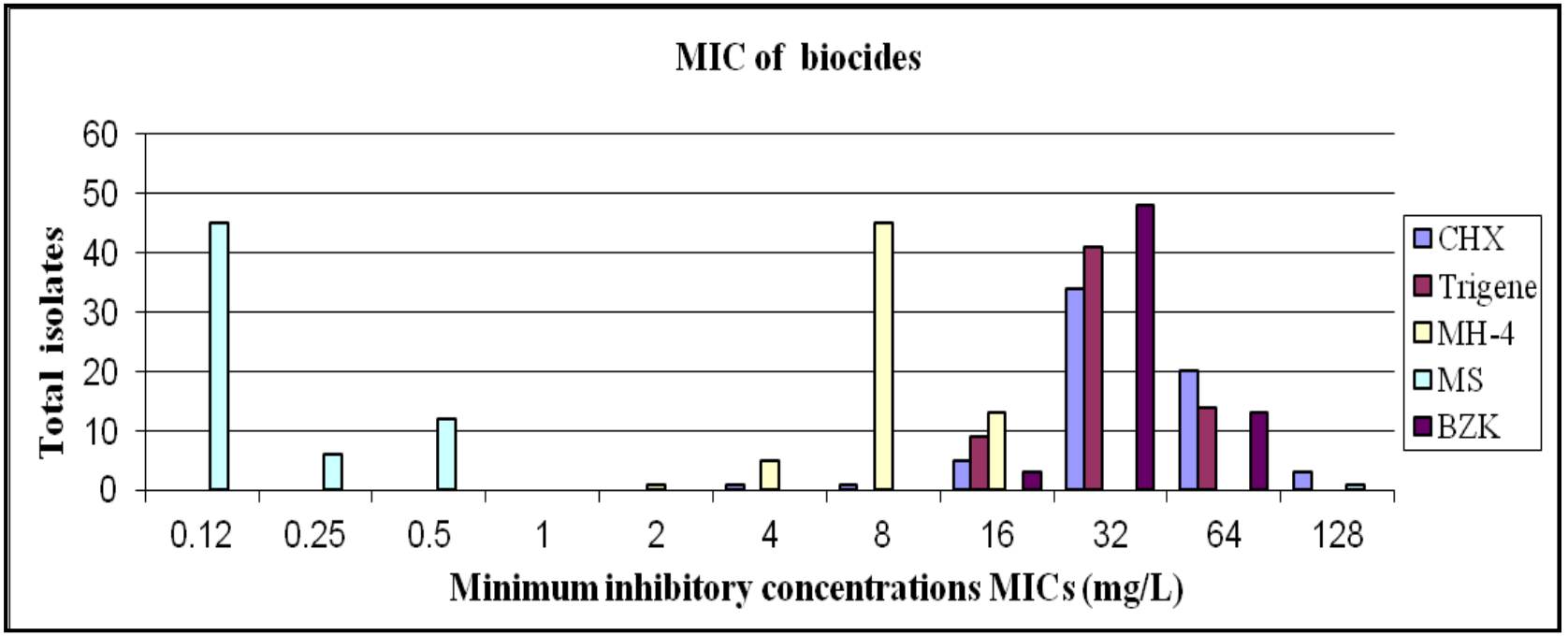


Figure 23. Distribution of MICs of biocides among 64 *K. pneumoniae* isolates, All biocides range started from 2mg/L to >128mg/L of MIC, except Mediscrub antiseptic begun from 0.12mg/L to 0.5mg/L of MIC.

CHX, Chlorhexidine; MH-4, MediHex-4; MS, Mediscrub; BZK, Benzalkonium chloride.

MICs of antibiotics

Number of isolates were resistant to the antibiotics tested particularly some of the β-lactams and the MICs of the antibiotics for 64 isolates are shown in (Table 11 and Figure 24). 3rd generation cephalosporins, colistin, gentamicin, polymyxin-B, trimethoprim and chloramphenicol antibiotics were resistance to 17, 34, 3, 1, 12, and 4 isolates respectively (Table 11 and Figure 24); however all *K. pneumoniae* isolates were sensitive to imipenem (MIC ≤ 2mg/L) and meropenem (MIC ≤ 0.12mg/L) (Table 11). The population was resistant to rifampicin with 49 isolates having MICs above the breakpoint; this was confirmed by the high MIC_{50} value (Table 11).

Out of the 64 *K. pneumoniae* isolates, 8 were resistant ceftazidime, 9 were resistant to cefotaxime and 17 were resistant to cefoxitin. Thirty-four were resistant to colistin, however most of isolates were susceptible to polymyxin-B antibiotic with the exception that one isolate was resistant to it, again supported by the MIC_{50} values (Table 11 and Figure 24). The ranges of MICs and the variations in MIC_{50} values of cephalosporins, colistin, gentamicin, trimethoprim and chloramphenicol varied widely, the MIC_{50} values spanned from 0.06 to 8mg/L. In general the level of resistance to the biocides was considerably greater than to the antibiotics (Table 11). The susceptibility details of all antibiotics are demonstrated in appendix A.

Table 11. The summary of MIC of antibiotics and the ranges of MIC_{50} and MIC_{90} values of antibiotics against 64 *K. pneumoniae* isolates

Antibiotics	Number of strains at each MIC (mg/L) of Antibiotics														MIC Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
	0.015	0.03	0.6	0.12	0.25	0.5	1	2	4	8	16	32	64	128			
Cefotaxime		14	22	13	2	5	-	-	-	-	-	**1**		**7**	0.03 – 128	0.06	128
Ceftazidime	-	-	-	8	27	12	8	-	-	-	-		**4**	**5**	0.12 – 128	0.25	64
Cefoxitin	-	-	-	-	-	-	-	1	20	26	**10**	**3**	**2**	**2**	2 – 128	8	32
Imipenem	-	-	-	15	28	11	10	-	-	-	-	-	-	-	0.12 – 1	0.25	1
Meropenem	43	19	2	-	-	-	-	-	-	-	-	-	-	-	0.015-0.06	0.015	0.03
Colistin	-	-	-	-	-	-	-	1	29	**34**	-	-	-	-	2 – 8	8	8
Rifampicin	-	-	-	-	-	-	-	-	-	1	14	**33**	**16**	-	8 – 64	32	64
Gentamicin	-	-	-	3	37	18	2	-	1	-	-	-	**3**	-	0.12 – 64	0.25	0.5
Polymyxin B	-	-	-	-	-	1	58	3	1	**1**	-	-	-	-	0.5 – 8	1	1
Trimethoprim	-	-	-	1	6	43	1	1	-	**3**	**1**	-	-	**8**	0.12 – 128	0.5	128
Chloramphenicol	-	-	-	-	-	-	-	-	42	16	2	-	-	**4**	4 – 128	4	8

Breakpoint; cefotaxime and trimethoprim (> 2mg/L); colistin, polymyxin-B and gentamicin (> 4mg/L); ceftazidime, cefoxitin, imipenem, meropenem and chloramphenicol (> 8mg/L); Rifampicin (> 16mg/L).

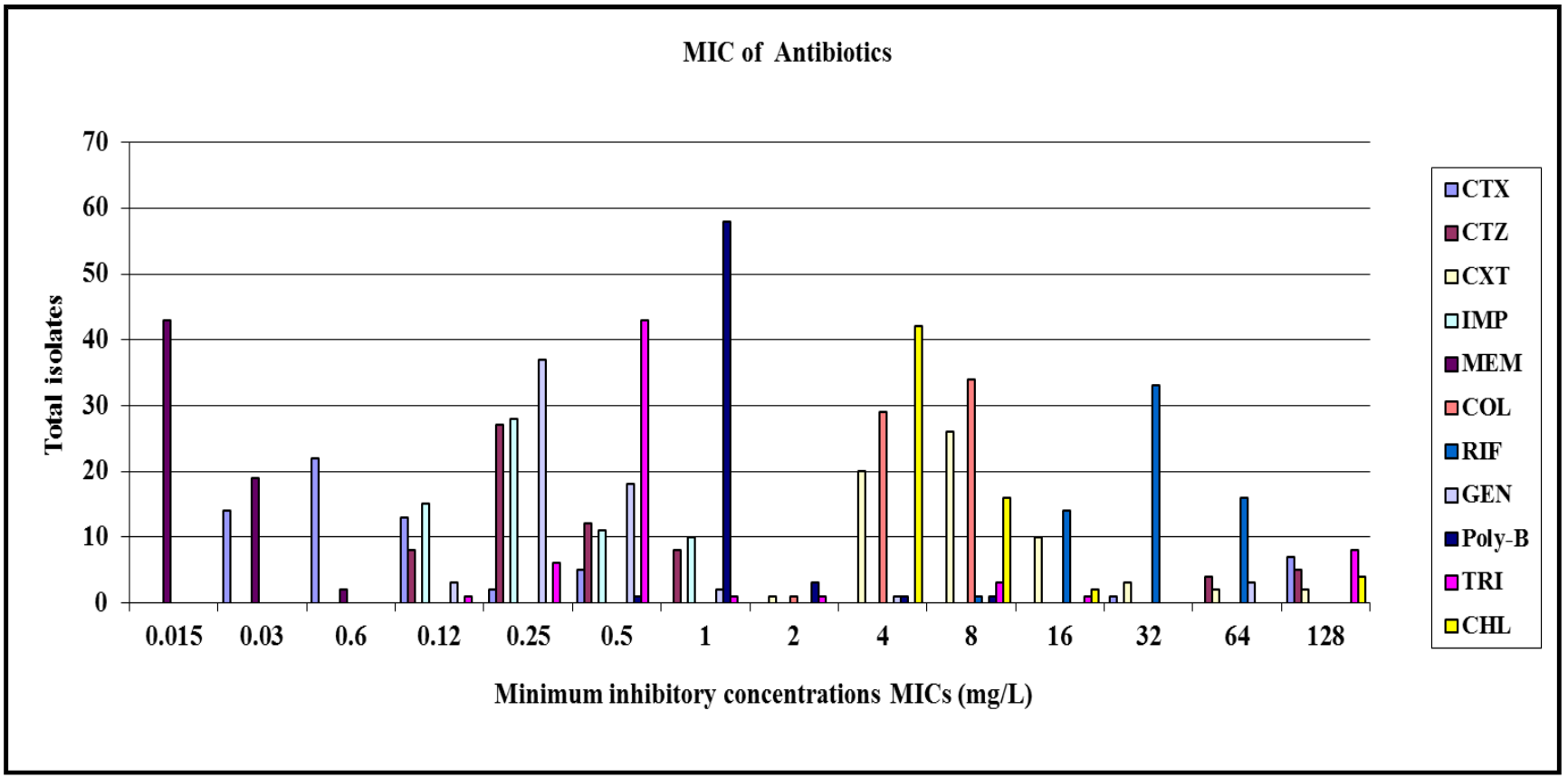


Figure 24. Distribution of antibiotics MICs for the 64 clinical *K. pneumoniae* isolates.

CTX, Cefotaxime; CTZ, Ceftazidime; CXT, Cefoxitin; IMP, Imipenem; MEM, Meropenem; COL, Colistin; RIF, Rifampicin; GEN, Gentamicin; Poly-B, Polymyxin B; TRI, Trimethoprim; CHL, Chloramphenicol.

Disc diffusion test

Further antibiotics were tested by the disc sensitivity test. All isolates were sensitive to Amikacin (AK), Apramycin (APR) and Cefotetan (CCT) antibiotics, but some isolates were resistant to some antibiotics (Table 12). *K. pneumoniae* isolates were resistant mainly to penicillin antibiotic, and the most of isolates (62) were insusceptible to ampicillin (AMP); followed carbenicillin and doxycycline antibiotics with 44 and 39 isolates insusceptible respectively, whereas 26 isolates were resistant to piperacillin. However, one isolate was resistant to netilmicin and tigecycline antibiotics individually (Table 12 and Figure 25).

Table 12. The number of *K. pneumoniae* isolates resistant to antibiotics determined by the disc diffusion method

Antibiotic discs (µg)	Number of isolates resistant
Ampicillin (25)	62
Carbenicillin (100)	44
Doxycycline (30)	39
Piperacillin (75)	26
Erythromycin (30)	7
Novobiocin (30)	4
Aztreonam (30)	2
Netilmicin (30)	1
Tigecycline (15)	1

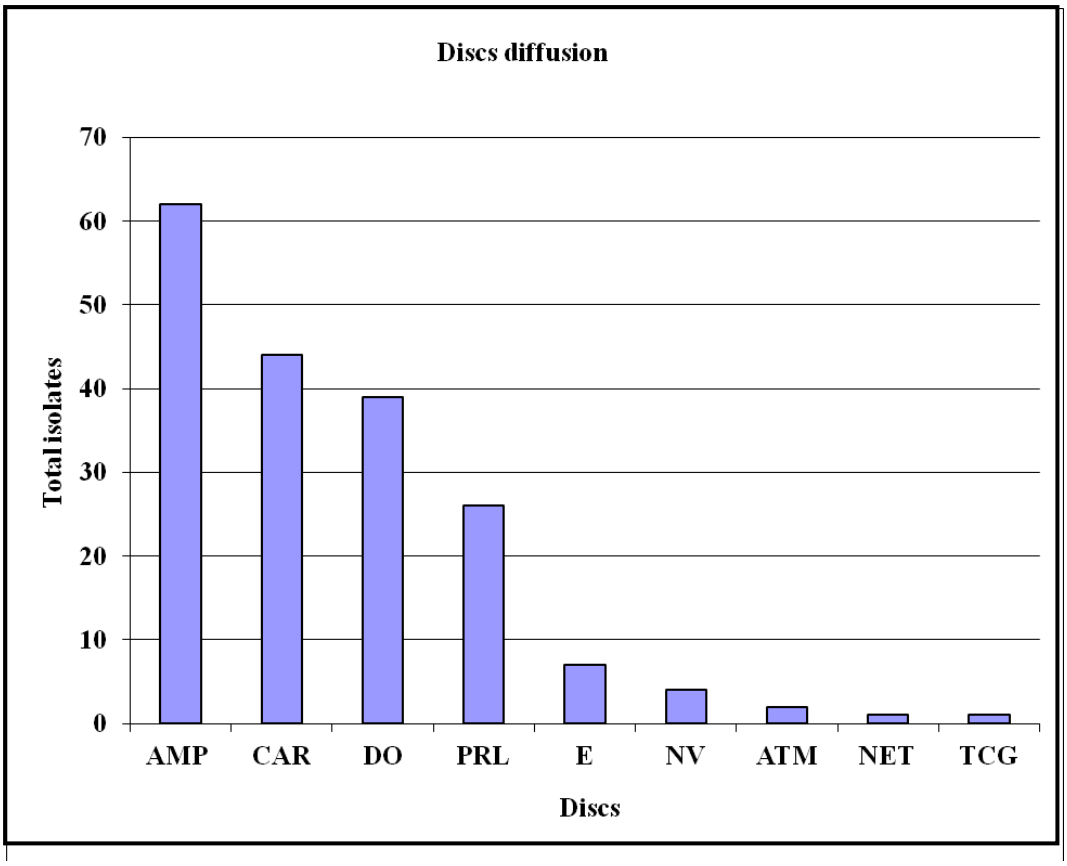


Figure 25. Distribution of antibiotic susceptibility test discs for 64 *K. pneumoniae* AMP, Ampicillin; ATM, Aztreonam; CAR, Carbenicillin; APR, Apramycin; DO, Doxycycline; NET, Netilmicin; PRL, Piperacillin; TCG, Tigecycline; E, Erythromycin; NV, Novobiocin.

Antiseptics resistance genes

In order to determine whether the decreased susceptibility could be correlated with specific resistance genes, PCR was used to amplify the antiseptic resistance genes *qac*E and *qac*ΔE1, these genes have been associated as playing an important role in decreasing susceptibility to antiseptics (reviewed by Russell, 2003). The cation efflux pump associated with chlorhexidine resistance gene, *cep*A, was described on only one occasion before (Fang *et al*, 2002) and the presence of this gene was also sought by PCR. The antiseptics resistance genes *cep*A, *qac*ΔE1 and *qac*E PCRs were performed with primers described earlier (Tables 5 and 6).

The primers pairs of Fang *et al* (2002) were used to identify the *cep*A gene. These primer pairs were used to amplify a 1051 base pair (bp) product with an annealing temperature 66ºC for 30s (Table 6) (Fang *et al*., 2002). In 56 isolates a gene fragment of 1051 base pairs was found with these primers indicating the presence of the *cep*A gene (Figure 26). The association between antiseptic resistance gene *cep*A and biocides insusceptibility is described below (Table 13).

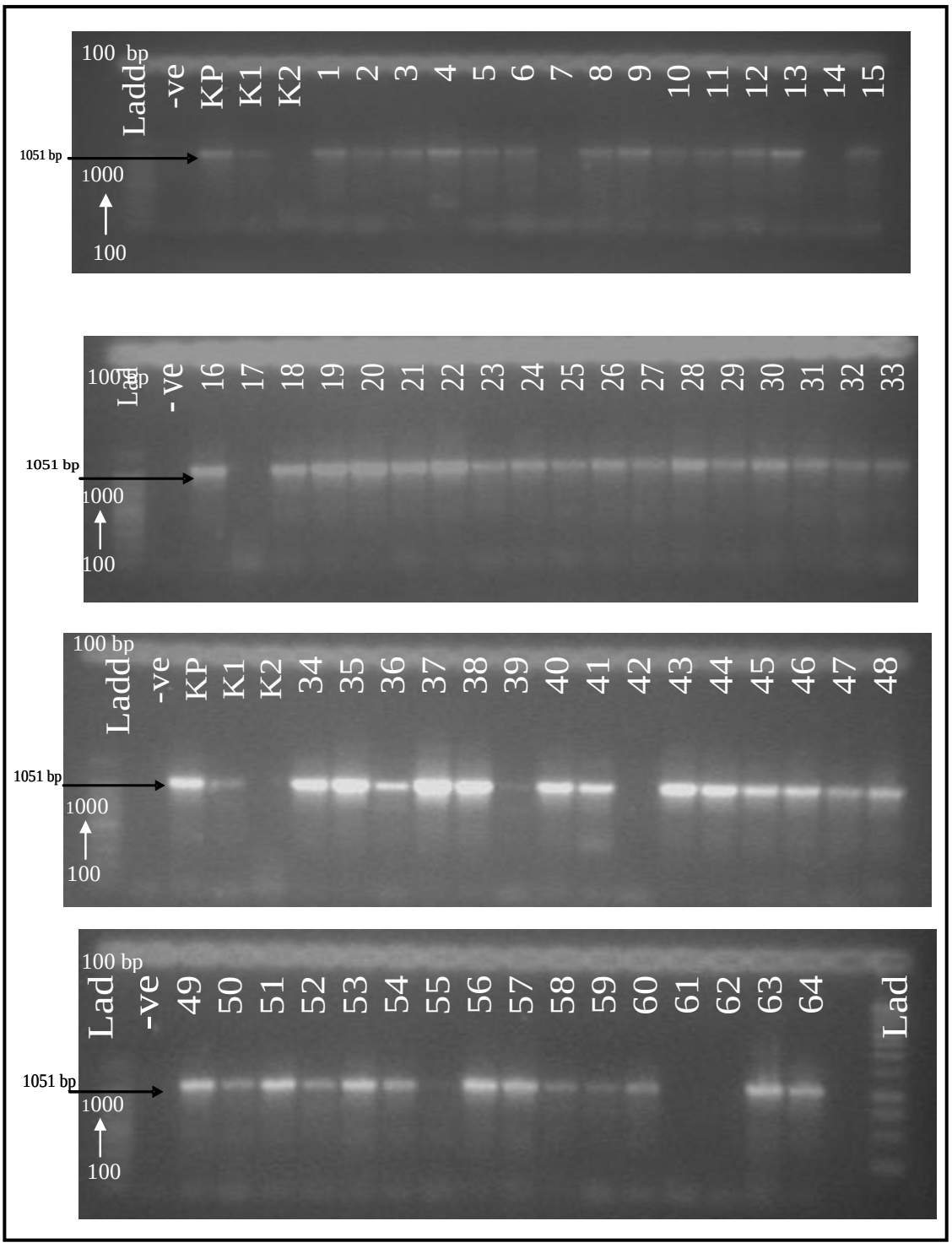


Figure 26. An agarose gel showing PCR amplification of *cep*A gene for 64 isolates of *K. pneumoniae* , A 100bp marker in lane 1, A negative control in lane 2, positive controls *K. pneumoniae* ATTC 13883 and *K1* in lanes 3 and 4 respectively, and *K2* in lane 5 (as negative control) .

The quaternary ammonium compound resistance genes *qac*ΔE1 and *qac*E were amplified according to primer pairs designed by Kazama *et al* (1998). The primer pairs of *qac*ΔE1 and *qac*E genes were used to amplify 370 and 350 bp fragments respectively, each having the same annealing temperature 49ºC for 40s (Table 5) (Kazama *et al*., 1998).

The *qac*ΔE1and *qac*E genes were amplified by PCR, the *qac*ΔE1 gene was detected in 34 isolates, while *qac*E was found in only one isolate (detailed in appendix A), and PCR amplification of *qac*ΔE1and *qac*E genes are shown in (Figures 27 and 28). The linkage between the presence of the *qac*ΔE1 and *qac*E genes and high level of biocides are shown in Table 13.

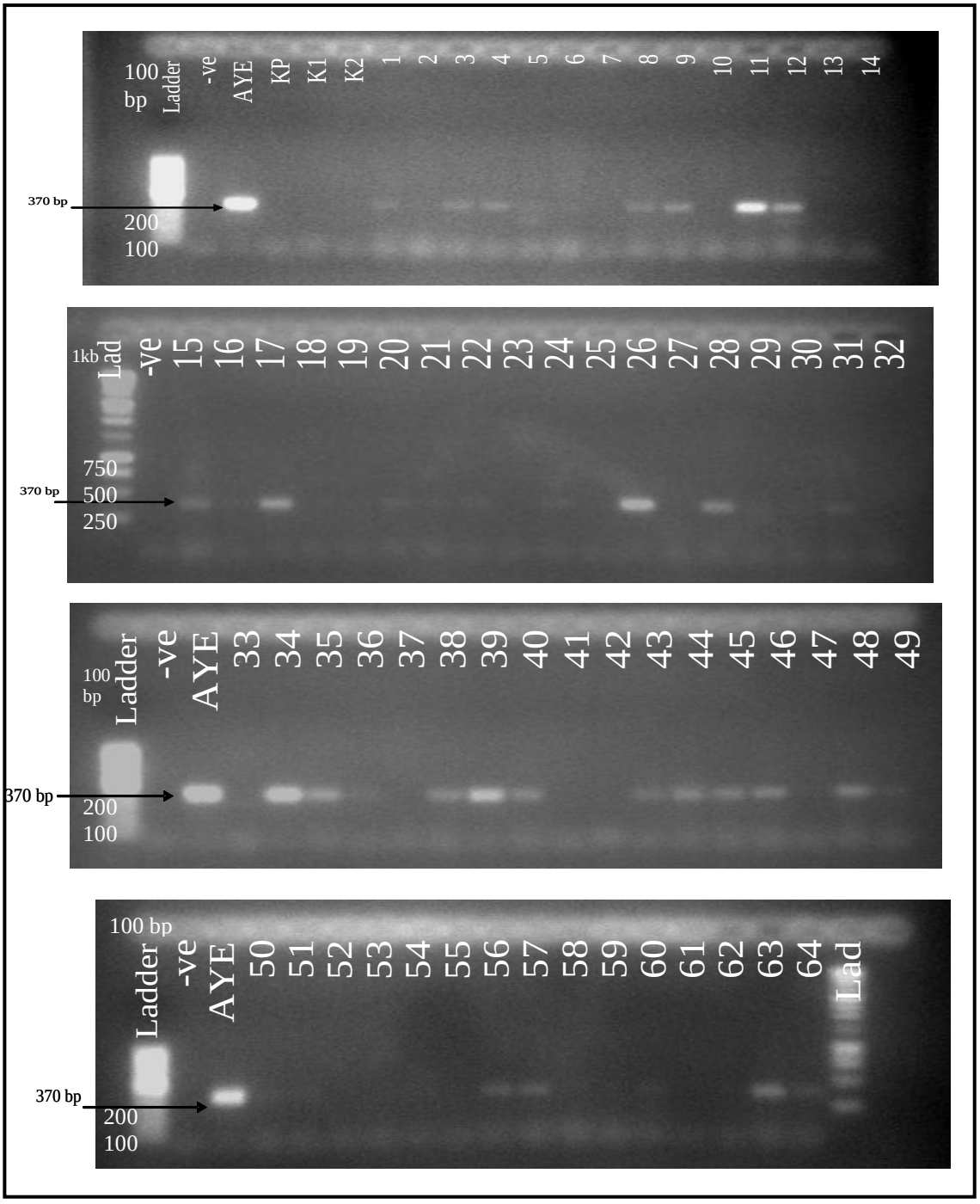


Figure 27. An agarose gel showing PCR amplification of *qac*ΔE1 gene for 64 isolates of *K. pneumoniae* , 100bp or 1kb markers in lane 1, A negative control in lane 2, A positive control *A. baumannii* ATCC 19606 in lane 3, and *K. pneumoniae* ATTC 13883, *K1* and *K2* in lanes 4, 5 and 6 respectively (as negative control).

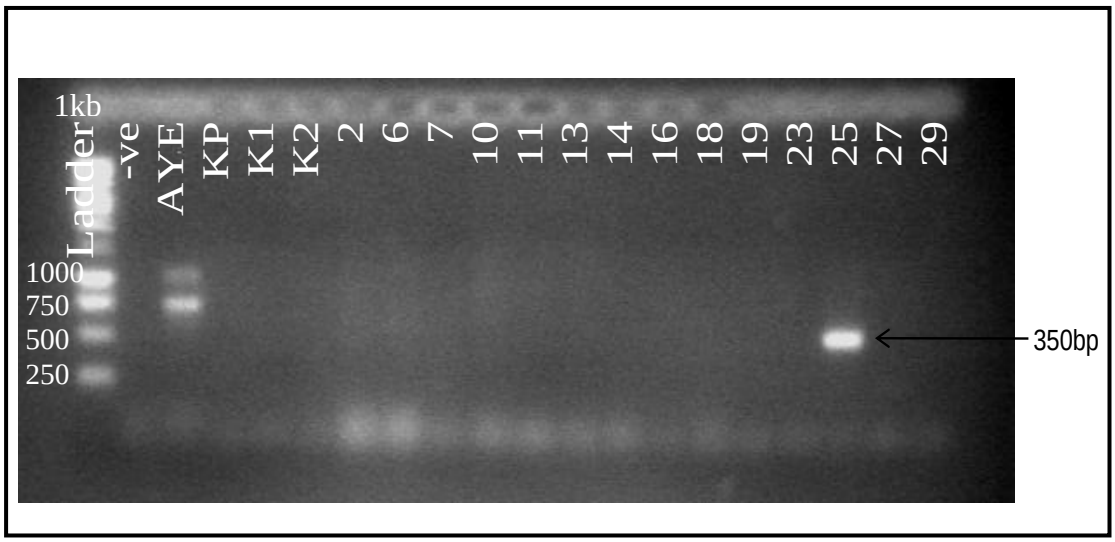


Figure 28. An agarose gel showing PCR amplification of *qac*E gene for 14 of 64 isolates of *K. pneumoniae*, A 1kb markers in lane 1, A negative control in lane 2, A positive control *A. baumannii* ATCC 19606 in lane 3, and *K. pneumoniae* ATTC 13883, *K1* and *K2* in lanes 4, 5 and 6 respectively (as negative control).

Table 13 associates the individual levels of Chlorhexidine, Trigene and Benzalkonium chloride susceptibility with the presence of the individual antiseptic resistance genes *cep*A, *qac*ΔE1 and *qac*E. Medihex-4 was not tested as its main component is 4% chlorhexidine and the strains were susceptible to Mediscrub so this biocide was not included either. High level resistance (MICs 32 – 128mg/L) of Chlorhexidine, Trigene and Benzalkonium chloride was found in 50, 49 and 53 isolates respectively; in each case the *cep*A gene was present, while *qac*ΔE1 and *qac*E antiseptics resistance genes were found in 31, 33 and 34 isolates for Chlorhexidine, Trigene and Benzalkonium chloride respectively. Every isolate that had an MIC of Trigene or Benzalkonium chloride (both containing quaternary ammonium compounds) ≥ 16mg/L possessed either the *qac*E or *qac*ΔE1 gene (Table 13). Thirty-two isolates had both *cep*A and *qac*ΔE1 genes, whereas just one isolate has both *cep*A and *qac*E gene and six isolates had no efflux pumps genes (Table 13).

Table 13. Association of individual MICs of Chlorhexidine, Trigene and Benzalkonium chloride with the presence of the *cep*A, *qac*ΔE1 and *qac*E genes

Biocide	MIC (mg/L)	No of strains	Antiseptics Resistance Genes					
			*cep*A	*qac*ΔE1	*qac*E	*cep*A +*qac*E	*cep*A+*qac*ΔE1	Absent *cep*A+*qac*ΔE1
Chlorhexidine	4	1						1
	8	1	1					
	16	5	5	4			4	1
	32	34	28	20			19	4
	64	20	19	9	1	1	8	
	128	3	3	1			1	
Trigene	16	9	7	1	1	1	1	2
	32	41	39	24			24	2
	64	14	10	9			7	2
Benzalkonium	16	3	3	1			1	
chloride	32	48	43	27	1	1	25	3
	64	13	10	6			6	3

qac, quaternary ammonium compounds; *cep*, cation efflux pump.

Efflux pump inhibitor and biocides resistance

The main function of efflux pump is to pump out various metabolites, usually those that are detrimental to the cell. This mechanism can play an important in bacterial resistance to antibiotics and biocides (Mahamoud *et al.*, 2007). Consequently, there are several compounds that can be administered with antibiotics or biocides to act as efflux pump inhibitors (EPIs). Reserpine, 1-(1-Naphthylmethyl)-piperazine (NMP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) can inhibit efflux pumps in Gram-negative bacteria (Garvey and Piddock, 2008).

The phenotypic effect of efflux pumps on biocides was determined by repeating the susceptibility tests in the presence of the EPIs. There was no impact of the EPIs reserpine and 1-(1-Naphthylmethyl)-piperazine (NMP) on the susceptibility of the five biocides. The presence of the EPI CCCP (10mg/L), on the other hand, provided a different response when it was examined on the MICs of the biocides. Although, it had no impact on Benzalkonium chloride, Trigene and Mediscrub (data not shown), it reduced the MICs of Chlorhexidine and Medihex-4 by between 2 and 128-fold (Table 14). In the case of Chlorhexidine, this was associated, except in 5 cases, with the presence of the *cep*A gene. The presence of CCCP had slightly less effect on Medihex-4 and this was associated, except in 8 cases, with the presence of the *cep*A gene. The *qac*ΔE1 gene was associated with less than half of the strains showing the reduction of Chlorhexidine and Medihex-4 MICs indicating that this gene does not play a role in biguanide susceptibility.

Table 14. The effect of CCCP (10mg/L) on the MICs of Chlorhexidine and Medihex-4 correlated to the presence of the *cep*A and *qac*ΔE1 genes

No of strains	Fold reduction of CHX MIC in 10mg/L CCCP	*cep*A	*qac*ΔE1	No of strains	Fold reduction MH-4 MIC in 10mg/L CCCP	*cep*A	*qac*ΔE1
1	8	-	-	1	2	+	+
1	8	+	-	2	4	-	-
3	8	+	+	4	4	+	-
1	16	-	-	1	8	-	-
3	16	+	-	1	8	-	+
3	16	+	+	6	8	+	+
11	32	+	-	2	16	-	-
2	32	-	+	1	16	-	+
15	32	+	+	11	16	+	-
1	64	-	-	21	16	+	+
7	64	+	-	1	32	-	-
9	64	+	+	7	32	+	-
2	128	+	-	5	32	+	+
1	128	+	+	1	64	+	+

CHX, Chlorhexidine, MH-4, Medihex-4

Minimum Bactericidal Concentrations of biocides

Minimum bactericidal concentration (MBC) value is defined as the lowest concentration that reduced the bacterial count by at least 99% inhibition. The MBC value was identified using the European standard test method EN 1040 quantitative suspension test described above.

Six isolates of *K. pneumoniae* isolated were chosen for their varying sensitivity to Chlorhexidine in appendix A, MBCs were calculated for the six isolates using the differences between the proportion of surviving colonies and a control (bacterial suspension was treated with PBS only). The 6 isolates of *K. pneumoniae* were tested against five biocides for assessment their MBCs. In this test, MBCs of the active component of five antibacterial disinfectants were measured, as stated by the manufacturer and the results are shown in Table 15.

The MBC of Trigene was the same for all strains at 1800mg/L of the active component (polymeric biguanide hydrochloride). The MBCs of Chlorhexidine ranged from < 360mg/L to 900mg/L, whereas those for MediHex-4, between < 36mg/L to 360mg/L, and Benzalkonium chloride, at 90mg/L, were lower. The MBC for Mediscrub was the highest for each strain at 9000mg/L. The presence of two efflux genes *cep*A and *qac*ΔE1 had no influence on the MBC values (Table 15).

Table 15. The Minimum Bactericidal Concentration (mg/L) expressed as the minimum concentration required to reduce the viable count of bacteria by 100-fold in 3 minutes according to the method of Kawamura-Sato *et al*. (2008).

Strain	Minimum Bactericidal Concentration (mg/L)					Efflux genes	
	CHX	Trigene	MH-4	MS	BZK	*cep*A	*qac*ΔE1
14	< 360	1800	< 36	9000	90	-	-
16	< 360	1800	< 36	9000	90	+	-
24	900	1800	360	9000	90	+	+
37	900	1800	360	9000	90	+	-
52	< 360	1800	< 36	9000	90	+	-
64	< 360	1800	< 36	9000	90	+	+

CHX, Chlorhexidine; MH-4, MediHex-4; MS, Mediscrub; BZK, Benzalkonium chloride.

The highest ratio of MBC/MIC was Mediscrub with ratio number between >18000 and > 75000, followed by Trigene has a ratio between >112 and >28, although Chlorhexidine, MediHex-4 and BZK, Benzalkonium chloride have a ratio range between 2 to 7 with 4 of 6 strains.

Table 16. Ratio of MBC/MIC of biocides against *K. pneumoniae.*

This is the ratio of the MBCs, determined in Table 15, divided by the MICs for the same six isolates, listed in Appendix A.

Strain	Ratio				
	CHX	Trigene	MH-4	MS	BZK
14	90	> 112	4	> 75000	3
16	45	> 56	4	> 18000	2
24	7	> 28	45	> 18000	3
37	7	> 56	45	> 75000	3
52	3	> 28	2	> 18000	2
64	6	> 28	2	> 70	3

CHX, Chlorhexidine; MH-4, MediHex-4; MS, Mediscrub; BZK, Benzalkonium chloride.

Assessment of bactericidal activity of disinfectants

Time killing test

The bactericidal activity was evaluated by time-killing assays, Time killing test were performed to assess the bactericidal effects of Chlorhexidine following the European standard test method EN 1040, with assay times of 1, 3, 5 and 10 minutes (Kawamura-Sato *et al*., 2008).

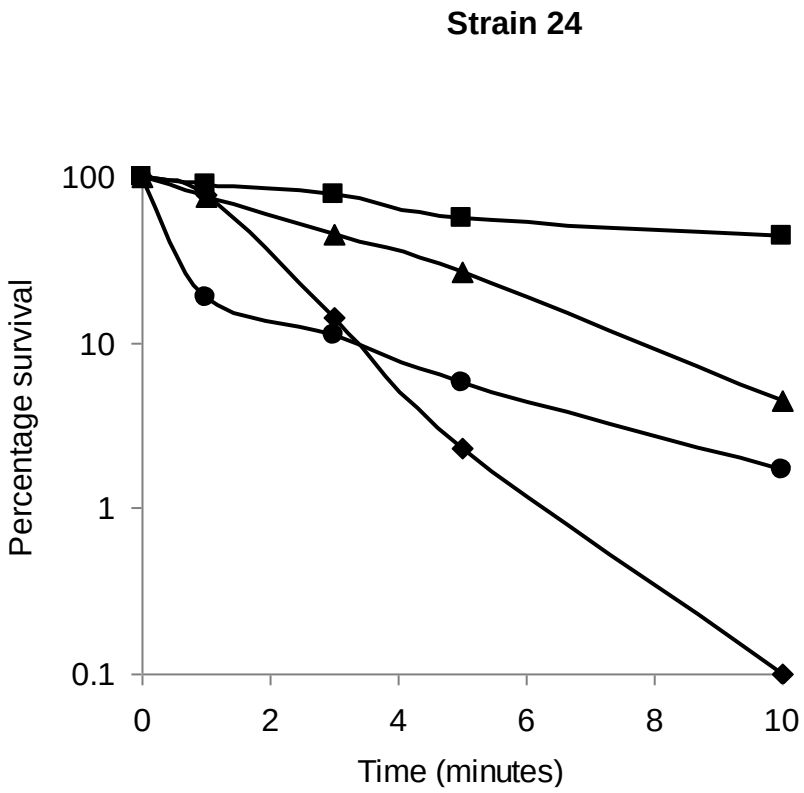
Chlorhexidine is the active component of many proprietary antiseptics. The activity of Chlorhexidine was measured at 40% of the lowest determined figure for the MBC (360mg/L), in order to obtain a drop in viability that could be precisely measured. The time for bacterial complete killing after exposure to 360mg/L of Chlorhexidine was 10 minutes to reduce the viable count of bacteria by three logarithms_{10}. This concentration of Chlorhexidine has the ability to reduce the viability of both *K. pneumoniae* strains 24 and 37 within 10 minutes (Figures 29 and 30).

Dirty test

This test was evaluated bactericidal activity, under conditions that simulated the presence of organic matter, in this case by using bovine serum albumin (BSA). The isolates were examined against Chlorhexidine at 360mg/L in presence of BSA 1%, 3% and 10% complying with the European standard test method EN 1276.

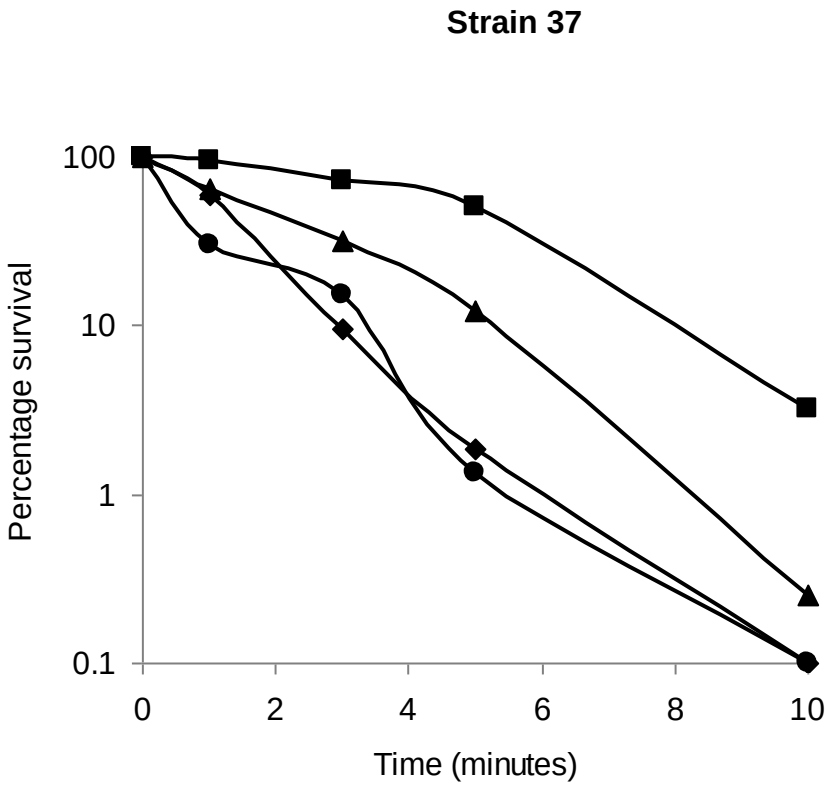
The six isolates were examined against Chlorhexidine at 40% of the lowest determined figure for the MBC (360mg/L) in the presence of BSA 1%, 3% and 10%, The effect of Chlorhexidine was almost eliminated for strain 24 once the concentration of BSA had reached 10% (Figure 29), the effect was considerably less in strain 37 (Figure 30). In general, as the concentration of BSA was increased, the ability of Chlorhexidine to kill the bacteria was reduced, BSA decreased the MBC value of Chlorhexidine and the rate of survival colonies was raised particularly with 10% BSA (Figures 29 and 30). In contrast, 10% BSA had no impact on the killing effect Chlorhexidine on strains14, 16, 52 and 64.

Figure 29. The effect of time on the bactericidal effect of Chlorhexidine (360mg/L) against *K. pneumoniae* strain 24 and the impact of increasing concentrations of bovine serum albumin.



The viability of strain 24 was monitored against time in the presence of Chlorhexidine at 40% of the MBC (♦). The experiment was repeated in the presence of bovine serum albumin at concentrations of 1% (●), 3% (▲) and 10% (■).

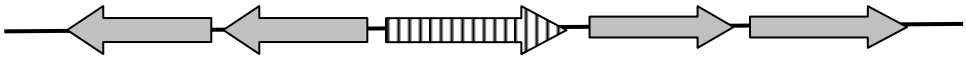
Figure 30. The effect of time on the bactericidal effect of Chlorhexidine (360mg/L) against *K. pneumoniae* strain 37 and the impact of increasing concentrations of bovine serum albumin.



The viability of strain 37 was monitored against time in the presence of Chlorhexidine at 40% of the MBC (♦). The experiment was repeated in the presence of bovine serum albumin at concentrations of 1% (●), 3% (▲) and 10% (■).

Genetic environment of the *cep*A antiseptic resistance gene

The *cep*A gene was amplified by PCR in 56 isolates (Figure 26). In 45 of these isolates we have now identified the 6-phosphofructokinase-1 (*pfk*A) downstream of *cep*A gene. In 40 of these isolates the methyltransferase gene (*men*G) was directly downstream of the *pfk*A gene (Figure 31). The periplasmic repressor gene (*cpx*P) was identified upstream of *cep*A in 47 isolates. The transcription regulator (*cpx*R) was directly upstream of the *cpx*P gene in 35 of these isolates (Figure 31).



*cpx*R *cpx*P *cep*A *pfk*A *men*G

Figure 31. Schematic representation of the genetic environment of the antiseptic resistance gene *cep*A in 35 isolates of *K. pneumoniae*.

Reverse transcription (RT) PCR

RT-PCR analysis of the *cep*A gene showed that it was expressed particularly in those strains with high MICs of Chlorhexidine (Figure 32 and Table 17). When these results were plotted against the $\log_{10}$ of the MIC, there was a correlation between the degree of expression and the MIC indicating that *cep*A contributed to active efflux mechanisms (Figure 33).

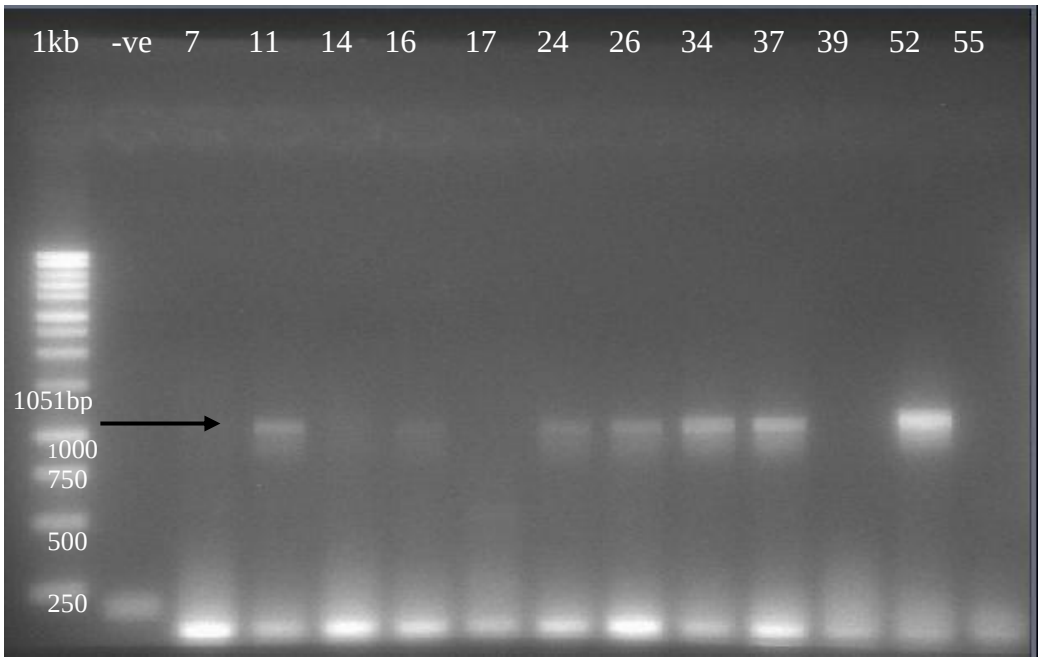


Figure 32. RT-PCR demonstrated the expression of isolates carried *cep*A11, 16, 24,26,34,37 and 52, A 1kb DNA ladder with the DNA band sizes (bp) are noted on the left in lane 1, and a negative control in lane 2.

Table 17. RT-PCR showing the expression level of the *cep*A gene in strains especially with high level MICs of Chlorhexidine

Isolates	MIC of Chlorhexidine (mg/L)	*cep*A carriage	Level of Expression (Arbitrary units)
16S control	-	-	100
7	32	-	108
11	32	+	142
14	4	-	114
16	8	+	119
17	64	-	107
24	128	+	147
26	32	+	137
34	32	+	169
37	128	+	174
39	32	-	106
52	128	+	222
55	32	-	103

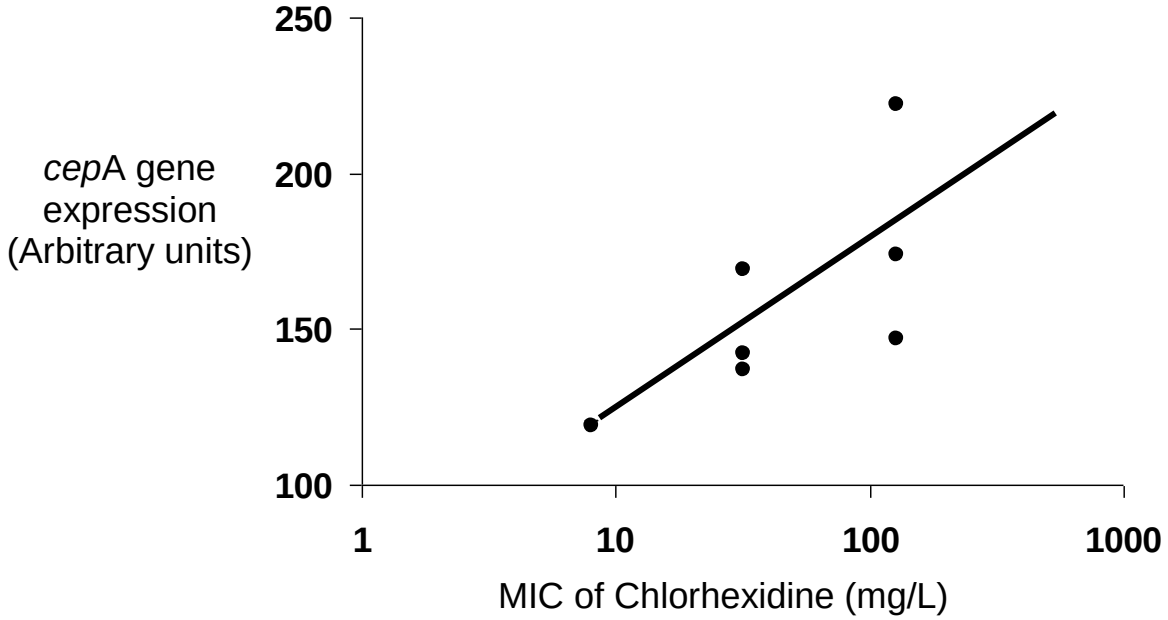


Figure 33. Correlation between the MIC of Chlorhexidine and *cep*A expression measured by RT-PCR.

Genetic environment of the *qac*ΔE1 antiseptic resistance gene

The *qac*ΔE1 gene was amplified by PCR in 34 isolates (Figure 27). The *sul*1sulfonamide resistance gene was detected directly downstream of the *qac*ΔE1 gene in 4 of 34 isolates (Figure 34 and 35). An open reading frame with no known function (*orf*5) was detected directly downstream of the *sul*1 gene in two isolates 11 and 17 (Figure 34 and 37); however isolate number 26 had the chromate resistance protein gene (*chr*A) downstream of the *sul*1 gene (Figure 34 and 39). The alignment sequences of the nucleotides of downstream *qac*ΔE1gene (*sul*1, *orf*5 and *chr*A are shown in Figures 36, 38 and 40 respectively.

The aminoglycoside adenyltransferase gene (*aad*A1), was found directly upstream of *qac*ΔE1 in 4 of 34 isolates possessing this gene (Figure 34 and 41), further analysis revealed that three of these isolates (11, 17 and 26) had the dihydrofolate reductase *dhfr*A1 gene directly upstream of *aad*A1(Figure 34 and 43). Two of these isolates (11 and 17) had an intergenic region (IGR) between the *aad*A1 and the *qac*ΔE1 genes, which was lacking in the other two isolates (26 and 34) (Figure 34 and 45). The nucleotide sequence alignments of the region upstream of *qac*ΔE1gene (*aad*A1, *dhfr*A1 and IGR) are shown in Figures 42, 44 and 46 respectively.

Isolate Numbers

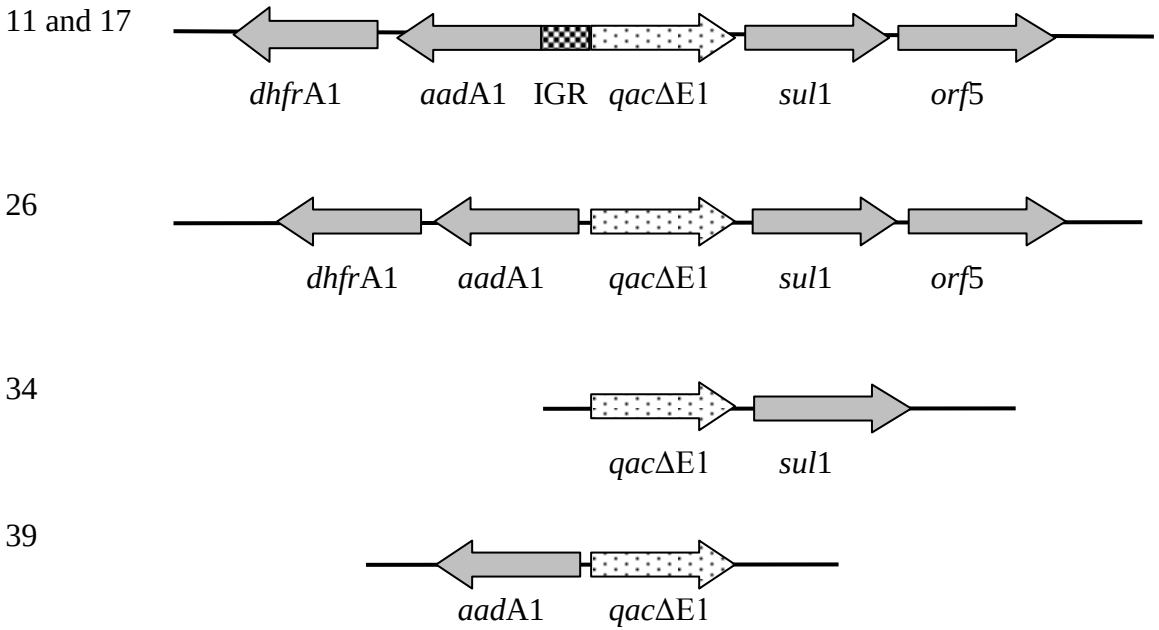


Figure 34. Schematic representation of the genetic environment of the antiseptic resistance gene *qac*ΔE1 in *K. pneumoniae*.

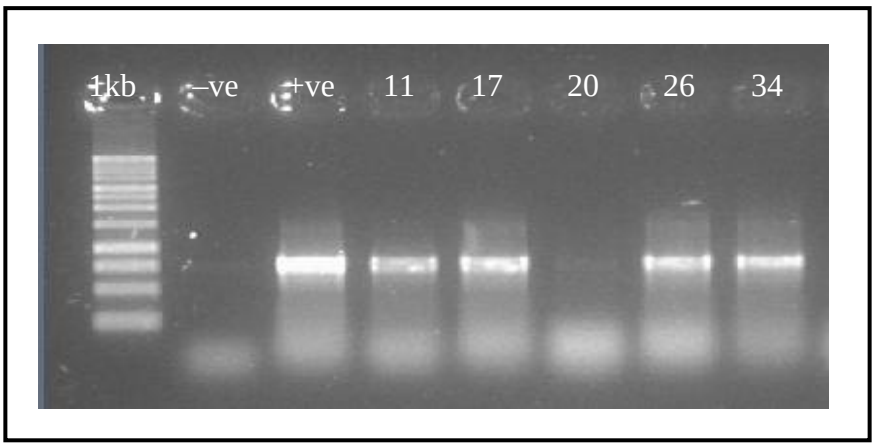


Figure 35. PCR amplification of four isolates of *K. pneumoniae* (11, 17 26 and 34) which carried the *qac*ΔE1→*sul*1 genes, A 1 kilo base DNA ladder in lane 1, A negative control in lane 2, lane 3 positive control *A. baumannii* ATCC 19606.

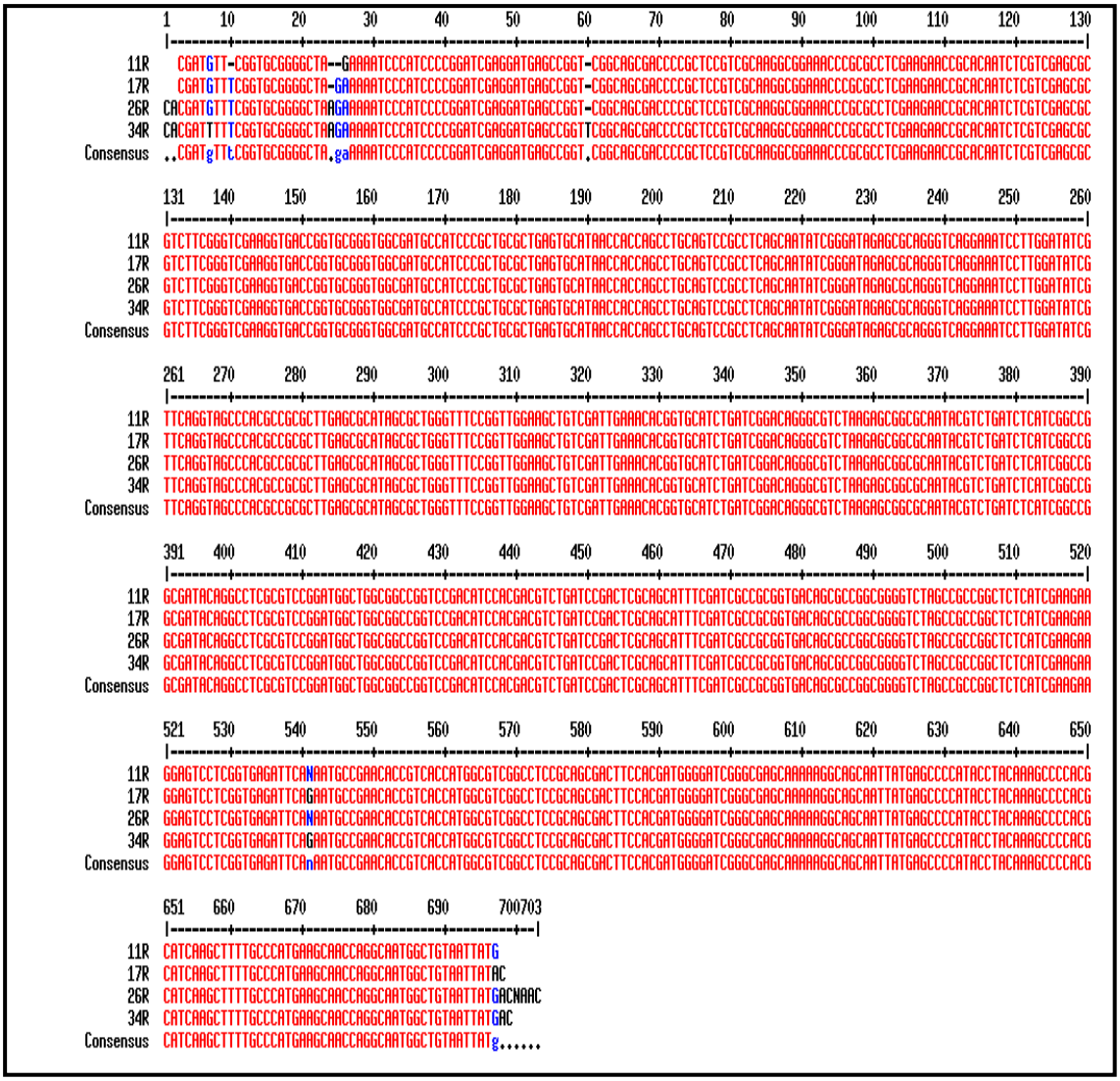


Figure 36. The alignment of nucleotide sequences of *qac*ΔE1→*sul*1 genes from four isolates of *K. pneumoniae* (11, 17 26 and 34).

The red coloured letters indicate nucleotide sharing consensus. The blue colured letters indicate low consensus areas.

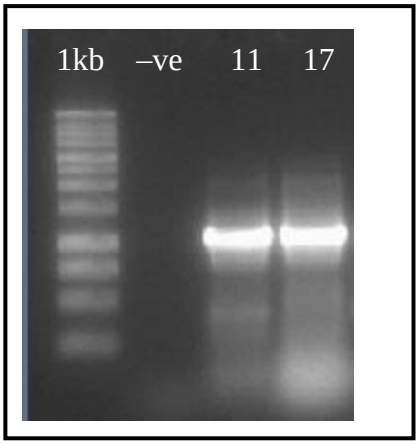


Figure 37. PCR amplification of two isolates *K. pneumoniae* (11 and 17) has *sul*1→*orf*5 genes, A 1kb DNA ladder is in lane 1, The negative control is in lane2.

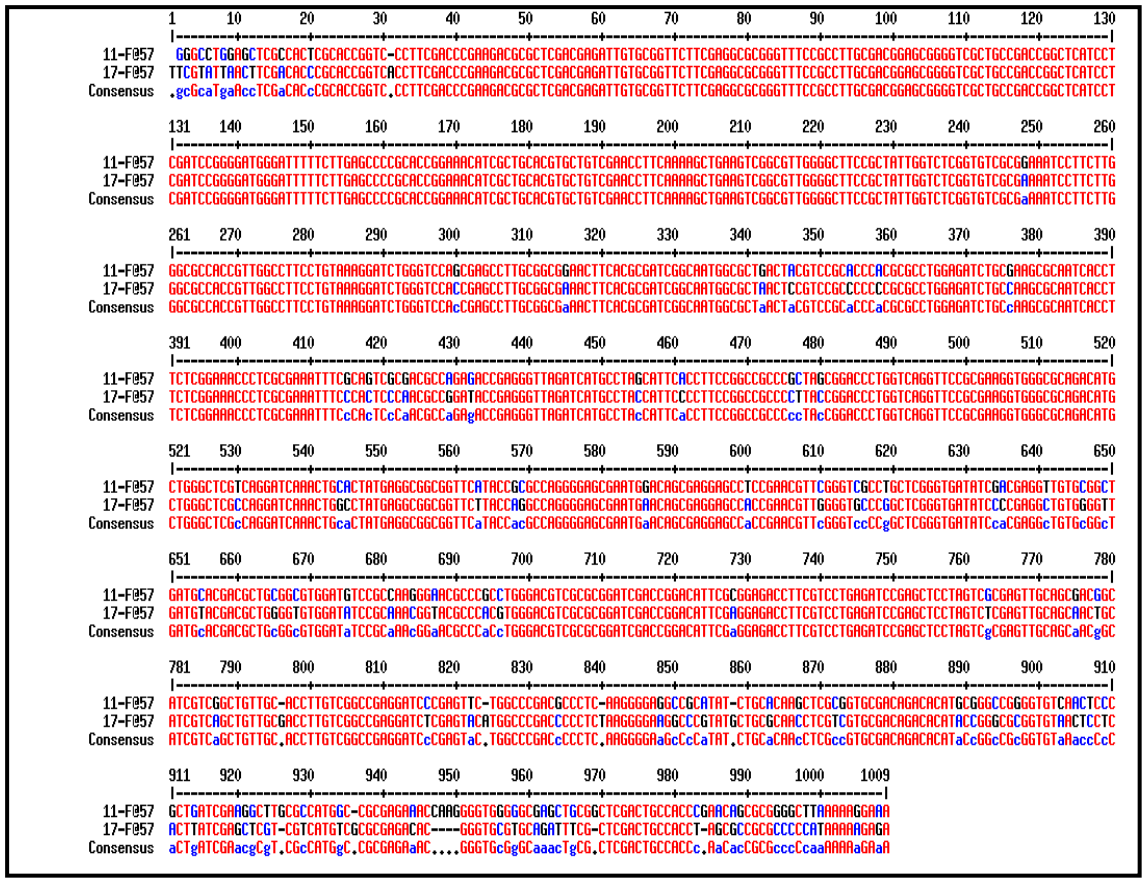


Figure 38. The nucleotide sequence alignments of *sul*1→*orf*5 genes from two isolates *K. pneumoniae* (11 and 17).

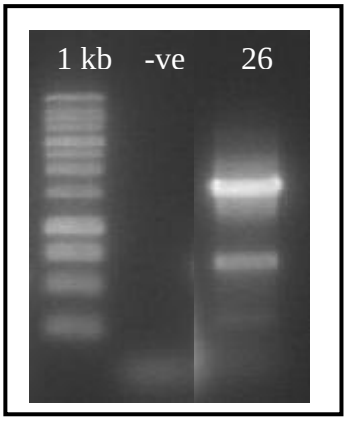


Figure 39. PCR amplification of isolates number 26 *K. pneumoniae* carried *sul*1→*chr*A genes, A ladder 1kb marker is in lane 1, A negative control is in lane 2.

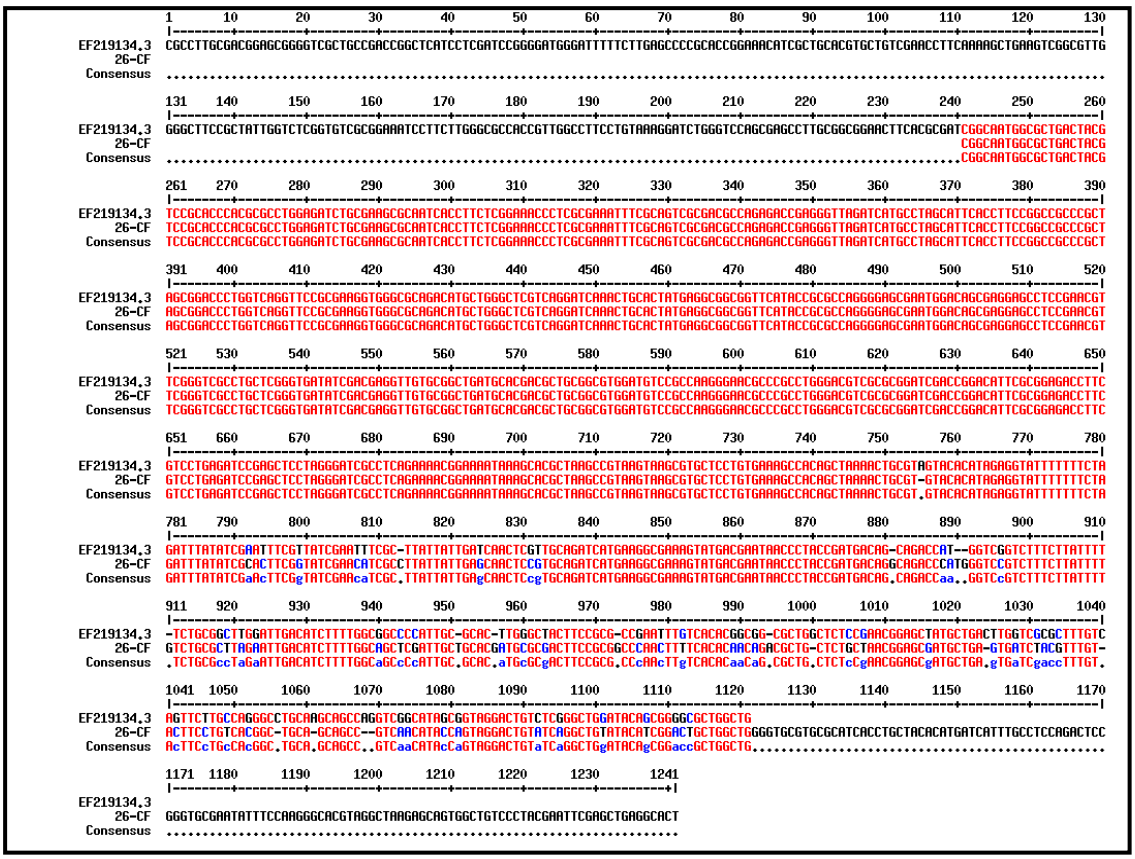


Figure 40. The nucleotide sequence of *sul*1→*chr*A genes from isolates number 26 *K. pneumoniae* aligned with *K. pneumoniae* EF21934.4.

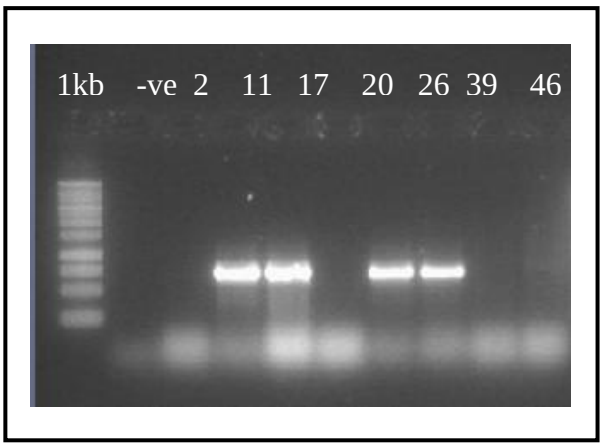


Figure 41. PCR amplification of four isolates of *K. pneumoniae* (11, 17, 26 and 39) are shown *aad*A1→*qac*ΔE1genes, A 1kb DNA ladder is in lane 1, A negative control is in lane 2.

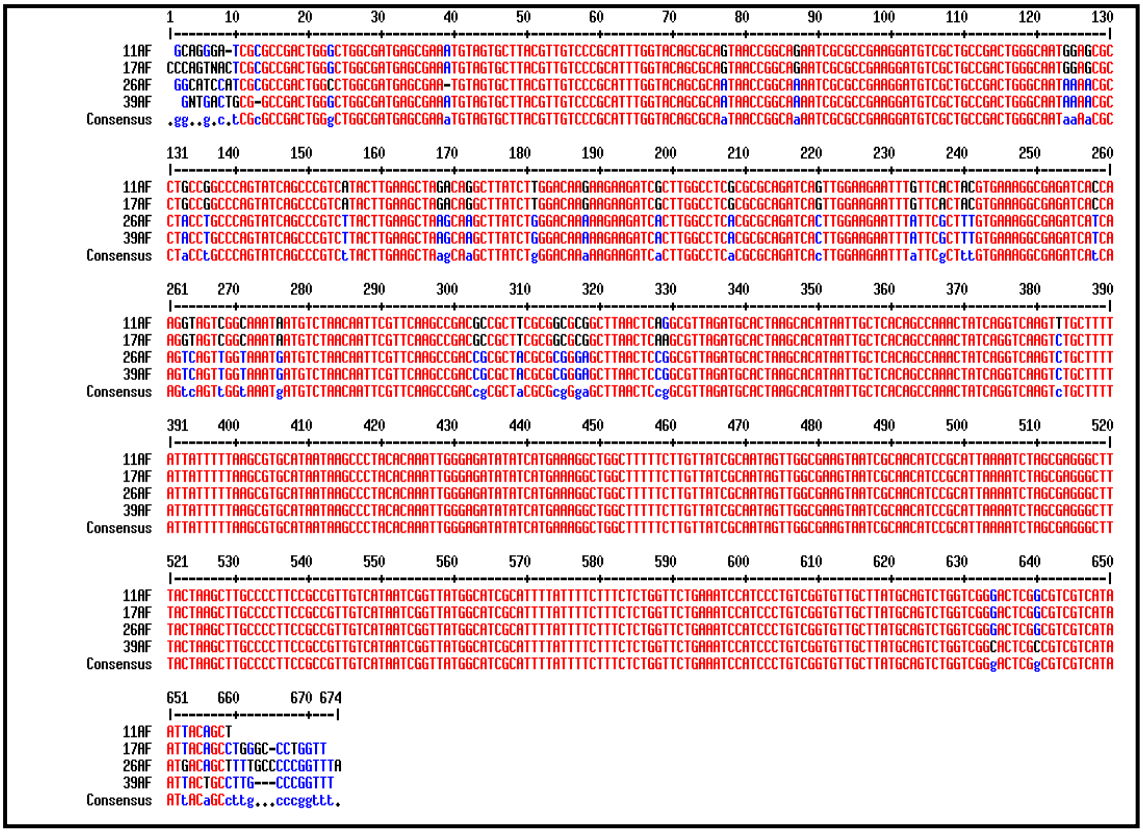


Figure 42. The alignment nucleotide sequences of *aad*A1→*qac*ΔE1genes from four isolates of *K. pneumoniae* (11, 17, 26 and 39).

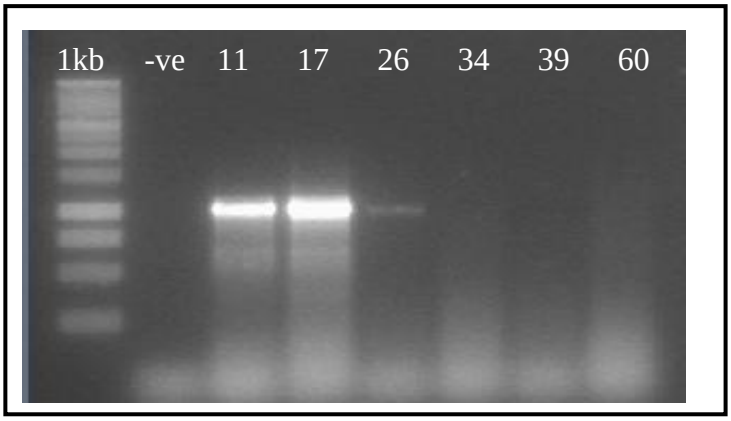


Figure 43. PCR amplification of three isolates of *K. pneumoniae* (11, 17and 26) showing the *dhfr*A1→*aad*A1 genes, A ladder 1kb marker is in lane 1, A negative control is in lane 2.

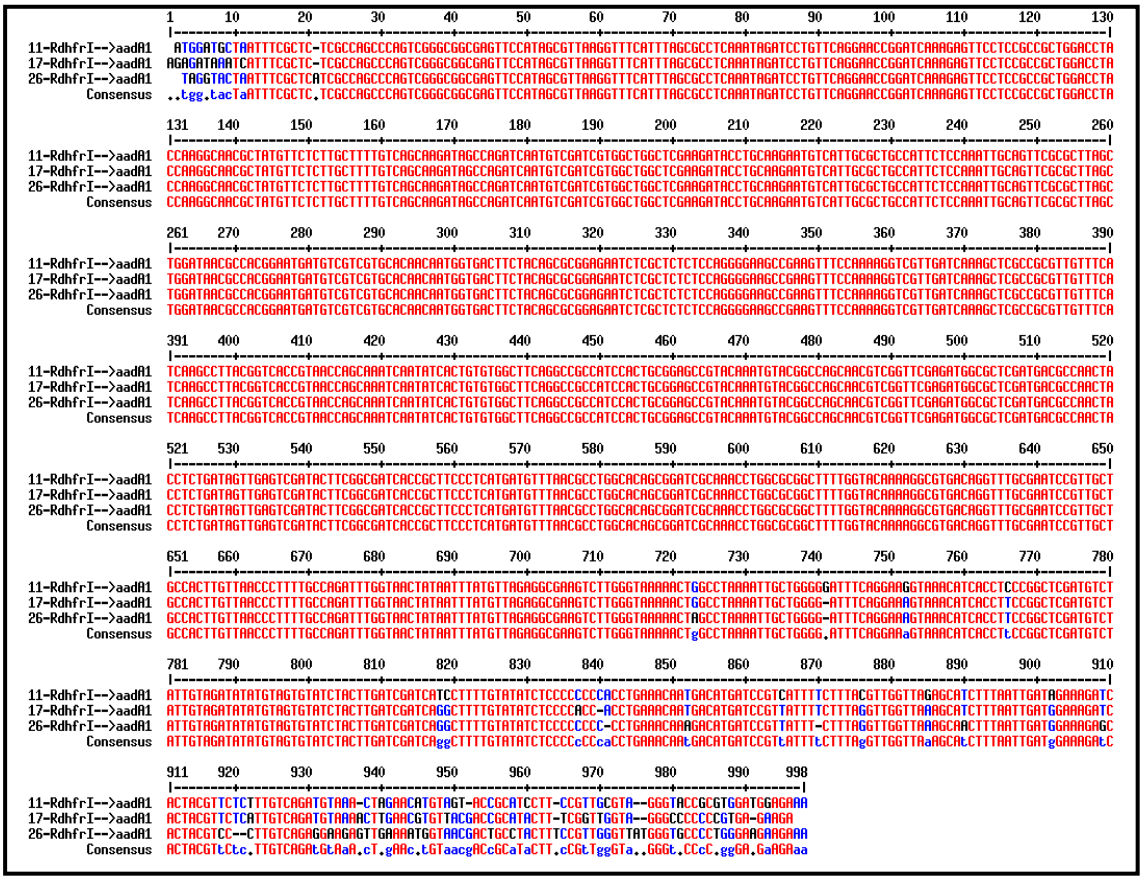


Figure 44. The nucleotide sequence alignments of *dhfr*A1→*aad*A1 genes from of three isolates of *K. pneumoniae* (11, 17and 26).

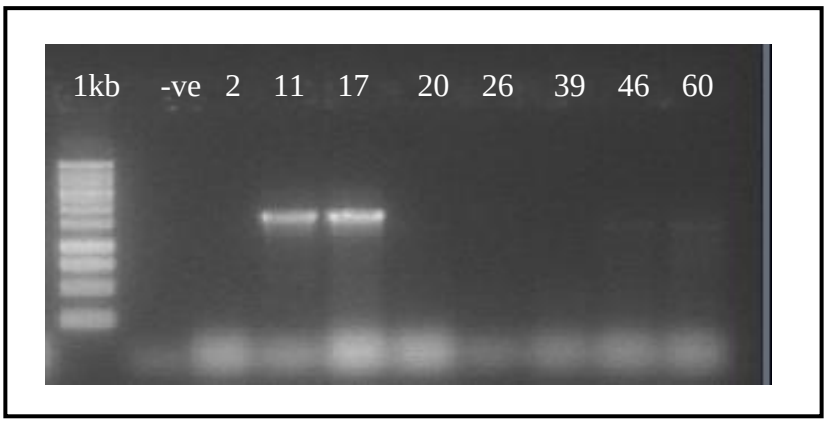


Figure 45. PCR amplification of two isolates of *K. pneumoniae* (11 and 17) which have an Intergenic region→ *aad*A1, A 1kb DNA ladder isin lane 1, A negative control is in lane 2.

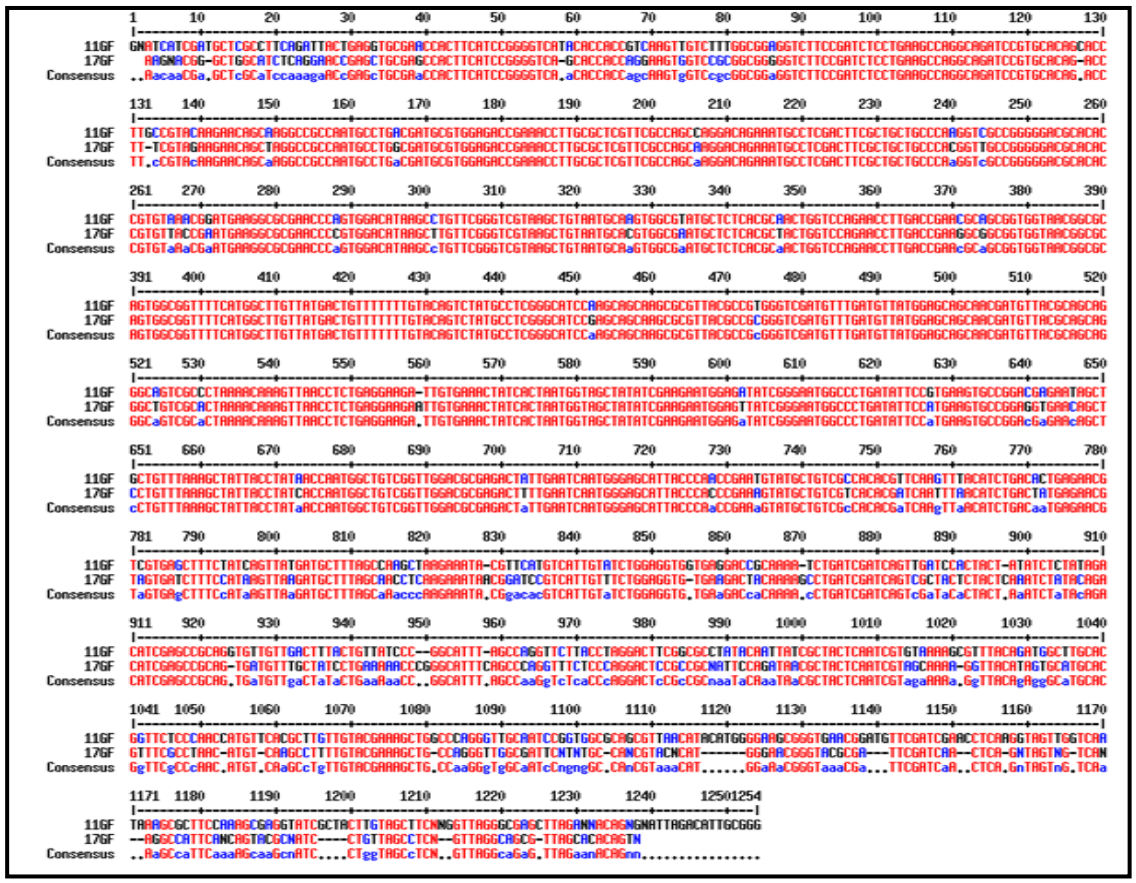


Figure 46. The alignment of nucleotide sequences of Intergenic region→ *aad*A1genes from two isolates of *K. pneumoniae* (11 and 17).

PFGE analysis

When examined by PFGE, isolates 26 and 39 showed virtually identical patterns (> 95%), suggesting that they are the same strain; whereas, isolates 11 and 34 demonstrated similar, but not identical, band patterns (80%) indicating that they are part of the same clonal group. Isolate 17 was an unrelated strain (Figures 47 and 48).

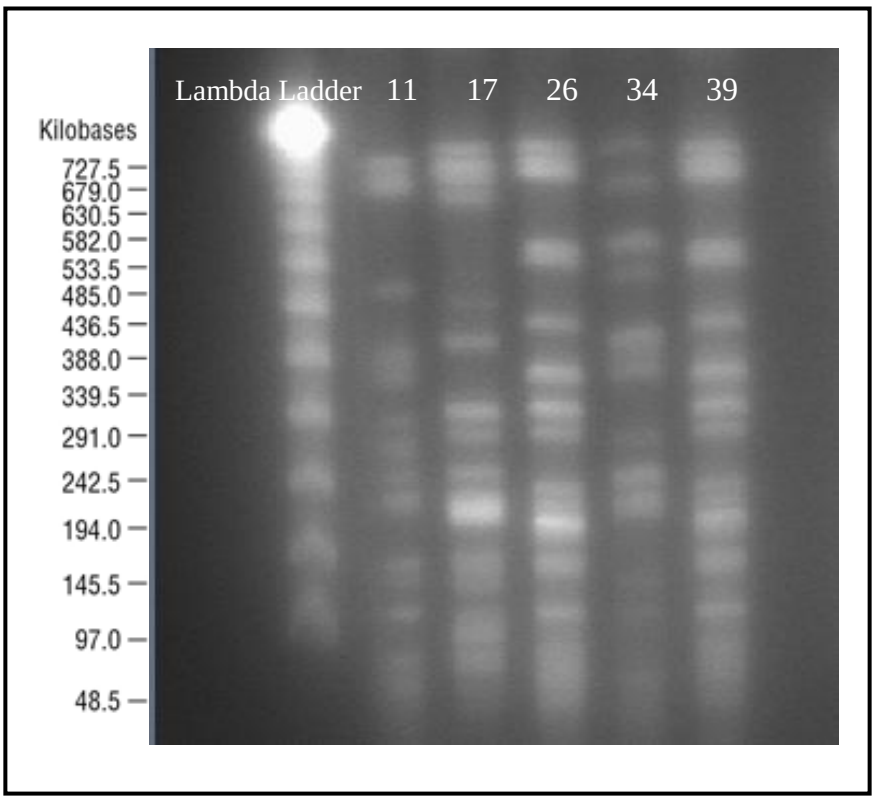


Figure 47. The pulsed-field typing of strains 11,17,26,34 and 39 after digestion with *XbaI*.

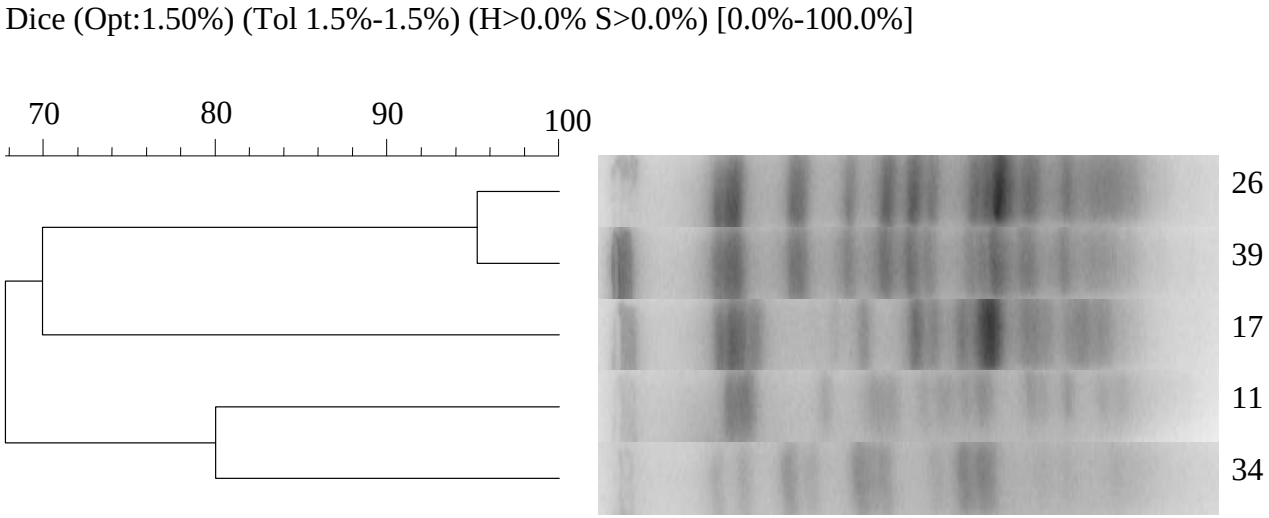


Figure 48. Cluster analysis of *K. pneumoniae* isolates 11,17,26,34 and 39 after pulsed-field gel electrophoresis.

Discussion and Conclusion

Discussion

The increasing incidence of *K. pneumoniae* infectious in hospitals worldwide has led to a greater awareness of the hazards of nosocomial infections and resulted in increased infection control measures.

Biocides are currently considered to be an essential component of infection control strategies in hospitals (Smith *et al*., 2008), and there is very little in the published literature about the effects of chlorhexidine and other antiseptics on clinical strains of *K. pneumoniae*. Block and Furman (2002) have observed a significant inverse relationship between the intensity of chlorhexidine use and the overall susceptibility of organisms (*S. aureus*, coagulase-negative staphylococci, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii* and *Candida albicans* to this antiseptic (Block and Furman, 2002, Smith *et al*., 2008). Furthermore, Koljalg *et al* (2002) found good correlation between chlorhexidine and antibiotic susceptibility in 70 distinct clinical isolates. They studied MBCs and MICs of small numbers of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *S. aureus* (not MRSA), *Streptococcus pyogenes* and *Enterococcus faecalis*. They noted that non-fermentative Gram-negative bacteria tolerated chlorhexidine at high concentrations better than the *Enterobactericeae* and that Gram-positive cocci, especially *S. pyogenes*, were the most susceptible (Koljalg *et al*., 2002).

The efficacy of five biocides, used extensively in hospitals, was screened against 64 clinical isolates of *K. pneumoniae*. The disinfectants showed susceptibility to Mediscrub except strain 64, the MIC being determined to the concentration of polymeric biguanide hydrochloride (triclosan). Mediscrub was the most effective biocide against *K. pneumoniae* compared with the others tested, with MICs between 0.12 and 0.5mg/L Mediscrub is used for washing hands and the surfactants, present to reduce the surface tension, clearly are also have an effect on the viability of the bacterial cells.

The main component of Medihex-4 is chlorhexidine gluconate and it was the MIC of this compound that was measured; however, the MICs of Medihex-4 were lower than that of the Chlorhexidine preparation. Medihex-4 also contains cocamidopropyl betaine and sodium lauryl sulphate as surfactants and these may also contribute to the reduced MICs found with this preparation. On the other hand, Benzalkonium chloride and Trigene were the least effective, the activity was also boosted by the surfactant sodium lauryl sulphate. The strains were less sensitive to Trigene, which also has polymeric biguanide hydrochloride as a main active component. Again the MICs were determined to the concentration of polymeric biguanide hydrochloride, but they were higher than those of Mediscrub.

The MICs of the polymeric biguanide hydrochloride antiseptics, Mediscrub and Trigene, were much lower than the MBCs, in some cases by 10,000-fold. The MBC of Mediscrub and Trigene was 9000mg/L and 1800mg/L respectively, 500-fold greater than the minimum inhibitory concentration (MIC); this strongly indicates that these antiseptics are mainly primarily bacteriostatic in their action against *K. pneumoniae* and to obtain a rapid kill, large quantities would have to be used. This suggests that in clinical concentrations they have little capacity to eradicate *K. pneumoniae*. Although the MIC of Benzalkonium chloride was high, the MBC was not much higher indicating that one the concentration threshold of inhibiting the bacterium had been reached, a three-fold increase was sufficient to kill the bacteria quickly.

On the other hand, the MBCs of Chlorhexidine and MediHex-4, which contains chlorhexidine, were < 360mg/L and < 36mg/L correspondingly, the differential between the MICs and the MBCs of the two chlorhexidine compounds was low and a 5-fold increase above the MICs of chlorhexidine was sufficient to elicit a rapid bactericidal response, which would be important in clinical practice.

The presence of organic matter, represented here by Bovine serum albumin, suggests that Chlorhexidine is very sensitive to its presence though the extent of this will vary from strain to strain. In order of that clinical isolates of *K. pneumoniae* may not respond to common biocides, which may not kill the bacteria especially in the presence of organic matter.

The presence of the *qac*ΔE1 and *cep*A genes is considered to be linked to high level MICs of biocides, the genetic environment of the two major antiseptic resistance genes, *qac*ΔE1 and *cep*A, in *K. pneumoniae*. About half of the isolates carried the *qac*ΔE1 gene and this was usually located upstream of the *sul*1 sulphonamide resistance gene and directly downstream of the aminoglycoside adenyltransferase gene (*aad*A1), which was flanked by the dihydrofolate reductase gene *dhfr*A1.

The *qac*ΔE1 gene appears to be part of a small resistance island suggesting that this gene is linked and migrates with antibiotic resistance genes. The close linkage of *qac* genes to antibiotic resistance genes has been identified before in resistance island. In the largest resistance island reported to date, in *A. baumanii* strain AYE, there are four *qac* genes in the 45 gene resistance island (Fournier *et al*., 2006), suggesting that there is strong selective pressure for these genes. The widespread carriage of *qac* genes in *K. pneumoniae*, and their linkage to antibiotic resistance genes suggests that widespread use of biocides, particularly as antiseptics, could select antibiotic resistant strains.

The *cep*A gene was much more prevalent than the *qac*ΔE1, it was present in 88% of the isolates. This gene has only once been associated with chlorhexidine resistance (Fang *et al*., 2002). In many isolates, downstream of *cep*A, there was a gene encoding 6-phosphofructokinase-1 (*pfk*A) and often a methyltransferase gene (*men*G) beyond that. More interestingly, in many of these isolates, the *cep*A gene was located behind *cpx*R/*cpx*P transcription regulator genes and these are responsible for the gene expression.

The results show that as the MIC increases so does the expression of *cep*A. The almost universal carriage of the *cep*A gene in *K. pneumoniae* and, as this study indicates, the expression of which is linked to the Chlorhexidine MIC indicates that much of the *K. pneumoniae* population carries the potential for resistance. If biocides are used as recommended, the concentration of the biocide should far exceed the MIC of even the resistant strains. However, many biocides are not used appropriately and this will select out resistant strains, as indicated by the almost universal carriage of the *cep*A gene.

The MIC assays performed in the presence of efflux pump inhibitor (EPI) cyanide m-chlorophenyl hydrazone (CCCP), showed that there was a considerable decrease in the Chlorhexidine MICs for almost all the strains and this was usually associated with the presence of *cep*A. On the other hand, there were reductions of the MediHex-4 MICs, but these were not as great as with Chlorhexidine, and not significantly associated with the presence of either the *cep*A or *qac* genes.

This suggests that efflux pumps, as yet undefined, are capable of exporting MediHex-4 but this does not play an important role in MediHex-4 susceptibility in these *Klebsiella* strains. The lack of influence of CCCP on the MICs of Benzalkonium chloride, Trigene and Mediscrub suggest that other resistance genes are more important, particularly for Benzalkonium chloride and Trigene. It was interesting to note though that for both these antiseptics there was a high proportion of resistant strains that had both the *cep*A and *qac*ΔE1 genes.

It is interesting to note that the proportion of reduced biocide susceptibility was high and the carriage of biocide resistance genes was also high. This suggests that the *Klebsiella* population in the Infirmary has been exposed to a considerable quantity of biocides and that reduced susceptibility is creeping into the population. However, the clinical importance of this may be doubtful as the concentration of biocides used is usually far greater than the MICs, even of the strains with reduced susceptibility.

This is likely to be become more important when other factors are taken into account, such as biofilm formation, which already compromise the activity of the biocide (Smith and Hunter, 2008,Tumah, 2009). Furthermore, this population was not noticeably resistant to the antibiotics tested and these results are in contrast with those of Koljalg *et al*.; however, in that study only a very small proportion of the strains tested were *Klebsiella* spp. (Koljalg *et al*., 2002). There is evidence in other species that biocide resistance genes can be closely linked to antibiotic resistance genes and, if that occurs in this species in the future, the use of biocides could select for antibiotic-resistant strains (Fournier *et al*., 2006).

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Appendix A

Minimum Inhibitory Concentrations (MICs) antibiotics, antiseptics, *cep*A and *qac*ΔE1 susceptibility of *K. pneumoniae.*

ID	MICs mg/ L															Disc diffusion test	Antiseptic resistance genes	
	Antiseptics					Antibiotics												
	CHX	Tri	MH-4	MS	BZK	CTX	CTZ	CXT	IMP	MEM	COL	RIF	GEN	Pol-B	TRI	CHL		*cep*A	*qac*ΔE1
1	32	32	8	0.12	32	0.12	0.25	16	0.12	0.015	4	64	0.5	1	0.5	8	AMP,CAR, E, DO, PRL	+	+
2	64	32	16	0.12	32	0.06	0.25	8	0.5	0.015	8	64	0.5	1	0.5	4	AMP, DO	+	-
3	32	32	8	0.12	32	0.03	0.12	8	0.25	0.015	8	32	0.25	1	0.5	4	AMP,CAR, PRL	+	+
4	32	32	8	0.12	64	0.12	0.25	4	0.25	0.015	8	16	0.5	1	0.5	4	AMP,CAR, DO	+	+
5	32	32	8	0.12	32	0.03	0.25	4	0.12	0.015	8	16	0.25	1	0.5	4	AMP,CAR, DO	+	+
6	64	32	8	0.12	32	0.03	0.5	8	1	0.015	8	64	0.25	1	0.5	8	AMP, DO	+	-
7	32	16	2	0.12	32	0.06	0.25	4	0.25	0.03	8	64	0.25	1	0.5	4	AMP	-	-
8	16	32	8	0.12	32	0.12	1	16	1	0.03	8	16	1	1	2	4	AMP,CAR, DO, PRL	+	+
9	32	32	8	0.12	32	0.12	0.25	8	1	0.015	8	32	0.5	1	0.5	8	AMP	+	+
10	64	32	8	0.12	32	>128	>128	>128	0.12	0.015	8	16	0.25	1	0.5	4	AMP,CAR, PRL	+	-
11	32	32	8	0.12	32	0.03	0.25	4	0.25	0.015	8	64	0.25	1	>128	4	AMP,CAR, DO	+	+
12	64	32	8	0.12	32	0.12	0.25	16	0.12	0.06	4	16	0.5	1	0.5	16	AMP, DO	+	+
13	32	32	8	0.25	32	0.03	0.12	4	0.12	0.015	4	32	0.25	1	0.5	8	AMP	+	-
14	4	16	8	0.12	32	0.12	0.25	8	0.12	0.015	4	8	0.25	1	0.12	8		-	-

15	32	64	8	0.5	64	32	128	64	0.25	0.015	8	16	0.12	1	>128	>128	AMP,CAR, DO, NV, PRL	+	+
16	8	32	8	0.5	64	0.12	0.5	8	0.12	0.015	8	64	0.25	1	0.25	4	AMP	+	-
17	64	64	8	0.12	32	0.25	1	16	0.25	0.015	8	64	0.25	1	>128	128	AMP, CAR, DO, PRL, NV	-	+
18	64	64	8	0.25	32	0.06	0.5	16	0.25	0.015	8	16	0.25	1	0.5	4	AMP, CAR, DO, PRL	+	-
19	64	32	8	0.12	32	0.06	0.25	4	0.25	0.015	8	32	0.25	1	0.5	4	AMP	+	-
20	32	32	16	0.5	32	0.06	0.12	8	0.25	0.015	8	32	0.25	1	0.5	4	AMP, CAR, DO	+	+
21	32	32	8	0.12	32	0.03	0.12	4	0.5	0.03	8	32	0.25	1	0.5	4	AMP, DO	+	+
22	64	32	8	0.12	32	0.12	0.25	4	0.5	0.015	8	32	0.25	1	0.5	4	AMP, CAR, DO, PRL	+	+
23	64	32	16	0.5	32	0.03	0.12	2	0.25	0.015	8	64	0.25	1	0.5	4	AMP, CAR, DO, PRL	+	-
24	128	64	8	0.5	32	0.03	0.5	4	0.25	0.03	8	64	0.25	1	0.5	8	AMP, CAR, DO, PRL, E	+	+
25	64	16	8	0.5	32	0.06	0.25	8	0.12	0.03	8	32	0.25	2	0.5	4	AMP, CAR	+	*qac*E
26	32	32	8	0.25	32	>128	>128	8	0.25	0.03	4	32	64	1	>128	4	ATM, AMP, CAR, DO, E, PRL	+	+
27	64	16	8	0.12	32	>128	64	8	0.5	0.03	8	32	0.12	1	0.5	8	AMP, CAR, DO, PRL	+	-
28	64	64	8	0.12	64	0.25	1	8	0.25	0.03	8	32	0.25	1	128	8	AMP, CAR, DO, PRL, E	+	+
29	32	32	8	0.5	32	0.06	1	16	0.25	0.015	8	64	0.25	1	0.5	4	AMP, CAR, PRL	+	-
30	32	32	8	0.12	32	0.06	1	8	1	0.03	4	64	1	1	0.5	8	AMP, CAR, DO, PRL	+	+
31	32	64	8	0.12	64	0.03	0.5	4	0.25	0.015	4	32	0.25	1	0.5	4	AMP, DO	+	+
32	32	64	16	0.5	32	0.12	0.12	4	0.12	0.015	4	32	0.25	1	0.5	4	AMP, DO	+	-

33	32	32	8	0.12	32	0.06	0.25	8	0.12	0.015	4	32	0.25	1	0.5	8	AMP, DO	+	-
34	32	32	8	0.12	32	0.06	0.25	8	0.25	0.03	4	16	0.12	1	>128	4	AMP, CAR, DO, PRL	+	+
35	32	32	4	0.12	32	0.12	0.25	8	0.5	0.03	8	16	0.25	1	0.5	4	AMP, CAR, DO, PRL	+	+
36	32	32	8	0.12	32	0.06	0.5	16	1	0.03	8	32	0.5	1	0.5	8	AMP, CAR, DO, PRL	+	+
37	128	32	8	0.12	32	0.06	0.5	8	0.12	0.015	8	32	0.5	1	0.5	8	AMP, CAR, DO, E	+	-
38	32	32	8	0.12	32	0.12	0.5	16	1	0.03	4	16	4	1	1	8	AMP, CAR, DO, PRL	+	+
39	32	64	4	0.25	32	>128	128	8	0.25	0.03	8	16	64	1	>128	4	ATM, AMP, CAR, DO, E,PRL	-	+
40	32	32	16	0.12	32	>128	64	8	0.12	0.03	8	32	64	1	>128	4	AMP, CAR	+	+
41	64	16	8	0.12	32	0.5	64	16	0.5	0.015	4	16	0.25	1	0.25	4	AMP	+	-
42	32	32	8	0.12	64	>128	64	4	0.25	0.015	4	32	0.25	1	0.5	8	AMP, CAR, DO, PRL	-	-
43	32	32	8	0.12	32	0.06	0.25	4	0.5	0.015	8	32	0.25	1	0.5	8	AMP	+	+
44	64	32	8	0.25	32	0.06	0.12	8	0.25	0.03	4	32	0.5	2	0.25	4	AMP, CAR, DO	+	+
45	64	32	16	0.25	32	0.12	0.25	4	0.12	0.015	8	32	0.25	1	0.5	4		+	+
46	64	16	16	0.25	32	0.06	0.25	8	0.25	0.03	4	32	0.25	4	0.5	4	AMP, CAR, DO	+	+
47	32	16	8	0.5	32	0.03	0.12	8	0.25	0.015	4	16	0.5	1	0.5	4	AMP, CAR	+	-
48	64	32	8	0.5	64	>128	128	>128	1	0.015	4	16	0.5	0.5	8	4	AMP, CAR, NV, PRL	+	+
49	16	32	4	0.5	16	0.06	0.25	4	0.5	0.015	4	64	0.5	2	0.5	4	AMP, CAR, DO	+	+
50	32	32	16	0.12	16	0.03	0.25	4	0.5	0.015	8	32	0.25	1	0.25	4	AMP	+	-
51	32	32	16	0.12	64	0.12	0.5	8	1	0.03	4	64	0.5	1	0.5	4	AMP	+	-
52	128	64	16	0.5	64	0.5	1	64	1	0.015	4	64	0.25	1	16	>128	AMP, CAR, DO, PRL, E, NV, TCG	+	-

53	64	16	16	0.12	32	0.03	0.5	32	0.25	0.015	4	32	0.25	1	0.5	4	AMP, CAR	+	-
54	64	32	8	0.12	16	0.06	0.25	8	1	0.015	4	32	0.25	1	0.5	4	AMP, CAR	+	-
55	32	64	8	0.12	64	0.03	0.25	4	0.25	0.015	4	32	0.25	1	0.5	4	AMP	-	-
56	16	64	8	0.12	64	0.5	0.5	32	0.25	0.015	4	32	0.25	1	8	128	AMP, CAR, DO, PRL	+	+
57	32	64	16	0.12	32	0.5	0.5	32	0.25	0.03	4	32	0.5	1	0.5	4	AMP, CAR	+	+
58	16	16	4	0.12	32	0.5	1	8	0.5	0.06	8	32	0.25	1	0.5	4	AMP, CAR, DO, PRL	+	-
59	32	32	4	0.12	32	0.06	0.25	16	0.12	0.015	4	32	0.5	1	0.5	8	AMP, CAR	+	-
60	32	32	8	0.12	64	0.06	0.25	4	0.25	0.015	4	32	0.5	1	0.5	4	AMP, CAR, DO	+	-
61	32	64	8	0.12	64	0.06	1	8	0.5	0.015	8	64	0.5	8	8	16	AMP, CAR, DO, PRL	-	-
62	32	32	8	0.12	32	0.03	0.25	4	0.25	0.015	2	32	0.25	1	0.25	4	AMP	-	-
63	16	32	8	0.12	32	0.06	0.25	4	0.25	0.015	4	32	0.5	1	0.25	4	AMP, CAR, DO, NET	+	+
64	64	64	16	>128	32	0.06	0.25	8	0.12	0.015	4	64	0.5	1	0.5	4	AMP, CAR	+	+

CHX, Chlorhexidine; MH-4, MediHex-4; MS, Mediscrub; BZK, Benzalkonium chloride; CTX, Cefotaxime; CTZ, Ceftazidime; CXT, Cefoxitin; IMP, Imipenem; MEM, Meropenem; COL, Colistin; RIF, Rifampicin; GEN, Gentamicin; Poly-B, Polymyxin B; TRI, Trimethoprim; CHL, Chloramphenicol.

a: Double dilution mg/L b: Disc diffusion test

Appendix B

Published papers and Abstracts of conferences

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Klebsiella pneumoniae susceptibility to biocides and its association with *cep*A, *qac*ΔE and *qac*E efflux pump genes and antibiotic resistance

A. Abuzaid, A. Hamouda, S.G.B. Amyes*

Molecular Chemotherapy, Centre for Infectious Diseases, University of Edinburgh, Edinburgh, UK

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SUMMARY

Background: Although antiseptics are some of the most widely used antibacterials in hospitals, there is very little information on reduced susceptibility to these biocides and its relationship with resistance to antibiotics.

Aim: To determine the relationship between reduced susceptibility to biocides and the carriage of antiseptic resistance genes, *cep*A, *qac*ΔE and *qac*E, as well as identifying the role of efflux pumps in conferring reduced susceptibility.

Methods: Susceptibility was assessed for five biocides: chlorhexidine, benzalkonium chloride, Trigene, MediHex-4, Mediscrub; and for 11 antibiotics against 64 isolates of *Klebsiella pneumoniae*. Susceptibility to all compounds was tested by the agar double dilution method (DDM) and the effect of efflux pumps on biocides determined by repeating the susceptibility studies in the presence of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). The presence of the *cep*A, *qac*ΔE and *qac*E genes was identified by polymerase chain reaction.

Findings: The bacteria were not widely antibiotic resistant though a few showed reduced susceptibility to cefoxitin, chloramphenicol and rifampicin and later-generation cephalosporins but not to carbapenems. Biocide susceptibility, tested by DDM, showed that 50, 49 and 53 strains had reduced susceptibility to chlorhexidine, Trigene and benzalkonium chloride, respectively. The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were found in 56, 34 and one isolates respectively and their effects as efflux pumps were determined by CCCP (10 mg/L), which decreased the minimum inhibitory concentrations (MICs) of chlorhexidine and Medihex-4 by 2–128-fold but had no impact on the MICs of benzalkonium chloride, Trigene and Mediscrub.

Conclusion: There was a close link between carriage of efflux pump genes, *cep*A, *qac*ΔE and *qac*E genes and reduced biocide susceptibility, but not antibiotic resistance, in *K. pneumoniae* clinical isolates.

Introduction

Klebsiella pneumoniae causes about 8% of hospital-acquired infections (nosocomial infections) including pneumonia, wound infections, diarrhoea and urinary tract infections.[1,2] The severity of these infections has increased as outbreaks have

* Corresponding author. Address: Molecular Chemotherapy, Medical Microbiology, University of Edinburgh, Room FU.427, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK. Tel.: +44 131 242 6652; fax: +44 131 242 6611.
E-mail address: s.g.b.amyes@ed.ac.uk (S.G.B. Amyes).

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occurred with multiply-resistant strains often producing extended-spectrum beta-lactamase enzymes (ESBLs), with associated increases in morbidity and mortality especially in intensive care units (ICUs), neonatal units and surgical wards.[1–6]

Hand contamination of hospital staff such as nurses and doctors is one of the most common modes of transmission of *K. pneumoniae* (17% ICU staff). Casewell and Phillips found that in order to combat these infections, the staff frequently washed their hands with cationic biocides (such as chlorhexidine), which produces a 98–100% reduction in the number of patients infected with *K. pneumoniae*.[7] Recently, a considerable effort has been made to improve standards of infection control within hospitals, leading to the increased use of biocides as disinfectants and antiseptics. In specific cases judicious biocide use has led to considerable reductions in numbers of *K. pneumoniae*; however, the general trend is that infections caused by multi-resistant *K. pneumoniae* are increasing, and this raises concerns that intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not just to themselves but also to antibiotics.[8–12]

It is difficult to define the clinical relevance of biocide resistance as the concentrations used clinically are often higher than those required to inhibit the organism. However, a series of mitigating factors, such as the local dilution policy, presence of organic matter, formation of biofilms and length of exposure, could comprise the effective concentration of the biocide so that resistance may become a problem.[13,14] With reference to biocides, it is therefore more appropriate to consider reductions in susceptibility rather than increases in resistance.

This study investigates the correlation between biocide susceptibility and its association with both the efflux pump genes, which have been linked to antiseptic 'resistance' (*cep*A, *qac*ΔE, *qac*E), and resistance to later-generation anti-Gram-negative antibiotics.[15]

Methods

Bacterial strains

Sixty-four strains of *K. pneumoniae* were isolated from different infection sites at Edinburgh Royal Infirmary from 2006 to 2008. Isolates were identified at the Infirmary by Vitek2 and confirmed by the API 20E strip (bioMérieux, Basingstoke, UK).

Minimum inhibitory concentration (MIC)

The antibiotics used were cefotaxime, cefoxitin, colistin, chloramphenicol, gentamicin, polymyxin B, rifampicin and trimethoprim (Sigma, Poole, UK), ceftazidime (GlaxoSmithKline, Brentford, UK), imipenem (Merck, Sharp & Dohme, Huddleston, UK), and meropenem (AstraZeneca, Luton, UK). The common hospital biocides used were 1% chlorhexidine gluconate, a member of the biguanide family, and 1% benzalkonium chloride, a quaternary ammonium compound (QAC), both supplied from Sigma. The commercial biocide preparation Trigene was tested; this has polymeric biguanide hydrochloride as the main active component. The cationic biocide MediHex-4, containing 4% chlorhexidine gluconate, and Mediscrub, containing 1% triclosan, were obtained from Medichem International (Sevenoaks, Kent, UK).[16]

Susceptibility to antibiotics was determined by double dilution method following the guidelines of the British Society for Antimicrobial Chemotherapy.[17,18] MICs were determined using the agar dilution method on Iso-Sensitest (IST) agar (Oxoid Ltd, Basingstoke, UK) and the agar plates were incubated in air at 37°C for 18–20 h.[17,18] *Escherichia coli* NCTC 1048, *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* NCTC 6571 were employed as control reference strains for determination of MIC.

Efflux pump inhibitor and reduced biocide susceptibility

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was purchased from Sigma. In order to determine whether an efflux pump was active, these pump inhibitors were dissolved in small volumes of dimethyl sulphoxide, and the volume made up with distilled water. This was then added to IST agar at 10 mg/L in plates containing increasing concentrations of biocide for minimum inhibitory concentration (MIC) determination. A decrease in biocide MIC indicated the presence of an efflux pump.

Polymerase chain reaction (PCR)

The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were detected by PCR with the primers and the annealing temperatures described below *cep*A primer pairs were designed to amplify 1051 base pair (bp) F5′CAACTCCTTCGCCTATCCCG3′, R5′TCAGGTCAGACCAAACGGCG3′ with annealing temperature 66°C for 30 s.[19] The *qac*ΔE and *qac*E primer pairs F5′GCCCTACACAAATTGGGAGA3, R5′CTGCGGTACCACTGCCAC AA3′; F5′GCCCTACACAAATTGGGAGA3′, R5′TTAGTGGGCACTT GCTTTGG3′ were designed to amplify 370 and 350 bp respectively, all having the same annealing temperature of 49°C for 40 s.[20] The PCR products were analysed on 1.5% of agarose (Fisher Scientific, Loughborough, UK). The gel was stained with GelRed and gel image was taken in tag image file format (JPEG files) with the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

Results

MICs of antibiotics and biocides

The susceptibility of the 64 isolates was determined by measuring the MIC of antibiotics and biocides. Some isolates were reported resistant to cefotaxime, ceftazidime, chloramphenicol, trimethoprim and rifampicin (Table I); however, most isolates were sensitive to third-generation cephalosporins and all were sensitive to imipenem (MIC $\leq$2 mg/L) and meropenem (MIC $\leq$0.12 mg/L) (Table I).

The ranges of MIC for each biocide are shown in Table I. In 51 of the 64 *K. pneumoniae* isolates, there was a considerable decrease in susceptibility to chlorhexidine, benzalkonium chloride and Trigene with MICs ranging from 32 to 128 mg/L and high MIC_{50} values (Table I). However, Medihex-4 showed a moderate level with MICs from 8 to 16 mg/L in 53 isolates; these values were about four-fold lower than for chlorhexidine reflecting the four-fold increase in chlorhexidine concentration

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Table I
Minimum inhibitory concentrations (MICs) of biocides and antibiotics against 64 *Klebsiella pneumoniae* isolates

Antibacterial agent	MIC (mg/L)		
	Range	MIC_{50}	MIC_{90}
Biocide			
Chlorhexidine	4–128	32	64
Benzalkonium chloride	16–64	32	64
Trigene	16–64	32	64
MediHex-4	2–16	8	16
Mediscrub	0.12–128	0.12	0.5
Antibiotics			
Cefotaxime	0.03–128	0.06	128
Ceftazidime	0.12–128	0.25	64
Cefoxitin	2–128	8	32
Imipenem	0.12–1	0.25	1
Meropenem	0.015–0.06	0.015	0.03
Colistin	2–8	8	8
Gentamicin	0.12–64	0.25	0.5
Chloramphenicol	4–128	4	8
Trimethoprim	0.12–128	0.5	128
Rifampicin	8–64	32	64
Polymyxin B	0.5–8	1	1

in this commercial product. Fifty-five isolates had MICs of Mediscrub ≤4 mg/L (Table I).

Antiseptic resistance genes

In order to determine whether the decreased susceptibility could be correlated with specific resistance genes, PCR was used to screen for the antiseptic resistance genes *cep*A, *qac*E and *qac*ΔE, which may have an important role in decreasing susceptibility to antiseptics.[21] Fifty-six isolates contained the *cep*A gene and, in every case, the size of gene fragment was 1051 bp. The *qac*E and *qac*ΔE genes were amplified by PCR; the *qac*ΔE gene was detected in 34 isolates; and *qac*E was found in only one isolate. Thirty-two isolates had both *cep*A and *qac*ΔE, whereas just one isolate had both *cep*A and *qac*E gene and six isolates had no efflux pump genes (Table II).

Table II associates the individual levels of chlorhexidine, Trigene and benzalkonium chloride susceptibility with the presence of individual antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E. Medihex-4 was not tested as its main component is 4% chlorhexidine; the strains were susceptible to Mediscrub, so this biocide was also excluded. High MICs (32–128 mg/L) of chlorhexidine, Trigene and benzalkonium chloride were found in 50, 49 and 53 isolates respectively. In each case the *cep*A gene was present, while *qac*ΔE and *qac*E antiseptic resistance genes were found in 31, 33 and 34 isolates for chlorhexidine, Trigene and benzalkonium chloride respectively.

The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were detected in 56, 34 and one isolates respectively, 32 isolates had both *cep*A and *qac*ΔE and one isolate had both *cep*A and *qac*E whereas six isolates had no antiseptic resistance genes. The association between carriage of the *cep*A, *qac*ΔE and *qac*E genes with significant reductions in biocide susceptibility of the clinical *K. pneumoniae* isolates was only partial, e.g. 51 of 56 isolates had efflux pump genes and had significant reductions in susceptibility (MIC 32–128 mg/L) to chlorhexidine, Trigene and benzalkonium chloride; however, six isolates did not have antiseptic resistance genes, but four of these six isolates had high MICs (32–64 mg/L) of chlorhexidine, Trigene and benzalkonium chloride.

Efflux pump inhibitors and reduced biocide susceptibility

The effects of CCCP (10 mg/L) on the MICs of the biocides was examined. It had no impact on benzalkonium chloride, Trigene and Mediscrub (data not shown); however, it reduced the MICs of chlorhexidine and Medihex-4 by between two- and 128-fold (Tables III and IV). In the case of chlorhexidine, this was associated, except in five cases, with the presence of the *cep*A gene. The presence of CCCP had slightly less effect on Medihex-4 and this was associated, except in eight cases, with the presence of the *cep*A gene. The *qac*ΔE gene was associated with less than half of the strains showing the reduction of chlorhexidine and Medihex-4 MICs.

Table II
Individual minimum inhibitory concentrations (MICs) of chlorhexidine, Trigene and benzalkonium chloride in the presence of *cep*A, *qac*ΔE and *qac*E genes

Biocide	MIC (mg/L)	No. of strains	Antiseptic resistance genes					
			*cep*A	*qac*ΔE	*qac*E	*cep*A + *qac*E	*cep*A + *qac*ΔE	NO *cep*A + *qac*ΔE
Chlorhexidine	4	1						1
	8	1	1					
	16	5	5	4			4	1
	32	34	28	20			19	4
	64	20	19	9	1	1	8	
	128	3	3	1			1	
Trigene	16	9	7	1	1	1	1	2
	32	41	39	24			24	2
	64	14	10	9			7	2
Benzalkonium chloride	16	3	3	1			1	
	32	48	43	27	1	1	25	3
	64	13	10	6			6	3

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Table III
Effect of CCCP (10 mg/L) on the minimum inhibitory concentration (MIC) of chlorhexidine correlated with the presence of *cep*A and *qac*ΔE genes

No. of strains	Fold reduction of chlorhexidine MIC in 10 mg/L CCCP	*cep*A	*qac*ΔE
1	8	−	−
1	8	+	−
3	8	+	+
1	16	−	−
3	16	+	−
3	16	+	+
11	32	+	−
2	32	−	+
15	32	+	+
1	64	−	−
7	64	+	−
9	64	+	+
2	128	+	−
1	128	+	+

CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

Discussion

The increasing incidence of *K. pneumoniae* infections in hospitals worldwide has led to greater awareness of the hazards of nosocomial infection and efforts to improve infection control using appropriate hygiene measures.

Biocides are currently considered to be an essential component of infection control strategies in hospitals and Block and Furman have observed a significant inverse relationship between the intensity of chlorhexidine use and overall susceptibility of organisms (*S. aureus*, coagulase-negative staphylococci, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter baumannii* and *Candida albicans*) to this antiseptic.[16,22] Koljalg *et al.* found a strong correlation between chlorhexidine and antibiotic susceptibility in MICs among 70 distinct clinical isolates of Gram-negative bacteria, comprising *Escherichia coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *S. aureus* (not MRSA), *Streptococcus pyogenes* and *Enterococcus faecalis*. They noted that non-fermentative bacteria tolerated chlorhexidine at high concentrations better than the Enterobacteriaceae and that Gram-positive cocci, especially *S. pyogenes*, were the most susceptible.[23]

In order to examine the effect on one species in detail, in this study the efficacy of five biocides, extensively used in hospitals, was screened against 64 clinical isolates of *K. pneumoniae*. Mediscrub was the most effective biocide against *K. pneumoniae*, with MICs between 0.12 and 0.5 mg/L, followed by MediHex-4; chlorhexidine, benzalkonium chloride and Trigene were the least effective.

These results may be explained in part by the fact that most of these unrelated *K. pneumoniae* strains carried the *cepA* efflux pump gene. The MIC assays performed in the presence of CCCP showed that there was a considerable decrease in the MICs of chlorhexidine for almost all the strains and this was usually associated with the presence of *cepA*. On the other hand, there were reductions in the MICs of MediHex-4, but these were not so great as with chlorhexidine, and were not significantly associated with the presence of either the *cepA* or *qac* genes. This suggests that efflux pumps, as yet undefined, are capable of exporting MediHex-4 but this does not play an important role in MediHex-4 susceptibility in these *Klebsiella* strains. The lack of influence of CCCP on the MICs of benzalkonium chloride, Trigene and Mediscrub suggests that other resistance genes are more important, particularly for benzalkonium chloride and Trigene. It was interesting to note though that for both these antiseptics there was a high proportion of strains, with reduced susceptibility, that had both the *cepA* and *qacΔE* genes.

It is interesting to note that the proportion of reduced biocide susceptibility was high and the carriage of biocide resistance genes was also high. This suggests that the *Klebsiella* population in the Infirmary has been exposed to a considerable quantity of biocides and that reduced susceptibility is creeping into the population. However, the clinical importance of this may be doubtful as the concentration of biocides used is usually far greater than the MICs, even of the strains with reduced susceptibility. This is likely to be become more important when other factors are taken into account, such as biofilm formation, which already compromise the activity of the biocide.[13,14] Furthermore, this population was not noticeably resistant to the antibiotics tested and these results are in contrast with those of Koljalg *et al.*; however, in that study only a very small proportion of the strains tested were *Klebsiella* spp.[23] There is evidence in other species that biocide resistance genes can be closely linked to antibiotic resistance genes and, if that occurs in this species in the future, the use of biocides could select for antibiotic-resistant strains.[24]

Table IV
Effect of CCCP (10 mg/L) on the minimum inhibitory concentration (MIC) of Medihex-4 (MH-4) correlated with the presence of *cep*A and *qac*ΔE genes

No. of strains	Fold reduction of MH-4 MIC in 10 mg/L CCCP	*cep*A	*qac*ΔE
1	2	+	+
2	4	−	−
4	4	+	−
1	8	−	−
1	8	−	+
6	8	+	+
2	16	−	−
1	16	−	+
11	16	+	−
21	16	+	+
1	32	−	−
7	32	+	−
5	32	+	+
1	64	+	+

CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

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Conflict of interest statement
None declared.

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Brief Communication

Bactericidal activity of five antiseptics on *Klebsiella pneumoniae* and its relationship to the presence of efflux pump genes and influence of organic matter

Abdulmonem Abuzaid, Ahmed Hamouda, Sebastian G. B. Amyes

Molecular Chemotherapy, Medical Microbiology, University of Edinburgh, UK

Klebsiella pneumoniae is responsible for about 8% of hospital-acquired infections. Biocides, including quaternary ammonium compounds (QACs) and biguanides, are frequently used in hospitals and there is evidence that their use has reduced hospital-acquired infections significantly.[1] The routine application of biocides could result in decreased susceptibility over time, which may eventually lead to the development of resistance to themselves and antibiotics.[2] This study assessed the bactericidal activity of five biocides, both under ideal conditions and in the presence of organic matter. The efficacy of biocide action was compared with the association of efflux pump genes that have been linked to antiseptic resistance (*cep*A, *qac*Δ*E*, *qac*E).[3]

Six isolates of *K. pneumoniae* isolated from patients in the Royal Infirmary Edinburgh were chosen for their varying sensitivity to chlorhexidine (CHX). The common hospital biocides used were 1% CHX gluconate, a member of biguanide family, and 1% benzalkonium chloride (BZK), a QAC, both were supplied from Sigma (Poole, UK), The commercial biocide preparations Trigene, the main component being polymeric biguanide hydrochloride, the cationic biocide Medihex-4 (MH-4), containing 4% CHX gluconate, and Mediscrub (MS), containing 1% polymeric biguanide hydrochloride, were obtained from Medichem International (Kent, UK).[4] The exact details and composition of the proprietary products may be found with reference to their accompanying literature.

Susceptibility studies were performed with each biocide; if it was a mixture the result was expressed as the concentration of the principal component, as listed above. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method on Iso-Sensitest agar (Oxoid Ltd, Basingstoke, UK) and the agar plates were incubated in air at 37°C for 24 hours.[5] Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration of the biocide required to kill 99.9% of the original bacteria within a given time (in this case 3 minutes).[6,7] Evaluation of MBC of the five disinfectants against *K. pneumoniae* was conducted according to the European standard test method EN 1040 on Tryptone Soya Agar (Oxoid).[7] One hundred µl of $\sim 10^8$ cfu/ml bacterial suspension was added to 900 µl biocide solution and the mixture was maintained at 20–22°C (room temperature) for 3 minutes, 100 µl of this mixture was transferred to 900 µl of neutralizer solution comprising 10% Tween 80, 0.1% histidine and 0.5% sodium thiosulphate (Sigma, Poole, UK), 3% lecithin soybean (MP Biomedicals, LLC Cambridge, UK) in Phosphate buffer saline (PBS, pH 7.4). This mixture was kept at 20–22°C for 3 minutes and diluted 1 : 10 serially with PBS and 100 µl aliquots of the diluted mixture was spread onto Tryptone Soya Agar plates and incubated at 37°C for 18–24 hours.[7] As a control, the toxicity of the neutralizer to the bacterial activity was evaluated by adding 100 µl of bacterial suspension to 900 µl of neutralizer.[7] In both cases, care was taken to measure the sensitivity to the active component of each antiseptic, as stated by the manufacturer, and this is the figure quoted in the results.

Time killing test were performed to assess the bactericidal effects of CHX (360 mg/l) following the European standard test method EN 1040, with assay times of 1, 3, 5 and 10 minutes.[7] This test was repeated to evaluate bactericidal activity, under conditions that simulated the presence of organic matter, in this case by using bovine serum albumin (BSA) (Roche Diagnostics Ltd, Burgess Hill, UK). The test was repeated with BSA (1, 3, and 10%) added complying with the European standard test method EN 1276.[7]

Correspondence to: Sebastian. G. B. Amyes, Molecular Chemotherapy, Medical Microbiology, University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland, UK. Email: s.g.b.amyes@ed.ac.uk

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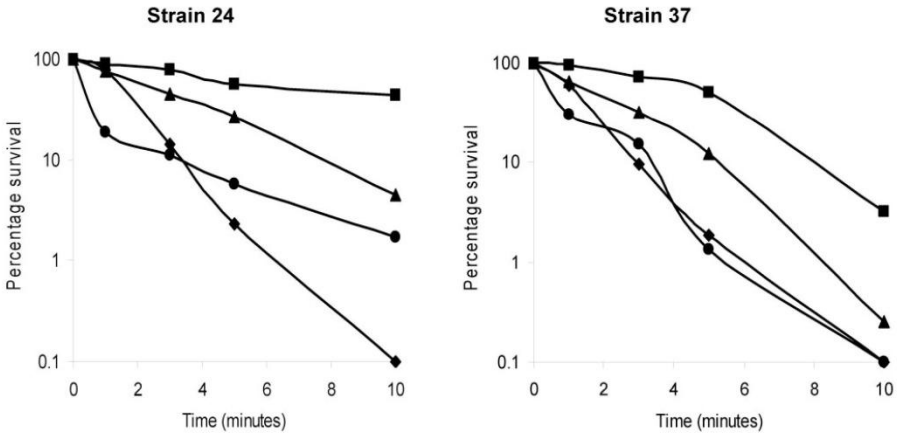

Figure 1 The effect of time on the bactericidal effect of chlorhexidine (360 mg/l) against *K. pneumoniae* strains 24 and 37 and the impact of increasing concentrations of bovine serum albumin. The viability of strains 24 and 37 was monitored against time in the presence of chlorhexidine at 40% of the MBC (◆). The experiment was repeated in the presence of bovine serum albumin at concentrations of 1% (●), 3% (▲), and 10% (■).

The *cep*A, *qac*ΔE, and *qac*E antiseptics resistance genes were amplified by PCR. The primers pairs of Fang *et al.* were used to identify the *cepA* gene, which encodes a cation efflux pump associated with CHX resistance[8] and the *qac*Δ*E* and *qac*E genes were amplified by the primer pairs of Kazama *et al.*[9]

All strains were sensitive to cefotaxime, ceftazidime, imipenem and meropenem; only strain 52 was resistant to cefoxitin. Strains 24 and 52 were resistant to pipericillin/tazobactam and only the latter could be considered multi-antibiotic resistant (Table 1). The MICs of the biocides showed that, in 4, 5, and 6 *K. pneumoniae* isolates, there was reduced susceptibility to CHX, Trigene and BZK respectively with MICs ranging from 32 to 128 mg/l (Table 1). However, MH-4 showed little variation in MICs (8–16 mg/l) in all isolates. On the other hand, five of the six isolates were susceptible to MS (MICs ≤4 mg/l) (Table 1). PCR detected the characteristic 1051 bp fragment of the amplified *cep*A gene in five isolates whereas the *qac*Δ*E* gene was detected in two isolates; both also had the *cep*A gene. One isolate had neither gene (Table 1). Most of the strains carrying the *cepA* gene had reduced susceptibility to CHX but it was not possible to relate susceptibility to the other antiseptics to the carriage of either the *cepA* or *qac*Δ*E* genes.

MBCs were calculated for the isolates using the differences between the proportion of surviving colonies and a control (bacterial suspension was treated with PBS only). The MBC of Trigene was the same for all strains at 1800 mg/l of the active component (polymeric biguanide hydrochloride). The MBCs of CHX ranged from <360 mg/l to 900 mg/l. whereas those for MH4, between <36 mg/l to 360 mg/l, and BZK, at 90 mg/l, were lower. The

Table 1 Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) of antiseptics, and the carriage of the *cep*A and *qac*Δ*E* efflux genes in *K. pneumoniae*

	Antiseptic												
	MIC (mg/l)					MBC (mg/l)					Efflux genes		
Strain no.	CHX	Tri	MH-4	MS	BZK	CHX	Tri	MH-4	MS	BZK	*cep*A	*qac*ΔE	Antibiotic resistance
14	4	16	8	0.12	32	<360	1800	<36	9000	90	−	−	
16	8	32	8	0.5	64	<360	1800	<36	9000	90	+	−	
24	128	64	8	0.5	32	900	1800	360	9000	90	+	+	CAR, DO, E, PRL
37	128	32	8	0.12	32	900	1800	360	9000	90	+	−	CAR, E, DO
52	128	64	16	0.5	64	<360	1800	<36	9000	90	+	−	CAR, CHL, CXT, DO, E, NV, PRL, TCG, TMP
64	64	64	16	>128	32	<360	1800	<36	9000	90	+	+	CAR

Note: CHX, Chlorhexidine; Tri, Trigene; MH-4, MediHex-4; MS, MediScrub; BZK, Benzalkonium chloride; CAR, Carbenicillin; CHL, Chloramphenicol; CXT, Cefoxitin; DO, Doxycycline; E, Erythromycin; NV, Novobiocin; PRL, Piperacillin/Tazobactam; TCG, Tigecycline; TMP, Trimethopim.

MBC for Mediscrub was the highest for each strain at 9000 mg/l. The presence of the two efflux genes had no influence on the MBC values (Table 1).

CHX is the active component of many proprietary antiseptics. The activity of CHX was measured at 40% of the lowest determined figure for the MBC (360 mg/l), in order to obtain a drop in viability that could be precisely measured over time. Even at these concentrations CHX had the ability to reduce the viability of both *K. pneumoniae* strains 24 and 37 within 10 minutes (Fig. 1). In both cases, the rate of kill was logarithmic in relation to time. When BSA was added, the ability of CHX to kill was reduced; virtually eliminated for strain 24 in 10% BSA though its effect was considerably less in strain 37 (Fig. 1). It is interesting to note that the presence of a small concentration of BSA (1%) initially promoted the killing effect; this is not considered to be an artefact because it was found to be repeatable with both strains.

There is very little in published literature about the effects of CHX and other antiseptics on clinical strains of *K. pneumoniae*. Koljalg *et al.*[10] found a correlation between CHX and antibiotic susceptibility in a mixed collection of clinical isolates. In our study, all strains showed reduced susceptibility to BZK and Trigene, the MIC of polymeric biguanide hydrochloride, the main active component, was measured. On the other hand all, except strain 64, were susceptible to Mediscrub, again the MIC of polymeric biguanide hydrochloride was measured but this activity may have been boosted by the surfactant sodium lauryl sulphate. Mediscrub is used for washing hands and the surfactants, present to reduce the surface tension, clearly are also having an effect on the viability of the bacterial cells. The main component of Medihex-4 is CHX gluconate and it was the MIC of this compound that was measured; however, the MICs of Medihex-4 were lower than that of the CHX preparation. Medihex-4 also contains cocamidopropyl betaine and sodium lauryl sulphate as surfactants and these may contribute to the reduced MICs found with this preparation.

Although most of the strains had reduced susceptibility to CHX, this could not be correlated with multiresistance to antibiotics. There was no correlation of reduced susceptibility to CHX with the presence of the *qacΔE* gene but all the strains showing reduced CHX susceptibility did possess the *cepA* gene.

The MICs of the polymeric biguanide hydrochloride antiseptics, Mediscrub and Trigene, were much lower than the MBCs, in some cases by 10 000-fold. This strongly indicates that these antiseptics are primarily bacteriostatic in their action against *K. pneumoniae* and to obtain a rapid kill, large quantities would have to be used. This suggests that, in clinical concentrations, they have little capacity to eradicate *K. pneumoniae*. Although the MIC of BZK was high, the MBC was not much higher indicating that once the concentration threshold of inhibiting the bacterium had been reached, a three-fold increase was sufficient to kill the bacteria quickly.

Similarly the differential between the MICs and the MBCs of the two CHX compounds was low and a 5-fold increase above the MICs of CHX was sufficient to elicit a rapid bactericidal response, which would be important in clinical practice. The presence of organic matter, represented here by BSA, suggests that CHX is very sensitive to its presence though the extent of this will vary from strain to strain. In conclusion, clinical *K. pneumoniae* may not respond to common biocides, which may not kill the bacteria especially in the presence of organic matter.

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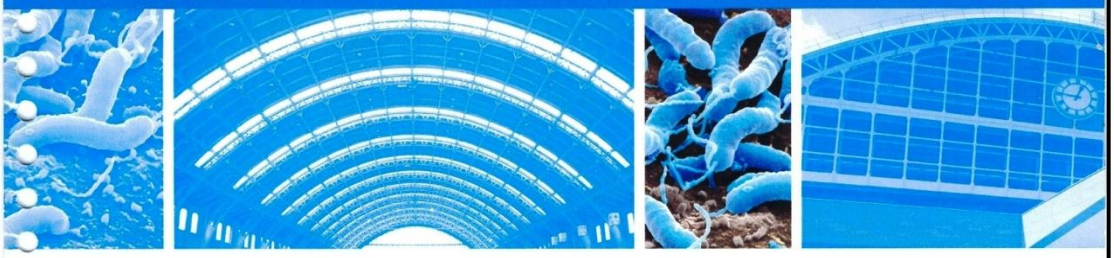

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Poster Session 1

0003	Listeria brain abscess: case report and review	*Dr Michael Mina* *Birmingham*
0004	Tumour necrosis factor receptor-1 associated periodic syndrome (TRAPS): a case report and review	*Dr Michael Mina* *Birmingham*
0005	Evaluation of the antibacterial activity of honeys (including Manuka and Sedr honeys) against bacteria causing opportunist infections	*Dr Sulaiman Alharbi* *Saudi Arabia*
0006	Growth and reproductive attributes of radionuclides 1 phytoremediators in the Mediterranean coastal black sands	*Dr Abdularhman Alatar* *Saudi Arabia*
0007	Inhibition effect of some soil streptomyces isolates against toxigenic *in vitro* isolate of *Fusarium verticillioides*	*Dr Muneera Alkahtani* *Saudi Arabia*
0014	A putative adhesin protein of *Salmonella*: a potential target for vaccine development?	*Mr Mahmoud Tawfick* *Leicester*
0016	*Clostridium perfirngens* endophthalmitis: a case report	*Dr Manjusha Narayanan* *Newcastle*
0017	Assessment of *Klebsiella pneumoniae* susceptibility to biocides and its association with cepA, qacΔE and qacE efflux pump genes and antibiotic resistance	*Mr Abdulmonem Abuzaid* *Edinburgh*
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0032	The role of CT PET in pyrexia of unknown origin (PUO)	*Dr Tehmina Bharucha* *London*
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Assessment of *Klebsiella pneumoniae* susceptibility to biocides and its association with *cep*A, *qac*ΔE and *qac*E efflux pump genes and antibiotic resistance

A.A.Y. Abuzaid, A. Hamouda, S.G.B. Amyes
Centre For Infectious Diseases, University of Edinburgh

Abstract

This study assessed the susceptibility of five biocides, chlorhexidine (CHX), Benzalkonium chloride (BZK), Trigene, MediHex-4 (MH-4), Mediscrub (MS) and 17 antibiotics against 64 isolates of *Klebsiella pneumoniae*. The relationship between resistance to biocides and the carriage of antiseptic resistance genes, *cep*A, *qac*ΔE and *qac*E was determined. Antimicrobial susceptibility was tested by the agar double dilution method (DDM) and disk diffusion methods. The bacteria were not widely resistant except to some beta–lactams. Biocides susceptibility, tested by DDM showed that 50, 49 and 53 strains were resistant to CHX, Trigene and BZK respectively. The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were found in 56, 34 and 1 isolates respectively and their effects as efflux pumps was determined by carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (10mg/L), which decreased the MICs of CHX and MH-4 by 2-128 fold but it had no impact on BZK, TG and MS. These results show there was a close link between carriage of efflux pump genes, *cep*A, *qac*ΔE and *qac*E genes and high-level biocide, but not antibiotic, resistance in K. pneumoniae clinical isolates

Aim

This study investigates the correlation between biocides use and its association with the genes responsible for antiseptics resistance efflux pump genes (*cep*A, *qac*ΔE, *qac*E) and resistance genes responsible for antibiotics resistance.

Methods and Materials

Bacterial strains: *Klebsiella pneumoniae* (64) were isolated from the Royal Infirmary in Edinburgh between 2006 and 2008.
MIC and disk diffusion: Antimicrobial susceptibility was tested by the agar double dilution method (DDM) and disk diffusion methods. following the guidelines of British Society for Antimicrobial Chemotherapy (BSAC)[1,2]. Biocides susceptibility was tested by DDM
Efflux pumps inhibitor and biocides resistance: Carbonyl cyanide m-chlorophenylhydrazone (CCCP) 10mg/L add to IST agar.
PCR: The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were detected by PCR. *cep*A primer pairs amplified 1051 pair base (bp) were described by Fang *et al* [3]. The *qac*ΔE and *qac*E primer pairs amplified 370 and 350 bp respectively, all having the same annealing temperature 49ºC for 40s, were described by Kazama *et al* [4].

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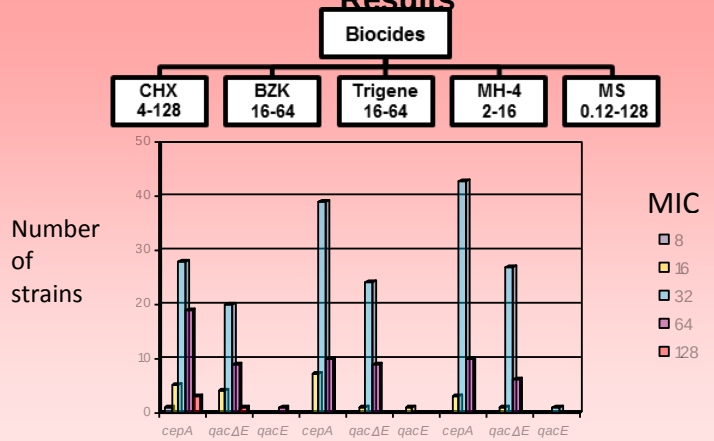
Results



Biocides susceptibility, tested by DDM showed that 50, 49 and 53 strains were resistant to CHX, Trigene and BZK respectively. All strains were resistant to MH-4 and sensitive to MS. The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were found in 56, 34 and 1 isolates respectively. There was an association of resistance with the *cep*A and, to a lesser extent, *qacΔE* gene. The role of these efflux pumps in conferring resistance was determined by carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (10mg/L), which decreased the MICs of CHX and MH-4 by 2-128 fold but it had no impact on BZK, TG and MS.

Conclusion

These results show there was a close link between carriage of efflux pump genes, *cep*A, *qac*ΔE and *qac*E genes and high-level biocide resistance in *K. pneumoniae* clinical isolates.

References

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