Rapid Detection of GES-type Extended-spectrum β-lactamases in *Pseudomonas aeruginosa* with a Peptide Nucleic Acid-based Real-time PCR Assay

CHRISTIAAN DE JAGER LABUSCHAGNE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Rapid Detection of GES-type Extended-spectrum β-lactamases in *Pseudomonas aeruginosa* with a Peptide Nucleic Acid-based Real-time PCR Assay

by

Christiaan De Jager Labuschagne

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

MSc (Medical Microbiology)

Department Medical Microbiology

Faculty of Health Sciences
University of Pretoria
Pretoria
South Africa

November 2007

I, the undersigned, declare that the thesis hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree.

Signature: _____

Date: _____

This thesis is dedicated to my friends and family

"There, but for the grace of GOD go I"

Unknown

Acknowledgements

I would like to sincerely thank:

Dr MM Ehlers, Department of Medical Microbiology, University of Pretoria/NHLS, for her professional guidance, assistance and encouragement to accomplish this project and dissertation successfully

Dr GF Weldhagen, Molecular Biology Laboratory, AMPATH National Laboratory Services, for his academic support and assistance in this project

Prof MG Dove, Department Medical Microbiology, University of Pretoria/NHLS, for his continued support of the project

Dr MM Kock, Department Medical Microbiology, University of Pretoria, for her help with the genotyping of the *Pseudomonas aeruginosa* isolates

My parents and family, for their support and belief in me

My colleagues and friends, for their encouragement and motivation

Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

Symbols

%	Percentage
°C	Degrees Celsius
β	Beta
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

Abbreviations

A	Adenosine
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AFLP	Amplified fragment length polymorphism
AIDS	Auto-immune deficiency syndrome
Ala	Alanine
AmpC	Chromosomal located cephalosporinase
Asn	Asparagine
ATP	Adenosine triphosphate
be	Base element
BEL	Belgium extended β-lactamase
bla	Beta-lactamase
bp	Base pair
C	Cytosine
ca	*circa*
CFU	Colony-forming unit
CISH	Chemiluminescent *in situ* hybridisation
CLSI	Clinical and laboratory standards institute
CRP	C-reactive protein
Co	Company
CS	Conserved segment
CSF	Cerebrospinal fluid
CTX-M	Cefotaximase
CVP	Central venous pressure catheter
Da	Dalton
-dF/dT	Negative derivative of fluorescence change compared to temperature
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ESBL	Extended-spectrum Beta-lactamase
FISH	Fluorescence *in situ* hybridisation
FRET	Fluorescence resonance energy transfer
g	Gram
G	Guanidine
GES	Guiana extended-spectrum
Glu	Glutamate
Gly	Glycine
h	Hour
hrs	Hours
IBC	Integron borne cephalosporinase
IC_{50}	50% inhibitory concentration
IMP	Beta-lactamase named after preferred substrate (imipenem)
In	Integron

kb	Kilo Base
kg	Kilogram
LCR	Ligase chain reaction
Leu	Leusine
LPS	Lipopolysaccharide
Ltd	Limited
Lys	Lysine
M	Molar
MATE	Multidrug and toxic compound extrusion
MFS	Major facilitator superfamily
Mg	Magnesium
mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
ml	Millilitre
mM	Millimolar
MW	Molecular weight
NaCl	Sodium chloride
NCCLS	National committee for clinical laboratory standards
nm	Nanometer
NMR	Nuclear magnetic resonance
nt	Nucleotide
ORF	Open reading frame
OXA	Oxacillinase
PBG	Poly-γ-benzylglutamate
PCR	Polymerase chain reaction
PCT	Procalcitonin
PER	Beta-lactamase named after original authors (P. Nordmann, E. Ronco, R. Labia)
PFGE	Pulse-field gel electrophoresis
PK/PD	Pharmacokinetics/pharmocodynamics
PNA	Peptide nucleic acid
PSE	*Pseudomonas*-specific β-lactamase
Pty	Property
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
rpm	Revolutions per minute
S	Serine
SBP	Soybean peroxidase
SDS	Sodium dodecyl sulfate
Ser	Serine
SHV	Sulphydryl variable
SMR	Small multidrug resistance
Spp	Species
SSCP	Single-strand conformational polymorphism
ssDNA	Single stranded deoxyribonucleic acid
STE	Sodium chloride Tris EDTA
T	Tyrosine
Taq	Polymerase enzyme named after *Thermus aquaticus*
TBE	Tris-Borate-EDTA
TD	Temperature difference
TE	Tris-EDTA
TEM	Beta-lactamase named after first patient isolated from (Temoneira)
T_m	Melting temperature
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
Trp	Tryptophan
U	Unit
UPGMA	Unweighted pair group method with average linkages

UV	Ultraviolet
VEB	Vietnamese extended-spectrum beta-lactamase
VIM	Verona integron-encoded metallo-β-lactamase
wt	Wild type

List of Conference Contributions

Ehlers MM, Labuschagne CDJ, Dove MG, Weldhagen GF (2006) The genetic location of *bla*$_{GES-1}$ and *bla*$_{GES-2}$ Extended-spectrum β-lactamase genes in *Pseudomonas aeruginosa* isolates collected in the Gauteng region of South Africa. *14th Biennial SASM Congress, CSIR, Pretoria.* 9-12 April 2006. Poster presentation

Labuschagne CDJ, Weldhagen GF, Dove MG, Ehlers MM (2006) Prevalence of GES-1 and GES-2 Extended-spectrum β-lactamases in *Pseudomonas aeruginosa* isolates collected in the Gauteng region of South Africa. *14th Biennial SASM Congress, CSIR, Pretoria*. 9-12 April 2006.Oral presentation

Labuschagne CDJ, Weldhagen GF, Dove MG, Ehlers MM (2006) Prevalence of GES-1 and GES-2 Extended-spectrum β-lactamases in *Pseudomonas aeruginosa* isolates collected in the Gauteng region of South Africa. *Faculty day, Faculty of Health Sciences, University of Pretoria*. 29 August 2006. Poster presentation

Labuschagne CDJ, Kock MM, Weldhagen GF, Dove MG, Ehlers MM (2006) Random Amplified Polymorphic DNA typing of ESBL positive *Pseudomonas aeruginosa* isolates recovered in the Gauteng region of South Africa. *Faculty day, Faculty of Health Sciences, University of Pretoria.* 29 August 2006. Poster presentation

Labuschagne CDJ, Weldhagen GF, Dove MG, Ehlers MM (2006) Emergence of GES-5 Extended-spectrum β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in South Africa. *MCBG Symposium, University of Witwatersrand*. 28 September 2006. Poster presentation

Rapid detection of GES-type extended-spectrum β-lactamases in *Pseudomonas aeruginosa* with a peptide nucleic acid-based real-time PCR assay

by

Christiaan De Jager Labuschagne

Promoter: Dr MM Ehlers (Medical Microbiology/NHLS)

Department: Medical Microbiology, Faculty of Health Sciences, University of Pretoria/NHLS

Degree: MSc (Medical Microbiology)

Summary

Extended-spectrum β-lactamases (ESBLs) constitute a major problem given their broad substrate specificity and ability to hydrolyse many of the extended-spectrum third-generation cephalosporins currently in use in hospital settings. Guiana extended-spectrum-type (GES-1 – GES-9) ESBL enzymes have mainly been found in *Pseudomonas aeruginosa* (*P. aeruginosa*) and only at a limited number of geographical sites, mainly France, Greece and South Africa. Detection of GES-type ESBL-producing *P. aeruginosa* isolates in the clinical microbiology laboratory using conventional methods is problematic with molecular methods yielding better results.

The aim of this study was to utilise various molecular techniques to determine the prevalence of GES-type ESBLs, characterise their genetic determinants and determine their clonal relatedness. The study further aimed to apply a sequence-selective, competitive PNA-based multiplex PCR in real-time for the identification and differentiation of GES-type enzymes.

The prevalence of GES-type ESBLs was determined successfully through DNA sequencing. An increase in GES-2 prevalence since 2000 was noted which emphasised the importance of constant surveillance to monitor antibiotic determinants, their spread and overall prevalence. The knowledge on prevalence could be used in turn to monitor the efficacy of infection

control measures and antibiotic regimens. Repeated sequencing confirmed the presence of *bla*$_{GES-5}$ in *P. aeruginosa* isolates. As far as could be established, this study reported the first occurrence of GES-5 in South Africa and was the second description of GES-5 in *P. aeruginosa*.

Application of a sequence-specific, competitive PNA-based multiplex PCR in real-time utilising SYBR Green was not suitable for the identification and differentiation of the *bla*$_{GES}$ genes. Although the method achieved different melting temperatures for the *bla*$_{GES}$ genes tested, these temperatures were not suitable for accurate differentiation. Melting temperatures obtained for the same *bla*$_{GES}$ gene varied and those for different genes overlapped. An approach exploiting the high temperature shift caused by the PNA-probe rather than its competitive nature might be more successful.

Random amplified polymorphic DNA typing has been described as a fast and simple method with high discriminatory power for the typing of *P. aeruginosa* and was thus used to determine the clonal relatedness of the *bla*$_{GES}$ positive *P. aeruginosa* isolates. The occurrence of identical or similar *P. aeruginosa* isolates producing ESBLs in a single hospital setting emphasised the importance of constant surveillance. The study further identified identical *P. aeruginosa* clones that occurred in different hospitals indicating spread from a common external reservoir into these hospitals.

The occurrence of highly drug-resistant *P. aeruginosa* in the environment has serious implications in a country with an ever increasing immune-compromised population. These finding were of concern since they demonstrated that acquired GES ESBLs can rapidly emerge and become a major cause of broad-spectrum β-lactam resistance among nosocomial pathogens. The information obtained in this study should be used to create awareness of the potential ESBL problem threatening current antimicrobial regimens in South Africa.

Vinnige identifikasie van GES-tipe uitgebreide-spektrum β-laktamases in *Pseudomonas aeruginosa* met ń peptied nukleiënsuur-gebaseerde ware-tyd PKR metode

deur

Christiaan De Jager Labuschagne

Promotor: Dr MM Ehlers (Geneeskundige Mikrobiologie/NHLS)
Departement: Geneeskundige Mikrobiology, Fakulteit van Gesondheidswetenskappe, Universiteit van Pretoria/NHLS
Graad: MSc (Geneeskundige Mikrobiologie)

Opsomming

Uitgebreide-spektrum β-laktamases (ESBLe) is ‘n groot probleem as gevolg van ‘n wye substraat spesifisiteit en die ensieme se vermoë om baie van die uitgebreide-spektrum derde-generasie cephalosporiene wat tans in hospitale gebruik word te hidroliseer. Guiana uitgebreide-spektrum-tipe (GES-1 – GES-9) ESBL ensieme kom hoofsaaklik in *Pseudomonas aeruginosa* (*P. aeruginosa*) voor en is tot dusvêr hoofsaaklik in geografiese liggings soos Frankryk, Griekeland en Suid-Afrika geïdentifiseer. Opsporing van GES-tipe ESBL-produserende *P. aeruginosa* isolate in die kliniese mikrobiologie labratorium, deur gebruik te maak van konvensionele metodes, is problematies terwyl molekulêre methodes beter resultate verskaf.

Die doel van die studie was om met behulp van verskeie molekulêre tegnieke die voorkoms van GES-tipe ESBLe, die genetiese karakterisering en kloneerings verwantskappe van *P. aeruginosa* te bepaal. Die studie het verder beoog om volgorde-spesifieke, PNA gebaseerde multipleks PKR in ware-tyd te gebruik vir die identifisering en onderskeiding van GES-tipe ensieme.

Die voorkoms van GES-tipe ESBLe was suksesvol deur middel van DNS basis volgorde-bepaling gedoen. 'n Verhoging in GES-2 voorkoms sedert die jaar 2000 beklemtoon die belangrikheid van voortdurende monitering van die teenwoordigheid van antibiotiese determinante, hul verspreiding en algehele voorkoms. Die inligting kan gebruik word om die doeltreffendheid van infeksie-beheer-maatreëls en antibiotika-kombinasies te kontroleer. Herhaalde DNS basis volgorde-bepaling het die voorkoms van bla_{GES-5} in *P. aeruginosa* isolate bevestig. Sover vasgestel kon word het die studie die eerste voorkoms van GES-5 in Suid-Afrika beskryf en was dit die tweede beskrywing van GES-5 in *P. aeruginosa*.

Die toepassing van 'n volgorde-spesifieke, PNA gebaseerde multipleks PKR in ware-tyd met "SYBR Green" was nie geskik vir die identifikasie en onderskeiding van bla_{GES} gene nie. Alhoewel die metode verskillende smelt temperature vir die onderskeie gene aangedui het, was die metode nie akkuraat nie. Smelt temperature verkry van dieselfde bla_{GES} geen het oorvleuel met die verkry vir ander bla_{GES} gene. Meer sukses mag dalk behaal word indien eerder op die groot verskil in temperatuur (geassosieer met die PNA merker) gefokus word as op die kompenterende aard van die merker.

Lukraak amplifikasie polymorfiese DNS tipering (RAPD) is beskryf as 'n vinnige en eenvoudige metode met hoë onderskeidings-vermoë vir die tipering van *P. aeruginosa* en was dus gebruik om die klonerings-verwantskap van die bla_{GES} positiewe isolate te bepaal. Die voorkoms van identiese en verwante *P. aeruginosa* isolate in 'n spesifieke hospitaal-opset beklemtoon die belangrikheid van konstante monitering van infeksie-beheer-maatreëls. Die studie het verder verwante *P. aeruginosa* isolate in verskillende hospitale geïdentifiseer wat verspreiding vanaf een eksterne reservoir aandui.

Voorkoms van hoogs antibiotika-weerstandige *P. aeruginosa* in the omgewing het ernstige implikasies vir 'n land wat 'n stygende hoeveelheid immuun-kompromitteerde pasïente het. Die bevindings is kommerwekkend aangesien dit aandui dat bekomde GES ESBLe vinnig kan verskyn en 'n groot oorsaak van uitgebreide-spektrum β-laktam-weerstandigheid in nosokomiale patogene veroorsaak. Die inligting bekom in die studie behoort gebruik te word om bewustheid te skep van die potensïele ESBL probleem wat die huidige antibiotikum skedule in Suid-Afrika bedreig te beklemtoon.

Chapter 1

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*), an opportunistic pathogen of humans, is a Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae* (Poole, 2005). *Pseudomonas aeruginosa* is amongst the most important causes of nosocomial infections, especially in burn victims and immunocompromised patients (Vahaboglu *et al.*, 1997). Due to some unique features of the outer membrane porins and chromosomal β-lactamases, *P. aeruginosa* is intrinsically less susceptible to several β-lactam antibiotics (Vahaboglu *et al.*, 1997; Jacoby and Munoz-Price, 2005).

In addition to the intrinsic resistance of Gram-negative bacteria, such as *P. aeruginosa,* these bacteria also produce enzymes namely β-lactamases, which are responsible for wide-spread β-lactam resistance (Dubois *et al*., 2002). These β-lactamases hydrolyse the amide bond of the four-membered characteristic β-lactam ring, rendering the antimicrobial ineffective (Waley, 1992). Four molecular classes of β-lactamases are known, dubbed A-D according to Ambler's classification (Ambler *et al*., 1991). Classes A and D include the so-called extended-spectrum β-lactamases [oxacillinase (OXA)-type; cefotaximase (CTX-M)-type; sulphydryl variable (SHV)-type; Temoneira (TEM)-type and Guiana extended-spectrum (GES)-type] (Poole, 2004). Class B β-lactamases include carbapenemases that hydrolyse most β-lactams, including carbapenems, while AmpC enzymes belong to the class C cephalosporinases (Poole, 2004). Beta-lactamase enzymes are able to target the extended-spectrum β-lactams and are therefore of concern in healthcare facilities (Poole, 2004).

The extended-spectrum β-lactamases (ESBLs) constitute a major problem in the use of β-lactam antibiotics to treat infections, given their broad substrate specificity and ability to hydrolyse many of the extended-spectrum third-generation cephalosporins (Poole, 2004). Extended-spectrum β-lactamase producing bacteria are typically resistant to penicillins, first- and second-generation cephalosporins as well as the third-generation oxyimino cephalosporins (e.g. ceftazidime, ceftriaxone) and monobactams (aztreonam) (Poole, 2004). The currently identified ESBLs in *P. aeruginosa* include the SHV-, TEM-, PER-, VEB-, and IBC/GES-types (Weldhagen *et al*., 2003; Jacoby and Munoz-Price, 2005).

The GES-type β-lactamases are of particular importance since these enzymes possess more than one of the different hydrolysis profiles (expanded-spectrum cephalosporins, carbapenems, cephamycins and monobactams) (Poirel *et al*., 2006). The first GES-type, GES-1 was identified in Paris (France) in 1999 from a French Guiana *Klebsiella pneumoniae* (*K. pneumoniae*) isolate and subsequently from a *P. aeruginosa* isolate (Poirel *et al*., 2000; Weldhagen *et al*., 2003). A mutation causing the substitution of asparagine (Asn) for glycine (Gly) at position 170 in GES-1 resulted in an enzyme resistant to clavulanic acid and able to hydrolyse imipenem (Poirel *et al.*, 2001). The enzyme was subsequently designated GES-2 (Poirel *et al.*, 2001). This GES-2 ESBL was discovered in 2000 in *P. aeruginosa* isolates in Pretoria, South Africa (Poirel *et al.*, 2001; Poirel *et al.*, 2002).

The GES-2 β-lactamase is the fourth example of a non-TEM-, non-SHV-type ESBL in *P. aeruginosa* and with a G + C content of non-*P. aeruginosa* origin (Poirel *et al.*, 2001). It is therefore believed to be the result of horizontal transfer of bla_{GES} genes between Gram-negative species (Poirel *et al.*, 2001). In addition, *P. aeruginosa* exhibits a strong bias for cytosine and guanine instead of adenine and thymine in the wobble position, strongly supporting the horizontal transfer theory (Philippon *et al*., 1997). The production of GES-2 β-lactamase is conferred by the gene bla_{GES-2}, which is a more potent gene in the GES/IBC (integron-borne cephalosporinase) β-lactamase family (Weldhagen, 2004a). Since 2000, GES-2 producing *P. aeruginosa* isolates have also been discovered in Argentina (Pasteran *et al*., 2004).

There are currently no tests recommended by the Clinical and Laboratory Standards Institute (CLSI; previously known as the NCCLS) for the detection of ESBLs in *P. aeruginosa* (Jacoby and Munoz-Price, 2005). Clinical laboratory detection (e.g. double disk synergy test and Etest ESBL strips) of ESBLs in *P. aeruginosa* is difficult and false-negative results are common (Finch, 1998; De Champs *et al*., 2002; Weldhagen *et al*., 2003). The following factors contribute to this: i) the presence of naturally occurring β-lactamases, such as chromosomally encoded AmpC (class C cephalosporinase) enzymes, which may be over expressed and therefore be responsible for false-negative results; ii) the simultaneous presence of metallo-enzymes with carbapenem-hydrolysing activities or with extended-spectrum oxacillinases; iii) relative resistance to inhibition by clavulanate; iv) the inoculum effect where the minimum inhibitory concentration (MIC) of extended-spectrum cephalosporins rise

as the inoculum increases, leading to false-negative results; v) and a combination of resistance mechanisms, such as efflux and impermeability of the cell wall (Bradford, 2001; Stürenburg and Mack, 2003; Weldhagen, 2004a).

These restrictions of conventional methods may be overcome with the application of molecular based methods, which directly target ESBL genes (Weldhagen *et al*., 2003). Molecular methods for the detection of ESBLs includes PCR with a series of primers designed for the recognition of class A β-lactamase genes in *P. aeruginosa* followed by sequencing of the amplification products (Weldhagen *et al*., 2003). Although sequencing of these PCR products can detect the ESBLs with great sensitivity, it is too expensive and time consuming to be used as a routine method in the diagnostic laboratory (Weldhagen *et al*., 2003).

A recently developed sequence-specific, peptide nucleic acid (PNA)-based multiplex PCR detection method seems to provide an accurate means to detect bla_{GES-2} compared to standard PCR and gene sequencing techniques (Weldhagen, 2004a). Peptide nucleic acids are deoxyribonucleic acid (DNA) homologues in which the phosphate backbone has been replaced with repetitive units of N-(2-aminoethyl) glycine, known as a polyamide backbone (Pellestor and Paulasova, 2004). The synthetic backbone of PNA confers unique properties to PNAs such as i) high stability binding to DNA and ribonucleic acid (RNA), ii) neutral charge, iii) invulnerability to nucleases and proteases and iv) higher thermal melting temperatures, which have been incorporated into a variety of applications, including antisense therapy and molecular detection methods (Pellestor and Paulasova, 2004).

The PNA-based multiplex PCR detection method relies on two features of PNA: i) its intrinsic high affinity for DNA and ii) and its inability to serve as a primer for DNA polymerases (Ørum *et al*., 1993). This PNA-based multiplex PCR uses a forward primer and reverse primer that is complementary to the desired mutant sequence, as well as a PNA-probe similar to the reverse primer, which is complementary to the wild type (wt) sequence (Weldhagen, 2004a). If a wt DNA template is present in the PCR reaction, the PNA-probe out-competes the reverse primer for binding to the template and no amplification occurs (Weldhagen, 2004a). The opposite occurs if a mutant sequence is present during the reaction, the primer will out-compete the PNA-probe and amplification will occur (Weldhagen, 2004a).

Apart from traditional PCR, real-time PCR can be used to perform the PNA-based multiplex PCR. Conventional PCR uses end point detection of amplification products, which relies upon gel electrophoresis to visualize bands (Mackay, 2004). In contrast real-time PCR allows the measurement of amplification products during amplification (Svanvik *et al.*, 2000). Amplification products are detected by the binding of a fluorescent reporter, which may be specific or non-specific (Svanvik *et al.*, 2000). Various reporters have been developed with the simplest assay using double-stranded DNA (dsDNA) specific dyes, such as the asymmetric cyanine dye SYBR Green (Svanvik *et al.*, 2000). These dyes, with no innate fluorescence, become intensely fluorescent when bound to dsDNA (Svanvik *et al.*, 2000). Since these free dyes do not recognise a sequence specific product it will bind to any double stranded product formed, which is exploited when performing a melting curve analysis (Svanvik *et al.*, 2000). To produce a melting curve, the amplicons are heated until dsDNA becomes single stranded DNA (Svanvik *et al.*, 2000). The resulting rapid decrease in fluorescence caused by the dissociation of the fluorogenic molecules is presented as a 'melting peak', using software (e.g. LightCycler Software 4, Roche) capable of calculating the negative derivative of fluorescence change compared to temperature *(-dF/dT*) (Mackay, 2004).

Pellestor and Paulasova (2004) reported that PNA has high thermal stability with single mismatches causing differences in melting temperatures as high as 15°C. The high thermal stability of PNAs should allow easy identification of single mismatches through melting curve analysis, since melting peaks may differ considerably (Jakimov *et al*., 2000; Pellestor and Paulasova 2004). Although the genes in the GES ESBL family differ only in a few point mutations, inclusion of a melting curve in the PNA-based multiplex PCR should allow the identification of GES variants based upon their different melting temperatures.

In a previous study conducted by Weldhagen (2004a) a PNA-probe together with conventional multiplex PCR were used to determine the presence of GES-1 and GES-2 ESBLs in *P. aeruginosa* isolates. The real-time PCR research performed by Weldhagen (2004b) utilised a fluorescence resonance energy transfer (FRET)-mediated mutation assay with sensor and anchor probes that detected and differentiated GES-1 from GES-2 (Weldhagen, 2004b). The identification of new GES variants may influence the specificity of the conventional PNA-probe exclusion multiplex PCR method developed by Weldhagen (2004a). According to published gene sequences it seems that the target region of the PNA-

probe might not be limited to only GES-1 and GES-2 variants (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001; Weldhagen 2004a; Poirel *et al*., 2005). Other variants such as bla_{GES-7}, bla_{GES-8} and bla_{GES-9} may contain the same target region causing false-positives during conventional multiplex PCR analysis (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001; Weldhagen, 2004a; Poirel *et al*., 2005).

In this study previous research conducted by Weldhagen (2004a; 2004b) was taken a step further and focused on the application of a PNA-probe to develop a rapid and sensitive real-time PCR-based ESBL detection method. The real-time PCR method with the use of SYBR Green may enable the clear distinction between different GES mutations (GES-1 and GES-2 as well as other GES variants) by means of the resulting melting curve analysis. This real-time PCR detection method has several advantages such as shorter run time estimated at 1:30 h and being more cost effective due to the use of inexpensive SYBR Green. Since it is a closed system, it would also minimize the risk of cross contamination (Mackay, 2004). The real-time PCR method could be further optimized for use as a routine diagnostic tool to detect antibiotic resistant genes of *P. aeruginosa* in the clinical setting. Real-time PCR showed important benefits when compared to the previously developed conventional multiplex PCR, which is time consuming (6-8 h), costly and which needs gel electrophoresis to finally analyse results (Weldhagen, 2004a; Weldhagen, 2004b).

Genomic fingerprinting methods have been employed as accurate methods for the typing of microorganisms (Speijer *et al*., 1999). Fingerprinting methods included pulse-field gel electrophoresis (PFGE), ribotyping and PCR-based techniques, such as random amplified polymorphic DNA (RAPD) typing and restriction fragment length polymorphism (RFLP) analysis (Speijer *et al*., 1999). These methods are epidemiologically important for recognising outbreaks of infection, detecting the cross-transmission of nosocomial pathogens and determining the source of infection (Olive and Bean, 1999). The RAPD typing has been shown to be as discriminatory as PFGE for the typing of *P. aeruginosa* while being suitable for cost effective and time-efficient typing of large numbers of isolates (Mahenthiralingam *et al*., 1996).

The aim of this study was to utilise a sequence-specific PNA-based multiplex PCR to determine the prevalence of GES-2 and other GES-type ESBLs in *P. aeruginosa*; furthermore a PNA-probe in real-time PCR was applied for the identification and discrimination of GES-type ESBLs in *P. aeruginosa*. The study aimed to apply RAPD typing as a fingerprinting method to investigate the clonal relationship of GES positive *P. aeruginosa* isolates identified during the study.

The objectives of this study were:

1. To determine the genetic location (chromosomal or plasmid) of *bla*$_{GES}$-type genes in *P. aeruginosa* isolates
2. To optimise competitive PNA-probe exclusion for use in real-time PCR for identification of GES-type ESBLs in *P. aeruginosa*
3. Comparison between conventional PNA-probe exclusion multiplex PCR and real-time PNA-probe exclusion PCR
4. Sequencing of selected PCR amplicons to confirm *bla*$_{GES}$ mutations
5. To determine the clonal relationship between selected isolates utilising RAPD analysis
6. Data analysis and construction of a phylogenetic tree

References:

Ambler P, Coulson AFW, Frère JM, Ghuysen JM, Joris B, Forsman M, Lévesque RC, Tirabi G, Waley SG (1991) A standard numbering scheme for the class A beta-lactamases. *Biochem. J*. **276:** 269-272.

Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*. **14:** 993-951.

De Champs C, Poirel L, Bonnet R, Sirot D, Chanal C, Sirot J, Nordmann P (2002) Prospective survey of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. *Antimicrob. Agents Chemother.* **46:** 3031-3034.

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of acc(3)-Ib/aac(6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **46:** 638-645.

Finch RG (1998) Antibiotic resistance. *J. Antimicrob. Chemother.* **42:** 125-128.

Giakkoupi P, Tzouvelekis LS, Tsakris A, Loukova V, Sofianou D, Tzelepi E (2000) IBC-1, a novel integron-associated class A beta-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. *Antimicrob. Agents Chemother.* **44:** 2247-2253.

Jacoby GA, Munoz-Price LS (2005) Mechanisms of disease: The new beta-lactamases. *N. Engl. J. Med.* **352:** 380-391.

Jakimov D, Četojević-Simin D, Kojić V, Mrdanović J (2000) Peptide nucleic acid (PNA)-more than anyone could imagine at the first sight. *Arch. Oncol.* **8:** 11-14.

Mackay IM (2004) Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* **10:** 190-212.

Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Spreet DP (1996) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34:** 1129-1135.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:** 1661-1669.

Ørum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley CJ (1993) Single base pair mutation analysis by PNA-directed PCR clamping. *Nucleic Acids Res.* **21:** 5332-5336.

Pasteran F, Faccone D, Campos K, Vazquez M, Rapoport M, Littvik A, Tokumoto M, Galas M (2004) Dissemination of GES extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* (PA) in Argentina, abstr. C2-1327, p. 105. Program Abstr. 44th *Intersci. Conf. Antimicrob. Agents Chemoth.* ASM, Washington, D.C.

Pellestor F, Paulasova P (2004) The peptide nucleic acids, efficient tools for molecular diagnosis (Review). *Int. J. Mol. Med.* **13:** 521-525.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Weldhagen GF, De Champs C, Nordmann P (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J. Antimicrob. Chemother.* **49:** 561-565.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Poirel L, Naas T, Nordmann P (2006) Pyrosequencing as a rapid tool for identification of GES-type extended-spectrum beta-lactamases. *J. Clin. Microbiol.* **44:** 3008-3011.

Poole K (2004) Resistance to beta-lactam antibiotics. *Cell Mol. Life Sci.* **61:** 2200-2223.

Poole K (2005) Mechanisms of antimicrobial resistance: Opportunities for new targeted therapies. *Adv. Drug Deliv. Rev.* **57:** 1443-1445.

Speijer H, Savelkoul PHM, Bonten MJ, Stobberingh EE, Tjhie JHT (1999) Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. *J. Clin. Microbiol.* **37:** 3654-3661.

Stürenburg E, Mack D (2003) Extended-spectrum beta-lactamases: Implications for the clinical microbiology laboratory, therapy, and infection control. *J. Infect.* **47:** 273-295.

Svanvik N, Stahlberg A, Sehlstedt U, Sjoback R, Kubista M (2000) Detection of PCR products in real time using light-up probes. *J. Anal. Biochem.* **287:** 179-182.

Vahaboglu H, Öztürk R, Aygun G, Coskunkan F, Yaman A, Kaygusuz A, Leblebicioglu H, Balik I, Aydin K, Otkun M (1997) Widespread detection of PER-1-type extended-spectrum beta-lactamases among nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* isolates in Turkey: a nationwide multicenter study. *Antimicrob. Agents Chemother.* **41:** 2265-2269.

Waley SG (1992) Beta-lactamase: *Mechanism of action*., p. 198–228. *In* M. I. Page (ed.), The chemistry of beta-lactams. A. and P. Blackie, London.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004a) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Weldhagen GF (2004b) Rapid detection and sequence-specific differentiation of extended-spectrum beta-lactamase GES-2 from *Pseudomonas aeruginosa* by use of a real-time PCR assay. *Antimicrob. Agents Chemother.* **48:** 4059-4062.

Chapter 2

Literature Review

2.1 Introduction

The genus *Pseudomonas* contains over 140 species, with more than 25 species associated with humans (Todar, 2002). The *Pseudomonas* spp. known to cause disease in humans are mostly associated with opportunistic infections and include *Pseudomonas aeruginosa* (*P. aeruginosa*), *P. fluorescens*, *P. putida*, *P. putrefaciens* and *P. stutzeri* (Todar, 2002). *Pseudomonas aeruginosa* has received the most attention because of the high frequency with which it is involved in human disease (Todar, 2002). Together with *P. maltophilia*, *P. aeruginosa* account for 80% of *Pseudomonas* spp. recovered from clinical specimens (Todar, 2002).

Pseudomonas aeruginosa is an important bacterial pathogen, often responsible for nosocomial infections and infections in immunocompromised patients (Gençer *et al*., 2002). Infections caused by *P. aeruginosa* are of particular concern in patients with severe burns, cancer, cystic fibrosis and acquired immuno-deficiency syndrome (AIDS) (Todar, 2002). The case fatality rate in these patients can be as high as 50% (Todar, 2002). The high fatality rate observed for *P. aeruginosa* infections in immunocompromised patients is due to a combination of weakened host defences, bacterial resistance to antibiotics and the production of extracellular bacterial enzymes and toxins (Todar, 2002).

Pseudomonas aeruginosa's resistance to antibiotics is often mediated by the production of enzymes such as β-lactamases (Bert *et al*., 2002). The extended-spectrum β-lactamases (ESBLs) constitutes a major problem in the use of β-lactam antibiotics to treat *P. aeruginosa* infections (Poole, 2004). This is due to the ESBLs broad substrate specificity and ability to hydrolyse many of the extended-spectrum third-generation cephalosporins (Poole, 2004). Extended-spectrum β-lactamase producing bacteria are typically resistant to penicillins, first- and second-generation cephalosporins as well as the third-generation oxyimino-cephalosporins (e.g. ceftazidime, ceftriaxone) and monobactams (aztreonam) (Poole, 2004). The ESBLs currently identified in *P. aeruginosa* include the Belgium extended (BEL) β-lactamases, Guiana extended-spectrum (GES) β-lactamases, Imipenemase (IMP)

β-lactamases, PER (named after original authors P. Nordmann, E. Ronco and R. Labia) β-lactamases, *Pseudomonas*-specific (PSE) β-lactamases, Sulfhydryl variable (SHV) β-lactamases, Temoneira (TEM) β-lactamases and Verona integron-encoded metallo-β-lactamase (VIM) (Jacoby and Munoz-Price, 2005; Poirel *et al*., 2005).

The detection of ESBL-producing *P. aeruginosa* isolates, using standard methods in the clinical microbiology laboratory is difficult (Weldhagen *et al*., 2003). Molecular techniques such as polymerase chain reaction (PCR)-based methods have proven more successful (Weldhagen *et al*., 2003). Apart from conventional PCR, real-time PCR can be used to perform multiplex PCR (Svanvik *et al.*, 2000) Various reporters have been developed for real-time PCR, with the simplest assay using double-stranded DNA (dsDNA) specific dyes, such as the asymmetric cyanine dye SYBR Green (Svanvik *et al.*, 2000). If non-specific reporters are used for real-time detection, inclusion of a post-PCR fluorescence-mediated DNA melting curve analysis is required to distinguish between the different products (Svanvik *et al.*, 2000).

The GES/IBC (Guiana Extended-spectrum/Integron-Borne Cephalosporinase) β-lactamase family is still relatively small and has only a few point mutations separating the genes in question (Weldhagen, 2004a), making it the ideal model for the development of the sequence-selective PNA-based real-time PCR method. The PNA-based PCR detection method relies on PNAs intrinsically high affinity for DNA and PNAs inability to serve as a primer for DNA polymerases (Ørum *et al*., 1993). The aims of the study were to: investigate the prevalence of GES-1 and GES-2 in *P. aeruginosa* strains utilising the competitive PNA-based multiplex PCR; investigate the genetic location of bla_{GES-1} and bla_{GES-2} genes identified; investigate the prevalence of GES-type ESBLs in *P. aeruginosa* utilising DNA sequencing; optimise the competitive PNA-based multiplex for use in real-time PCR utilising SYBR Green; investigate the clonal relatedness of the bla_{GES} positive *P. aeruginosa* isolates through random amplified polymorphic DNA (RAPD) analysis. The dissemination of β-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections due to ESBL-producing *P. aeruginosa* (Weldhagen *et al.*, 2003). Due to this, the continuous monitoring of antibiotic resistance genes and search for novel antibiotic resistance genes in bacteria is essential to

enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings (Sardelic *et al.*, 2003).

2.2 Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was first described as a distinct bacterial species at the end of the nineteenth century (Campa *et al*, 1993). Although *P. aeruginosa* was described as an infective agent by 1889, the importance of the bacterium as a human pathogen, especially in hospitalised patients, did not emerge until the second half of the twentieth century (Campa *et al*, 1993). Presently, *P. aeruginosa* is considered one of the most important causes of nosocomial infections in hospital settings (Todar, 2002).

2.2.1 Morphology, growth and metabolism of *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative non-sporulating bacillus, ubiquitous in soil and water environments (Römling *et al*., 1994; Todar, 2002). The rods vary in length from 1.5 to 3 µm, are straight or slightly curved and occur single, in pairs or in short chains (Pollack, 2000). Most strains are motile by means of one or more polar flagella (Todar, 2002).

The bacterium has a strictly respiratory metabolism, but can grow in the absence of oxygen if nitrate is available as an electron acceptor (Todar, 2002). *Pseudomonas aeruginosa* grows optimally at 37°C, but can survive and multiply over a wide temperature range (20°C - 42°C) (Pollack, 2000). Growth at specifically 42°C distinguishes *P. aeruginosa* from other *Pseudomonas* species (Pollack, 2000). *Pseudomonas aeruginosa* possesses metabolic versatility, enabling the bacterium to grow on the simplest media supplying only acetate for carbon and ammonium sulphate for nitrogen (Pollack, 2000; Todar, 2002). The bacterium is able to utilise over 75 organic compounds (e.g. glucose, glycerol and tryptone) and requires no organic growth factors (e.g. vitamins and amino acids) (Pollack, 2000; Todar, 2002). These properties contribute to *P. aeruginosa's* role as an opportunistic pathogen (Pollack, 2000).

2.2.2 Epidemiology of *P. aeruginosa*

In addition to being an inhabitant of soil, water and plants, the bacterium may also be found on the skin of healthy people (Nester *et al.*, 2001; Todar, 2002). *Pseudomonas aeruginosa* is introduced and re-introduced into the hospital setting on shoes, ornamental plants, flowers and produce as well as from visitors and patients transferred from other facilities (Nester *et al*., 2001; Todar, 2002). Once introduced into the hospital *P. aeruginosa* can persist in places where there is dampness or water (Nester *et al*., 2001). *Pseudomonas aeruginosa* has the ability to form biofilms greatly enhancing the ability of the bacterium to adhere to and survive on environmental surfaces, medical devices and the airways of patients with chronic lung disease (particularly cystic fibrosis patients) (Navon-Venezia *et al*., 2005).

Numerous reservoirs for *P. aeruginosa* exist in the hospital setting such as disinfectants (e.g. quaternary ammonium compounds), food, sinks, taps, mops, ointments, hospital equipment (respirators), eye drops and contact lens solutions as well as cosmetics (Nester *et al*., 2001; Todar, 2002). *Pseudomonas aeruginosa* has even been isolated from the throat and stool of non-hospitalised patients (Todar, 2002). The gastrointestinal carriage rates can increase from 5% in non-hospitalised patients to 20% in hospitalised patients within 72 h after admission (Todar, 2002). This increase may be due to person-to-person (nursing personnel-to-patient) spread of the bacterium (Wilson and Dowling, 1998).

2.2.3 Pathogenesis and virulence of *P. aeruginosa*

Infection caused by *P. aeruginosa* may occur in three stages: i) bacterial attachment and colonisation, ii) local infection and iii) blood stream dissemination and systemic disease (Todar, 2002). The overall effect of *P. aeruginosa* infection is to prevent healing by causing tissue damage, which increases the risk of the development of septic shock (Nester *et al*., 2001). Most infections caused by *P. aeruginosa* are both invasive and toxigenic due to the bacterium's ability to produce toxins, specifically Exotoxin A (Todar, 2002). Virulence factors in *P. aeruginosa* and their biological activities were summarized in Table 2.1.

Table 2.1: Virulence factors of *P. aeruginosa* associated with disease (Wilson and Dowling, 1998)

Virulence factor	Biological action
Mucoid exopolysaccharide (alginate)	Adherence to epithelium; barriers to phagocytes; inhibits antibody and complement binding
Protease enzymes	Tissue damage; epithelial cell tight junction separation; degrades fibronectin; cleaves antibodies creating non-functional blocking antibodies; inactivates α_1-antiproteinase; complement components and cytokines; cleave C3b receptors from neutrophils; stimulates mucous secretion
Exotoxin A	Cytotoxic by inhibiting protein synthesis; toxic to macrophages; T cell mitogen; inhibits granulocytes and macrophage progenitor cell proliferation
Lipopolysaccharide	Dominant antigenic determinant on cell surface; loss of sugar unit side chains during chronic infection creates "rough" LPS and serum sensitivity; less potent endotoxin properties than other Gram-negative species
Pigments (pyocyanin, pyoverdin)	Inhibits ciliary beat; siderophores; toxic to other bacterial species and human cells; enhance oxidative metabolism of neutrophils; inhibits lymphocyte proliferation
Phospholipase C	Haemolysis; tissue damage; destroys surfactant
Rhamnolipid	Inhibits ciliary beat; stimulate mucus secretion; affect ion transport across epithelium; Haemolysis
Pili	Adherence to epithelium
Lipase	Tissue damage
Histamine	Impair epithelial integrity
Exoenzyme S	Cytotoxic; Adherence to epithelium
Leukocidin	Cytotoxic to neutrophils and lymphocytes

Since *P. aeruginosa* is an opportunistic pathogen, it does not cause infection in the absence of impaired host defences (Wilson and Dowling, 1998). In those cases where infection does occur, loss of the integrity of a physical barrier to infection (e.g. skin, mucous membrane) or underlying immune deficiency (e.g. neutropenia, immunosuppression) is usually present (Tang *et al*., 1996). However, despite the opportunistic nature of *P. aeruginosa,* the wide array of potential virulence factors that have been described contribute to the bacterium's pathogenicity in the compromised patient (Wilson and Dowling, 1998).

2.2.4 Clinical manifestation of *P. aeruginosa* infections

Pseudomonas aeruginosa seldom infects healthy tissues, yet there is scarcely any tissue this opportunistic pathogen cannot infect if the hosts tissue defences are compromised in some manner (e.g. due to burns) (Tang *et al*., 1996; Todar, 2002). Since the first reported case of *P. aeruginosa* in 1890 this bacterium has been associated with various diseases such as urinary tract infections, respiratory tract infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections and gastrointestinal infections (e.g. necrotising enterocolitis) (Pollack, 2000; Todar, 2002). Bacteremia due to *P. aeruginosa* is most prevalent in patients with malignancy, chemotherapy, AIDS, burn wound sepsis and diabetes

(Römling *et al*., 1994; Tang *et al*., 1996). Predisposing conditions in patient populations especially those susceptible to *P. aeruginosa* infections include placement of intravenous lines, severe burns, urinary tract catheterisation, surgery, trauma and premature birth (Hauser and Sriram, 2005).

2.2.5 Treatment of *P. aeruginosa* infections

Surgical debridement and topical therapy with antibacterial agents such as silver sulfadiazine, is often suggested for the prevention of infection with *P. aeruginosa* in burn victims (Nester *et al*., 2001; Todar, 2002). Treatment with antibiotics is however, recommended when infection does occur (Nester *et al*., 2001). Table 2.2 describes some of the antibiotics used in the treatment of *P. aeruginosa* infections.

Care should be taken in the choice of antibiotic for the treatment of *P. aeruginosa* infection since both synergistic and antagonistic effects have been noted between certain antibiotics (Hauser and Sriram, 2005). Synergistic effects have been noted in combinations of i) antipseudomonal extended-spectrum penicillins with fluoroquinolones and ii) antipseudomonal β-lactams (e.g., penicillin, cephalosporin) with aminoglycosides (Hauser and Sriram, 2005). Two-drug combination therapy with antibiotics that fall within the above stated groups are thus recommended for treatment of *P. aeruginosa* infections (Hauser and Sriram, 2005). Apart from two-drug combination therapy three-drug combinations have been recommended consisting of a carbapenem (e.g., imipenem, meropenem), an antipseudomonal quinolone and an aminoglycoside (Hauser and Sriram, 2005).

Table 2.2: Classes of antibiotics used against *P. aeruginosa* infections (Wilson and Dowling, 1998; Hauser and Sriram, 2005)

Drug	Comment
Antipseudomonal penicillin (Tazocin®)	Piperacillin-Tazobactam combination. Negatively influenced by aminoglycosides. High allergic potential. Minimizes emergence of multi-drug resistant gram-negative rods
4th generation cephalosporin (Cefepime)	Also known as Maxipime®. Effective for use against most strains of Ceftazidime-resistant *P. aeruginosa*. Suitable for proven serious systemic *P. aeruginosa* infections, febrile neutropenia or use in cystic fibrosis
Carbapenem (Imipenem)	Rapidly hydrolysed by dehydropeptidase I (present on renal tubular cells). Combined with cilastatin (reversible inhibitor of dehydropeptidase I). Combination with chloramphenicol decrease antimicrobial effects
Cephalosporin (Ceftazidime)	Inhibits bacterial growth through binding of penicillin-binding proteins. Ceftazidime levels may be increased by probenecid. Is a third generation cephalosporin. Show high penetration into the subarachnoid space (ideal for treating meningitis)
Quinolone (Ciprofloxacin)	Bactericidal effect in both actively dividing and dormant cells. Inhibits DNA synthesis. Available in both oral and intravenous formulations. Shows greater activity than levofloxacin and gatifloxacin
Aminoglycoside (Tobramycin)	Potentiates effect of extended-spectrum penicillins. Toxicity due to accumulation. Do not penetrate well into lungs and bronchopulmonary secretions. Inactive in acidic environments. Associated with nephrotoxicity and ototoxicity
Monobactam (Aztreonam)	Incompatible in solutions containing Vancomycin or Metronidazole. Safe for use in penicillin allergic patients

Antagonism has been demonstrated in dual β-lactam combinations (Hauser and Sriram, 2005). One such combination is the use of imipenem plus piperacillin (Hauser and Sriram, 2005). In this case, imipenem is a potent inducer of AmpC β-lactamases, but is resistant to the enzymatic activity (Hauser and Sriram, 2005). Piperacillin does not induce AmpC β-lactamase, but is degraded by the enzyme when produced in high amounts (Hauser and Sriram, 2005). Thus, when these two β-lactams are given together, imipenem induces AmpC β-lactamase production, resulting in the degradation of piperacillin (Hauser and Sriram, 2005). Apart from antagonistic reactions, mono-therapy is only recommended in cases involving febrile patients with neutropenia, where ceftazidime or a carbapenem (e.g. imipenem, meropenem) is used (Hauser and Sriram, 2005). Nosocomial infection is difficult to treat, since *P. aeruginosa* is frequently resistant to multiple antibacterial agents (Nester *et al*., 2001). Due to this, antibiotic susceptibility tests are done to guide the selection of an effective treatment regimen, which is usually administered intravenously in high doses (colistin, 2.5 to 5.0 $mg^{-1}.kg^{-1}.day$; polymyxin B, 2.5 to 3.0 $mg^{-1}.kg^{-1}.day$) until the infection is cleared (Mandell *et al*., 2005).

Empirical therapy in a patient with nosocomial sepsis who is at risk of *P. aeruginosa* infection should include an antipseudomonal β-lactam (Hauser and Sriram, 2005). Based on pharmacokinetics/pharmacodynamics (PK/PD) the preferred agents include imipenem (500 mg six hourly), cefepime (2 g eight hourly) or ceftazidime (2 g eight hourly) (Mandell *et al*., 2005). As soon as susceptibility results for the *P. aeruginosa* isolate are available, antibiotics should be changed accordingly. In case of multi-resistant *P. aeruginosa*, colistin (2.5 to 5.0 $mg^{-1}.kg^{-1}.day$) is recommended. Antibiotic therapy should be continued until the patient is fever free for three days and the patient's infection-markers (e.g. normal CRP; PCT < 0.5; normal while cell count) are stabilised (Mandell *et al*., 2005).

2.3 Mechanisms of antibiotic resistance in *P. aeruginosa*

The mechanisms involved in antibiotic resistance of bacteria may be divided into three distinct types: inactivation of the drug (enzymatic e.g. β-lactamases); modification of the target of action; and reduction in the concentration of the drug that reaches the target without modification of the drug itself (Hogan and Kolter, 2002; Poole, 2005). *Pseudomonas aeruginosa* exhibits natural, intrinsic and acquired resistance to antibiotics, which will be discussed in detail (sections 2.3.1 and 2.3.2) (Nakae *et al*., 1999; De Freitas and Barth, 2002; Kugelberg *et al*., 2005).

2.3.1 Intrinsic resistance of *P. aeruginosa* to antibiotics

Pseudomonas aeruginosa is intrinsically resistant to a wide range of antibiotics (Kugelberg *et al*., 2005). This intrinsic resistance is mainly due to low outer membrane permeability and the presence of several drug efflux systems (Nakae *et al*., 1999; Kugelberg *et al*., 2005). The reduced outer membrane permeability was thought to play a key role in intrinsic resistance but this is now attributed to synergy between a low-permeability outer membrane and active efflux from the cell (Schweizer, 2003). In those isolates exhibiting reduced outer membrane permeability, reduced permeability is caused by porin deficiency, with the OprD porin commonly lost in *P. aeruginosa* (Poole, 2004).

To date five families of bacterial efflux systems have been described from different species (e.g. *Staphylococcus aureus*, *Escherichia coli*, *P. aeruginosa*, *Lactobacillus lactis*) (Figure 2.1): the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) family; the resistance-nodulation-division (RND) family; the multidrug and toxic compound extrusion (MATE) family; and the small multidrug resistance (SMR) family (Schweizer, 2003; Poole, 2004). The RND family is described as the most significant regarding export of clinically important antimicrobials (Poole, 2004). Resistance-nodulation-division efflux systems in *P. aeruginosa* include MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexJK, MexXY and CzrAB-OmpN (Schweizer, 2003). The substrate spectrum for many of these pumps is wide and includes β-lactams, β-lactam inhibitors, fluoroquinolones, tetracycline, chloramphenicol, novobiocin, macrolides, ethidium bromide, sodium dodecyl sulfate (SDS), vanadium, erythromycin, cadmium and zinc (Brooun *et al*., 2000; Schweizer, 2003).

Pseudomonas aeruginosa also harbors an inducible chromosomally encoded β-lactamase responsible for intrinsic resistance (Livermore, 2002). The enzyme is referred to as AmpC β-lactamase (class C cephalosporinase) and is capable of degrading many β-lactams even though it is naturally expressed at very low levels (Livermore, 2002). Antibiotics such as cephalothin and ampicillin may induce AmpC production (Livermore, 2002).

In addition to efflux-pumps and AmpC production, biofilm formation may contribute to the intrinsic resistance of *P. aeruginosa* (Hogan and Kolter, 2002; Hauser and Sriram, 2005; Poole, 2005). The factors that contribute to biofilm-associated antibiotic resistance include: slow growth rates; decreased diffusion of antibiotics through the biofilm; accumulation of enzymes (polysaccharases, proteases and β-lactamases) that contribute to resistance and the activation of stress responses in bacteria within the biofilm (Gilbert *et al*., 2002; Hogan and Kolter, 2002).

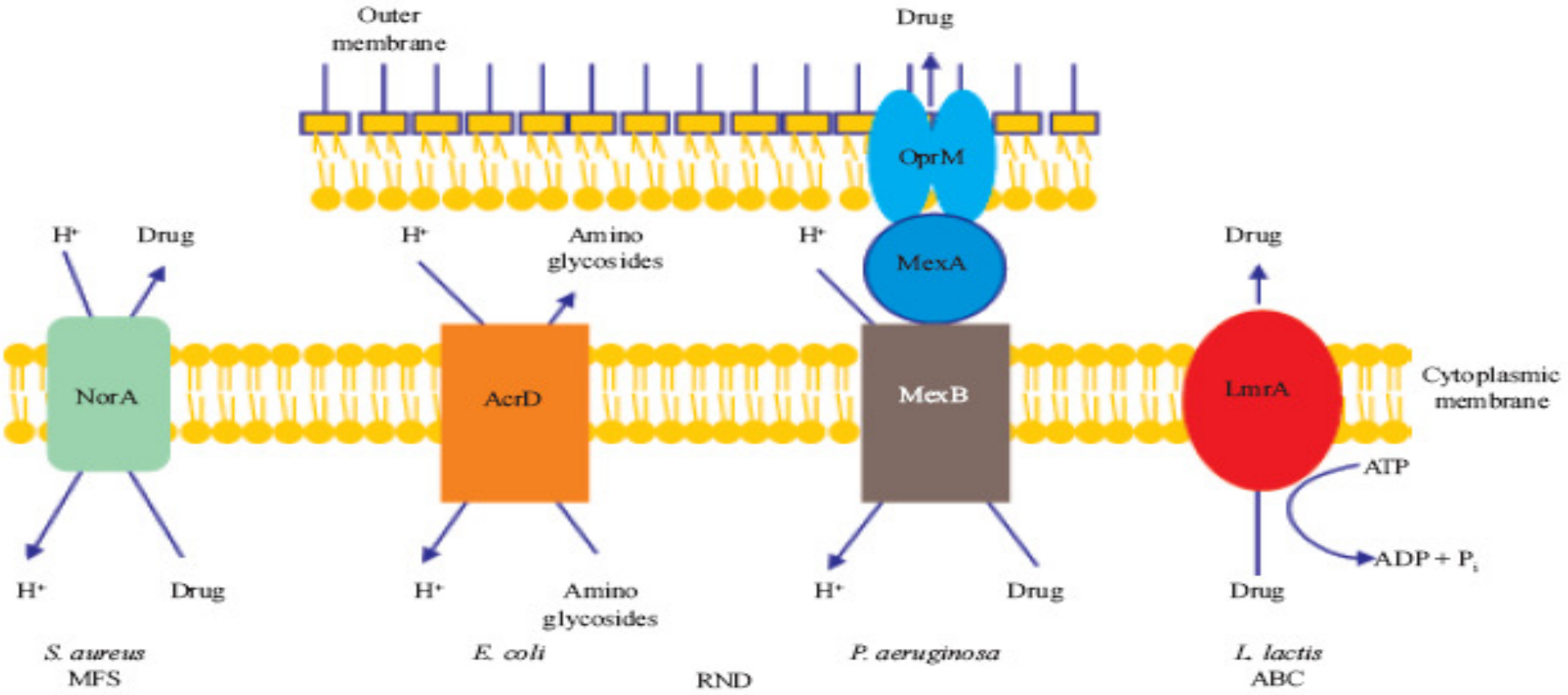

Figure 2.1: Schematic illustration of the main types of bacterial drug efflux-pumps in *Staphylococcus aureus*, *Escherichia coli*, *P. aeruginosa* and *Lactobacillus lactis*. Illustrated are NorA, a member of the major facilitator superfamily (MFS); AcrD and MexAB-OprM, two members of the resistance-nodulation-division (RND) family and LmrA, a member of the ATP-binding cassette (ABC) family. All systems extrude drugs in an energy dependant manner, either by using proton motif force or ATP. The two other types of efflux systems found in bacteria, multidrug and toxic compound extrusion (MATE) and small multidrug resistance (SMR), are structurally similar to the MFS but were designated as distinct families, based on phylogenetic diversity (MATE) or size (SMR) (Schweizer, 2003)

2.3.2 Acquired resistance to antibiotics in *P. aeruginosa*

Specific antibiotic resistance mechanisms can be acquired through mutation of the bacterial genome (Hogan and Kolter, 2002). A second method for antibiotic resistance acquisition is gaining additional genes through horizontal gene transfer (Hogan and Kolter, 2002).

2.3.2.1 Mutations causing resistance of *P. aeruginosa* to antibiotics

Various antibiotics are designed to overcome the inherent defences of *P. aeruginosa* and are active against most isolates. These antibiotics include penicillins, cephalosporins, carbapenems, monobactams, aminoglycosides, fluoroquinolones and polymyxins (Livermore, 2002). However, bacterial strains may still develop resistance to these compounds through mutations (Livermore, 2002). Examples include fluoroquinolone resistance gained due to mutations to topoisomerases II and IV (regulate over- and under-winding of the DNA double helix and resolve nucleic acid knots and tangles), depression of the chromosomal AmpC β-lactamase reduces susceptibility to penicillins and cephalosporins, while up-regulation of MexAB-OprM compromises fluoroquinolones, penicillins, cephalosporins and meropenem (Froelich-Ammon and Osheroff, 1995; Livermore, 2002). Similarly other efflux systems may be up-regulated through mutations, resulting in resistance to fluoroquinolones, β-lactams and aminoglycosides (Livermore, 2002). Mutations in RNA polymerases and DNA gyrase may result in resistance to the rifamycins and quinolones, respectively (Lambert, 2005).

2.3.2.2 Horizontal gene transfer as acquisition mode of antibiotic resistance by *P. aeruginosa*

Genes conferring antibiotic resistance can be exchanged among bacterial populations (Carattoli, 2001; Hogan and Kolter, 2002). The principal mechanisms involved in genetic exchange among bacteria are transformation (incorporation of free DNA carrying resistance genes from the environment), transduction (transfer of genetic material between pathogenic species by bacteriophages) and conjugation (DNA transfer during bacterial mating) (Roy, 1999; Carattoli, 2001). The vehicles for genetic exchange involved in these mechanisms are plasmids, transposons and integrons (Figure 2.2.) (Roy, 1999; Rice, 2001; Poole, 2004).

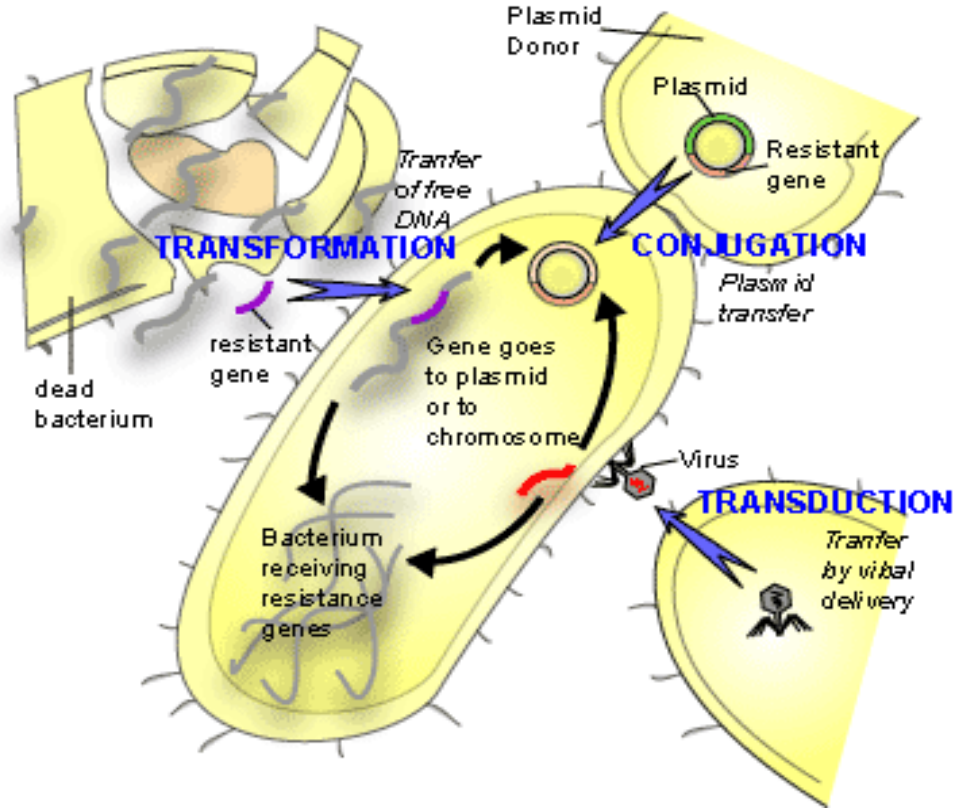

Figure 2.2: Illustration of the principal mechanisms involved in genetic exchange among bacteria showing transformation, conjugation and transduction (Yim, 2007)

Plasmids are extrachromosomal double stranded DNA, usually circular and varying in size from a few kilobases to a 10th or more of the size of the bacterial chromosome (Jacoby and Munoz-Price, 2005). Plasmids were divided into F (sex factor) and R (resistance) plasmids, but many R-plasmids were found to encode F-like replication or transfer genes (Roy, 1999). Most of the antibiotic resistance genes are disseminated by conjugative R-plasmids (Roy, 1999). These resistance plasmids carry the resistance genes, often organised into integrons or carried on transposons (Jacoby and Munoz-Price, 2005). Transposons, also known as jumping genes, are responsible for the dissemination of several antibiotic and heavy metal resistance genes (Roy, 1999).

Integrons may be defined as elements that contain the genetic determinants of the components of a site-specific recombination system that recognises and captures mobile gene cassettes (Fluit and Schmitz, 1999). An integron includes the gene for an integrase (*int*), which is responsible for mobilising and inserting gene cassettes by a site-specific recombination mechanism into an adjacent recombination site (*attI*) (Fluit and Schmitz, 1999).

To date four classes of integron have been described, differing in the nature of the integrase gene and the overall structure (Poirel *et al*., 2000). These integrons are divided into resistance integrons (classes 1 to 3) and super integrons (class 4) (Fluit and Schmitz, 2004). The resistance integrons carry gene cassettes encoding resistance against antibiotics (e.g. β-lactams and aminoglycosides) and disinfectants (e.g. quarternary ammonium compounds) and may be located on either the chromosome or plasmids (Fluit and Schmitz, 2004). Super integrons on the other hand are located on the chromosome and contain gene cassettes that encode a variety of functions and proteins such as chloramphenicol acetyltransferase, fosfomycin resistance protein, haemagglutinin and lipoproteins (Fluit and Schmitz, 2004).

The majority of integrons described are class 1 integrons (Figure 2.3) (Fluit and Schmitz, 1999). The class 1 integrons possess a 5'-conserved segment (5'-CS) that contains the integrase gene (*intI1*), a recombination site (*attI1*) and most commonly, a 3'-conserved segment (3'-CS) (Poirel *et al*., 2000). The 3'-CS carries *qacE*Δ*1*, the functional deletion derivative of the *qacE* gene (disinfectant resistance) the *sulI* gene (sulphonamide resistance) and an open reading frame (ORF) of unknown function (ORF5) (Poirel *et al*., 2000).

Within the 5'-CS and 3'-CS segments the variable region of the integron is made up of gene cassettes, usually antibiotic resistance genes such as β-lactamases (Poirel *et al*., 2000). The gene cassettes are associated with a 59-base element (59-be) located downstream of the gene of the integrated gene cassette (Poirel *et al*., 2000). The 59-be's (also known as *attC*) are not highly conserved and vary in length from 57 to 141 base pairs (bp) (Fluit and Schmitz, 1999; Poirel *et al*., 2000). The 59-be's are all bounded by a core site (GTTRRRY) at the recombinant crossover point and an inverse core site (RYYYAAC) at the 3' end of the inserted gene (Fluit and Schmitz, 1999; Poirel *et al*., 2000).

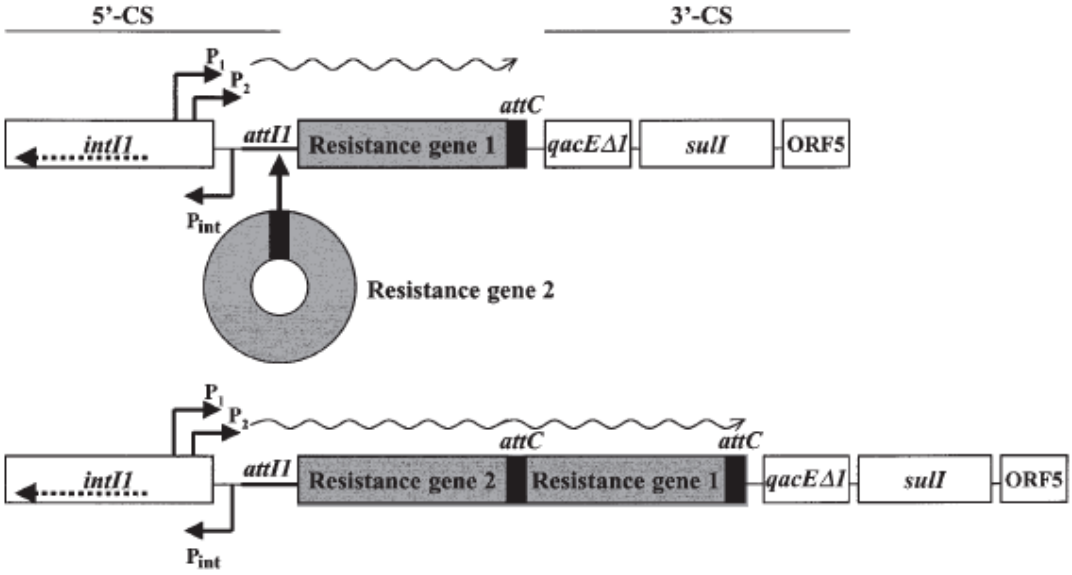

Figure 2.3: Schematic representation of a class 1 integron with a model for gene cassette acquisition. The process by which a circularized gene cassette (resistance gene 2) is inserted at the attI site in a class 1 integron containing a resistance gene cassette (resistance gene 1) is outlined in the figure. Genes and open reading frames in the 5'-and 3'-CS of a schematic class 1 integron are indicated by boxes. Resistance gene cassette inserted within the integrons is indicated by grey boxes and vertical black bars represent attC recombination sites. P_1 and P_2 are integron-associated promoters; P_{int} is the intergrase gene (*intI*1) promoter. The *qacE*Δ1 and *sulI* genes in the 3'-CS confer resistance to quaternary ammonium compounds and sulfonamides, respectively (Fonseca *et al.*, 2005)

The resistance genes captured by integrons often encode enzymes conferring antibiotic resistance through drug inactivation (Hogan and Kolter, 2002; Poole, 2004). These enzymes include β-lactamases, aminoglycoside-modifying enzymes, chloramphenicol acetyltransferases as well as tetracycline- and macrolide-inactivating enzymes (Schweizer, 2003). Various acquired β-lactamases and aminoglycoside-modifying enzymes are prevalent in *P. aeruginosa* (Livermore, 2002).

2.4 The extended-spectrum β-lactamases in bacteria

The production of β-lactamases is a major mechanism of β-lactam resistance in *P. aeruginosa* (Weldhagen *et al.*, 2003). These hydrolytic enzymes disrupt the amide bond (Figure 2.4) of the characteristic four-membered β-lactam ring, rendering the antimicrobial ineffective (Poole, 2004). Ambler and colleagues (1991) have classified β-lactamases into four molecular classes, A-D.

The enzymes, able to target the extended-spectrum β-lactams, are of particular concern for healthcare facilities (Poole, 2004). These enzymes include the AmpC (class C cephalosporinase) enzymes, the so-called extended-spectrum β-lactamases (ESBLs) (classes A and D) and the carbapenemases that hydrolyse most β-lactams, including the carbapenems (classes A, B and D) (Poole, 2004).

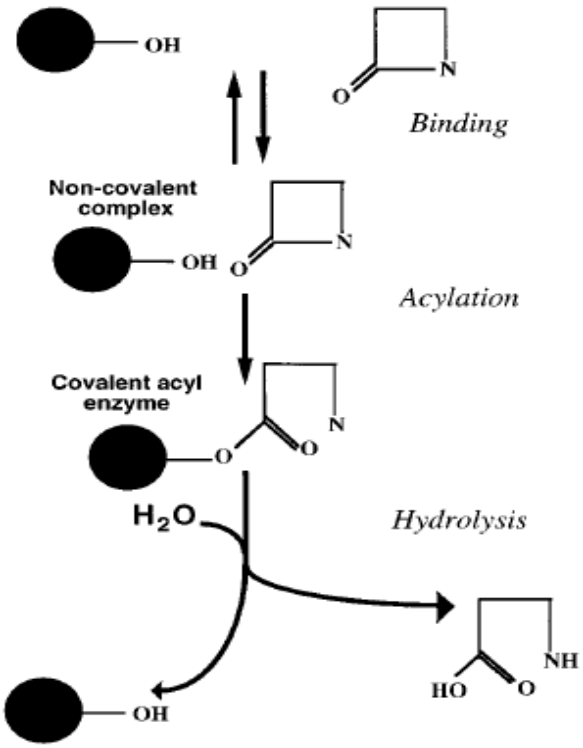

Figure 2.4: Schematic representation of an active site serine β-lactamase hydrolysing the β-lactam ring of a β-lactam antibiotic. The β-lactamase associates non-covalently with the antibiotic to yield a non-covalent Michaelis complex. The β-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and the hydrolysed, inactive drug. This mechanism is utilised by β-lactamases of molecular classes A, C and D, but class B enzymes utilise a zinc ion to attack the β-lactam ring (Waley, 1992)

Various ESBLs have been found in *P. aeruginosa* and can be divided into three groups namely penicillinase-derivates (class A β-lactamases), metallo-enzymes (class B enzymes) and oxacillinases (class D enzymes) (Nordmann and Guibert, 1998). Stürenburg and Mack (2003) defined ESBLs as molecular class A or D β-lactamases that: i) are able to hydrolyse oxyimino cephalosporins at a rate equal to or higher than 10% of that for benzylpenicillin, ii) have an active-site serine and iii) are generally inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam.

2.4.1 Characterisation of ESBLs in bacteria

Most ESBLs belong to the Ambler's molecular class A (Ambler *et al*., 1991; Bradford, 2001). The class A enzymes are characterised by a serine residue at the active site, a molecular mass of approximately 29 000 Da and preferential hydrolysis of penicillins (Bradford, 2001). However, the molecular classification scheme does not sufficiently differentiate the different types of class A enzymes (Bradford, 2001).

Bush and colleagues (1995) devised a classification scheme using the biochemical properties of the enzyme, the molecular structure and the nucleotide sequence of the genes to place β-lactamases into functional groups. With this scheme ESBLs were defined as β-lactamases capable of hydrolysing oxyimino cephalosporins that were inhibited by clavulanic acid (Bush *et al.*, 1995). According to this classification scheme ESBLs were placed into functional group 2be (Bush *et al.*, 1995).

2.4.2 Types of ESBLs in bacteria

Most ESBLs arose from the TEM or SHV enzymes through point mutations at selected loci giving rise to the extended-spectrum phenotype (Bradford, 2001). The nomenclature of β-lactamases is not standardised (Jacoby and Munoz-Price, 2005). The β-lactamase SHV denotes a variable response to sulphydryl inhibitors and TEM was named after the patient Temoneira, from whom the first sample was obtained (Jacoby and Munoz-Price, 2005).

2.4.2.1 TEM-type ESBLs (class A) in bacteria

The enzyme, TEM-1, is the most commonly encountered β-lactamase in Gram-negative bacteria which is responsible for up to 90% of ampicillin resistance in *E. coli* (Bradford, 2001). There are more than 130 TEM enzymes recognised at present (Jacoby and Munoz-Price, 2005). The TEM enzymes have been reported in genera of the family *Enterobacteriaceae* such as *Enterobacter aerogenes*, *Proteus* spp. and *Salmonella* spp. (Bradford, 2001). The TEM-type ESBLs have been found in non-*Enterobacteriaceae* Gram-negative bacteria such as TEM-42 in *P. aeruginosa* and TEM-17 in *Capnocytophaga ochracea* (Bradford, 2001).

2.4.2.2 SHV-Type ESBLs (class A) in bacteria

The enzyme SHV-1 is related to TEM-1 with 68% amino acid homology and has a similar structure (Jacoby and Munoz-Price, 2005). There are relatively few derivatives of SHV-1 compared to TEM-1 with approximately 50 varieties currently recognized (Jacoby and Munoz-Price, 2005). Amongst the SHV variants many are characterised by the substitution of a serine (Ser) for a glycine (Gly) at position 238 and a substitution of lysine (Lys) for glutamate (Glu) at position 240 (Bradford, 2001). It has been noted that the Ser at position 238 is critical for efficient hydrolysis of ceftazidime and the Lys residue at position 240 is critical for efficient hydrolysis of cefotaxime (Bradford, 2001). The SHV-type ESBLs are most prevalent in *Klebsiella pneumoniae* but these enzymes have also been identified in *Citrobacter diversus*, *E. coli* and *P. aeruginosa* (Bradford, 2001; Weldhagen *et al*., 2003).

2.4.2.3 Cefotaximase-Type ESBLs (class A) in bacteria

Cefotaximase (CTX-M) exhibits greater activity against cefotaxime than against ceftazidime (Jacoby and Munoz-Price, 2005). A unique feature of these enzymes is that they are better inhibited by tazobactam than by sulbactam or clavulanate, probably due to increased active site flexibility (Bradford, 2001; Wilke *et al*., 2005). The CTX-M enzymes are the most common group of ESBLs that do not belong to the SHV or TEM families (Jacoby and Munoz-Price, 2005). The CTX-M enzymes share approximately 40% homology with TEM- and SHV-type ESBLs (Bradford, 2001).

Currently more than 40 CTX-M enzymes are known with CTX-M-2, CTX-M-3 and CTX-M-14 being most widespread (Jacoby and Munoz-Price, 2005). The CTX-M ESBLs have mainly been identified in strains of *Salmonella enterica* serovar *Typhimurium*, *E. coli* and other species of *Enterobacteriaceae* (Bradford, 2001). The CTX-M-type enzymes have not been identified in *P. aeruginosa* and are so far restricted to the family *Enterobacteriaceae* (Weldhagen *et al*., 2003).

2.4.2.4 Oxacillinase-Type ESBLs (class D) in bacteria

The Oxacillin-hydrolysing (OXA-type) ESBLs belong to molecular class D and according to the classification scheme of Bush and colleagues (1995), functional group 2d (Bradford, 2001). Currently, twelve OXA-type ESBLs are known, derived from OXA-1, OXA-2 or OXA-10 by amino acid substitutions (N143S; G157D; A124T); often adjacent to active site residues (Trp 154 and Leu 155), affecting their orientation and thus the characteristics of the enzymes (Paetzel *et al*., 2000; Jacoby and Munoz-Price, 2005). The OXA-10 derived ESBLs often carry substitutions at Gly 157 (e.g. G157D) that are responsible for resistance to ceftazidime (Poole, 2004). The OXA-type ESBLs exhibit high hydrolytic activity against oxacillin and cloxacillin, confer resistance to ampicillin and cephalothin and are poorly inhibited by clavulanic acid (Bradford, 2001). The OXA-type ESBLs in contrast to most of the other ESBLs are mainly found in *P. aeruginosa* (Bradford, 2001).

2.4.2.5 Metallo-enzyme Type ESBLs (class B) in bacteria

The metallo-enzymes hydrolyse most β-lactams including ceftazidime, cefsulodin, cefepime and cefpirome (Nordmann and Guibert, 1998). All class B enzymes including metallo-enzymes are not inhibited by clavulanic acid or tazobactam (Nordmann and Guibert, 1998). Metallo-enzymes in *P. aeruginosa* are exemplified by imipenemase (IMP-1) (Nordmann and Guibert, 1998). The IMP-1 enzyme is often termed as a carbapenemase since it hydrolyses imipenem and meropenem (Nordmann and Guibert, 1998). The gene coding the IMP-1 enzyme is transferred through plasmids and integrons (Nordmann and Guibert, 1998).

2.4.2.6 Novel ESBL groups in bacteria

In addition to the established enzyme families, unusual enzymes of non-TEM, non-SHV and non-OXA lineages have been described (Stürenburg and Mack, 2003). These novel enzymes have mainly been found in *P. aeruginosa* at a limited number of geographical sites (Bradford, 2001; Jacoby and Munoz-Price, 2005). The enzymes that fall into this group include PER-1 in France, Italy and Turkey; VEB-1 and VEB-2 in Southeast Asia; GES-1, GES-2 and GES-8 (IBC-2) in France, Greece and South Africa (Jacoby and Munoz-Price, 2005). Other uncommon ESBLs such as Brazil extended-spectrum β-lactamase (BES-1), GES-7 (IBC-1), SFO-1 (named after *Serratia fonticola* on the basis of the high degree of homology to the

β-lactamase of *S. fonticola*) and TLA-1 have been found only in the family *Enterobacteriaceae* (Matsumoto and Inoue, 1999; Bonnet *et al*., 2000; Jacoby and Munoz-Price, 2005).

2.4.2.7 GES-IBC Type ESBLs in bacteria

Since the GES-IBC β-lactamase family will be investigated in this study for the development of the sequence-selective PNA-based real-time PCR method, it will be discussed in more detail. The GES-IBC β-lactamase family consists thus far of GES-1 to GES-9 (Poirel *et al*., 2005). The first GES-type namely GES-1 was identified in Paris (France) in 1999 from a French Guiana *Klebsiella pneumoniae* isolate collected in 1998 (Poirel *et al*., 2000; Weldhagen, 2004b). Subsequently GES-1 was also identified from a *P. aeruginosa* isolate collected in South Africa in 2001 (Poirel *et al*., 2000; Weldhagen, 2004b). In 2000 IBC-1 (GES-7) was detected in an *Enterobacter cloacae* strain isolated in a Greek hospital (Vourli *et al*., 2004). The IBC-1 (GES-7) enzyme differed from GES-1 by a single amino acid residue (Lys instead of Glu at Ambler's position 104) (Vourli *et al*., 2004). A Glu substitution for Lys at position 98 in IBC-1 resulted in IBC-2 (GES-8) (Mavroidi *et al*., 2001).

The IBC-2 (GES-8) enzyme differs from GES-1 with a Leu instead of an Ala at position 120 (Mavroidi *et al*., 2001). The substitution of Asn for Gly at position 170 in GES-1 resulted in GES-2 (Bradford, 2001; Poirel *et al*., 2001; Jacoby and Munoz-Price, 2005). Further point mutations in the GES-IBC family resulted in the formation of GES-3, GES-4, GES-5, GES-6 and GES-9 (Poirel *et al*., 2005). The differences in the GES enzymes amino acid sequences are illustrated in Figure 2.5 and their substrate profiles are summarised in Table 2.3.

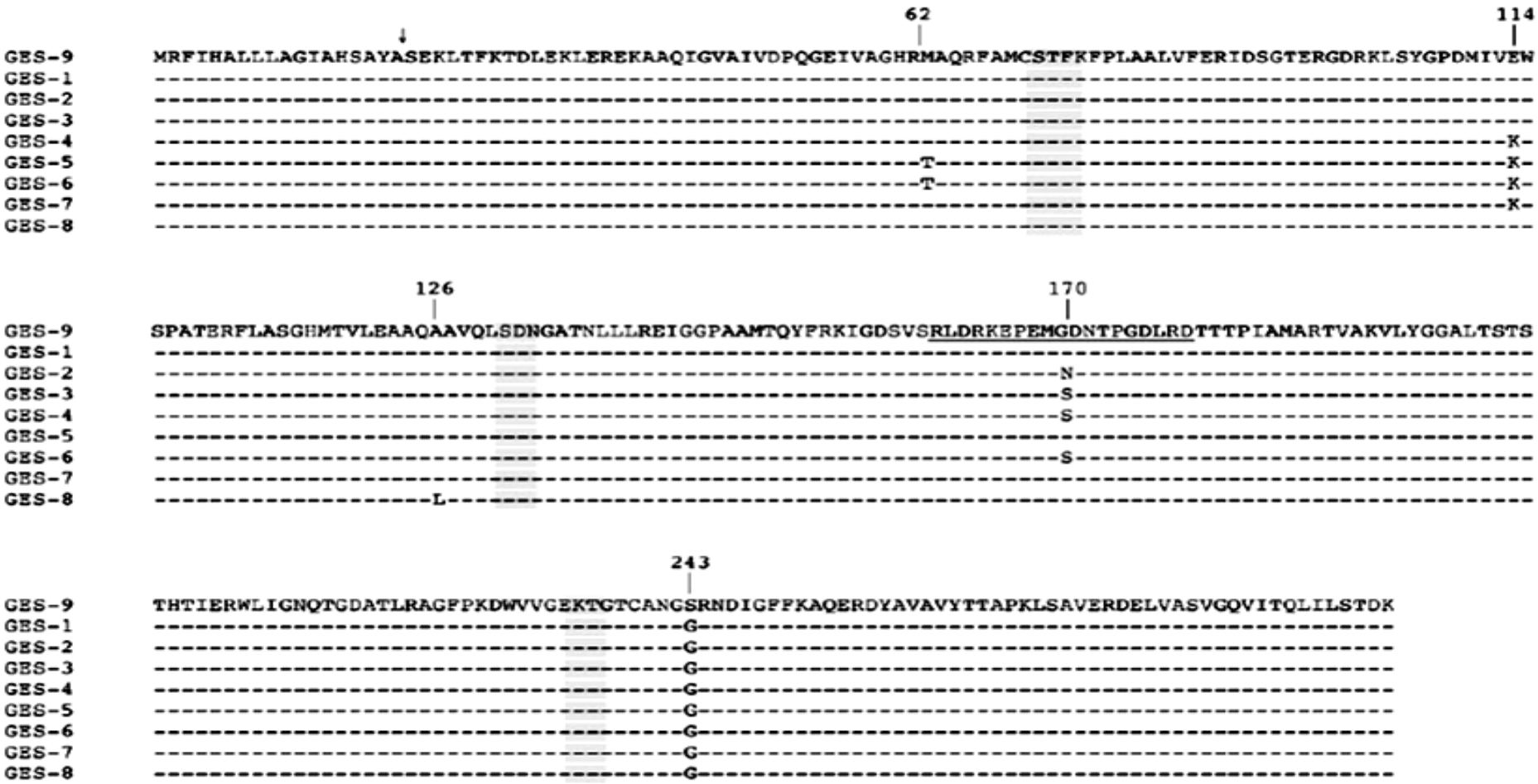

Figure 2.5: **Comparison of the amino acid sequence of β-lactamase GES-9 of *P. aeruginosa* DEJ to that of other GES enzymes. GES nominations correspond to an update of the GES nomenclature, with GES-1, GES-2, GES-3, GES-4, GES-5 and GES-6 and include name changes for IBC-1 and IBC-2, named here GES-7 and GES-8, respectively (Lee and Jeong, 2005). Numbering of β-lactamases is according to Ambler *et al.* (1991). The vertical arrow indicates the putative cleavage site of the leader peptide of the mature β-lactamases. The amino acid residues that are part of the omega loop of Ambler class A β-lactamases are underlined (Poirel *et al.*, 2005)**

Table 2.3: Substrates and inhibitors of GES-type β-lactamases of Gram-negative bacteria

Enzyme	Substrates	Inhibitors	References
GES-1	Aztreonam; Ceftazidime; Cephamycins; Extended-spectrum Cephalosporins	Cefotaxin; Clavulanic acid; Tazobactam; Imipenem	Poirel *et al*., 2000
GES-2	Ceftazidime; Cefotaxime; Carbenicillin; Imipenem; Ureido-penicillins; Extended-spectrum Cephalosporins	Clavulanic acid; Tazobactam	Poirel *et al*., 2001
GES-3	Ceftazidime; Cefotaxime; Piperacillin; Aztreonam; Extended-spectrum Cephalosporins	Clavulanic acid; Tazobactam; Sulbactam	Wachino *et al*., 2004a
GES-4	Cefminox; Moxalactam; Cefmetazole; Imipenem; Cephamycins; Meropenem	Clavulanic acid; Tazobactam; Sulbactam	Wachino *et al*., 2004b
GES-5	Ticarcillin; Piperacillin; Tobramycin; Amikacin; Imipenem Ceftazidime; Cefotaxime	Clavulanic acid; Tazobactam	Vourli *et al*., 2004
GES-6	Piperacillin; Ticarcillin; Ceftazidime; Imipenem; Cefotaxime	Clavulanic acid; Tazobactam	Vourli *et al*., 2004
GES-7 (IBC-1)	Ceftazidime; Cefotaxime	Clavulanic acid; Tazobactam; Imipenem	Giakkoupi *et al*., 2000
GES-8 (IBC-2)	Ceftazidime; Oxyimino-Cephalosporins	Clavulanic acid; Tazobactam; Imipenem	Mavroidi *et al*., 2001
GES-9	Aztreonam; Amino-penicillins; Ureido-penicillins; Narrow-spectrum Cephalosporins; Extended-spectrum Cephalosporins	Clavulanic acid; Tazobactam; Sulbactam; Imipenem; Cefotaxin	Poirel *et al*., 2005

Confusion in the nomenclature or numbering of antibiotic resistance genes has occurred with several mistakes in the nomenclature of GES-type enzymes (Lee and Jeong, 2005). To clarify the nomenclature the following has been proposed: i) to maintain the current denomination concerning the fully characterised GES-3 and GES-4 enzymes published by Wachino and colleagues (2004a; 2004b); ii) to rename variants published by Vourli and colleagues (2004) as GES-5 and GES-6 (for GES-3 and GES-4 respectively); iii) to rename the IBC-1 and IBC-2 variants identified in Greece (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001) as GES-7 and GES-8, respectively (Lee and Jeong, 2005). This study will follow these proposed corrections and refer to the GES-type enzymes as explained.

2.5. Clinical microbiological techniques for ESBL detection

It is important that laboratories be aware of the importance of bacteria producing ESBLs and how best to detect these ESBL-producing bacteria. Ignorance can lead to treatment failures in patients receiving inappropriate antibiotic treatment and outbreaks of multidrug-resistant pathogens, which will require expensive control measures (Pitout *et al*., 2005). The

methodologies discussed in this section are divided into phenotypic- and genotypic based techniques.

2.5.1 Phenotypic based techniques for ESBL detection

Conventional methods include phenotypic based techniques such as culture methods which are used in the clinical microbiology laboratory for the detection of ESBLs (Florijn *et al*., 2002). Culture techniques are based on solid media for single colony isolation and liquid media for cell enumeration (Nester *et al*., 2001). Horse or sheep's blood agar is a suitable solid medium for single colony isolation of *P. aeruginosa* (Vercauteren *et al*., 1997). Brain heart infusion broth has successfully been used for the enumeration of *P. aeruginosa* cells (Speijer *et al*., 1999).

There are currently no tests recommended by the Clinical and Laboratory Standards Institute (CLSI) for the detection of ESBLs in *P. aeruginosa* (Jacoby and Munoz-Price, 2005). Clinical laboratory detection of ESBLs in *P. aeruginosa* is difficult (Finch, 1998; Weldhagen *et al*., 2003). The following factors contribute to this: i) the presence of naturally occurring β-lactamases, such as the chromosomally encoded AmpC (class C cephalosporinase) enzymes, which may be over expressed and therefore be responsible for false-negative results; ii) the simultaneous presence of metallo-enzymes with carbapenem-hydrolysing activities or with extended-spectrum oxacillinases; iii) the relative resistance to inhibition by clavulanate; iv) and a combination of resistance mechanisms, such as efflux and impermeability of the cell wall (Thomson, 2001; Weldhagen *et al*., 2003; Stürenburg *et al*., 2004). Another contributing factor associated with the difficulties in ESBL detection is the inoculum effect (Bradford, 2001; Stürenburg and Mack, 2003). The inoculum effect is when the minimum inhibitory concentration (MIC) of extended-spectrum cephalosporins rises as the inoculum increases (Bradford, 2001; Stürenburg and Mack, 2003), which may lead to false-negative results.

Several tests (e.g. double-disk approximation test, three-dimensional test, Etest ESBL strips) used in clinical microbiology to detect ESBLs, employ a β-lactamase inhibitor (e.g. clavulanate) in combination with an oxyimino-cephalosporin (e.g. ceftazidime, cefotaxime) (Bradford, 2001). The β-lactamase inhibitor inhibits the ESBL by binding the enzymes active site, resulting in the reduction of resistance to the cephalosporin, which is seen as an enhanced

zone of inhibition towards the cephalosporin disc (Bradford, 2001). Table 2.4 summarises some of the advantages and disadvantages associated with the different clinical microbiology techniques used for ESBL detection. Figures 2.6, 2.7, 2.8 and 2.9 illustrate results for three commonly used ESBL tests.

Table 2.4: Phenotypic based techniques for the detection of ESBLs in Gram-negative bacteria

Test	Advantages	Disadvantages	References
Double-disk approximation test	Easy to use and interpret. Cost-effective	Distance of disk placements not standardized. Positives may be overlooked if disks are too far apart or if the inoculum is too large	Bradford, 2001; Stürenburg and Mack, 2003
Three-dimensional test	Sensitive and easy to interpret	Labour intensive and technically challenging	Bradford, 2001
Etest ESBL strips	Easy to use	Not as sensitive as double-disk test. Not always easy to interpret. Unable to differentiate between K1 hyper-production and ESBLs	Bradford, 2001; Stürenburg and Mack, 2003; Stürenburg *et al*., 2004
Vitek ESBL test	Easy to use and interpret	Reduced sensitivity	Bradford, 2001; Stürenburg and Mack, 2003

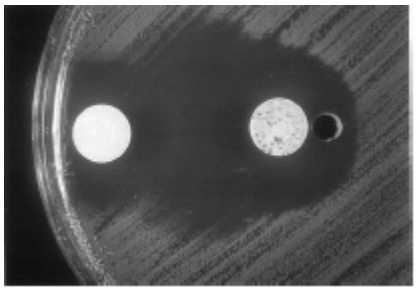

Figure 2.6: Positive double-disk approximation test. The central disk contains aztreonam and the disk on the border of the plate contains amoxycillin plus clavulanic acid. An extension of the inhibition zone around the aztreonam disk towards the clavulanate-containing disk can be observed. The well close to the aztreonam disk contains the inoculum for the three-dimensional test, which in this case, is negative (Vercauteren et al., 1997)

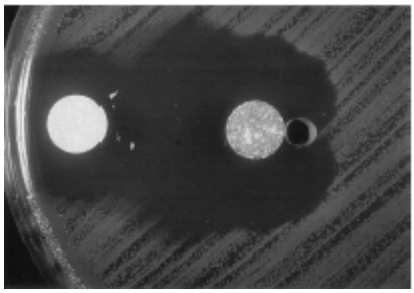

Figure 2.7: Positive three-dimensional test. The central disk contains cefepime. The cup alongside the central disk is filled with the three-dimensional test inoculum. Growth of the test organism appears behind the cup and reaches this cup, so that a heart-shaped distortion of the inhibition zone around the cefepime disk can be observed (Vercauteren et al., 1997)

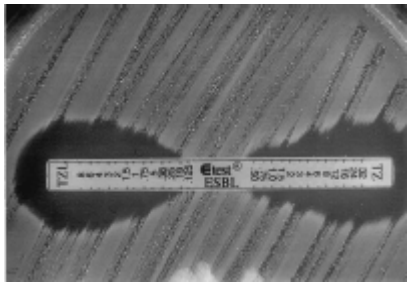

Figure 2.8: Negative ESBL Etest. The MIC of ceftazidime (<0.50 µg.ml^{-1}) can be read at the end of the strip marked "TZ". On the opposite end, marked "TZL", the MIC of ceftazidime plus clavulanic acid (0.19 µg.ml^{-1}) can be determined. This results in a ratio of the ceftazidime MIC/ceftazidime-clavulanate MIC equal to or less than 2. The cut-off value of 8 is not reached, thus the test organism is negative for ESBL production (Vercauteren et al., 1997)

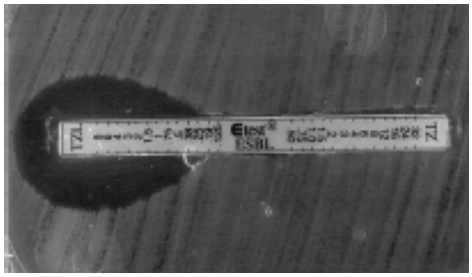

Figure 2.9: Positive ESBL Etest. The MIC of ceftazidime (>32 µg.ml^{-1}) can be read on the end of the strip marked "TZ". On the opposite end, marked "TZL", the MIC of ceftazidime plus clavulanic acid (0.19 µg.ml^{-1}) can be determined. This results in a ratio of the ceftazidime MIC/ceftazidime-clavulanate MIC equal to or greater than 253. The cut-off value of 8 is reached and the test organism is suspected of ESBL production (Vercauteren et al., 1997)

The phenotypic based methods (e.g. double-disk approximation test, Etest) seem like an attractive methodology for ESBL detection due to ease of use and low cost when compared to molecular methods. These techniques however, cannot differentiate the type of ESBL or be used reliably in ESBL detection in *P. aeruginosa* (as explained before). Molecular methods may offer a more accurate alternative since these methods are not affected by other resistance mechanisms (e.g. efflux) or the inoculum effect.

2.5.2 Molecular techniques for ESBL detection

The clinical microbiology techniques mentioned above can only presumptively identify the presence of an ESBL (Bradford, 2001). The identification of specific ESBLs in clinical isolates is more complicated (Bradford, 2001). Initially isoelectric point determination was sufficient to identify the ESBL present (Bradford, 2001). However, with various ESBLs present today, many with the same isoelectric point, this method is no longer useful

(Bradford, 2001). Currently, various molecular techniques are available to identify specific ESBLs (Sundsfjord *et al*., 2004).

2.5.2.1 The Polymerase chain reaction for ESBL detection

Advances made in polymerase chain reaction (PCR) technology and other molecular based DNA signal and target amplification techniques in the last 20 years, have resulted in these techniques becoming key procedures in molecular diagnostics (Elnifro *et al*., 2000). These techniques are conceptually simple, sensitive and specific and can be automated (Elnifro *et al*., 2000). Polymerase chain reaction-based techniques are now common in research laboratories and their use in the routine diagnostic laboratory setting is increasing (Elnifro *et al*., 2000).

The PCR reaction involves two oligonucleotide primers, which flank the target DNA sequence that is to be amplified (Sambrook and Russell, 2001). After the target DNA has been denatured the primers hybridise to opposite strands of the DNA in such an orientation that DNA synthesis by the polymerase enzyme proceeds through the region between the two primers (Sambrook and Russell, 2001). Extension creates two double-stranded target regions, each of which can again be denatured, ready for the second cycle of hybridisation and extension (Sambrook and Russell, 2001). The third cycle generates two double-stranded molecules that comprise precisely the target region (Sambrook and Russell, 2001). Repeated cycles of denaturation, hybridisation and extension create a rapid exponential accumulation of the specific target fragment of DNA (Sambrook and Russell, 2001). Conventional PCR uses end point detection of amplification products, which relies upon gel electrophoresis of the specific nucleic acids in the presence of ethidium bromide (DNA intercalating dye) followed by visual or densitometric analysis of the resulting bands after irradiation with ultraviolet light (Mackay, 2004).

The use of PCR in the diagnostic laboratory is limited by cost and sometimes the availability of adequate test sample volume if the sample is to be tested for a range of different pathogens (Elnifro *et al*., 2000). A variant of PCR, multiplex PCR has been described to overcome these shortcomings and to increase the diagnostic capacity of PCR (Elnifro *et al*., 2000). Multiplex PCR is when more than one target sequence is amplified during the same PCR reaction by including more than one pair of primers in the reaction (Elnifro *et al*., 2000). Multiplex PCR

has the potential of considerable savings of time and has been applied in many areas of nucleic acid diagnostics, including gene deletion analysis, mutation and polymorphism analysis, quantitative analysis and RNA detection (Elnifro *et al*., 2000). A multiplex PCR has been described for the detection and sequence differentiation of GES-1 and GES-2 in *P. aeruginosa* (Weldhagen, 2004a). This technique will be described in more detail under PNA-directed PCR clamping (section 2.6.2.1).

2.5.2.2 Real-time PCR for ESBL detection

In contrast to conventional PCR, real-time PCR combines PCR chemistry with fluorescent probe detection, allowing the measurement of amplification products during the amplification reaction (Svanvik *et al.*, 2000; Espy *et al*., 2006). Real-time PCR has previously been described as rapid-cycle real-time PCR, homogenous PCR and kinetic PCR (Espy *et al*., 2006). With features such as excellent sensitivity and specificity, low contamination risk, ease of performance and speed, real-time PCR is an attractive alternative option compared to conventional PCR (Espy *et al*., 2006). In Table 2.5 the advantages and disadvantages of conventional PCR and real-time PCR are compared.

Table 2.5: Advantages and disadvantages of conventional and real-time PCR methods (Johnson, 2000; Mackay, 2004)

Conventional PCR			**Real-time PCR**	
Advantages	**Disadvantages**		**Advantages**	**Disadvantages**
Detects low concentration of target DNA	Fairly expensive reagents and disposables		Detects low concentration of target DNA	High start-up cost
Fine discrimination between closely related variants	Carry-over contamination resulting in false positives		Fine discrimination between closely related variants	Need sequence data for primer design
	Need separate pre- and post-PCR areas (at least three areas)		Carry-over contamination minimized	Need post PCR analysis in order to evaluate amplicon size
	Need sequence data for primer design		Increased speed	
	Laborious post-PCR handling step		Cost effective on a per-run basis	
	Nested PCR increases risk of contamination (PCR vessel opened)		Increased number of cycles may circumvent need for nested PCR	

Monitoring the PCR reaction in real-time has been made possible by the labelling of primers, oligonucleotide probes or amplicons with molecules capable of fluorescing (Mackay, 2004). A change in signal is produced by these fluorescent labels following direct interaction with or hybridisation to the amplicon (Mackay, 2004). The signal produced is related to the amount of amplicon present during each cycle and the amount of specific amplicon increases during the PCR reaction (Mackay, 2004).

Various detection strategies are available for real-time PCR with the simplest assay using double-stranded DNA (dsDNA) specific dyes such as the asymmetric cyanine dye SYBR Green (Svanvik *et al.*, 2000). The dsDNA specific dyes, with no fluorescence of their own, become intensely fluorescent when the dyes bind to dsDNA (Svanvik *et al.*, 2000). These free dyes do not recognise a sequence specific product and will bind to any double stranded products formed during the reaction (Svanvik *et al.*, 2000).

If non-specific reporters are used for real-time detection, inclusion of a post-PCR fluorescence-mediated DNA melting curve analysis is required to distinguish between the different products (Svanvik *et al.*, 2000). To produce the melting curve, the amplicons are heated until dsDNA denatures and becomes single-stranded DNA (ssDNA) (Figure 2.10a) (Mackay, 2004). The resulting rapid decrease in fluorescence caused by the dissociation of the fluorogenic molecules is presented as a specific melting peak using software capable of calculating the negative derivative of the fluorescence change with temperature (Figure 2.10b; Figure 2.10c) (Mackay, 2004). Nucleotide changes in the target DNA shifts the melting peak (greater G + C content results in higher melting temperatures; greater A + T content results in lower melting temperatures) (Mackay, 2004). This shift is reproducible for the same nucleotide changes and can be used diagnostically to genotype microbial templates (Mackay, 2004).

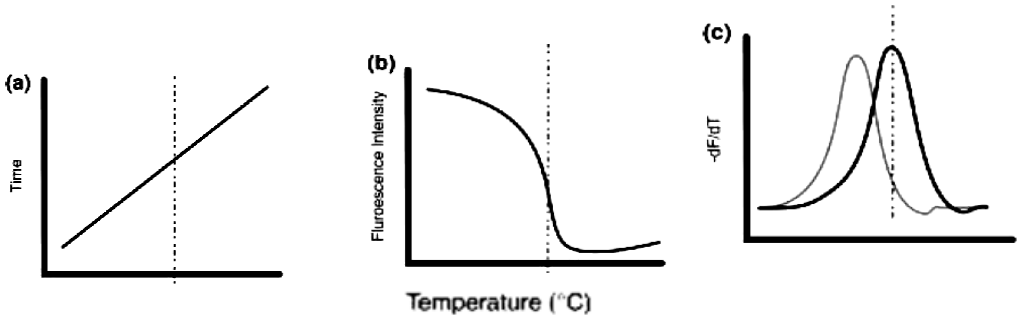

Figure 2.10: **Fluorescence-mediated melting curve analysis. (a) At the completion of a real-time PCR the reaction can be cooled to a temperature below the expected T_m of the oligonucleotide probes (or amplicons when using free dyes) and then slowly reheated to above 90°C at a fraction of a degree/second (Y-axis represents time; X-axis represents temperature); (b) During the reheating process, fluorescence is constantly measured (Y-axis represents fluorescence intensity; X-axis represents temperature); (c) Software calculates the negative derivative of the fluorescence with temperature, producing a clear melting peak (black peak) that indicates the T_m of the oligonucleotide probe or the TD of melting dsDNA (when using free dyes) (Y-axis represents –dF/dT; X-axis represents temperature). If one or more nucleotide changes are present they produce a different T_m or TD from the target (grey peak) (Mackey, 2004)**

Apart from the free dyes other chemistries are available with hybridisation probes (Figure 2.11), molecular beacons (Figure 2.12) and TaqMan probes (Figure 2.13) as the most popular chemistries (Espy *et al*., 2006). The three chemistries mentioned rely on the transfer of light energy between two adjacent dye molecules, a fluorophore and a quencher, a process known as fluorescence resonance energy transfer (FRET) (Espy *et al*., 2006). The quencher molecule absorbs light from the fluorophore transforming it into heat (or a different wavelength in the case of hybridisation probes) (Espy *et al*., 2006).

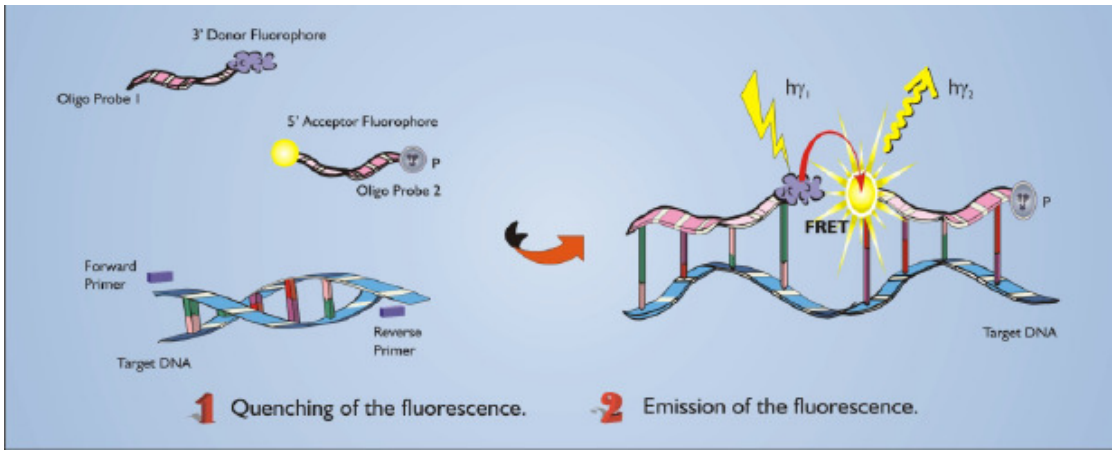

Figure 2.11: **Schematic representation of the hybridisation probe chemistry depicting the probe pair before and after binding to the target DNA (http://www.sigmaaldrich.com /img/assets/20760/PP_FluorescentProbes.pdf)**

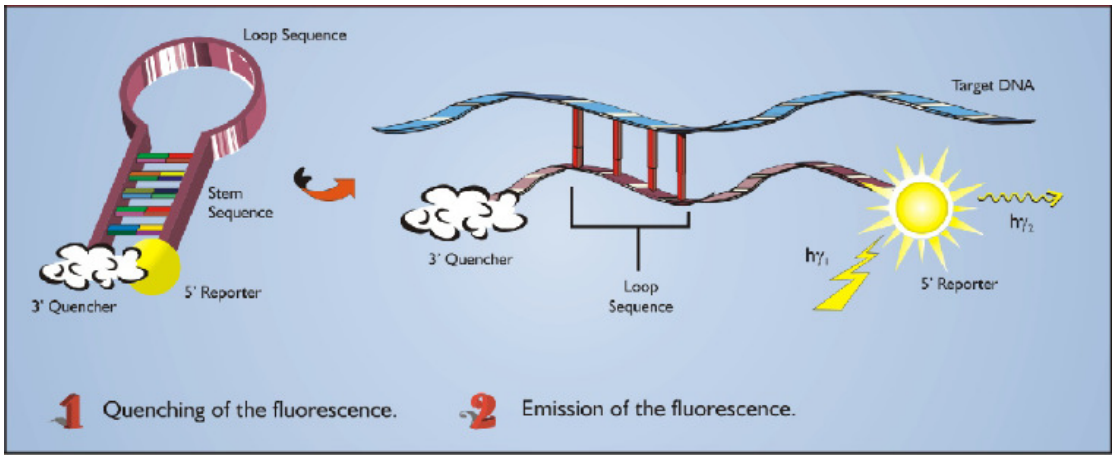

Figure 2.12: Schematic representation of the molecular beacon chemistry depicting the probe before and after binding to the target DNA (http://www.sigmaaldrich.com /img/assets/20760/PP_FluorescentProbes.pdf)

Hybridisation probes have been utilised previously for the detection and differentiation of GES-1 and GES-2 (Weldhagen, 2004b). In the study a 3' fluorescein isothiocyanate- labelled sensor probe and 5' LCRed640-labelled anchor probe was utilised forming a hybridisation probe pair (Weldhagen, 2004b).

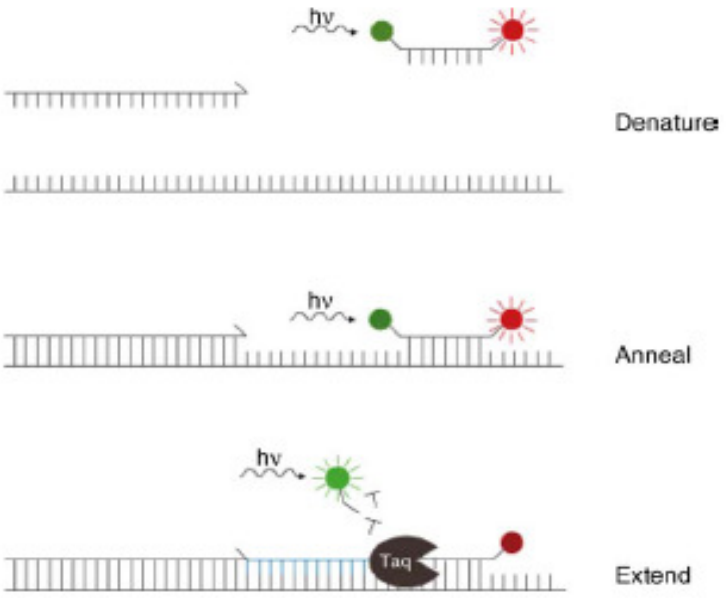

Figure 2.13: Schematic representation of the TaqMan chemistry depicting the probe before and after extension (Gunson *et al*., 2006)

In the case of unbound probes the sensor probe emits green light at 530 nm and the anchor probe emits no fluorescence (Weldhagen, 2004b). Upon hybridisation (sensor and anchor probes in a head-to-tail conformation) FRET occurs with light being transformed from the fluorescein by the LCRed640 dye into red light at 640 nm (Weldhagen, 2004b). The reaction is monitored in the 640 nm wavelength channel to be able to follow and interpret the hybridisation events (Weldhagen, 2004b). A post-amplification melting curve analysis was performed to differentiate between *bla*$_{GES-1}$ and *bla*$_{GES-2}$ (Weldhagen, 2004b). The nucleotide difference between *bla*$_{GES-1}$ and *bla*$_{GES-2}$ resulted in two distinct melting peaks specific for each enzyme. The melting peaks produced were depicted in Figure 2.14.

The mutational region targeted by the hybridisation probes to differentiate *bla*$_{GES-1}$ from *bla*$_{GES-2}$ is no longer adequate (Weldhagen, 2004b). With the description of new *bla*$_{GES}$ variants (*bla*$_{GES-3}$ to *bla*$_{GES-9}$) the region targeted can no longer identify *bla*$_{GES-1}$ and *bla*$_{GES-2}$ accurately. The *bla*$_{GES-3}$, *bla*$_{GES-8}$ and *bla*$_{GES-9}$ genes possess the same bases at position 492 (G) and 493 (G) as *bla*$_{GES-1}$ and might give the same melting peak meant to differentiate *bla*$_{GES-1}$ from other *bla*$_{GES}$ genes. Thus the hybridisation probe method is no longer adequate for the differentiation of *bla*$_{GES-1}$ and *bla*$_{GES-2}$.

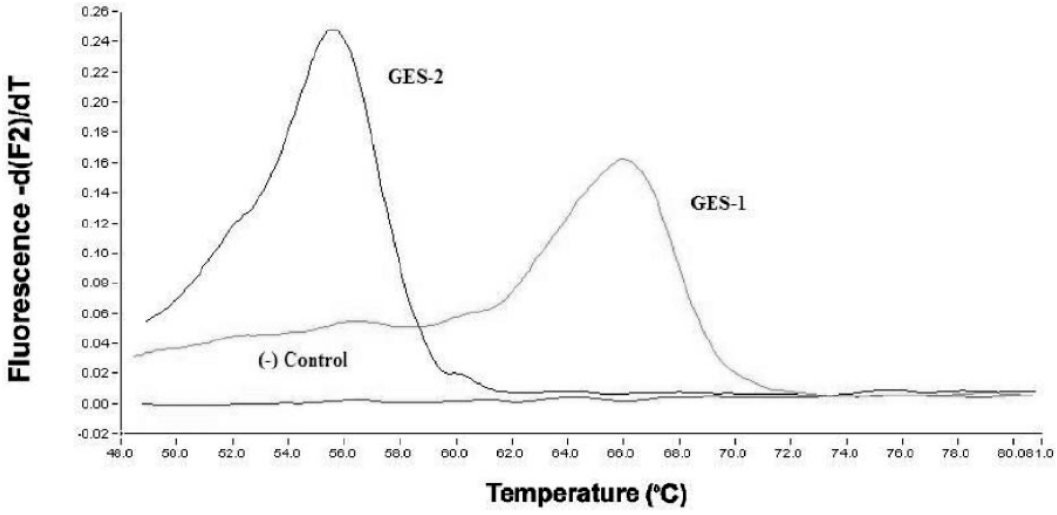

Figure 2.14: Melting curve analysis for *bla*$_{GES-1}$, *bla*$_{GES-2}$ and ATCC 25922 (*bla*$_{GES/IBC}$ template-negative control). The melting curve results are plotted as the negative derivative of fluorescence F2 [(*-d(F2)/dT*)] versus temperature. The T_m difference between an exact sensor probe match (GES-1) and a two nucleotide mismatch (GES-2) is clearly visible. The negative control did not produce a melting peak (Weldhagen, 2004b)

Real-time PCR provides a versatile, fast, specific and accurate tool to be used in molecular diagnostic techniques (Espy *et al*., 2006). The ever increasing number of newer and smaller platforms that allow the use of smaller reaction volumes may help in space and money saving in the laboratory (Espy *et al*., 2006). Although hybridisation probes provided an effective technique for distinguishing *bla*$_{GES-1}$ and *bla*$_{GES-2}$ from each other, the description of new *bla*$_{GES}$ genes (Poirel *et al*., 2005) with the same mutations in the targeted region rendered this method unreliable. Hybridisation probe chemistry is a relatively expensive chemistry to use compared to the simple SYBR Green dye assays and can only distinguish between those two variants in the GES/IBC ESBL family.

2.5.2.3 Application of molecular methods for ESBL detection in Gram-negative bacteria

The phenotypic methods may be limited by poor sensitivity, slow-growth or poorly viable organisms, narrow detection windows, complex interpretation, immunosuppression, antimicrobial therapy, high levels of background and non-specific cross-reactions (Mackay, 2004). Molecular methods based on the genotypic characteristics of microorganisms may overcome some of these limitations.

The increasing number of additional subtypes within each ESBL-family has placed strict limitations on molecular techniques with regard to their ability to cover the whole range of variants within each family (Sundsfjord *et al*., 2004). Nucleic acid sequencing is still the gold standard for ESBL-typing and recent developments within rapid sequencing techniques could probably make this approach more readily available and cost-effective for ESBL-typing (Sundsfjord *et al*., 2004). Apart from conventional sequencing, pyrosequencing has been used for the identification of GES type ESBLs (Poirel *et al*., 2006). Pyrosequencing was described as a reliable technique that allows fast identification of short DNA sequences (Poirel *et al*., 2006). The technique takes 3 hours to perform from DNA extraction to sequencing result (Poirel *et al*., 2006). Due to the costs involved, sequencing is not suitable for routine ESBL-typing.

Table 2.6: Molecular techniques for the detection of ESBLs in Gram-negative bacteria (Bradford, 2001; Weldhagen *et al*., 2003)

Test	Advantages	Disadvantages
DNA probes	Specific for a gene family (e.g. TEM or SHV)	Labour intensive. Cannot distinguish between the enzyme variants
PCR	Easy to perform. Specific for a gene family (e.g. TEM, SHV, PER, VEB, GES)	Cannot distinguish between the enzymes variants. Quality of template is important to avoid false-negative results
Oligotyping	Detects specific TEM variants	Labour intensive. Require specific oligonucleotide probes. Cannot detect new variants
PCR-Restriction Fragment Length Polymorphism (RFLP)	Easy to perform. Able to detect specific nucleotide changes	To be able to detect nucleotide change, the change in sequence must result in altered restriction (pattern change)
PCR-Single-strand conformational polymorphism (SSCP)	Able to distinguish between a number of SHV variants	Require special electrophoresis conditions
Ligase chain reaction (LCR)	Able to distinguish between a number of SHV variants	Require a large number of oligonucleotide primers
Nucleotide sequencing	The Gold standard. Can detect all variants	Labour intensive and expensive. Technically challenging. Manual methods can be difficult to interpret

Overall molecular methods for ESBL detection are complex and challenging due to the diversity of phenotypic expression and genotypes (Sundsfjord *et al*., 2004). The use of molecular methods was mainly restricted to reference laboratories and for molecular epidemiological studies due to the high costs involved and the need for skilled laboratory personnel (Sundsfjord *et al*., 2004).

2.5.2.4 Genotyping methods of *P. aeruginosa* strains

Genomic fingerprinting methods are considered the most accurate methods for the typing of microorganisms for epidemiological purposes (Speijer *et al*., 1999). Typing is an epidemiologically important tool utilised for recognising outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of infection, recognising particularly virulent strains and monitoring vaccination programmes (Olive and Bean, 1999).

Genomic fingerprinting methods include pulsed-field gel electrophoresis (PFGE), ribotyping (RFLP analysis of rRNA genes for differentiating between species and strains) and PCR-based fingerprinting methods (Speijer *et al*., 1999; Bradford, 2001). Due to the availability of various typing methods there is no general agreement on the optimal typing strategy to be used for a given pathogen (Renders *et al*., 1996). Phenotypic methods such as antibiogram

patterns and pyocin typing have been used (Menon *et al*., 2003). These phenotypic methods however, are time consuming and inconsistent (Menon *et al*., 2003).

The methods used currently are mostly PCR-based and include random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis and amplified fragment length polymorphism (AFLP) analysis (Struelens, 1998; Olive and Bean, 1999; Speijer et al., 1999). Table 2.7 summarises some of the genomic fingerprinting techniques, their principles, advantages and disadvantages.

The RAPD technique is used widely for typing of *P. aeruginosa* (Speijer *et al*., 1999; Menon *et al*., 2003). This method is suitable for high throughput, has a high resolving power and the ability to detect differences along the whole genome (Holt and Cote, 1998). It's robustness make this method the best for the typing of *P. aeruginosa* (Holt and Cote, 1998; Speijer *et al*., 1999; Olive and Bean, 1999; Campbell *et al*., 2000; Menon *et al*., 2003; Ortiz-Herrera *et al*., 2004).

Table 2.7: Genomic fingerprinting methods for typing of *P. aeruginosa*

Technique and principal	Advantages	Disadvantages	References
RAPD: Incorporates a single arbitrary designed oligonucleotide primer in an amplification reaction to generate DNA fragment polymorphisms (Annealing site variation)	Least hands-on time. Suitable for high throughput situations. High resolving power when using polyacrylamide-urea gels. Detects differences along whole genome making it useful over long periods. Reproducible, simple and robust	Discriminatory power less than AFLP and Macrorestriction analysis. Moderate intralaboratory reproducibility. Standardization is difficult	Campbell *et al*., 2000; Holt and Cote, 1998; Speijer *et al*., 1999; Menon *et al*., 2003; Ortiz-Herrera *et al*., 2004; Olive and Bean, 1999
RFLP: Use polymorphism seen in restriction enzyme recognition sites, within a particular genetic locus of interest, to form gel bands that differ in size between unlike strains	Simple and reproducible	Moderate discrimination	Olive and Bean, 1999; Struelens *et al.*, 1998
Macrorestriction analysis: Isolates are digested with *SpeI* in agarose blocks and the resulting fragments separated by PFGE (Restriction site variation)	Highly reproducible. High discriminatory power. Considered reference method for majority of nosocomial pathogens	Time consuming. Use agarose gels resulting in lower resolving power. Difficult	Speijer *et al*., 1999; De Freitas and Barth, 2002; Thong *et al*., 2004
AFLP: Based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion	High resolving power when using polyacrylamide-urea gels. Highly discriminatory and reproducible	Require purified DNA. Expensive setup and running cost	Olive and Bean, 1999; Speijer *et al*., 1999; Pirnay *et al*., 2002

2.6 Peptide nucleic acids and its use in molecular diagnostics

Peptide nucleic acids (PNA) originated during the 1980s from efforts by Nieslen and Buchardt to develop new nucleic acid sequence-specific reagents (Nielsen *et al*., 1991). With flow linear dichroism Nielsen and Buchardt observed that α-helical poly-γ-benzylglutamate (PBG) could form stacked complexes with aromatic chromophores (Ray and Norden, 2000). Based on this observation it was proposed that PBG with alternating nucleobases and acridine moieties instead of phenyls, might bind sequence-selectively to duplex DNA by combined Hoogsteen base pair formation and intercalation with the helix backbone in the major groove of DNA (Ray and Norden, 2000). The *ad interim* name peptide nucleic acid was thus given to the proposed peptide nucleobase compound (Ray and Norden, 2000).

2.6.1 Peptide nucleic acid chemistry and properties

Today's PNAs are DNA analogues in which the sugar-phosphate backbone is replaced by a peptide amide bond backbone (Gangamani *et al*., 1997; Jakimov *et al*., 2000). The peptide backbone is made up by repetitive units of *N*-(2-aminoethyl)glycine to which the purine and pyrimidine bases are attached via a methyl carbonyl linker (Pellestor *et al*., 2004). The methyl carbonyl linker can be used to connect unusual nucleotide bases to this backbone at the amino nitrogen position (Ray and Norden, 2000). Peptide nucleic acid oligomers are synthesised by conventional tBoc- or Fmoc-solid support peptide chemistry (Ray and Norden, 2000).

Peptide nucleic acid oligomers hybridise with high efficiency and sequence specificity to Watson-Crick complementary DNA, RNA or PNA oligomers and four PNA structures have been solved to date (Nielsen, 1998; Pellestor and Paulasova, 2004). These structures are a PNA-RNA duplex, a PNA-DNA duplex, a PNA_2-DNA triplex and a PNA-PNA duplex, elucidated with nuclear magnetic resonance (NMR) methods and X-ray crystallography (Nielsen, 1998). Peptide nucleic acids were originally designed for sequence specific recognition of dsDNA via triplex binding in the major groove of the DNA double helix (Nielsen, 1998). The observation has been made that homopyrimidine PNAs prefer binding to the homopurine duplex DNA target by strand displacement via formation of an internal PNA-DNA-PNA triplex (Nielsen, 1998).

The uncharged synthetic backbone of PNA provides it with unique hybridisation characteristics (Pellestor *et al*., 2004). Since binding is not hindered by electrostatic repulsion, PNA hybridises to its target nucleic acid sequence with higher stability reflected by a higher thermal melting temperature as compared to the corresponding DNA-DNA or DNA-RNA duplexes (Pellestor and Paulasova, 2004). The polyamide backbone allows for hybridisation virtually independently of the salt concentration (Pellestor and Paulasova, 2004). This hybridisation property can be exploited when targeting DNA or RNA sequences involved in secondary structures, which are destabilised by low ionic strength (Pellestor and Paulasova, 2004). Peptide nucleic acids are dependant on pH, with more stable binding at neutral to slightly acidic pH (Pellestor and Paulasova, 2004).

Peptide nucleic acids have a high chemical and biological stability and are not degraded by nucleases or proteases resulting in a long lifetime, both *in vivo* and *in vitro* (Nielsen, 1998; Jakimov *et al*., 2000; Pellestor *et al*., 2004). Polymerases do not recognise PNAs, thus PNA cannot directly be used as a primer or be copied (Pellestor and Paulasova, 2004).

2.6.2 Peptide nucleic acids as diagnostic tool for genetic determinants

The development of PNA oligomers has led to the development of new applications for PNA, especially in diagnostics (Ray and Norden, 2000). The unique binding properties of PNA have been employed in the detection of genetic mutation and mismatch analysis (Ray and Norden, 2000) and could therefore be applied in similar analysis of GES type genes.

2.6.2.1 PNA-directed PCR clamping

The basis for the PNA-directed PCR clamping technique is the higher specificity of PNA binding to DNA, higher stability of a PNA-DNA duplex compared to the corresponding DNA-DNA duplex and it's inability to act as a primer for DNA polymerases (Ray and Norden, 2000). Peptide nucleic acid-based multiplex PCR uses a forward primer and reverse primer that is complementary to the desired mutant sequence, as well as a PNA-probe similar to the reverse primer, which is complementary to the wild type (wt) sequence (Weldhagen, 2004a). If a wt DNA template is present in the PCR reaction, the PNA-probe out-competes the reverse primer for binding to the template and no amplification occurs (Weldhagen, 2004a). The opposite can occur if a mutant sequence is present during the reaction, the

primer will then out-compete the PNA-probe and amplification will occur (Weldhagen, 2004a). Peptide nucleic acid-directed PCR clamping has been used by Weldhagen (2004a) to identify the GES-1 and GES-2 ESBLs in *P. aeruginosa*. Figure 2.15 schematically illustrates the PNA-directed PCR clamping method used by Weldhagen (2004a). Figure 2.16 depicts the end point gel electrophoresis results (conventional PCR) for the PNA-directed PCR clamping method used for GES-1 and GES-2 identification and differentiation.

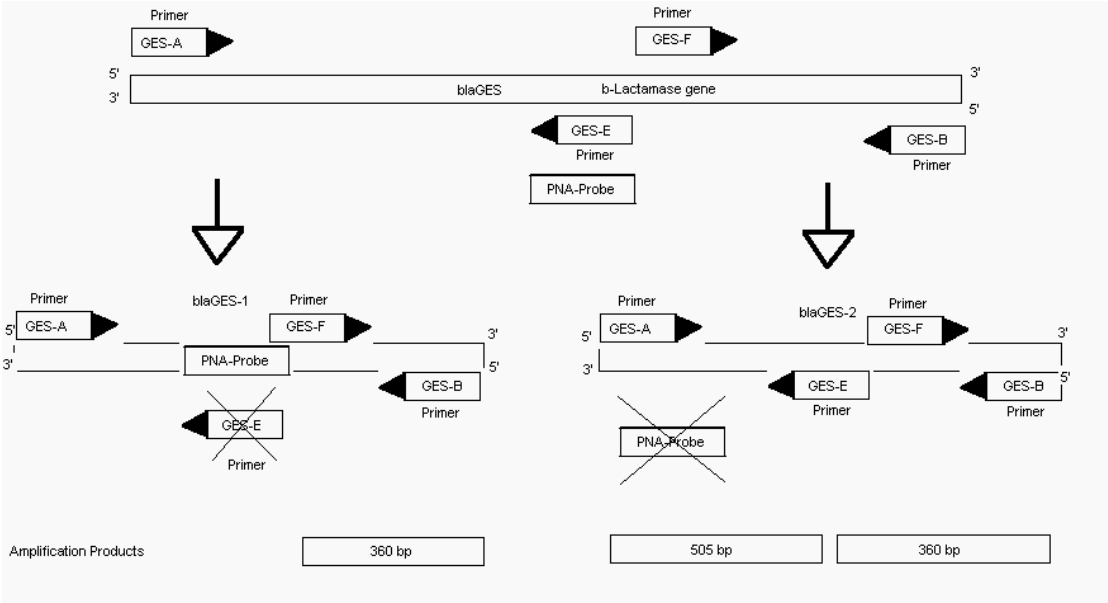

Figure 2.15: Schematic representation of the PNA-directed PCR clamping method used in the identification of GES-1 and GES-2 in *P. aeruginosa* (Weldhagen, 2004a)

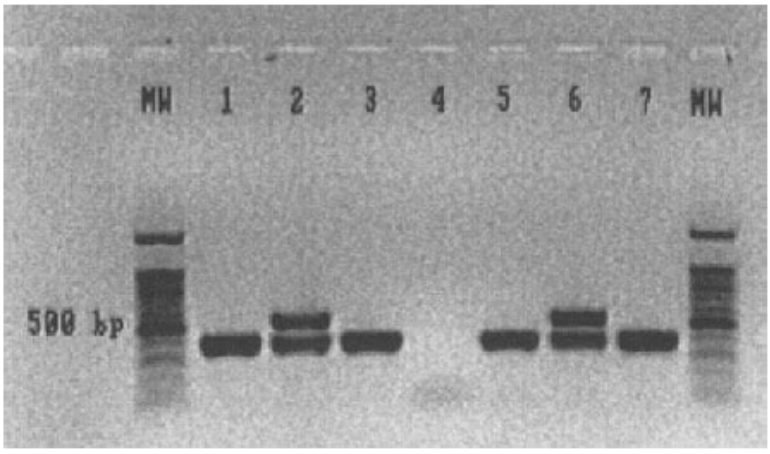

Figure 2.16: Gel electrophoresis result of a typical PNA-based multiplex PCR. MW, 100 bp marker. Lane 1, GES-1; Lane 2, GES-2; Lane 3, GES-1; Lane 4, template negative; Lane 5, GES-1; Lane 6, GES-2; Lane 7, GES-1 (Weldhagen, 2004a)

Iwamoto and Sonobe (2004) successfully used PNA-mediated competitive PCR clamping for the detection of mutations in the rifampin resistance-determining region in *Mycobacterium tuberculosis* (*M. tuberculosis*). Von Wintzingerode and colleagues (2000) described PCR clamping as a useful supplement to conventional PCR amplification in rDNA-based studies of microbial diversity.

2.6.2.2 Other PNA applications in molecular diagnostics

Various PNA applications are currently available for molecular diagnostics. The following is a short summary of four commonly used PNA-based methods. Fluorescence *in situ* hybridisation (FISH) using PNA-probes combines the advantages of microscopy for elucidation of morphological information with the target specificity provided by molecular methods in a way which is easily adaptable to current microscopic techniques (Stender *et al*., 1999). Peptide nucleic acid FISH has been successful used in the laboratory diagnosis of *M. tuberculosis* (Stender *et al.*, 2002).

The second method, chemiluminescent *in situ* hybridisation (CISH), utilises a soybean peroxidase (SBP)-labelled PNA-probe targeted to specific rRNA sequences for the direct detection of microorganisms on membrane filters (Stender *et al*., 2002). The CISH method has been described for the identification of *P. aeruginosa* in bottled water, *E. coli* in municipal water and *Dekkera bruxellensis* in wine (Stender *et al*., 2002). The third method, PNA molecular beacons, relies on a PNA oligomer labelled with a fluorescent dye at one terminus and a quencher molecule at the other (Brandt and Hoheisel, 2004). The fourth method, PNA light-up probes, consists of a PNA oligomer labelled with a dye that is usually coupled to a flexible linker at the N-terminal end (Brandt and Hoheisel, 2004). Upon hybridisation of the probe, the fluorescence quantum yield of the dye increases (Brandt and Hoheisel, 2004). Light-up PNA-probes have been demonstrated successfully for quantification of real-time PCR and show discriminatory power down to single base pair level (Brandt and Hoheisel, 2004).

In conclusion, ESBLs in *P. aeruginosa* present a serious problem in the use of β-lactam antibiotics (Poole, 2004). The accurate detection of ESBLs is important for the monitoring of new antibiotic resistance genes and is essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings (Sardelic *et al*.,

2003). The detection of ESBLs in *P. aeruginosa* is difficult with factors such as the presence of naturally occurring β-lactamases, relative resistance to inhibition by clavulanate and the occurrence of multiple resistance mechanisms, confounding efforts (Finch, 1998; Weldhagen *et al*., 2003). Molecular methods overcome these problems by detecting the ESBL genes. The purpose of the study was to develop a PNA-directed PCR clamping method for use in real-time PCR to provide a rapid and accurate method that could be optimised as a routine diagnostic technique for use in the clinical microbiology setting.

2.7 Summary

Pseudomonas aeruginosa, a Gram-negative aerobic rod, is an important opportunistic bacterial pathogen, often responsible for nosocomial infections and infections in immunocompromised patients (Gençer *et al*., 2002; Todar, 2002). Infections caused by *P. aeruginosa* are of particular concern in patients with severe burns, cancer and cystic fibrosis where the case fatality rate can be as high as 50% (Todar, 2002).

The ESBLs are a problem when using β-lactam antibiotics to treat infections, given their broad substrate specificity and ability to hydrolyse many of the extended-spectrum third-generation cephalosporins (Poole, 2004). Bacteria that produce extended-spectrum β-lactamases are typically resistant to the penicillins, first- and second-generation cephalosporins as well as the third-generation oxyimino cephalosporins (e.g. ceftazidime, ceftriaxone) and monobactams (aztreonam) (Poole, 2004).

The ESBLs identified in *P. aeruginosa* include the GES β-lactamases and the SHV β-lactamases (Jacoby and Munoz-Price, 2005). The first GES-type ESBL, GES-1 was identified in France in 1999 from a French Guiana *K. pneumoniae* isolate and subsequently from a *P. aeruginosa* isolate (Poirel *et al*., 2000; Weldhagen *et al*., 2003). The Asn170Gly mutational substitution resulted in the formation of GES-2 (Bradford, 2001; Poirel *et al*., 2001; Jacoby and Munoz-Price, 2005). The ESBL GES-2 was discovered in 2000 in *P. aeruginosa* isolates in Pretoria, South Africa (Poirel *et al*., 2001).

The detection of ESBL-producing *P. aeruginosa* isolates, using standard methods in the clinical microbiology laboratory is difficult and false-negative results are common (Weldhagen *et al*., 2003). The factors contributing to this difficulty include naturally occurring β-lactamases, relative resistance to inhibition by clavulanic acid and a combination of resistance mechanisms (Weldhagen *et al*, 2003). Molecular techniques such as PCR-based methods followed by sequencing of amplification products have proven more successful (Weldhagen *et al*., 2003). Sequencing is however an expensive technique not suitable in settings where resources are limited. The recently developed sequence-selective, PNA-based multiplex PCR detection method seems to provide an accurate means to detect $bla_{\text{GES-2}}$ compared to standard PCR and gene sequencing techniques (Weldhagen, 2004a).

Peptide nucleic acids are deoxyribonucleic acid (DNA) homologues in which the phosphate backbone has been replaced with repetitive units of N-(2-aminoethyl) glycine, known as a polyamide backbone (Pellestor and Paulasova, 2004). The PNA-based multiplex PCR detection method relies on the intrinsic high affinity of PNA for DNA and its inability to serve as a primer for DNA polymerases (Ørum *et al*., 1993). Real-time PCR can be used to perform the PNA-base multiplex PCR. A multitude of reporters are available for real-time PCR, with the simplest assay using double-stranded DNA (dsDNA) specific dyes, such as the asymmetric cyanine dye SYBR Green (Svanvik *et al.*, 2000). The inclusion of a post-PCR fluorescence-mediated DNA melting curve analysis is required to differentiate between amplification products when non-specific reporters are used (Svanvik *et al.*, 2000). The melting temperatures obtained from the melting curve can be used to differentiate between the GES variants.

Genomic fingerprinting is considered the most accurate method for the typing of microorganisms for epidemiological purposes (Speijer *et al*., 1999). Strain classification or subtyping is important epidemiologically for recognising outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the infection, recognising particularly virulent strains of microorganisms and monitoring vaccination programmes (Olive and Bean, 1999). With various typing methods available there is no general agreement on the optimal typing strategy to be used for a given pathogen (Renders *et al*., 1996). The most frequently used methods include RAPD and PFGE, often used in conjunction for more representative results (Speijer *et al*., 1999). Difficulties in

standardisation and intralaboratory reproducibility are amongst the most common problems with these typing methods (Olive and Bean, 1999).

The GES/IBC (Guiana extended-spectrum/Integron-borne cephalosporinase) β-lactamase family is still relatively small and has only a few point mutations separating the genes in question, making it the ideal model for the development of the sequence-selective PNA-based real-time PCR method (Weldhagen, 2004a). The PNA-based real-time method may be used for the monitoring of new antibiotic resistance genes (such as GES-2) in bacteria, which is essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings (Sardelic *et al.*, 2003). This study aimed to determine the prevalence of GES-type extended-spectrum β-lactamases in *P. aeruginosa* strains, to optimise a PNA-probe exclusion method for real-time PCR and to determine the clonal relationship of these *P. aeruginosa* strains utilising random amplified polymorphic DNA (RAPD) analysis.

2.8 References

Ambler P, Coulson AFW, Frère JM, Ghuysen JM, Joris B, Forsman M, Lévesque RC, Tirabi G, Waley SG (1991) A standard numbering scheme for the class A beta-lactamases. *Biochem. J*. **276:** 269-272.

Bert F, Branger C, Lambert-Zechowsky N (2002) Identification of PSE and OXA β-lactamase genes in *Pseudomonas aeruginosa* using PCR-restiction fragmnet length polymorphism. *J. Antimicrob. Chemother.* **50**:11-18.

Bonnet R, Sampaio JLM, Chanal C, Sirot D, De Champs C, Viallard JL, Labia R, Sirot J (2000) A novel class A extended-spectrum beta-lactamase (BES-1) in *Serratia marcescens* isolated in Brazil. *Antimicrob. Agents Chemother*. **44:** 3061-3068.

Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*. **14:** 993-951.

Brandt O, Hoheisel JD (2004) Peptide nucleic acids on microarrays and other biosensors. *Trends Biotechnol.* **22:** 617-622.

Brooun A, Liu S, Lewis K (2000) A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **44:** 640-646.

Bush K, Jacoby GA, Medeiros AA (1995) A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* **39:** 1211-1233.

Campa M, Bendinelli M, Friedman H (1993) *Pseudomonas aeruginosa* as an opportunistic pathogen, p 2-8, 223-226. Plenum Press, New York.

Campbell M, Mahenthiralingam E, Speert D (2000) Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **38:** 4614-4615.

Carattoli A (2001) Importance of integrons in the diffusion of resistance. *Vet. Res*. **32:** 243-259.

De Freitas ALP, Barth AL (2002) Antibiotic resistance and molecular typing of *Pseudomonas aeruginosa*: Focus on imipenem. *Braz. J. Infect. Dis.* **6:** 1-7.

Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE (2000) Multiplex PCR: Optimization and application in diagnostic virology. *Clin. Microbiol. Rev.* **13:** 559-570.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JDC, Wengenack NL, Rosenblatt JE, Cockerill FR, Smith TF (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* **19:** 165-256.

Finch RG (1998) Antibiotic resistance. *J. Antimicrob. Chemother.* **42:** 125-128.

Fluit AC, Schmitz FJ (1999) Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur. J. Clin. Microbiol. Infect. Dis.* **18:** 761-770.

Fluit AC, Schmitz FJ (2004) Resistance integrons and super-integrons. *Clin. Microbial. Infect.* **10:** 272-288.

Florijn A, Nijssen S, Schmitz FJ, Verhoef J, Fluit AC (2002) Comparison of E-test and double disk diffusion test for detection of extended spectrum β-lactamases. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**:241-243.

Fluorescent probes for real-time quantitative PCR. Accessed 11 April 2007. [Online] http://www.sigmaaldrich.com/img/assets/20760/PP_FluorescentProbes.pdf.

Fonseca EL, Vieira VV, Cipriano R, Vicente ACP (2005) Class 1 integrons in *Pseudomonas aeruginosa* isolates from clinical settings in Amazon region, Brazil. *FEMS Immunol. Med. Microbiol.* **44:** 303-309.

Froelich-Ammon SJ, Osheroff N (1995) Topoisomerase poisons: Harnessing the dark side of enzyme mechanism. *J. Biol. Chem.* **270:** 21429-21432.

Gangamani BP, Kumar VA, Ganesh KN (1997) 2-Aminopurine peptide nucleic acids (2-apPNA): intrinsic fluorescent PNA analogues for probing PNA-DNA interaction dynamics. *Chem. Commun.* **19:** 1913-1914.

Gençer S, Ak Ö, Benzonana N, Bat rel A, Özer S (2002) Susceptibility patterns and cross resistance of antibiotics against *Pseudomonas aeruginosa* in a teaching hospital of Turkey. *Annal. Clin. Microbiol. Antimicrob.* **1:** 2-5

Giakkoupi P, Tzouvelekis LS, Tsakris A, Loukova V, Sofianou D, Tzelepi E (2000) IBC-1, a novel integron-associated class A beta-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. *Antimicrob. Agents Chemother.* **44:** 2247-2253.

Gilbert P, Allison DG, McBain AJ (2002) Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J. Appl. Micro. Symp. Suppl.* **92**:98S-110

Gunson RN, Collins TC, Carman WF (2006) Practical experience of high throughput real-time PCR in the routine diagnostic virology setting. *J. Clin. Virol.* **37:** 355-367.

Hauser AR, Sriram P (2005) Severe *Pseudomonas aeruginosa* infections: Tackling the conundrum of drug resistance. *Postgrad. Med.* **117:** 41–48.

Hogan D, Kolter R (2002) Why are bacteria refractory to antimicrobials? *Curr. Opin. Microbiol.* **5:** 472-477.

Holt SM, Cote GL (1998) Differentiation of dextran-producing *Leuconostoc* strains by a modified randomly amplified polymorphic DNA protocol. *Appl. Environ. Microbiol.* **64:** 3096-3098.

Iwamoto T, Sonobe T (2004) Peptide-nucleic acid-mediated competitive PCR clamping for detection of rifampin-resistant *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **48:** 4023-4026.

Jacoby GA, Munoz-Price LS (2005) Mechanisms of disease: The new beta-lactamases. *N. Engl. J. Med.* **352:** 380-391.

Jakimov D, Četojević-Simin D, Kojić V, Mrdanović J (2000) Peptide nucleic acid (PNA)-more than anyone could imagine at the first sight. *Arch. Oncol.* **8:** 11-14.

Johnson JR (2000) Development of polymerase chain reaction-based assays for bacterial gene detection. *J. Microbiol. Meth.* **41:** 201-209.

Kugelberg E, Löfmark S, Wretlind B, Andersson DI (2005) Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **55:** 22-30.

Lambert PA (2005) Bacterial resistance to antibiotics: Modified target sites. *Adv. Drug Deliv. Rev.* **57:** 1471-1485.

Lee SH, Jeong SH (2005) Nomenclature of GES-type extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* **49:** 2148-2150.

Livermore DM (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: Our worst nightmare? *Clin. Infect. Dis.* **34:** 634-640.

Mackay IM (2004) Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* **10:** 190-212.

Mandell GL, Bennett JE, Dolin R (2005) *Principles and practice of infectious diseases*, 6th edition, volume 2. Elsevier, USA.

Matsumoto Y, Inoue M (1999) Characterization of SFO-1, a plasmid mediated inducible class A β-lactamase from *Enterobacter cloacae*. *Antimicrob. Agents Chemother.* **43:** 307-313.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Menon PK, Eswaran SP, Pant SS, Bharadwaj R, Nagendra CA (2003) Random amplification of polymorphic DNA based typing of *Pseudomonas aeruginosa*. *MJAFI.* **59:** 25-28.

Nakae T, Nakajima A, Ono T, Saito K, Yoneyama H (1999) Resistance to beta-lactam antibiotics in *Pseudomonas aeruginosa* due to interplay between the MexAB-OprM efflux pump and beta-lactamase. *Antimicrob. Agents Chemother.* **43:** 1301-1303.

Navon-Venezia S, Ben-Ami R, Carmeli Y (2005) Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Curr. Opin. Infect. Dis.* **18:** 306-313.

Nester EW, Anderson DG, Roberts CE, Pearsall NN, Nester MT (2001) *Microbiology: A human perspective*, 3rd ed. McGraw-Hill, New York, N.Y.

Nielsen PE, Egholm M, Berg RH, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with thymine-substituted polyamide. *Science* **254:** 1497-500.

Nielsen PE (1998) Structural and biological properties of peptide nucleic acid (PNA). *Pure & Appl. Chem.* **70:** 105-110.

Nordmann P, Guibert M (1998) Extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **42:** 128-131.

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:** 1661-1669.

Ortiz-Herrera M, Gerónimo-Gallegos A, Cuevas-Schacht F, Pérez-Fernández L, Coria-Jiménez R (2004) RADP-PCR characterization of *Pseudomonas aeruginosa* strains obtained from cystic fibrosis patients. *Salud Publica Mex.* **46:** 149-157.

Ørum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley CJ (1993) Single base pair mutation analysis by PNA-directed PCR clamping. *Nucleic Acids Res.* **21:** 5332-5336.

Paetzel M, Danel F, De Castro L, Mosimann SC, Page MGP, Strynadka NCJ (2000) Crystal structure of the class D β-lactamase OXA-10. *Nat. Struct. Biol.* **7:** 918-925.

Pellestor F, Paulasova P (2004) The peptide nucleic acids, efficient tools for molecular diagnosis (Review). *Int. J. Mol. Med.* **13:** 521-525.

Pellestor F, Paulasova P, Macek M, Hamamah S (2004) The peptide nucleic acids: A new way for chromosomal investigation on isolated cells? *Human Reprod.* **19:** 1946-1951.

Pirnay JP, De Vos D, Cochez C, Bilocq F, Vanderkelen A, Zizi M, Ghysels B, Cornelis P (2002) *Pseudomonas aeruginosa* displays an epidemic population structure. *Environ. Microbiol.* **4:** 898-911.

Pitout JDD, Nordmann P, Laupland KB, Poirel L (2005) Emergence of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) in the community. *J. Antimicrob. Chemother.* **56:** 52-59.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Poirel L, Naas T, Nordmann P (2006) Pyrosequencing as a rapid tool for identification of GES-type extended-spectrum beta-lactamases. *J. Clin. Microbiol.* **44:** 3008-3011.

Pollack M (2000) *Pseudomonas aeruginosa*, p. 2310-2335. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and practice of infectious diseases*, 5th ed. Churchill Livingstone, Edinburgh, Scotland.

Poole K (2004) Resistance to beta-lactam antibiotics. *Cell Mol. Life Sci.* **61:** 2200-2223.

Poole K (2005) Mechanisms of antimicrobial resistance: Opportunities for new targeted therapies. *Adv. Drug Deliv. Rev.* **57:** 1443-1445.

Ray A, Norden B (2000) Peptide nucleic acid (PNA): Its medical and biotechnical applications and promise for the future. *FASEB J.* **14:** 1041-1060.

Renders N, Römling U, Verbrugh H, Van Belkum A (1996) Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. *J. Clin. Microbiol.* **34:** 3190-3195.

Rice L (2001) Evolution and clinical importance of extended-spectrum beta-lactamases. *CHEST.* **119:** 391-396.

Römling U, Wingender J, Müller H, Tümmler B (1994) A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl. Environ. Microbiol.* **60:** 1734-1738.

Roy PH (1999) Horizontal transfer of genes in bacteria. *Microbiol. Today.* **26:** 168-170.

Sambrook J, Russell RW (2001) *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sardelic S, Pallecchi L, Punda-Polic V, Rossolini GM (2003) Carbapenem-Resistant *Pseudomonas aeruginosa*-carrying VIM-2 metallo-beta-lactamase determinants, *Croatia. Emerg. Infect. Dis.* **9:** 1022-1023.

Schweizer HP (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: Unanswered questions. *Genet. Mol. Res.* **1:** 48-62.

Speijer H, Savelkoul PHM, Bonten MJ, Stobberingh EE, Tjhie JHT (1999) Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. *J. Clin. Microbiol.* **37:** 3654-3661.

Stender H, Mollerup TA, Lund K, Petersen KH, Hongmanee P, Godtfredsen SE (1999) Direct detection and identification of *Mycobacterium tuberculosis* in smear-positive sputum samples by fluorescence *in situ* hybridization (FISH) using peptide nucleic acid (PNA) probes. *Int. J. Tuberc. Lung Dis.* **3:** 830-837.

Stender H, Fiandaca M, Hyldig-Nielsen JJ, Coull J (2002) PNA for rapid microbiology. *J. Microbiol. Meth.* **48:** 1-17.

Struelens MJ, De Gheldre Y, Deplano A (1998) Comparative and library epidemiologic typing systems: Outbreak investigations versus surveillance systems. *Infect. Control Hosp. Epidemiol.* **19:** 565-569.

Stürenburg E, Mack D (2003) Extended-spectrum beta-lactamases: Implications for the clinical microbiology laboratory, therapy, and infection control. *J. Infect.* **47:** 273-295.

Stürenburg E, Lang M, Horstkotte MA, Laufs R, Mack D (2004) Evaluation of the MicroScan ESBL plus confirmation panel for detection of extended-spectrum β-lactamases in clinical isolates of oxyimino-cephalosporin-resistant Gram-negative bacteria. *J. Antimicrob. Chemother.* **54:** 870-875.

Sundsfjord A, Simonsen GS, Haldorsen BC, Haaheim H, Hjelmevoll S, Littauer P, Dahl KH (2004) Genetic methods for detection of antimicrobial resistance. *APMIS.* **112:** 815-837.

Svanvik N, Stahlberg A, Sehlstedt U, Sjoback R, Kubista M (2000) Detection of PCR products in real time using light-up probes. *J. Anal. Biochem.* **287:** 179-182.

Tang HB, Dimango E, Bryan R, Gambello M, Iglewski BH, Goldburg JB, Prince A (1996) Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model infection. *Infect. & Immun.* **64:** 37-43.

Thomson KS (2001) Controversies about extended-spectrum and AmpC beta-lactamases. *Emerg. Infect. Dis.* **7:** 333-336.

Thong KL, Lai KS, Ganeswrie R, Puthucheary SD (2004) Pulsed-field gel electrophoresis of multidrug-resistant and –sensitive strains of *Pseudomonas aeruginosa* from a Malaysian hospital. *Jpn. J. Infect. Dis.* **57:** 206-209.

Todar K (2002) *Pseudomonas aeruginosa*. Todar's Online Textbook of Bacteriology. [Online.] http://testbookofbacteriology.net/pseudomonas.html.

Vercauteren E, Descheemaeker P, Ieven M, Sanders CC, Goosseens H (1997) Comparison of screening methods for detection of extended-spectrum beta-lactamases and their prevalence among blood isolates of *Escherichia coli* and *Klebsiella* spp in a Belgian teaching hospital. *J. Clin. Microbiol.* **35:** 2191-2197

Von Wintzingerode F, Landt O, Ehrlich A, Göbel UB (2000) Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity. *Appl. Environ. Microbiol.* **66:** 549-557.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004a) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004b) Molecular characterization of a cephamycin-hydrolyzing and inhibitor resistant class A beta-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. *Antimicrob. Agents Chemother.* **48:** 2905-2910.

Waley SG (1992) *Beta-lactamase: Mechanism of action*., p. 198–228. *In* M. I. Page (ed.), The chemistry of beta-lactams. A. and P. Blackie, London.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004a) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Weldhagen GF (2004b) Rapid detection and sequence-specific differentiation of extended-spectrum beta-lactamase GES-2 from *Pseudomonas aeruginosa* by use of a real-time PCR assay. *Antimicrob. Agents Chemother.* **48:** 4059-4062.

Wilke MS, Lovering AL, Strynadka NCJ (2005) Beta-lactam antibiotic resistance: A current structural perspective. *Curr. Opin. Microbiol.* **8:** 525-533.

Wilson R, Dowling RB (1998) *Pseudomonas aeruginosa* and other related species. *Thorax.* **53:** 213-219.

Yim G (11 April 2007) Attack of the superbugs: antibiotic resistance. The science creative quarterly. [Online.] http://www.scq.ubc.ca/?p=410

Chapter 3

Prevalence of GES-2 and other GES-type extended-spectrum β-lactamases in *Pseudomonas aeruginosa* isolates collected in the Gauteng region of South Africa

The editorial style of the Journal of Clinical Microbiology was used in this chapter.

3.1 Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is among the most important causes of nosocomial infections, especially in burn victims and immunocompromised patients. Extended-spectrum β-lactamases (ESBLs) constitute a major problem in the use of β-lactam antibiotics to treat infections, given their broad substrate specificity and ability to hydrolyse many of the extended-spectrum third-generation cephalosporins currently in use in hospital settings. Guiana extended-spectrum (GES)-type β-lactamase enzymes are of importance since these enzymes have mainly been found in *P. aeruginosa* and only at a limited number of geographical sites, primarily France, Greece and South Africa. The aim of this study was to determine the prevalence of bla_{GES-2} and other bla_{GES}-types in 100 *P. aeruginosa* clinical isolates utilising a sequence-specific, peptide nucleic acid (PNA)-based multiplex PCR method and DNA sequencing. Results indicated that 50% (n = 50) of the *P. aeruginosa* isolates tested positive for the presence of bla_{GES-2} while 3% (n = 3) tested positive for the presence of other bla_{GES}-types. Deoxyribonucleic acid sequencing remains the only reliable method for accurate identification of all nine bla_{GES} genes. An initial study conducted in 2000 revealed that 2% of the *P. aeruginosa* isolates had a GES-2 ESBL. It is evident from the current study that there was a definite increase in GES-2 ESBL occurrence in the Gauteng region of South Africa over a 4-year period (2000-2004). This emphasised the importance of constant surveillance of *P. aeruginosa* clinical isolates to determine the prevalence of antibiotic resistance genes. The high prevalence of GES-2 ESBLs in the Gauteng area may have serious health implications, threatening the efficacy of antibiotics currently used against *P. aeruginosa* infection.

3.2 Introduction

Extended-spectrum β-lactamases (ESBLs) of the Guiana extended-spectrum (GES)-type have increasingly been reported in Gram-negative rods such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*). Guiana extended-spectrum-type ESBLs have been identified from various geographical locations including Argentina, Brazil, France, French Guiana, Greece, Portugal and South Africa (Dubois *et al*., 2002; Poirel *et al*., 2002; Duarte *et al*., 2003; Castanheira *et al*., 2004; Vourli *et al*., 2004; Wachino *et al*., 2004a; Wachino *et al*., 2004b; Pasteran *et al*., 2005).

The first described GES-1 β-lactamase was in France in a *K. pneumoniae* isolate obtained from a 1-month-old girl previously hospitalised in French Guiana (Poirel *et al*., 2000). Subsequently β-lactamase GES-1 was identified in *P. aeruginosa* in France and *K. pneumoniae* in Portugal (Dubois *et al*., 2002; Duarte *et al*., 2003). The GES-1 enzyme encoded by bla_{GES-1} exhibits strong activity against most β-lactams (amoxycillin, ticarcillin, piperacillin, cephalothin and ceftazidime), except aztreonam and imipenem (Poirel *et al*., 2000). Inhibition studies (IC_{50}) have shown GES-1 to be inhibited by clavulanic acid (5 µM), tazobactam (2.5 µM) and imipenem (0.1 µM) (Poirel *et al*., 2000).

Beta-lactamase GES-2 was originally described in a *P. aeruginosa* isolate obtained from a blood culture during May 2000 in South Africa (Poirel *et al*., 2001). Subsequently GES-2 has also been identified in *P. aeruginosa* isolates from Argentina (Pasteran *et al*., 2004). Compared to GES-1, GES-2 contains a 2 base pair (bp) substitution (nucleotide positions 493 and 494) leading to a single glycine (Gly) to asparagine (Asn) change at Ambler's position 170, inside the omega loop of the catalytic site of the mature protein (Poirel *et al*., 2001). This amino acid change is thought to be the cause of more efficient hydrolysis of imipenem and GES-2 being less susceptible to inhibitors such as clavulanic acid (Poirel et al., 2001). The occurrence of GES-2 confers the ability to *P. aeruginosa* to hydrolyse extended-spectrum cephalosporins, as well as imipenem to a minor extent (Poirel *et al*., 2001). This antibiotic resistance mechanism causes *P. aeruginosa* to be highly resistant in the clinical setting (Poirel *et al.*, 2002; Weldhagen *et al.*, 2003).

Several point mutations along the *bla*$_{GES}$ gene have resulted in GES-type ESBLs with distinct hydrolysis profiles from GES-1 and GES-2 (Poirel *et al*., 2002; Castanheira *et al*., 2004; Vourli *et al*., 2004; Poirel *et al*., 2006). One such nucleotide substitution at position 493 resulted in a Gly170serine (Ser) change identified in GES-3, GES-5 and GES-6 (Poirel *et al*., 2006). The isolates with the Gly170Ser change hydrolyse carbapenems at a low level similar to that for GES-2, which also hydrolyses cephamycins (Poirel *et al*., 2006). These isolates are weakly susceptible to Ambler class A β-lactamase inhibitors (Poirel *et al*., 2006). The most recent GES-type to be identified, GES-9, possessed a nucleotide substitution at position 709 that resulted in a Gly243Ser change (Poirel *et al*., 2005). The GES-9 enzyme did not hydrolyse carbapenems, but exhibited broadened activity towards monobactams (e.g. aztreonam) (Poirel *et al*., 2005).

Detection of GES-type ESBL-producing *P. aeruginosa* isolates in the clinical microbiology laboratory using conventional methods was difficult (Weldhagen *et al*., 2003). This was due to factors such as the presence of naturally occurring β-lactamases, for instance the chromosomally encoded AmpC (class C cephalosporinase) enzyme, which might be over expressed, leading to false negative results (Weldhagen *et al*., 2003). Other factors included the simultaneous presence of metallo-enzymes with carbapenem-hydrolysing activities or with extended-spectrum oxacillinases, the relative resistance to inhibition by clavulanate and a combination of resistance mechanisms, such as efflux and impermeability of the bacterial cell wall (Weldhagen *et al*., 2003).

Molecular based methods for the detection of ESBLs included PCR with a series of primers designed for the recognition of class A, β-lactamase genes (e.g. GES-1A and GES-1B for *bla*$_{GES}$ genes) in *P. aeruginosa* followed by sequencing of the amplification products (Weldhagen *et al.*, 2003). Although DNA sequencing could be seen as a "Gold standard" and sequencing of these PCR products could detect the ESBLs with great sensitivity and specificity, sequencing was too expensive to be used as a routine method (Weldhagen *et al.*, 2003).

Previously, more traditional methods such as isoelectric focusing analysis and double-disk synergy tests were used (Bradford, 2001; Weldhagen *et al.*, 2003). Techniques such as isoelectric focussing analysis may just indicate the presence of acquired β-lactamases rather

than identify the specific ESBL (Weldhagen *et al*., 2003), while the double-disk synergy test has proven to be an unreliable detection method (Bradford, 2001; De Champs *et al*., 2002; Weldhagen *et al.*, 2003).

A recently developed sequence-specific competitive peptide nucleic acid (PNA)-based multiplex PCR detection method provided an accurate means to detect and differentiate *bla*$_{GES-2}$ from other *bla*$_{GES}$-types compared to standard PCR and gene sequencing techniques (Weldhagen, 2004). The method relied on PNA's intrinsic high affinity for DNA and its inability to serve as a primer for DNA polymerases (Ørum *et al*., 1993). This multiplex PCR used a forward primer in conjunction with a reverse primer complementary to the desired mutant sequence (*bla*$_{GES-2}$), as well as a PNA-probe similar to the reverse primer, but complementary to the wild type (wt) sequence (*bla*$_{GES-1}$) (Weldhagen, 2004). In the event of a wt DNA template (*bla*$_{GES-1}$) present in the PCR reaction, the PNA-probe out-competed the reverse primer for binding and no amplification occurred (Weldhagen, 2004). Conversely, the opposite happened when a mutant sequence (*bla*$_{GES-2}$) was present during the reaction (Weldhagen, 2004). The reverse primer would out-compete the PNA-probe resulting in amplification of a 505 bp product (Weldhagen, 2004). The PCR method used by Weldhagen (2004) included an internal amplification control that resulted in the amplification of a 360 bp product when a *bla*$_{GES}$ template was present.

The dissemination of GES-type β-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections due to ESBL-producing *P. aeruginosa* (Weldhagen *et al.*, 2003). Due to this, the monitoring of new antibiotic resistance genes in bacterial species was essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings (Sardelic *et al.*, 2003).

This study aimed to determine the prevalence of *bla*$_{GES-2}$ and other *bla*$_{GES}$-types in 100 *P. aeruginosa* isolates collected over the Gauteng region of South Africa utilising a competitive PNA-based multiplex PCR. Deoxyribonucleic acid sequencing was used to confirm the identity of *bla*$_{GES}$ genes identified during the study since novel GES-types might be overlooked with the PNA-based multiplex PCR method.

3.3 Materials and Methods

3.3.1 Bacterial strains

One hundred *P. aeruginosa* clinical isolates were collected and identified by two private pathology laboratories in Gauteng, South Africa. The *P. aeruginosa* isolates were collected between 2004 and 2005 from the Gauteng region of South Africa. Bacterial isolates were obtained from various specimen types including pus swabs, urine, irrigation fluids and sputum. Routine antibiograms were performed according to Clinical Laboratory Standards Institute (CLSI) susceptibility testing standards. Resistance to ceftazidime was a requirement for inclusion in the study and was determined with a disk diffusion assay, as previously described (CLSI, 2005). A bla_{GES-2}-producing isolate, *P. aeruginosa* GW-1 (Poirel *et al.*, 2001) and bla_{GES-1}-producing isolate, *K. pneumoniae* ORI-1 (Poirel *et al.*, 2000), were used as positive controls during multiplex PCR assays and DNA sequencing.

3.3.2 Whole-cell DNA extraction

Extraction of whole-cell DNA was performed by a precipitation-based method, as described previously (Philippon *et al.*, 1997). Briefly; one single colony-forming unit (CFU) of *P. aeruginosa* was resuspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated overnight at 37°C (WBM SPL 25 Labcon shaking water bath, Laboratory Marketing Services, Maraisburg) while shaking at 50 revolutions per minute (rpm). One ml of the overnight culture was pelleted in a centrifuge at 3 000 × g (Labofuge 400R, Heraeus Instruments, Germany) for 10 min at 4°C. The pellet was resuspended in 1 ml sterile distilled water and re-pelleted as described above. The resulting pellet was resuspended in 500 µl STE buffer containing 75 mM NaCl (Promega, Madison, WI), 20 mM Tris (Sigma Chemical Co., St. Louis, MO) and 25 mM EDTA (Promega, Madison, WI) at pH 7.5.

Cells were lysed by adding 25 µl of a 20% SDS (Promega, Madison, WI) solution (pH 12.45) and 1 µl of a 50 $mg.ml^{-1}$ stock solution lysozyme (Sigma Chemical Co., St. Louis, MO) with incubation at 37°C (QBT2 heating block, Grant Instruments Ltd., Cambridge, United Kingdom) for one hour. On completion of cell lysis, 220 µl of 5 M NaCl (Promega, Madison, WI) was added followed by the addition of 700 µl chloroform / isoamylalcohol (24:1) (Sigma Chemical Co., St. Louis, MO) to separate DNA and protein phases. Following centrifugation at 5 000 × g (Z233 M-2, Hermle Labortechnik, Wehingen, Germany) for 10 min at room

temperature (25°C), the upper phase containing whole-cell DNA was removed and transferred to a new tube. The DNA was precipitated with 700 µl isopropanol (Merck, Darmstadt, Germany) at -20°C for 1 h or overnight. The precipitated DNA was pelleted by centrifugation at 5 000 × g (Z233 M-2, Hermle Labortechnik, Wehingen, Germany) for 10 min at room temperature (25°C) followed by two washing steps with 800 µl of a 70% ethanol solution (Merck, Darmstadt, Germany). After air-drying, the DNA pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI) containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4. Five hundred µl of the extracted DNA was stored at 4°C and -20°C until further analysis.

3.3.3 PCR amplification of bla$_{GES}$ genes

Polymerase chain reaction analysis was performed with the *bla*$_{GES}$ specific primer set GES-1A and GES-1B (Poirel *et al*., 2001) using whole-cell DNA extracted from the 100 *P. aeruginosa* isolates as template. The primers were described in Table 3.1. Primer synthesis and purification was conducted by Inqaba Biotechnical Industries Pty. Ltd., South Africa. The reaction consisted of 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI), 0.8 µl of each primer (20 mM), 4 µl whole-cell DNA as template and molecular grade water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The PCR cycle previously described by Weldhagen (2004a), for sequence-specific, PNA-based multiplex PCR was used as described below (section 3.3.4). The amplicons obtained (864 bp) were analysed by gel electrophoresis as described above (section 3.3.4).

3.3.4 Sequence-specific competitive PNA-based multiplex PCR

Two sets of primer pairs were used in a multiplex PCR assay together with a *bla*$_{GES-2}$ specific PNA-probe (Applied Biosystems, Rotkreutz, Switzerland) (Table 3.1). The selected primer pairs were specific for the detection of *bla*$_{GES}$ and amplified 360- and 505 bp products respectively. The multiplex PCR mix (reaction volume 50 µl) consisted of 10 X Mg-free PCR buffer, 1.5 mM $MgCl_2$, a 200 µM concentration of each deoxynucleoside triphosphate, 1.25 U of Taq DNA polymerase (Promega, Madison, WI), a 0.32 µM concentration of each primer, 0.32 µM PNA-probe and 2 µl DNA template.

Multiplex PCR amplification was performed on a GeneAmp 9600 thermocycler (Perkin Elmer Cetus, Emeryville, CA). The PCR cycle programme consisted of an initial denaturation step at 95°C for 2 min, followed by 35 amplification cycles each comprising a

denaturation step at 95°C for 30 s, followed by an annealing step at 50°C for 1 min and an extension step at 72°C for 1 min. After the completion of 35 amplification cycles, a final extension step was performed at 72°C for 5 min.

3.3.5 Verification of DNA extractions and PCR products

All DNA extractions were verified by gel electrophoresis at 2 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 1 h in a 1% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (Promega, Madison, WI) (0.5 µl.ml^{-1}) in 1 X TBE running buffer [containing 45 mM Tris-borate and 1 mM EDTA at pH 8.3 (Promega, Madison, WI)]. Amplicons were electrophoresed on a 1% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (Promega, Madison, WI) (0.5 µl.ml^{-1}) at 4 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 45 min in 1 X TBE running buffer (pH 8.3), with a 100 bp DNA ladder (Promega, Madison, WI) as molecular size marker. All agarose gels were visualized under UV illumination (TFM-26 Ultraviolet Transilluminator, UVP, Upland, CA) (Sambrooks and Russell, 2001) and the images captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA).

3.3.6 DNA sequencing of bla_{GES} genes

The *bla_{GES}* specific PCR amplicons were sequenced with the GES-1A forward primer using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) to determine the *bla_{GES}* identity. Sequencing was performed by Inqaba Biotechnical Industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Polymerase chain reaction products obtained with the *bla_{GES}* specific PCR from *P. aeruginosa* GW-1 (Poirel *et al.*, 2001) and *K. pneumoniae* ORI-1 (Poirel *et al.*, 2000) were used as sequencing controls.

3.5 Results and Discussion

The sequence-selective competitive PNA-based multiplex PCR assay described by Weldhagen (2004) was used to determine the prevalence of *bla_{GES-2}* and other *bla_{GES}*-types in clinical isolates of *P. aeruginosa*. The amplification products generated during the PNA-

based multiplex PCR were subjected to gel electrophoresis after which two distinct patterns were detected. The presumptive *bla*$_{GES-1}$ possessing *P. aeruginosa* clinical isolates and the control strain (*K. pneumoniae* ORI-1) produced a 360 bp internal amplification control band. The *bla*$_{GES-2}$ possessing *P. aeruginosa* clinical isolates and the control strain (*P. aeruginosa* GW-1) produced two distinct bands of 360 bp and 505 bp, respectively, clearly distinguishing presumptive *bla*$_{GES-1}$ isolates from *bla*$_{GES-2}$ isolates. The two amplification profiles (a single 360 bp band for presumptive GES-1; a 360 bp and 505 bp band for GES-2) resulted due to the competitive nature of the PNA-probe (Weldhagen, 2004). Results indicated that three (3%) of the 100 *P. aeruginosa* clinical isolates tested positive for a possible *bla*$_{GES-1}$ when analysed with competitive PNA-based multiplex PCR. Thirty-two percent (n = 32) of isolates tested positive for *bla*$_{GES-2}$ with this technique. These results corresponded with the results obtained by Weldhagen (2004).

A third band of 864 bp was observed for all the isolates analysed that did not occur in the work conducted by Weldhagen (2004). This band is the result of amplification directly between forward primer GES-1A and reverse primer GES-1B, amplifying the whole *bla*$_{GES}$ gene. The amplification of the whole *bla*$_{GES}$ gene may be due to a stemloop secondary structure (Weldhagen, 2004), interfering with the binding of reverse primer GES-E, favouring amplification between GES-1A and GES-1B. Amplification of the whole gene did not seem to interfere with the overall result of the multiplex PCR, which is determined by amplification of a 360 bp product (presumptive *bla*$_{GES-1}$) or a 360 bp product together with a 505 bp product (*bla*$_{GES-2}$). A gel electrophoresis result depicting the banding patterns found was shown in Figure 3.1.

The stemloop structure (Figure 3.2) has been noted in the secondary structure of the *bla*$_{GES}$ gene situated at positions 466 – 470 (CGGCT) and 482 – 486 (AGCCG) of the coding region (Weldhagen, 2004). The stemloop has a melting temperature of 82°C, creating a possible steric hindrance during annealing of the reverse primer GES-E to the closely situated target site (Weldhagen, 2004). Weldhagen (2004) described the inclusion of an initial denaturation step of 2 min at 95°C in the amplification protocol and permitting the PNA-probe to bind earlier to the target sequence relative to the competing reverse primer (GES-E) to overcome the steric interference caused by the stemloop. The PNA-probe binds earlier since antiparallel PNA-DNA duplexes form more rapid in comparison to the duplexes formed in parallel binding mode (Ørum *et al.*, 1997). In addition, the PNA-probe was designed to bind in a

highly thermostable, antiparallel mode, with the resulting PNA-DNA duplex impairing the amplicon interstrand-reassociation, further stabilizing the reverse primer-binding site on the DNA template (Weldhagen, 2004). The formation of the 864 bp product, when performing the PNA-based multiplex PCR in this study, indicated that the steric interference by the stemloop was not completely overcome. Prolonging the initial denaturation step to 10 min might help to overcome the steric interference caused by the stemloop structure.

Performing the *bla*$_{GES}$ specific PCR utilising GoTaq Green Master Mix (Promega, Madison, WI) on whole-cell DNA revealed 53 of the 100 *P. aeruginosa* isolates to possess a *bla*$_{GES}$ gene. All 32 isolates identified as *bla*$_{GES-2}$ positive with the PNA-based multiplex PCR were confirmed to be *bla*$_{GES-2}$ by means of DNA sequencing. A further 17 isolates were shown to possess *bla*$_{GES-2}$ genes through DNA sequencing, initially identified as *bla*$_{GES}$ negative with the PNA-based multiplex PCR. Sequencing of the amplicons obtained from the possible *bla*$_{GES-1}$ positive isolates revealed these genes to be *bla*$_{GES-5}$ with a 99% identity to the *bla*$_{GES-5}$ gene (GenBank accession number AY494717) published by Vourli and co-workers (2004). Both the *bla*$_{GES-1}$ [*K. pneumoniae* ORI-1 (Poirel *et al.*, 2000)] and *bla*$_{GES-2}$ [*P. aeruginosa* GW-1 (Poirel *et al.*, 2001)] sequencing controls gave positive results after DNA sequencing, confirming the fidelity and accuracy of the PCR and sequencing reactions.

Attempts to amplify the *bla*$_{GES}$ gene in Promega Taq in Buffer A (Promega, Madison, WI) produced little product. Sufficient amplification was achieved using GoTaq Green Master Mix (Promega, Madison, WI). GoTaq Green Master Mix (Promega, Madison, WI) gave better results compared to Promega Taq in Buffer A, as exemplified in the additional 17 isolates identified to be *bla*$_{GES-2}$ positive using GoTaq Green Master Mix (Promega, Madison, WI). Optimised master mixes were thus a better choice of PCR reagents to be used in screening projects, resulting in greater sensitivity. The PNA-based multiplex PCR technique has been reported to be sensitive to template quality (e.g. high salt concentrations), which might have resulted in the false-negative results obtained (Weldhagen, 2004).

The sequence specific, PNA-based multiplex PCR was designed to detect two bp mismatches between *bla*$_{GES-1}$ and *bla*$_{GES-2}$ at position 492 and 493 but could not detect point mutations outside this chosen mutation site (Weldhagen, 2004). To date nine types of GES enzymes (GES-1 to GES-9) have been described (Poirel *et al.*, 2005). Table 3.2 indicated the nucleotide differences between these nine *bla*$_{GES}$ variants. The shaded columns in Table 3.2

represent the nucleotides detected by PNA-based multiplex PCR. The PNA-based multiplex PCR amplified a 360 bp internal control band when a GG motif was present at position 492 and 493, while an AA motif at the same position resulted in 360 bp and 505 bp bands were amplified due to the competitive nature of the PNA-probe (Weldhagen, 2004). The *bla*$_{GES-3}$, *bla*$_{GES-7}$, *bla*$_{GES-8}$ and *bla*$_{GES-9}$ genes all possess GG at position 492 and 493 and should result in the same gel electrophoresis profile as *bla*$_{GES-1}$. The *bla*$_{GES-4}$, *bla*$_{GES-5}$ and *bla*$_{GES-6}$ genes with AG at 492 and 493 should result in the destabilisation of the PNA-probe and give the same gel electrophoresis profile as *bla*$_{GES-2}$. The sequence-selective, PNA-based multiplex PCR could no longer identify and differentiate between *bla*$_{GES-1}$ and *bla*$_{GES-2}$, but rather distinguish between those *bla*$_{GES}$ genes with a GG motif at position 492 and 493 from those with mismatches at the same position (Poirel *et al*., 2006).

The misidentification of *bla*$_{GES-5}$ as *bla*$_{GES-1}$ was unexpected. The PNA-based method functioned on the basis of competitive exclusion between a PNA-probe and reverse primer GES-E (Weldhagen, 2004). The reverse primer GES-E [5'-GTGTGTTGTCG**TT**CATCTC-3'] and the PNA-probe [5'-GTTGTCG**CC**CATCTC-3'] differed by two nucleotides (Weldhagen, 2004). The two bp difference was sufficient to allow competitive exclusion between the primer and probe. The binding region on the *bla*$_{GES-5}$ gene [5'-GTGTGTTGTCG**TC**CATCTC-3'] possessed a single nucleotide mismatch compared to the PNA-probe and reverse primer and should have been sufficient to allow competitive exclusion of the PNA-probe and allow binding of reverse primer GES-E. The one bp difference might not be sufficient to allow competitive exclusion between the primer and PNA-probe, since PNA-DNA binding is stronger than DNA-DNA binding due to the synthetic back-bone of PNA molecules (Ørum *et al*., 1993). Nucleotide sequencing thus remains the only reliable method for distinguishing all nine *bla*$_{GES}$ variants.

An initial study conducted in 2000 on 361 specimens from South Africa, indicated that 2% of the *P. aeruginosa* isolates studied possessed a *bla*$_{GES-2}$ gene (Poirel *et al*., 2002). In comparison with the current study where 50% of *bla*$_{GES}$ genes were found to be *bla*$_{GES-2}$ positive there was a definite increase in *bla*$_{GES-2}$ occurrence in the Gauteng region of South Africa amongst *P. aeruginosa* isolates. This emphasised the importance of constant surveillance of *P. aeruginosa* clinical isolates to determine the prevalence of antibiotic resistance genes. The high prevalence of *bla*$_{GES-2}$ suggested a possible selective pressure in the Gauteng clinical setting that might favour *bla*$_{GES-2}$ over *bla*$_{GES-5}$ in *P. aeruginosa*. The

high prevalence of the ESBL GES-2 in the Gauteng area may have serious health implications threatening the efficiency of antibiotics (e.g. ceftazidime, imipenem) currently used against *P. aeruginosa* infections at various clinical settings.

3.6 Conclusions

The increase in occurrence of the GES-2 ESBL since 2000 emphasizes the importance of constant surveillance of *P. aeruginosa* clinical isolates to determine the prevalence of antibiotic resistance genes. The dissemination of these β-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections (e.g. septicaemia) due to ESBL-producing *P. aeruginosa* (Weldhagen *et al.*, 2003).

Continuous monitoring of emerging antibiotic resistance genes in bacteria such as *P. aeruginosa* is therefore essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings. The identification of new bla_{GES} variants with mutations outside the target region and similar mutations inside the target region of the PNA-based multiplex PCR resulted in this method being able to only divide the bla_{GES} genes into two groups instead of differentiating between bla_{GES-1} and bla_{GES-2}. The 360 bp amplification product indicated group 1 consisting of bla_{GES-1}, bla_{GES-3}, bla_{GES-7}, bla_{GES-8} and bla_{GES-9} while amplification of a 360 bp together with a 505 bp product indicated group 2 consisting of bla_{GES-2}, bla_{GES-4}, bla_{GES-5} and bla_{GES-6}. This method may be useful, however, to distinguish GES-types with carbapenemase activity from those that do not possess this quality. Indicating group 1 or group 2 with the PNA-based multiplex PCR may be unreliable as shown with the identification of bla_{GES-5} in group 1 instead of group 2 through the high binding stability of PNA-DNA hybrids. Nucleotide sequencing thus remaines the only reliable method for distinguishing all nine bla_{GES} variants.

Further research should be directed towards the development of cost effective and reliable methods for the rapid identification and differentiation of the nine bla_{GES} variants currently identified and possible future variants that may arise. Molecular detection methods that may be useful include real-time PCR detection and possibly pyrosequencing; however, these methods are still in developmental stages (Poirel *et al.*, 2006). Strategies should be employed to optimise the use of antibiotics in order to balance the need to provide adequate initial

antibiotic coverage to high-risk patients, with avoidance of unnecessary antibiotic utilisation which can promote resistance (Chlebicki and Oh, 2004). Possible strategies to optimise antibiotic utilisation may be the use of antibiotic administration protocols, antibiotic rotation, de-escalation therapy in highly selected patients or restriction in hospital formularies (Chlebicki and Oh, 2004).

3.7 References

Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*. **14:** 993-951.

Castanheira M, Mendes RE, Walsh TR, Gales AC, Jones RN (2004) Emergence of the extended-spectrum beta-lactamase GES-1 in a *Pseudomonas aeruginosa* strain from Brazil: Report from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* **48:** 2344-2345.

Chlebicki MP, Oh HML (2004) Extended-spectrum beta-lactamases in clinical isolates of *Esherichia coli* and *Klebsiella spp.* in a Singapore Hospital: Clinical spectrum. *Ann. Acad. Med. Singapore.* **33:** 302-306.

Clinical and Laboratory Standards Institue (2005) *Performance standards for antimicrobial disk susceptibility tests*, 8th ed. Approved standard M2-A8. Clinical and Laboratory Standards Institute, Wayne, PA.

De Champs C, Poirel L, Bonnet R, Sirot D, Chanal C, Sirot J, Nordmann P (2002) Prospective survey of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. *Antimicrob. Agents Chemother.* **46:** 3031-3034.

Duarte A, Boavida F, Grosso F, Correia M, Lito LM, Cristino JM, Salgado MJ (2003) Outbreak of GES-1 beta-lactamase-producing multidrug resistant *Klebsiella pneumoniae* in a university hospital in Lisbon, Portugal. *Antimicrob. Agents Chemother.* **47:** 1481-1482.

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of acc(3)-Ib/aac(6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **46:** 638-645.

Ørum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley CJ (1993) Single base pair mutation analysis by PNA-directed PCR clamping. *Nucleic Acids Res.* **21:** 5332-5336.

Ørum H, Kessler C, Kock T (1997) *Nucleic acid amplification technologies: Application to disease diagnosis*, p. 29-48. *In* H. Lee, S. Morse, and Ø. Olsvik (ed.). Peptide nucleic acids, 1st ed., Eaton Publishing, St. Natick, M.A.

Pasteran F, Faccone D, Campos K, Vazquez M, Rapoport M, Littvik A, Tokumoto M, Galas M (2004) Dissemination of GES extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* (PA) in Argentina, abstr. C2-1327, p. 105. Program Abstr. 44th *Intersci. Conf. Antimicrob. Agents Chemoth.* ASM, Washington, D.C.

Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vázquez M, Procopio A (2005) Novel variant (bla_{VIM-11}) of the metallo-beta-lactamase bla_{VIM} family in a GES-1 extended-spectrum-beta-lactamase-producing *Pseudomonas aeruginosa* clinical isolate in Argentina. *Antimicrob. Agents Chemother.* **49:** 474-475.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Weldhagen GF, De Champs C, Nordmann P (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J. Antimicrob. Chemother.* **49:** 561-565.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Poirel L, Naas T, Nordmann P (2006) Pyrosequencing as a rapid tool for identification of GES-type extended-spectrum beta-lactamases. *J. Clin. Microbiol.* **44:** 3008-3011.

Sambrook J, Russell RW (2001) *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sardelic S, Pallecchi L, Punda-Polic V, Rossolini GM (2003) Carbapenem-Resistant *Pseudomonas aeruginosa*-carrying VIM-2 metallo-beta-lactamase determinants, *Croatia. Emerg. Infect. Dis.* **9:** 1022-1023.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004a) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004b) Molecular characterization of a cephamycin-hydrolyzing and inhibitor resistant class A beta-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. *Antimicrob. Agents Chemother.* **48:** 2905-2910.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Table 3.1: PNA-based multiplex PCR primer and probe characteristics

Primer or Probe	Sequence (5'→3')	T_m (°C)	Position (nt) on *bla*$_{GES}$	Function	Source or Reference
GES-1A	ATGCGCTTCATTCACGCAC	56.6	01-19	Forward primer	Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
GES-1B	CTATTTGTCCGTGCTCAGG	56.4	846-864	Reverse primer	Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
GES-E	GTGTGTTGTCGTTCATCTC	51.8	487-505	Reverse primer (complementary to *bla*$_{GES-2}$)	Weldhagen, 2004
GES-F	CCTGGCGACCTCAGAGATAC	57.4	505-524	Forward primer	Weldhagen, 2004
PNA-probe	GTTGTCGCCCATCTC	70	487-501	Complimentary to *bla*$_{GES-1}$ and *bla*$_{IBC}$	Weldhagen, 2004

Table 3.2: Nucleotide differences between the different *bla*$_{GES}$ genes

bla$_{GES}$ gene	Nucleotide position										
	53	166	176	291	357	358	359	492	493	557	708
bla$_{GES-1}$	G	T	T	G	G	C	T	G	G	T	G
bla$_{GES-2}$	G	T	T	G	G	C	T	A	A	T	G
bla$_{GES-3}$	G	C	C	A	G	C	T	G	G	T	G
bla$_{GES-4}$	G	C	C	A	G	C	T	A	G	T	G
bla$_{GES-5}$	A	T	T	G	G	C	T	A	G	T	G
bla$_{GES-6}$	A	T	T	A	G	C	T	A	G	T	G
bla$_{GES-7}$	A	T	T	A	G	C	T	G	G	T	G
bla$_{GES-8}$	A	T	T	G	C	T	G	G	G	T	G
bla$_{GES-9}$	G	T	T	G	G	C	T	G	G	C	A

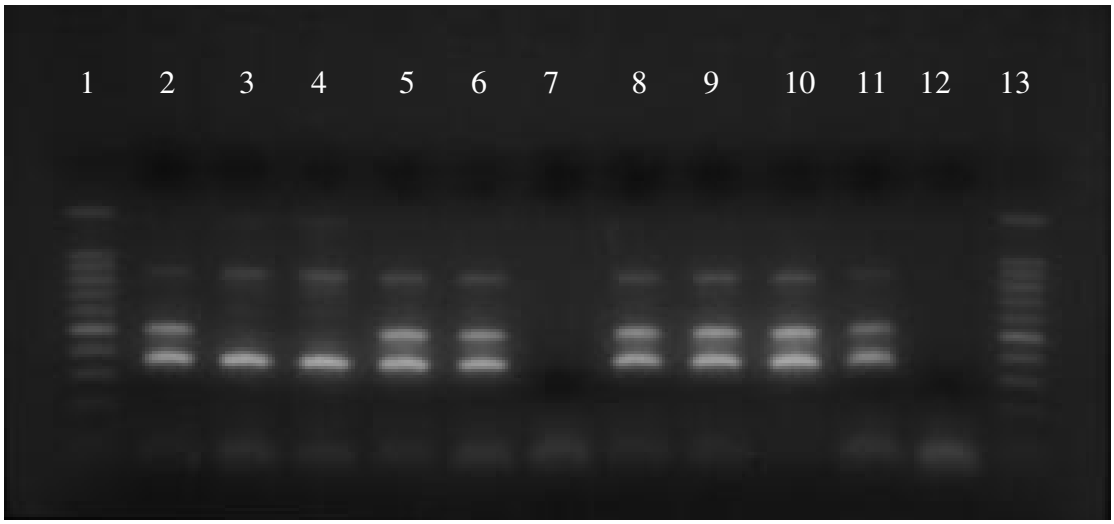

Figure 3.1: Gel electrophoresis result of the PNA-based multiplex PCR exhibiting results of seven clinical isolates. Lane 1: 100 bp marker (Promega, Madison, WI); Lane2, *bla*$_{GES-2}$ control; Lane 3, *bla*$_{GES-1}$ control; Lane 4, isolate 1; Lane 5, isolate 2; Lane 6, isolate 3; Lane 7, negative control; Lane 8, isolate 4; Lane 9, isolate 5; Lane 10, isolate 6; Lane 11, isolate 7; Lane 12, template negative control; Lane 13, 100 bp marker

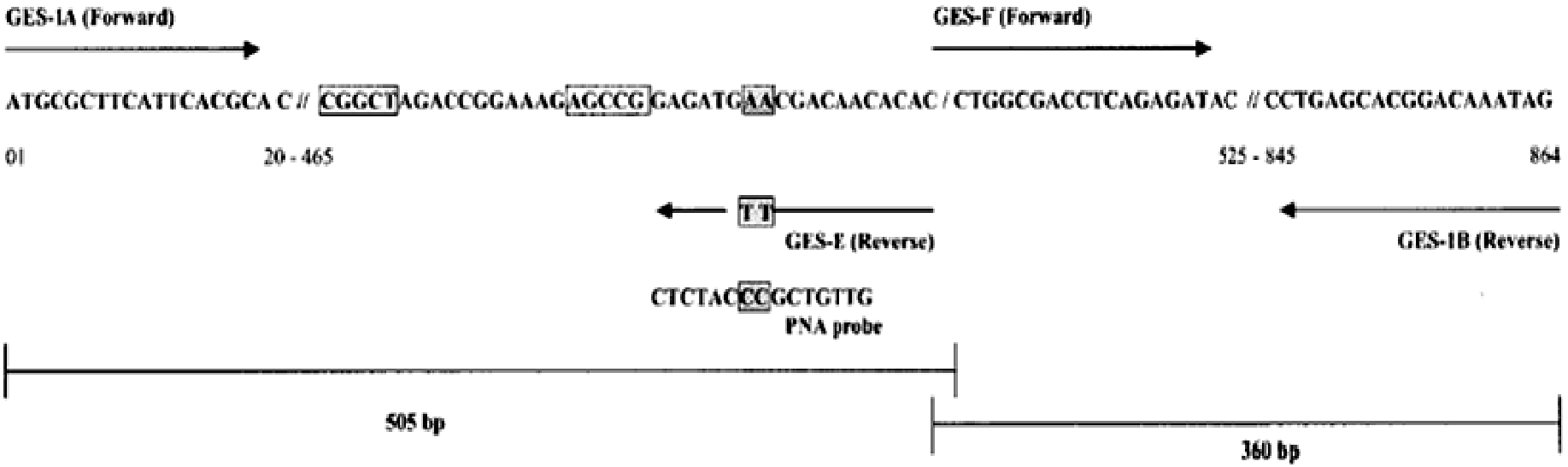

Figure 3.2: Schematic diagram depicting the annealing sites of the amplification primers (GES-1A, GES-1B, GES-E, GES-F) and the PNA-probe relative to the *bla*$_{GES-2}$ gene sequence, (nt 1 – 864). The relevant point mutations were shown in shaded boxes. A stemloop structure between nt 466 - 470 and nt 482 - 486 was depicted in open boxes. Amplification products (360 and 505 bp respectively) were depicted by straight lines. The diagram was depicted in a 5'-3' sequence direction (Weldhagen, 2004)

Chapter 4

Emergence of GES-5 extended-spectrum β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in South Africa

The editorial style of the Journal of Clinical Microbiology was used in this chapter.

4.1 Abstract

Extended-spectrum β-lactamases (ESBLs) have been reported in members of the family *Enterobacteriaceae* since the 1980s, but since 2000 also in *Pseudomonas aeruginosa* (*P. aeruginosa*). The ESBLs detected in *P. aeruginosa* thus far include types of Ambler class A (TEM, SHV, PER and GES) and class D (OXA). To date, nine GES enzymes have been described (GES-1 to GES-9). The first GES-5 ESBL enzyme was described in 2004 from an *Escherichia coli* (*E. coli*) clinical isolate originating from a hospital in Athens, Greece. The aim of this study was to further investigate *P. aeruginosa* isolates found to be bla_{GES-5} positive in a previous GES-type ESBL prevalence study. Repeated sequencing confirmed the bla_{GES} genes found in *P. aeruginosa* isolates P29, P48 and Db24 as bla_{GES-5}. Sequencing of bla_{GES} amplified from *P. aeruginosa* isolate P28 identified both bla_{GES-2} and bla_{GES-5}. Analyses of the determined nucleotide sequences revealed the bla_{GES-5} gene to be preceded by two putative promoter regions previously described as P1 (-35[TGGACA]; -10[TAAAGCT]) and P2 (-35[TTGTTA]; -10[TACAGT]). This study described the first isolation of bla_{GES-5} in South Africa and the second description of bla_{GES-5} in *P. aeruginosa*. The description of GES-5 in three highly related *P. aeruginosa* isolates and one unrelated isolate from two different provinces in South Africa strongly suggested horizontal gene transfer of bla_{GES-5} to *P. aeruginosa* from an unknown bacterial host species. The presence of bla_{GES-5} indicates that the gene may possibly be widespread in South Africa. Beta-lactam antibiotic selective pressure in the South African clinical setting may facilitate the spread of bla_{GES} genes and their establishment in pathogenic bacteria such as *P. aeruginosa* with integrons and mobile genetic elements playing a critical role in the process.

4.2 Introduction

Since the 1980s, extended-spectrum β-lactamases (ESBLs) such as the SHV- and TEM-types have been reported in members of the family Enterobacteriaceae (Wang *et al*., 2006). The presence of ESBLs has been described in *P. aeruginosa* and includes ESBL types of class A (GES, PER, SHV, TEM and VEB) and class D (OXA) (Weldhagen *et al*., 2003; Wang *et al*., 2006). To date nine GES enzymes have been described (GES-1 to GES-9) (Poirel *et al*., 2005b). A nomenclature update has been proposed for the GES-like enzymes, such as GES-3 and GES-4 described by Vourli and colleagues (2004) to be renamed to GES-5 and GES-6 respectively and IBC-1 and IBC-2 renamed as GES-7 and GES-8 respectively (Lee and Jeong, 2005). Among the described GES-type ESBLs, GES-1, GES-2, GES-5, GES-7 and GES-8 have been reported in *P. aeruginosa* (Mavroidi *et al*., 2001; Poirel *et al*., 2001; Castanheira *et al*., 2004; Poirel *et al*., 2005b; Wang *et al*., 2006).

Vourli and colleagues (2004) first described the GES-5 ESBL in 2004. The enzyme was discovered in an *Escherichia coli* (*E. coli*) clinical isolate originating from a hospital in Athens (Vourli *et al*., 2004). The bla_{GES-5} gene (GenBank accession number AY494717) occurred as part of the variable region of a class 1 integron and was preceded by an *accA4* gene cassette identical to that found in the previously described GES-7-encoding integrons, including In*111* (Vourli *et al*., 2004). A serine (Ser) for glycine (Gly) substitution at Ambler's position 170 was observed in GES-5, which is presumably on the omega loop in the enzyme, inducing structural changes that optimise docking of the hydroxyethyl moiety of carbapenems (Vourli *et al*., 2004).

A year later GES-5 was described in a *Klebsiella pneumoniae* (*K. pneumoniae*) isolate from a hospital in Bundang city, Korea (Ryoo *et al*., 2005). The *K. pneumoniae* isolate produced both the GES-5 and a SHV-12 ESBL (Ryoo *et al*., 2005). The bla_{GES-5} described by Ryoo and colleagues (2005) in *K. pneumoniae* differed from the bla_{GES-5} described by Vourli and co-workers (2004) in *E. coli* by only one silent mutation (G→A) at nucleotide position 54 (Jeong *et al*., 2005). The Korean bla_{GES-5} was described as being part of a class 1 integron (Jeong *et al*., 2005).

The GES-5 ESBL has also been described in China (Wang *et al*., 2006). The bla_{GES-5} gene was identified in a *P. aeruginosa* clinical isolate collected in 2004 from blood at the PLA

304th Hospital in Beijing (Wang *et al*., 2006). This was the first description of *bla*$_{GES-5}$ occurring in *P. aeruginosa* (Wang *et al*., 2006). The *bla*$_{GES-5}$ gene (GenBank accession number AY953375) described by Wang and co-workers (2006) contained three silent mutations at nucleotide positions 117 (C→A), 435 (G→C) and 513 (G→T) compared with *bla*$_{GES-5}$ from *E. coli* (GenBank accession number AY494717) (Wang *et al*., 2006). The authors of the Chinese *bla*$_{GES-5}$ did not have any comment on associated integrons with the gene (Wang *et al*., 2006). A *bla*$_{GES-5}$ was reported in a *P. aeruginosa* clinical isolate from France at the 15th European Congress of Clinical Microbiology and Infectious Diseases by Poirel and co-workers (2005a). The enzyme with increased activity towards aztreonam was later described as *bla*$_{GES-9}$, the latest addition to the GES ESBL family (Poirel *et al*., 2005b).

Genomic fingerprinting methods are regarded as accurate methods for the typing of microorganisms and are important epidemiologically for recognising outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the infection, recognising particularly virulent strains of organisms and monitoring vaccination programmes (Speijer *et al*., 1999; Olive and Bean, 1999). Genetic fingerprinting methods include pulse-field gel electrophoresis (PFGE), ribotyping and polymerase chain reaction (PCR)-based techniques, such as random amplified polymorphic DNA (RAPD) typing and restriction fragment length polymorphism (RFLP) analysis (Speijer *et al*., 1999). The RAPD typing PCR-based methodology has been shown to be as discriminatory as pulse-field gel electrophoresis (PFGE) for the typing of *P. aeruginosa* (Mahenthiralingam *et al*., 1996). Mahenthiralingam and co-workers (1996) demonstrated primer 277, primer 275 and primer 272 to be suitable for the amplification of reproducible discriminatory polymorphisms of *P. aeruginosa*. Campbell and co-workers (2000) evaluated primer 272 on 600 samples for RAPD analysis of *P. aeruginosa* and reported RAPD analysis using this primer as being robust, simple and highly reproducible.

The aim of this study was to further investigate the *P. aeruginosa* clinical isolates found to be *bla*$_{GES-5}$ positive in the prevalence study (Chapter 3) through RAPD analysis and DNA sequencing. The study further investigated the association of the *bla*$_{GES-5}$ genes identified with class 1 integrons.

4.3 Materials and Methods

4.3.1 Bacterial strains

Three clinical isolates of *P. aeruginosa* (P29, P48, Db24) were analysed. The isolates were obtained from a study which determined the prevalence of GES-2 and other GES-type ESBLs in *P. aeruginosa* isolates with a sequence-selective PNA-based multiplex PCR (Chapter 3). A fourth *P. aeruginosa* clinical isolate (P28) suspected of possessing a bla_{GES-5} gene was included in the study. The *P. aeruginosa* isolates were collected and identified by two private pathology laboratories in Gauteng, South Africa, during 2004/2005.

4.3.2 Whole-cell DNA extraction

Whole-cell DNA was obtained with a precipitation-based method as previously described (Chapter 3.3) (Philippon *et al*., 1997). Extracted DNA was stored at 4°C and -20°C before analysis.

4.3.3 PCR amplification of bla_{GES} genes

Polymerase chain reaction analysis was performed with the bla_{GES} specific primer set GES-1A (Forward primer; 5'-ATGCGCTTCATTCACGCAC-3') and GES-1B (Reverse primer; 5'-CTATTTGTCCGTGCTCAGG-3') (Poirel *et al*., 2001) using whole-cell DNA extracted from the four *P. aeruginosa* isolates (P28, P29, P48 and Db24) as template. The primers were synthesised and purified by Inqaba Biotechnical Industries Pty. Ltd., South Africa. The reaction consisted of 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI), 0.8 µl of each primer (20 mM), 4 µl whole-cell DNA as template and molecular grade water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The PCR cycle programme previously described by Weldhagen (2004b), for sequence-specific, PNA-based multiplex PCR was used on the GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA). In short the cycle programme consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles each comprising a denaturation step at 95°C for 30 s followed by an annealing step at 50°C for 1 min and an extension step at 72°C for 1 min. The programme was completed with a final extension step performed at 72°C for 5 min. The primers used were described in Table 4.1.

4.3.4 PCR amplification of class 1 integrons

Polymerase chain reaction analysis was performed with a class 1 integron specific primer set Int-E (Forward primer, 5'-AGGATGCGAACCACTTCATC-3') and Int-F (Reverse primer, 5'-CGGTGCCTGAGTCAATTCTT3-') (Weldhagen, 2004a) to investigate the association of the *bla*$_{GES-5}$ genes identified with class 1 integrons. The primers Int-E and Int-F were synthesised and purified by Integrated DNA Technologies Inc., Coralville, Iowa. The amplification conditions were as described above for the amplification of *bla*$_{GES}$ genes. The primers used were described in Table 4.1.

4.3.5 Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA analysis was performed on nine isolates using whole-cell DNA as template to determine the clonal relationship of isolates according to the modified method of Mahenthiralingam and colleagues (1996). The isolates were typed using three different primers to obtain a banding pattern representative of the whole genome. The primers were primer 272 (5'-AGCGGGCCAA-3'), primer 275 (5'-CCGGGCAAGC-3') and primer 277 (5'-AGGAAGGTGC-3') (Mahenthiralingam *et al.*, 1996). The primers were synthesised and purified by Inqaba Biotechnical Industries Pty. Ltd., South Africa. The method was standardised by using GoTaq Green Master Mix (Promega, Madison, WI) for PCR reactions. The reaction mixture consisted of 0.375 µl primer (20 mM), 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI), 4 µl whole-cell DNA as template and molecular grade sterile water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The following cycle programme was utilised and run on a GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA): 4 cycles of denaturation at 94°C for 5 min, annealing at 36°C for 5 min and extension at 72°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 1 min. The programme was completed by a final extension step at 72°C for 10 min.

Banding patterns were obtained by electrophoresis at 80 V (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 3 hrs in a 1.5% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (0.5 µg.ml^{-1}) (Promega, Madison, WI) and run in a 1 X TBE running buffer (pH 8.3) (Promega, Madison, WI). Molecular size standards (100 bp – 10 000 bp) were included on all gels (O'GeneRuler DNA Ladder Mix, Fermentas). The agarose gels were visualized under UV illumination (TFM-26 Ultraviolet Transilluminator, UVP, Upland, CA) and the images captured using the DigiDoc-It imaging system (UVP, Upland, CA). The primers used were described in Table 4.1.

4.3.6 Gel Electrophoresis of PCR products

The PCR products of the *bla*$_{GES}$ specific and class 1 integron specific PCRs were analysed by electrophoresis at 4 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 1 h in a 1% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (0.5 µg.ml^{-1}) in a 1 X TBE running buffer (pH 8.3) (Promega, Madison, WI). The agarose gels were visualized under UV illumination (TFM-26 Ultraviolet Transilluminator, UVP, Upland, CA) and the images captured using the DigiDoc-It imaging system (UVP, Upland, CA). Amplicon size of the *bla*$_{GES}$ specific PCR was verified with a 100 bp DNA ladder (Promega, Madison, WI). Amplicon size of the integron specific PCR was verified with the O'GeneRuler DNA Ladder Mix (100 bp to 10 000 bp) (Fermentas).

4.3.7 DNA sequencing of bla$_{GES}$ genes

Both strands of the *bla*$_{GES}$ specific PCR amplicons were sequenced using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) to determine the *bla*$_{GES}$ identity. Polymerase chain reaction products obtained from the class 1 integron specific PCR for *P. aeruginosa* isolates P29, P48 and Db24 were sequenced using the IntE forward primer to determine the sequence region preceding the *bla*$_{GES}$ genes. Sequencing was performed by Inqaba Biotechnical Industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia).

4.4 Results and Discussion

The *P. aeruginosa* clinical isolates (P28, P29 and P48) were isolated from irrigation fluids collected from a hospital in the Gauteng province, South Africa, in 2004/2005. *Pseudomonas aeruginosa* isolate Db24 was isolated from a urine sample collected in the Limpopo province, South Africa, in 2004/2005.

Polymerase chain reaction analyses with the class 1 integron-specific primers on isolates P29, P48 and Db24 produced amplicons and the sequencing analysis revealed the *bla*$_{GES-5}$ genes to be associated with class 1 integrons. Analyses of the determined nucleotide sequences obtained for *P. aeruginosa* isolates P29 and P48 revealed the *bla*$_{GES-5}$ gene to be preceded by two putative promoter regions described as P1 (-35[TGGACA]; -10[TAAAGCT]) and P2 (-35[TTGTTA]; -10[TACAGT]) (Figure 4.1) by Poirel and co-workers (2000). Analysis of the nucleotide sequence obtained from *P. aeruginosa* isolate Db24 revealed the same putative promoter P1 (Figure 4.2). The second promoter P2, however had a T→C mutation on the last base pair of the -10 promoter sequence.

Random amplified polymorphic DNA analysis of the *bla*$_{GES-5}$ positive isolates revealed all three to be separate strains, with P29 and P48 closely related. Other *P. aeruginosa* isolates (P15, P16, P25, P27 and P40) collected from the same hospital as *P. aeruginosa* isolates P29 and P48 had the same RAPD fingerprint as P29, but were negative for the *bla*$_{GES-5}$ gene. These isolates were however *bla*$_{GES-2}$ positive. *Pseudomonas aeruginosa* isolate P28, obtained from the same hospital, presented the same RAPD fingerprint as P29. Sequence analysis of P28 revealed a *bla*$_{GES-2}$ gene. Repeated sequencing, however, revealed this isolate to possess both *bla*$_{GES-2}$ and *bla*$_{GES-5}$, shown in Figure 4.3. The dendogram depicting the clonal relatedness of isolates mentioned above was given in Figure 4.4.

Sequencing analysis of the amplicons obtained from the *bla*$_{GES}$ specific PCR performed on *P. aeruginosa* isolates P29, P48 and Db24 confirmed the *bla*$_{GES}$ genes to be *bla*$_{GES-5}$ with a 99% identity to the *bla*$_{GES-5}$ gene (GenBank accession number AY494717) published by Vourli and co-workers (2004). Ryoo and colleagues (2005) described a *bla*$_{GES-5}$ in *K. pneumoniae* that differed from the *bla*$_{GES-5}$ described by Vourli and co-workers (2004) in *E. coli* by only one silent mutation (G→A) at nucleotide position 54 (Jeong *et al*., 2005). The

sequencing results indicated that the *bla*$_{GES-5}$ genes identified in this study possessed a G at nucleotide position 54 similar to the Greek *bla*$_{GES-5}$ (Vourli *et al*., 2004).

Figure 4.5 illustrates the world distribution of *bla*$_{GES}$ genes currently identified including the *bla*$_{GES-5}$ genes. The first GES ESBL, *bla*$_{GES-1}$, was identified in a *K. pneumoniae* isolate collected in 1998 in French Guiana (Poirel *et al*., 2000). Following this first identification, GES-1 ESBL was also reported in France (from *P. aeruginosa* isolated in 1999) (Dubois *et al*., 2002), Portugal (from *K. pneumoniae* isolated in 1999) (Correia *et al*., 2003; Duarte *et al*., 2003), South Africa (from *P. aeruginosa* isolated between 1998 and 2001) (Weldhagen, 2004b), Argentina (from *P. aeruginosa* isolated in 2002) (Pasteran *et al*., 2005) and Brazil (from *P. aeruginosa*) (Castanheira *et al*., 2004). The GES-2 ESBL was first identified in South Africa from a *P. aeruginosa* isolate obtained from a blood culture in 2000 (Poirel *et al*., 2001). The *bla*$_{GES-2}$ gene was subsequently reported in a *P. aeruginosa* isolate in Argentina (Pasteran *et al*., 2004). The GES-3 and GES-4 ESBLs were identified in two different *K. pneumoniae* isolates collected in 2002 and have so far only been described in Japan (Wachino *et al*., 2004a; Wachino *et al*., 2004b).

The GES-5 ESBL was first described in an *Escherichia coli* isolate in Greece (Vourli *et al*., 2004). The GES-5 ESBL has subsequently been reported in Korea (from *K. pneumoniae* isolated in 2003) (Ryoo *et al*., 2005), China (from *P. aeruginosa* isolated in 2004) (Wang *et al*., 2006) and now South Africa (from *P. aeruginosa* collected between 2004 and 2005). The GES-6 ESBL was first described in Greece from a *K. pneumoniae* isolated in Athens (Vourli *et al*., 2004). No subsequent reports of GES-6 have been published so far. The GES-7 ESBL, previously named IBC-1, has only been described in *Enterobacter cloacae*, obtained from a hospital in Thessaloniki, Greece in 1999 (Giakkoupi *et al*., 2000). The ESBL GES-8, previously known as IBC-2, was characterised from a *P. aeruginosa* isolate obtained from a hospital in Thessaloniki, Greece in 1998 (Mavroidi *et al*., 2001). No subsequent reports of this ESBL have been published. The most recent addition to the GES ESBL family was GES-9, described in France from a *P. aeruginosa* isolate obtained from a rectal swab in 2004 (Poirel *et al*., 2005b).

4.5 Conclusions

This study described the first isolation of *bla*$_{GES-5}$ in South Africa and is the second description of *bla*$_{GES-5}$ in *P. aeruginosa* (Wang *et al*., 2006). The description of GES-5 in three highly related *P. aeruginosa* isolates and one unrelated isolate from two provinces in South Africa strongly suggested horizontal gene transfer of *bla*$_{GES-5}$ to *P. aeruginosa* from another bacterial host species. The presence of the *bla*$_{GES-5}$ gene in two provinces indicated that the genes may be widespread in South Africa but with a relatively low prevalence (4 GES-5 positive isolates out of 100 isolates).

The β-lactam selective pressure in the South African clinical setting facilitates the spread of *bla*$_{GES}$ genes and their establishment in pathogenic bacteria such as *P. aeruginosa*, with integrons and mobile elements playing a critical role in the process (Giakkoupi *et al*., 2000). The emergence of GES-5 in South Africa suggests that current antibiotic regimens select for ESBLs. Surveillance programmes monitoring the occurrence and prevalence of ESBLs may help in the optimisation of antibiotic administration protocols to prevent antibiotic resistance through emergence of novel resistance genes.

4.6 References

Campbell M, Mahenthiralingam E, Speert D (2000) Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **38:** 4614-4615.

Castanheira M, Mendes RE, Walsh TR, Gales AC, Jones RN (2004) Emergence of the extended-spectrum beta-lactamase GES-1 in a *Pseudomonas aeruginosa* strain from Brazil: Report from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* **48:** 2344-2345.

Correia M, Boavida F, Crosso F, Salgado MJ, Lito LM, Cristino JM, Mendo S, Duarte A (2003) Molecular characterization of a new class 3 integron in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **47:** 2838-2843.

Duarte A, Boavida F, Grosso F, Correia M, Lito LM, Cristino JM, Salgado MJ (2003) Outbreak of GES-1 beta-lactamase-producing multidrug resistant *Klebsiella pneumoniae* in a university hospital in Lisbon, Portugal. *Antimicrob. Agents Chemother.* **47:** 1481-1482.

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of acc(3)-Ib/aac(6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **46:** 638-645.

Giakkoupi P, Tzouvelekis LS, Tsakris A, Loukova V, Sofianou D, Tzelepi E (2000) IBC-1, a novel integron-associated class A beta-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. *Antimicrob. Agents Chemother.* **44:** 2247-2253.

Jeong SH, Hong SG, Song JS, Lee JH, Lee SH (2005) First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. *Antimicrob. Agents Chemother.* **49:** 4809-4810.

Lee SH, Jeong SH (2005) Nomenclature of GES-type extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* **49:** 2148-2150.

Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Spreet DP (1996) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34:** 1129-1135.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:** 1661-1669.

Pasteran F, Faccone D, Campos K, Vazquez M, Rapoport M, Littvik A, Tokumoto M, Galas M (2004) Dissemination of GES extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* (PA) in Argentina, abstr. C2-1327, p. 105. Program Abstr. 44th *Intersci. Conf. Antimicrob. Agents Chemoth.* ASM, Washington, D.C.

Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vázquez M, Procopio A (2005) Novel variant (bla_{VIM-11}) of the metallo-beta-lactamase bla_{VIM} family in a GES-1 extended-spectrum-beta-lactamase-producing *Pseudomonas aeruginosa* clinical isolate in Argentina. *Antimicrob. Agents Chemother.* **49:** 474-475.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005a) GES-5, a novel variant of the extended-spectrum beta-lactamases with increased hydrolysis of aztreonam identified in a *Pseudomonas aeruginosa* clinical isolate in France, abstr. 1133_215. 15th *Europ. Congr. Clin. Microbiol. Infect. Dis.* April 2-5, 2005. European Society of Clinical Microbiology and infectious Diseases, Copenhagen, Denmark.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005b) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Ryoo NH, Kim E, Hong SG, Park YJ, Lee K, Bae IK, Song EH, Jeong SH (2005) Dissemination of SHV-12 and CTX-M-type extended-spectrum beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* and emergence of GES-3 in Korea. *J. Antimicrob. Chemother.* **56:** 698-712.

Speijer H, Savelkoul PHM, Bonten MJ, Stobberingh EE, Tjhie JHT (1999) Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. *J. Clin. Microbiol.* **37:** 3654-3661.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004a) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004b) Molecular characterization of a cephamycin-hydrolyzing and inhibitor resistant class A beta-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. *Antimicrob. Agents Chemother.* **48:** 2905-2910.

Wang C, Cai P, Chang D, Mi Z (2006) A *Pseudomonas aeruginosa* isolate producing the GES-5 extended-spectrum beta-lactamase. *J. Antimicrob. Chemother.* **57:** 1261-1262.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004a) Integrons and beta-lactamases: A novel perspective on resistance. *Int. J. Antimicrob. Agents.* **23:** 556-562.

Weldhagen GF (2004b) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Table 4.1: Characteristics of primers used for RAPD analysis, *bla*$_{GES}$ specific PCR and integron-specific PCR

Primer*	Sequence	T_m (°C)	Function	Reference
RAPD analysis				
272	5'-AGCGGGCCAA-3'	43.6	Typing	Mahenthiralingam *et al.*, 1996
275	5'-CCGGGCAAGC-3'	47.7	Typing	Mahenthiralingam *et al.*, 1996
277	5'-AGGAAGGTGC-3'	39.5	Typing	Mahenthiralingam *et al.*, 1996
***bla*$_{GES}$ specific PCR**				
GES-1A	5'-ATGCGCTTCATTCACGCAC-3'	56.6	Forward primer	Poirel *et al.*, 2001
GES-1B	5'-CTATTTGTCCGTGCTCAGG3'	56.4	Reverse primer	Poirel *et al.*, 2001
Integron specific PCR				
Int-E	5'AGGATGCGAACCACTTCATC3'	55	Forward primer	Weldhagen, 2004a
Int-F	5'CGGTGCCTGAGTCAATTCTT3'	55.2	Reverse primer	Weldhagen, 2004a

* Primers Int-E and Int-F were synthesised and purified by Integrated DNA Technologies Inc., Coralville, Iowa. All other primers were synthesised and purified by Inqaba Biotechnical Industries Pty. Ltd., South Africa

GCGCGAAGATTCTTCTCTGGTTTACTCCTGGAGACCAGCCCAGTACCGTGCAC
AGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGG
AGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTC
GCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC
-35 **P1** **-10**
ACGAACCCAG**TGGACA**TAAGCCTGTTCGGTTCG**TAAGCT**GTAATGCAAGTAG
CGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTA
← *intI1* **-35** **P2** **-10**
ACGGCGCAGTGGCGGTTTTCATGGC**TTGTTA**TGACTGTTTTTTTGGGG**TACAG**
TCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTT
GATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA
Core Site RBS bla_{GES-5} →
AAGTTAGACGGGCGTACAAAGATAATTTCCATCTCAAGGGATCACCATGCGC
TTCATTCACGCACTATTACTGGCAGGGATCGCTCACTCTGCATATGCGTCGGA
AAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACC
GAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCG
CTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTT
CATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTA
GCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTA
GCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGC
AATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGA
AAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTAC
GCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGA
CGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGG
AGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAA
ACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCA
GGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCC
GTAGAACGTGACGAATTAGTTGCCTCTGTCGGTC

Figure 4.1: Schematic representation of the gene sequence derived from isolates P29 and P48. The putative promoters, P1 and P2 were indicated in bold face. The core site for recombination, ribosomal binding site (RBS), bla_{GES-5} start codon and 5' end of *intI1* were indicated

CCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACG
ATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCC
TCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGA
-35 **P1** **-10**
TGAAGGCACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATG
CAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGC
← *intI1* **-35** **P2**
GGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGG
-10
GGTACAGCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTC
GATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCC
Core Site RBS
TAAAACAAAGTTAGACGGGCGTACAAAGATAATTTCCATCTCAAGGGATCAC
bla$_{GES-5}$ →
CATGCGCTTCATTCACGCACTATTACTGGCAGGGATCGCTCACTCTGAATATG
CGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAA
AGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCG
GGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCT
AGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGA
AAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCG
GTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTG
CAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGAC
CTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTA
GACCGGAAAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGAT
ACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGG
CGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAAC
CAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTG
GAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTT
TAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAA
CTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTC

Figure 4.2: Schematic representation of the gene sequence derived from isolate Db24. The putative promoters, P1 and P2 were indicated in bold face. The mutation on the P2 -10 sequence was indicated in bold face. The core site for recombination, ribosomal binding site (RBS), *bla*$_{GES-5}$ start codon and 5' end of *intI1* were indicated

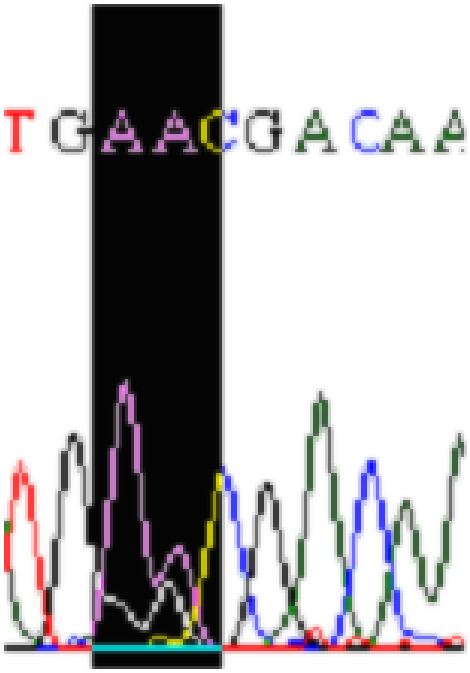

Figure 4.3: Electropherogram depicting the mixed nucleotides G and A at position 493 of the *bla*$_{GES}$ gene obtained from isolate P28

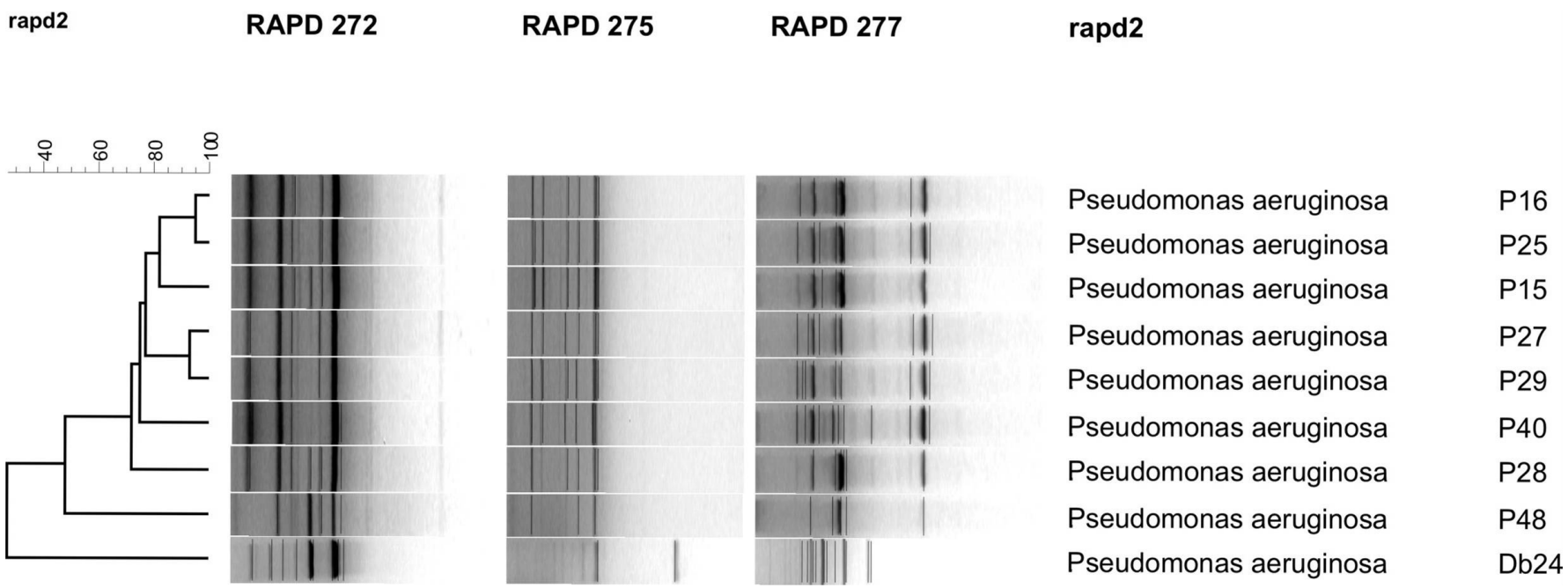

Figure 4.4: Dendogram depicting the clonal relationship between isolates P16, P25, P15, P27, P29, P40, P28, P48 and Db24 after RAPD analysis was conducted with primer 272, 275 and 277

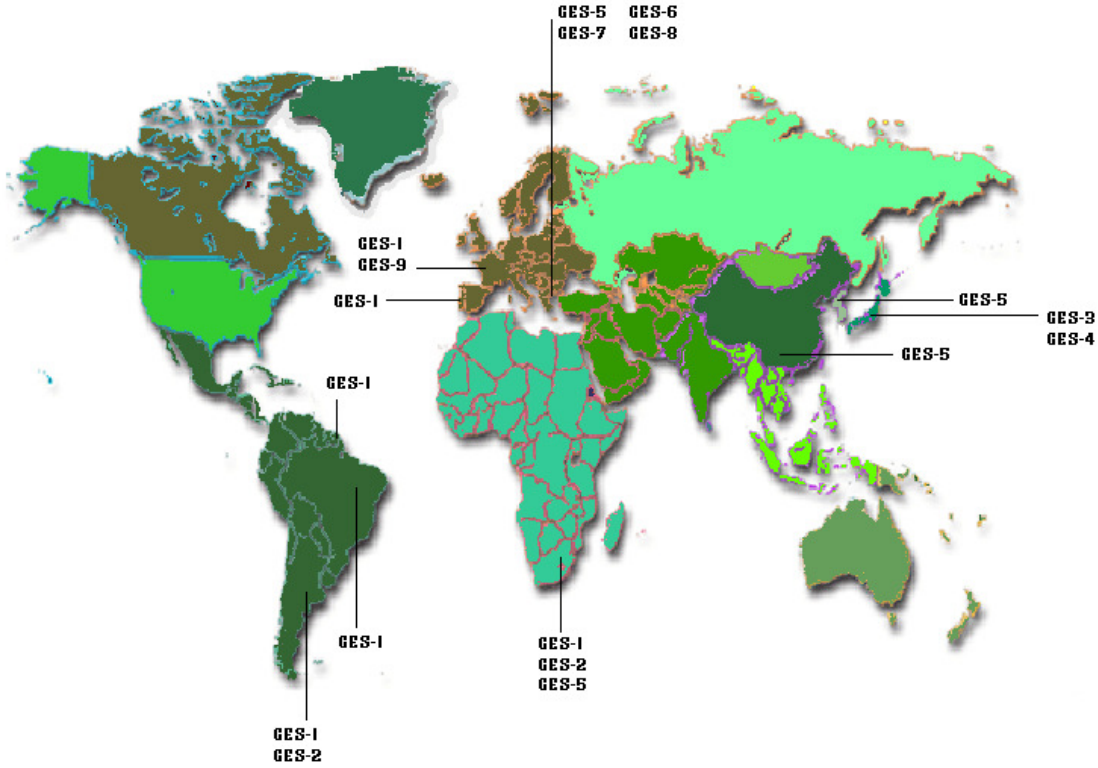

Figure 4.5: Schematic representation of the world distribution of GES ESBLs described between 2000 and 2006

Chapter 5

Genetic location and integron association of *bla*$_{GES-2}$ and *bla*$_{GES-5}$ extended-spectrum β-lactamase genes in *Pseudomonas aeruginosa* isolates collected in the Gauteng region of South Africa

The editorial style of the Journal of Clinical Microbiology was used in this chapter.

5.1 Abstract

Extended-spectrum β-lactamases (ESBLs) are considered one of the most important resistance mechanisms against penicillin and cephalosporins. The genes encoding these enzymes are often carried by multidrug-resistance plasmids and are capable of horizontal transfer among different species. Guiana extended-spectrum-type ESBL enzymes have mainly been found in *Pseudomonas aeruginosa* (*P. aeruginosa*) and only at a limited number of geographical sites, mainly France, Greece and South Africa. The aim of this study was to investigate whether *P. aeruginosa* carries the genes *bla*$_{GES-2}$ and *bla*$_{GES-5}$ on plasmids or on the chromosome. Results indicated that in 26 of a total of 28 GES-2-producing isolates, *bla*$_{GES-2}$ was found on both a plasmid and chromosomal location. In two isolates *bla*$_{GES-2}$ was located on plasmids only; there was no *bla*$_{GES-2}$ genes located on the chromosome exclusively. Results further indicated that all *bla*$_{GES-5}$ genes identified in this study were located on plasmids with none occurring on the chromosome. Both *bla*$_{GES-2}$ and *bla*$_{GES-5}$ are known to be associated with class 1 integrons, which could explain the presence of *bla*$_{GES-2}$ genes on both plasmids and chromosomes. The presence of the *bla*$_{GES-2}$ gene on both plasmid and chromosomal DNA may possibly be the result of integron association with transposons. The maintenance of *bla*$_{GES-2}$ on both the chromosome and plasmids in the same *P. aeruginosa* isolate indicated a possible selective pressure for the *bla*$_{GES-2}$ gene in the South African clinical setting.

5.2 Introduction

Pseudomonas aeruginosa, an aerobic Gram-negative rod, is ubiquitous in the environment (Poole, 2004). In the hospital setting this nosocomial pathogen affects severely ill patients and causes a wide spectrum of infections from skin and wound infections to septicaemia (Navon-Venezia *et al*., 2005). With broad substrate specificity and the ability to hydrolyse many extended-spectrum third-generation cephalosporins, the extended-spectrum β-lactamases (ESBLs) constitute a major problem in the use of β-lactam antibiotics to treat infections (Poole, 2004). Extended-spectrum β-lactamases occur frequently in clinical strains of enterobacteria but are less common in *P. aeruginosa* (Mavroidi *et al*., 2001). Ambler class A ESBLs currently identified in *P. aeruginosa* include the GES-, LBT-, PER-, SHV-, TEM- and VEB-types (Weldhagen *et al*., 2003).

Antimicrobial resistance in *P. aeruginosa* is a consequence of various mechanisms, from intrinsic resistance to gene acquisition by horizontal transfer, mediated by genetic mobile elements (Fonseca *et al*., 2005). Plasmids, transposons and integrons are vehicles and structures for the mobilisation, acquisition and spread of antibiotic resistance genes (e.g. bla_{GES}) (Fonseca *et al*., 2005). Integrons have been described as naturally occurring gene expression elements by which bacteria can acquire resistance genes (Carattoli, 2001). Integrons have been divided into four classes based on the homology of the integrase proteins (40-60% amino acid identity) (Carattoli, 2001).

Class 1 integrons represent the most common structure observed in clinical isolates (Carattoli, 2001). Elements belonging to class 1 are characterised by the presence of two conserved segments, the 5'-conserved segment (5'-CS) and the 3'-conserved segment (3'-CS) (Carattoli, 2001). The 5'-CS contains the promoter, the *intI* gene (integrase) and the *attI* site, while the 3'-CS codes for *sul1* (confers resistance to sulphonamides), *qacEΔ1* (confers resistance to quaternary ammonium compounds) and ORF5 (encodes a protein of unknown function) (Carattoli, 2001). Antibiotic resistance gene cassettes are integrated between the 5'-CS and 3'-CS at the *attI1* site (Carattoli, 2001).

A variety of transposable elements have been identified in bacterial pathogens that contribute to the bacterial genome by excision and insertion of DNA fragments from a donor site to other non-homologous target sites (Carattoli, 2001). Transposons are mobile genetic elements encoding the necessary machinery to promote self-translocation (Carattoli, 2001). Transposons are known to contribute to integron mobility, such as the Tn*21*-transposable element that often carries class 1 integrons (Carattoli, 2001). The association of a highly efficient gene-capture and expression system such as integrons, together with the capacity for vertical and horizontal mobility provided by transposons, represents an important combination in the natural flux of resistance genes (Carattoli, 2001). Furthermore, integrons borne on transposons have been described as being preferentially incorporated into conjugative plasmids, which guarantees a broad-host range horizontal transfer of the resistance determinants carried on the integron (Carattoli, 2001).

According to the literature, *bla*$_{SHV}$ and *bla*$_{TEM}$ genes encoding for SHV- and TEM-type enzymes, are typically located on plasmids in the *Enterobacteriaceae* (Livermore, 1995). The spread of plasmids carrying these genes may be limited by species-related plasmid replication (Weldhagen *et al*., 2003). However, a plasmid location of genes encoding ESBLs of the SHV and TEM series has been reported in *P. aeruginosa* and simultaneously in enterobacterial species from the same patients (Mugnier *et al*., 1996; Marchandin *et al*., 2000; Chanawong *et al*., 2001). The *bla*$_{PER}$- and *bla*$_{VEB}$-like genes have been reported as being mostly plasmid-encoded in the *Enterobacteriaceae*, while these enzymes are mostly chromosome-encoded in *P. aeruginosa* isolates (Vahaboglu *et al*., 1996; Vahaboglu *et al*., 1997; Girlich *et al*., 2001; Vahaboglu *et al*., 2001). The *bla*$_{GES-2}$ gene was reported to be part of a class 1 integron located on a self-transferable 100 kb plasmid (Poirel *et al*., 2001). The first *bla*$_{GES-5}$ gene described in Greece from a *Escherichia coli* (*E. coli*) clinical isolate, was reported to be part of a class 1 integron located on a 13 kb plasmid (Vourli *et al*., 2004). A subsequent report of *bla*$_{GES-5}$ in Korea described the gene as being part of a class 1 integron located on a >70 kb plasmid (Jeong *et al*., 2005). No data have been published on the genetic location of a LBT-type EBSL found in *P. aeruginosa* (Rejiba *et al*., 2002).

The dissemination of β-lactamases among *Enterobacteriaceae* may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections (e.g. septicaemia) caused by ESBL-producing *P. aeruginosa* (Weldhagen *et al.*, 2003). The specific genetic locations of *bla*$_{GES-2}$ and *bla*$_{GES-}$

$_5$ ESBL genes may shed some light on the possible transmission of these genes and their genetic stability in the selected *P. aeruginosa* clinical isolates. In this study the genetic location of the *bla*$_{GES-2}$ and *bla*$_{GES-5}$ ESBL genes were investigated. A sequence-selective, competitive peptide nucleic acid (PNA)-based multiplex PCR method (Weldhagen, 2004) was selected to detect and differentiate *bla*$_{GES-2}$ and *bla*$_{GES-5}$ genes on *P. aeruginosa* chromosomal and plasmid DNA.

5.3 Materials and Methods

5.3.1 Bacterial Strains

Thirty-one clinical isolates of *P. aeruginosa* were included in this study. These isolates were obtained from a previous study (Chapter 3) that investigated the prevalence of GES-2 and GES-type ESBLs in 100 clinical isolates of *P. aeruginosa*. All thirty-one isolates tested positive for either *bla*$_{GES-2}$ or *bla*$_{GES}$ with a competitive PNA-based multiplex PCR in the prevalence study conducted on whole-cell DNA (Chapter 3). The *P. aeruginosa* isolates were collected between 2004 and 2005 in the Gauteng province of South Africa.

5.3.2. Plasmid DNA extraction

Extraction of plasmid DNA was performed as previously described (Crosa *et al.,* 1994). Briefly, a single colony of *P. aeruginosa* pure culture was resuspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated (WBM SPL 25 Labcon shaking water bath, Laboratory Marketing Services, Maraisburg) overnight at 37°C while shaking at 50 rpm. One ml of the overnight culture was pelleted in a centrifuge (Labofuge 400R, Heraeus instruments, Germany) at 3 000 × g for 10 min at 4°C. The pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI) containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4 and pelleted as above. The resulting pellet was resuspended in 40 µl TE buffer (Promega, Madison, WI) (pH 7.4). Cells were lysed by adding 600 µl of a 4% SDS solution (Promega, Madison, WI) at pH 12.45 with incubation (QBT2 heating block, Grant Instruments Ltd., Cambridge, United Kingdom) at 37°C for 30 min. The lysed cell mixture was neutralised by adding 30 µl of 2 M Tris (Sigma Chemical Co., St. Louis, MO) at pH 7.0. After neutralisation, chromosomal DNA and protein was precipitated by adding 240 µl of a 5 M NaCl (Promega, Madison, WI) solution and incubated on ice (0°C) for 4 h. After incubation the mixture was centrifuged (Z233 M-2, Hermle Labortechnik, Wehingen,

Germany) at 5 000 × g for 10 min at room temperature (25°C). The supernatant containing plasmid DNA was transferred to a new tube. The DNA was precipitated with 700 µl isopropanol (Merck, Darmstadt, Germany) at -20°C for 1 h or overnight. The precipitated DNA was pelleted by centrifugation (Z233 M-2, Hermle Labortechnik, Wehingen, Germany) at 5 000 × g for 10 min at room temperature (25°C) followed by two washing steps (addition of 800 µl 70% ethanol followed by centrifugation (Z233 M-2, Hermle Labortechnik, Wehingen, Germany) at 5 000 × g for 5 min at room temperature (25°C) with 800 µl of a 70% ethanol solution (Merck, Darmstadt, Germany). After air drying the DNA pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI) containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4. Five hundred µl of the extracted DNA was stored at 4°C and -20°C respectively, until further analysis.

5.3.3 Whole-cell DNA extraction

Extraction of whole-cell DNA was performed by a precipitation-based method in combination with a repeated final washing step with 70% ethanol to improve template quality by desalting as described previously (Chapter 3.3) (Philippon *et al*., 1997). Extracted DNA was resuspended in TE buffer (pH 7.4) (Promega, Madison, WI) and stored in 500 µl aliquots at -20°C and -70°C.

5.3.4 Chromosomal DNA extraction

Extraction of chromosomal DNA from *P. aeruginosa* was accomplished by spooling the chromosomal DNA onto a class rod from extracted whole-cell DNA (Clabots *et al*., 1993). The whole-cell DNA extraction was performed as described above. Spooled chromosomal DNA was resuspended in 200 µl TE buffer (pH 7.4) (Promega, Madison, WI) and stored in 100 µl aliquots at - 20°C and -70°C until further analysis.

5.3.5 Competitive PNA-based multiplex PCR

Peptide nucleic acid-based multiplex PCR amplification and detection of amplicons was performed as described in Chapter 3. The PNA-based multiplex PCR amplification was conducted on a GeneAmp PCR thermocycler system 9600 (Perkin Elmer Cetus, Emeryville, CA). Primer and probe characteristics were given in Table 5.1.

5.3.6 Class 1 integron specific PCR

A primer pair annealing to both the *IntI* (Int-E, Forward primer, 5'-AGGATGCGA ACCACTTCATC-3') and bla_{GES} (Int-F, Reverse primer, 5'-CGGTGCCTGAGT CAATTCTT-3') genes of class 1 integrons were used to investigate the association of bla_{GES} genes identified in this study with class 1 integrons (Weldhagen, 2006). The primers Int-E and Int-F were synthesised and purified by Integrated DNA Technologies Inc., Coralville, Iowa. The class 1 integron specific PCR reaction mix consisted of 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI), 0.1 µM of each primer (20 µM), 2 µl plasmid DNA as template and water to a final volume of 25 µl. Plasmid DNA extracted from *P. aeruginosa* GW-1 (Poirel *et al.*, 2001) was used as positive control. Water served as negative control. The PCR cycling conditions were as described in Chapter 3 (section 3.3.3). The amplicons were analysed with gel electrophoreses as described in section 5.3.7.

5.3.7 Verification of DNA extractions and PCR products

Deoxyribonucleic acid (DNA) extractions and class 1 integron specific PCR products were visualized by electrophoresis in 1% agarose gels (Pronadisa, Madrid, Spain) containing 0.5 $\mu l.ml^{-1}$ ethidium bromide (Promega, Madison, WI) as previously described (Chapter 3.3.5). Gel images were captured with the DigiDoc-It imaging system (UVP, Upland, CA). The amplicon size was verified with O'GeneRuler DNA Ladder Mix (100 bp to 10 000 bp) (Fermentas).

5.4 Results and Discussion

Sequence-selective, competitive PNA-based multiplex PCR was performed on both chromosomal and plasmid DNA obtained from 31 GES positive *P. aeruginosa* isolates to determine the genetic location of bla_{GES} genes. These isolates were found to be GES positive during a previously conducted GES-2 and GES-type prevalence study that performed the sequence-selective competitive PNA-based multiplex PCR on whole-cell DNA (see Chapter 3). Although the PNA-based multiplex PCR may only be able to divide bla_{GES} genes into two groups as discussed in chapter 3, the method could be used in this study to differentiate bla_{GES-2} and bla_{GES-5} since the bla_{GES} genes present in the isolates studied, have previously been identified through DNA sequencing. The PNA-based multiplex PCR method was used to identify the bla_{GES} genes in either plasmid or chromosomal DNA.

The results of the PNA-based multiplex PCR performed on both plasmid and chromosomal DNA were tabulated in Table 5.2. Gel electrophoresis results depicting the PNA-based multiplex PCR amplification products were given in Figure 5.1. Among the *bla*$_{GES-2}$ positive isolates, 26 of a total of 28 studied isolates carried the gene on both a chromosomal and plasmid DNA location. In the remaining two isolates, *bla*$_{GES-2}$ was found to occur on a plasmid only. There were no isolates identified that carried a *bla*$_{GES}$ gene exclusively on the chromosome. These results indicated that the *bla*$_{GES-2}$ gene was located on the chromosome and a plasmid in the majority of *P. aeruginosa* isolates tested (26/28). The *bla*$_{GES-2}$ gene only remained plasmid-borne in 2 of the *P. aeruginosa* isolates tested. All PCRs conducted on chromosomal DNA for *bla*$_{GES-5}$ positive isolates were negative. The three *P. aeruginosa* isolates identified to possess the *bla*$_{GES-5}$ gene, carried the gene exclusively on a plasmid location. The class 1 integron-specific PCR revealed all but two isolates (Db27, Db57) to be associated with class 1 integrons. A gel electrophoresis result obtained for the class 1 integron-specific PCR was given in Figure 5.2. The two *bla*$_{GES-2}$ genes not associated with class 1 integrons were present on both plasmid and chromosomal DNA (Db27, Db57).

According to the literature, *bla*$_{TEM}$ and *bla*$_{SHV}$ genes are typically located on plasmids in *Enterobacteriaceae* (Livermore, 1995). A plasmid location of genes encoding ESBLs of the TEM and SHV series have been reported in *P. aeruginosa* and simultaneously in enterobacterial species from the same patients (Mugnier *et al*., 1996; Marchandin *et al*., 2000; Chanawong *et al*., 2001). Although *bla*$_{VEB}$- and *bla*$_{PER}$-like genes have been reported as being mostly plasmid encoded in *Enterobacteriaceae*, these genes seemed to be mostly located on the chromosome in *P. aeruginosa* (Vahaboglu *et al*., 1996; Vahaboglu *et al*., 1997; Girlich *et al*., 2001; Vahaboglu *et al*., 2001). These reports hint at a tendency of *P. aeruginosa* to carry β-lactamase genes on the chromosome. This tendency was reflected in this study with 26 of 31 GES-type ESBL positive *P. aeruginosa* isolates that carried the β-lactamase gene on the chromosome.

The results obtained from this study were in agreement with literature reporting *bla*$_{GES}$ genes to be either plasmid or chromosome encoded in *P. aeruginosa* (Weldhagen *et al*., 2003). The *bla*$_{GES-2}$ gene identified in South Africa from *P. aeruginosa* GW-1 was reported to be part of a 100 kb plasmid containing a class 1 integron (Poirel *et al*., 2001). Subsequently, a nosocomial outbreak of GES-2 positive *P. aeruginosa* was described in South Africa (Poirel *et al*., 2002). The identified *P. aeruginosa* isolates harboured a 150 kb conjugative plasmid

containing a class 1 integron (Poirel *et al*., 2002). The plasmid location of *bla*$_{GES-5}$ found in three isolates of *P. aeruginosa* in this study may be the result of a gene being carried on a self-transferable or conjugative plasmid. The same conclusion may be applicable to the *bla*$_{GES-2}$ gene found to be plasmid-borne in two *P. aeruginosa* isolates. This study is the first to document the *bla*$_{GES-2}$ gene on a bacterial chromosome and the first to desribe this gene on both the chromosome and plasmid in the same *P. aeruginosa* isolate. Further studies may be necessary to examine the nature of *bla*$_{GES}$-carrying plasmids in *P. aeruginosa*.

Besides a plasmid location, antibiotic resistance genes have been identified as gene cassettes and as part of class 1 integrons in *P. aeruginosa* (Weldhagen *et al*., 2003). Although the genes encoding β-lactamases of Ambler class B (metallo-enzymes) and Ambler class D (oxacillinases) are usually located in class 1 integrons, the class A ESBLs are not typically associated with these genetic determinants (Weldhagen., 2004). The VEB-, GES-, CTX-M- and PSE-types are the only class A ESBLs with a known class 1 integron association (Weldhagen, 2004). Amongst the GES ESBLs, *bla*$_{GES-1}$ has been found to be part of a class 3 integron in a Portuguese isolate of *K. pneumoniae* (Correia *et al*., 2003). Integrons play a major role in the spread of antibiotic resistance due to their ability to integrate gene cassettes and their typical location on mobile genetic elements such as plasmids and transposons (Dubois *et al*., 2002). The association of integrons with transposons may provide an additional means of mobility to antibiotic resistance genes (Dubois *et al*., 2002).

The chromosomal and plasmid location of *bla*$_{GES-2}$ genes observed in 26 *P. aeruginosa* isolates may be a result of a class 1 integron associating with a transposon, facilitating the spread of the gene between plasmids and the bacterial chromosome. The maintenance of the *bla*$_{GES-2}$ gene on both the chromosome and plasmids and thus an increased gene copy number, may indicate selective pressure in the South African clinical setting. *Pseudomonas aeruginosa* isolates with a high degree of GES-2 β-lactamase production may experience a selective advantage in environments where imipenem is used extensively. The high occurrence of *bla*$_{GES-2}$ compared to *bla*$_{GES-5}$ may further indicate the need and adaptive ability of the bacterium to survive imipenem treatments. The *bla*$_{GES-5}$ genes identified in this study were all plasmid-borne which is in agreement with the literature that has reported *bla*$_{GES-5}$ to occur on plasmids in *E. coli* and *K. pneumoniae* (Vourli *et al*., 2004; Jeong *et al*., 2005).

5.5 Conclusion

Given that *bla*$_{GES}$ genes are integron located, it is possible that their presence in *P. aeruginosa* may result from horizontal transfer from Gram-negative aerobes, known to be sources of integrons, which may be present in the same ecological/nosocomial niche (Weldhagen *et al*., 2003). The majority of *bla*$_{GES-2}$ genes identified in this study were located on both the bacterial chromosome and plasmids. The presence of the *bla*$_{GES-2}$ gene on both plasmid and chromosomal DNA may be a result of integron association with transposons facilitating the spread of *bla*$_{GES-2}$ genes (Dubois *et al*., 2002). The maintenance of *bla*$_{GES-2}$ on both the chromosome and plasmids in the same bacterial isolate indicate a selective pressure for this gene in the South African clinical setting. Thus caution should be exercised when treating *P. aeruginosa* infections with imipenem in South African hospitals.

5.6 References

Carattoli A (2001) Importance of integrons in the diffusion of resistance. *Vet. Res*. **32:** 243-259.

Chanawong A, M'Zali FH, Heritage J, Lulitanond A, Hawkey PM (2001) SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum beta-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. *J. Antimicrob. Chemother.* **48:** 839-852.

Clabots CR, Johnson S, Bettin KM, Mathie PA, Mulligan ME, Schaberg DR, Peterson LR, Gerding DN (1993) Development of a rapid and efficient endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. *J. Clin. Microbiol.* **31:** 1870-1875.

Correia M, Boavida F, Crosso F, Salgado MJ, Lito LM, Cristino JM, Mendo S, Duarte A (2003) Molecular characterization of a new class 3 integron in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **47:** 2838-2843.

Crosa JH, Tolmasky ME, Actis LA, Falkow S (1994) *Plasmids*, p. 365-386. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, N. R. Kreig (ed.), *Methods for general and molecular bacteriology*. ASM Press, Washington, D.C.

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of acc(3)-Ib/aac(6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **46:** 638-645.

Fonseca EL, Vieira VV, Cipriano R, Vicente ACP (2005) Class 1 integrons in *Pseudomonas aeruginosa* isolates from clinical settings in Amazon region, Brazil. *FEMS Immunol. Med. Microbiol.* **44:** 303-309.

Girlich D, Poirel L, Leelaporn A, Karim A, Tribuddharat C, Fennewald M, Nordmann P (2001) Molecular epidemiology of the integron-located VEB-1 extended-spectrum beta-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. *J. Clin. Microbiol.* **39:** 175-182.

Jeong SH, Hong SG, Song JS, Lee JH, Lee SH (2005) First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. *Antimicrob. Agents Chemother.* **49:** 4809-4810.

Livermore DM (1995) Beta-lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev*. **8:** 557-584.

Marchandin H, Jean-Pierre H, De Champs C, Sirot D, Darbas H, Perigauly PF, Carriére C (2000) Production of a TEM-24 plasmid-mediated extended-spectrum beta-lactamase by a clinical isolate of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44:** 213-216.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Mugnier P, Dubrous P, Casin I, Arlet G, Collatz E (1996) A TEM-derived extended-spectrum beta-lactamase in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **40:** 2488-2493.

Navon-Venezia S, Ben-Ami R, Carmeli Y (2005) Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Curr. Opin. Infect. Dis.* **18:** 306-313.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Weldhagen GF, De Champs C, Nordmann P (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J. Antimicrob. Chemother.* **49:** 561-565.

Poole K (2004) Resistance to beta-lactam antibiotics. *Cell Mol. Life Sci.* **61:** 2200-2223.

Rejiba S, Limam F, Belhadj C, Belhadj O, Ben-Mahrez K (2002) Biochemical characterization of a novel extended-spectrum beta-lactamase from *Pseudomonas aeruginosa* 802. *Microb. Drug Resist.* **8:** 9-13.

Vahaboglu H, Dodanli S, Eroglu C, Öztürk R, Soyletir G, Yildirim I, Akan V (1996) Characterization of multiple-antibiotic resistant *Salmonella typhimurium* strains: Molecular epidemiology of PER-1-producing isolates and evidence for nosocomial plasmid exchange by a clone. *J. Clin. Microbiol.* **34:** 2942-2946.

Vahaboglu H, Öztürk R, Aygun G, Coskunkan F, Yaman A, Kaygusuz A, Leblebicioglu H, Balik I, Aydin K, Otkun M (1997) Widespread detection of PER-1-type extended-spectrum beta-lactamases among nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* isolates in Turkey: a nationwide multicenter study. *Antimicrob. Agents Chemother.* **41:** 2265-2269.

Vahaboglu H, Coskunkan F, Tansel O, Öztürk R, Sahin N, Koksal I, Kocazeybek B, Tatman-Ötkun M, Leblebicioglu H, Ozinel MA, Akalin H, Kocagoz S, Korten V (2001) Clinical importance of extended-spectrum beta-lactamase (PER-1-type) producing *Acinetobacter* spp. and *Pseudomonas aeruginosa* strains. *J. Med. Microbiol.* **50:** 642-645.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Weldhagen GF (2006) Genetic stability of class 1 integron-borne *bla*$_{GES}$-type genes under short-term *in vitro* antibiotic stress. *Int. J. Antimicrob. Agents*, **28**:477-483.

Table 5.1: Peptide Nucleic acid-based multiplex PCR primer and probe characteristics

Primer or Probe	Sequence 5'→3'	T_m (°C)	Position (nt) on *bla*$_{GES}$	Product size	Reference
GES-1A Forward primer	ATGCGCTTCATTC ACGCAC	56.6	01-19	505 bp	Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
GES-E Reverse primer complementary to *bla*$_{GES-2}$	GTGTGTTGTCGTT CATCTC	51.8	487-505		Weldhagen, 2004
GES-F Forward primer	CCTGGCGACCTC AGAGATAC	57.4	505-524	360 bp	Weldhagen, 2004
GES-1B Reverse primer	CTATTTGTCCGTG CTCAGG	56.4	846-864		Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
PNA-probe Complimentary to *bla*$_{GES-1}$ and *bla*$_{IBC}$	GTTGTCGCCCATC TC	70	487-501	na*	Weldhagen, 2004

* not applicable

Table 5.2: PNA-based multiplex PCR results for both plasmid and chromosomal DNA templates

Pseudomonas aeruginosa Isolate	ESBL gene identified	Genetic location
Db1	bla_{GES-2}	Chromosomal & Plasmid
Db2	bla_{GES-2}	Chromosomal & Plasmid
Db5	bla_{GES-2}	Chromosomal & Plasmid
Db6	bla_{GES-2}	Chromosomal & Plasmid
Db8	bla_{GES-2}	Chromosomal & Plasmid
Db10	bla_{GES-2}	Chromosomal & Plasmid
Db17	bla_{GES-2}	Plasmid
Db18	bla_{GES-2}	Chromosomal & Plasmid
Db20	bla_{GES-2}	Chromosomal & Plasmid
Db24	bla_{GES-5}	Plasmid
Db25	bla_{GES-2}	Chromosomal & Plasmid
Db26	bla_{GES-2}	Chromosomal & Plasmid
Db27	bla_{GES-2}	Chromosomal & Plasmid
Db31	bla_{GES-2}	Chromosomal & Plasmid
Db32	bla_{GES-2}	Plasmid
Db33	bla_{GES-2}	Chromosomal & Plasmid
Db34	bla_{GES-2}	Chromosomal & Plasmid
Db35	bla_{GES-2}	Chromosomal & Plasmid
Db36	bla_{GES-2}	Chromosomal & Plasmid
Db40	bla_{GES-2}	Chromosomal & Plasmid
Db41	bla_{GES-2}	Chromosomal & Plasmid
Db42	bla_{GES-2}	Chromosomal & Plasmid
Db48	bla_{GES-2}	Chromosomal & Plasmid
Db50	bla_{GES-2}	Chromosomal & Plasmid
Db51	bla_{GES-2}	Chromosomal & Plasmid
Db52	bla_{GES-2}	Chromosomal & Plasmid
Db57	bla_{GES-2}	Chromosomal & Plasmid
Db59	bla_{GES-2}	Chromosomal & Plasmid
P23	bla_{GES-2}	Chromosomal & Plasmid
P29	bla_{GES-5}	Plasmid
P48	bla_{GES-5}	Plasmid

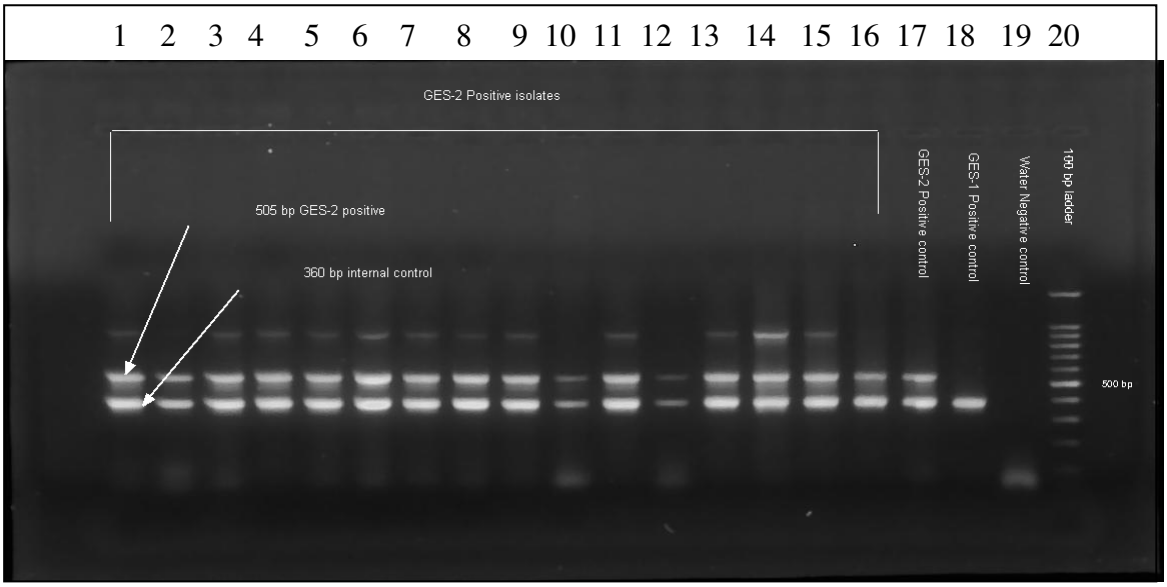

Figure 5.1: Gel electrophoresis depicting competitive PNA-based multiplex PCR amplification products for isolates Db1, Db2, Db5, Db6, Db8, Db10, Db18, and Db25. Lane 1-8: Chromosomal DNA as template. Lane 9-16: Plasmid DNA as template. Lane 17: GES-2 positive control (505- & 360 bp). Lane 18: GES-1 positive control (360 bp). Lane 19: Water negative control. Lane 20: 100 bp DNA ladder

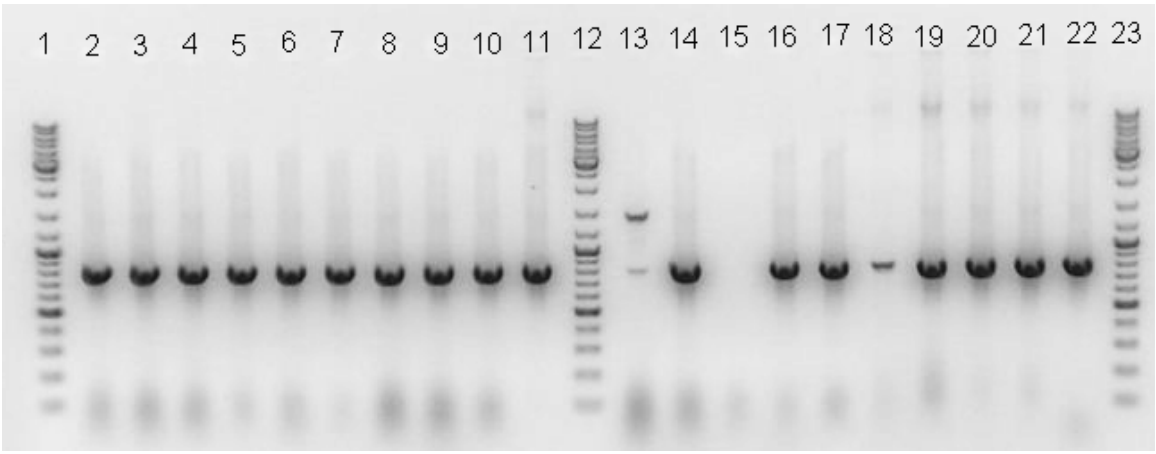

Figure 5.2: Gel electrophoresis depicting class 1 integron specific PCR amplification products. Lane 1: Marker. Lane 2: Db1, Lane 3: Db2. Lane 4: Db5. Lane 5: Db6. Lane 6: Db8. Lane 7: Db10. Lane 8: Db17. Lane 9: Db18. Lane 10: Db20. Lane 11: Db24. Lane 12: Marker. Lane 13: Db25. Lane 14: Db26. Lane 15: Db27. Lane 16: Db31. Lane 17: Db32. Lane 18: Db33. Lane 19: Db34. Lane 20: Db35. Lane 21: Db36. Lane 22: Db40. Lane 23: DNA ladder (100 bp to 10 000 bp)

Chapter 6

Expansion of a sequence-selective, competitive, PNA-based multiplex conventional PCR into real-time utilising SYBR Green for the detection of *bla*$_{GES}$ in *Pseudomonas aeruginosa*

The editorial style of the Journal of Clinical Microbiology was used in this chapter.

6.1 Abstract

Pseudomonas aeruginosa is of clinical importance due to innate multidrug resistance and other drug resistance determinants such as extended-spectrum β-lactamases (ESBLs). The detection of ESBLs is difficult and may often lead to false reporting of *P. aeruginosa* as being susceptible to certain antipseudomonal penicillin or cephalosporins resulting in inappropriate antimicrobial therapy. The GES class A ESBL family characteristically hydrolyse ceftazidime with higher efficiency than other oxyimino-β-lactams and currently consist of nine enzymes named GES-1 to GES-9. A competitive PNA-based multiplex PCR method was recently developed for the detection and differentiation of *bla*$_{GES-1}$ and *bla*$_{GES-2}$. The description of *bla*$_{GES}$ genes with mutations in and outside the PNA-probe target site has made this method unreliable. This study aimed to apply a PNA-based multiplex PCR assay in real-time PCR utilising SYBR Green. The use of SYBR Green made melting curve analysis possible and allowed single base pair mutations to be detected. Such a technique combined the cost effectiveness of conventional PCR with the sensitivity and speed of real-time PCR. However, the competitive PNA-based multiplex PCR method in real-time was unsuccessful. The melting temperatures obtained showed little variation between GES variants with melting temperature overlapping for different GES variants. Future research should be focused towards methods such as simple probe technology and pyrosequencing to develop a cost effective, rapid and sensitive assay for detection of ESBLs in bacteria such as *P. aeruginosa* in which sensitivity testing is unreliable.

6.2 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, seldom causing disease in healthy individuals. Infections caused by this bacterium are usually encountered in hospitalised patients (Poole, 2004). Such infections are a serious problem especially in cancer and cystic fibrosis patients as well as burn victims, with a case fatality rate of 50% (Poole, 2004). *Pseudomonas aeruginosa* is of clinical importance due to innate multidrug resistance and an ability to acquire high-level multidrug resistance (Schweizer, 2003). The inherent multidrug resistance is a result of an inducible chromosome-encoded Amp-C β-lactamase conferring resistance to those β-lactams that induce this enzyme and are hydrolysed by the Amp-C enzyme (e.g. cephalothin and ampicillin) (Livermore, 2002). Efflux systems such as MexAB-OprM furthermore contributed to *P. aeruginosa*'s inherent antibiotic resistance by removing agents such as β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides and tetracycline (Livermore, 2002).

In addition to the increased expression of AmpC β-lactamases and efflux systems, extended-spectrum β-lactamases (ESBLs) confer resistance to expanded-spectrum cephalosporins in *P. aeruginosa* (Navon-Venezia *et al*., 2005). The presence of ESBLs in *P. aeruginosa* may have important clinical implications since these enzymes confer resistance to all penicillin and cephalosporins but are difficult to detect phenotypically (Navon-Venezia *et al.*, 2005). The detection of these enzymes (ESBLs) may lead to false reporting of *P. aeruginosa* as being susceptible to certain antipseudomonal penicillin or cephalosporins and consequently, to inappropriate antimicrobial therapy (Navon-Venezia *et al.*, 2005).

Since the late 1980's a global spread of ESBLs derived from TEM- and SHV-type penicillinases capable of hydrolysing the oxyimino-cephalosporins have been noticed (Wachino *et al*., 2004). This was mainly observed in the *Enterobacteriaceae*, including *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) (Wachino *et al*., 2004). Besides the TEM- and SHV-types, various non-TEM and non-SHV type class A β-lactamases, exhibiting extended-spectrum activities, have been reported in a variety of Gram-negative bacilli (Wachino *et al*., 2004). Extended-spectrum β-lactamases have been reported increasingly for *P. aeruginosa* and may belong to various families including: the TEM- and SHV-types, which are common among *Enterobacteriaceae*, the PER-type from Turkey, the VEB-type from Southeast Asia and more recently, the GES-types which have

been reported from various parts of the world including Brazil, France, Greece, Korea and South Africa (Wachino *et al*., 2004; Jeong *et al*., 2005; Poirel *et al*., 2005). The GES class A ESBL family characteristically hydrolyses ceftazidime with higher efficiency than other oxyimino-β-lactams (Vourli *et al*., 2004).

To date nine GES-type ESBL enzymes have been identified, named GES-1 to GES-9 (Poirel *et al*., 2005). Among the GES ESBLs, GES-1 was the first report of the GES-type class A β-lactamases (Poirel *et al*., 2000). The GES-1 enzyme was produced by *K. pneumoniae*, isolated from a child transferred from French Guiana to France in 1998 (Poirel *et al*., 2000). The GES-2 enzyme was identified from a patient in the Academic Hospital of Pretoria, South Africa and differed from GES-1 by a single amino acid change, located in the active site of the enzyme (Weldhagen *et al*., 2003). Apart from its ESBL activity, GES-2 displays weak carbapenemase activity, which may partly contribute to decreased susceptibility of *P. aeruginosa* to carbapenems (Navon-Venezia *et al*., 2005). Should the GES-2 enzymes be combined with an additional mechanism, such as low permeability or efflux, the strain may become carbapenem resistant (Navon-Venezia *et al*., 2005). The most recent GES variant, GES-9, was identified in a *P. aeruginosa* isolated from a rectal swab of a patient hospitalised at the Bicetre hospital, France in March 2004 (Poirel *et al*., 2005). The GES-9 enzyme differed from GES-1 by only a glycine (Gly)-to-serine (Ser) change at Ambler position 243 and exhibited activity towards aztreonam (Poirel *et al*., 2005). The bla_{GES} genes have mainly been identified as being part of class 1 integrons, with the exception of bla_{GES-1}, found in a *K. pneumoniae* strain from Portugal, embedded in a class 3 integron (Poirel *et al*., 2005).

The double-disk synergy tests with clavulanate and extended-spectrum cephalosporins are sensitive and specific for the detection of ESBLs in *Enterobacteriaceae*, but have performed poorly in *P. aeruginosa* with frequent false-negative results (Weldhagen *et al*., 2003). Therefore, molecular techniques such as PCR with primers targeting the class A β-lactamase genes may offer a more sensitive method for ESBL detection (Weldhagen *et al*., 2003). Sequencing of the amplification products is needed to further differentiate between all the genes in a β-lactamase family, substantially adding to the cost of such an experiment (Weldhagen, 2004a). Weldhagen (2004a) described a PNA-based multiplex PCR for the detection and differentiation of bla_{GES-1} and bla_{GES-2}. The PNA-based method offered a partial alternative to sequencing by detecting the mutations between bla_{GES-1} and bla_{GES-2} (Weldhagen, 2004a). However, the description of new bla_{GES} genes with mutations in and

out-side the target site of the PNA-based assay have made the assay unreliable for the effective differentiation between *bla*$_{GES-1}$ and *bla*$_{GES-2}$. Weldhagen (2004b) described a real-time PCR assay utilizing FRET-probes to distinguish between *bla*$_{GES-1}$ and *bla*$_{GES-2}$. The real-time PCR assay can however, only distinguish between *bla*$_{GES-1}$ and *bla*$_{GES-2}$. The description of new *bla*$_{GES}$ genes may influence this technique, since the FRET-probes can only distinguish between mutations that occur at the binding site of the probes.

This study aimed to extend the PNA-based multiplex PCR method to real-time PCR utilising SYBR Green. SYBR Green has the advantage of allowing melting curve analysis, which may permit the rapid and simultaneous detection of single base pair mutations distinguishing *bla*$_{GES}$ genes.

6.3 Materials and Methods

6.3.1 Bacterial strains

Five *P. aeruginosa* isolates (Db1; Db18, Db26; Db36; Db50) identified to possess *bla*$_{GES-2}$, two *P. aeruginosa* isolates (Db24; P48) identified to possess *bla*$_{GES-5}$ and one *P. aeruginosa* isolate (P28) possessing both the *bla*$_{GES-2}$ and *bla*$_{GES-5}$ genes were included in this study. The control bacterial strains used in this study were listed in Table 6.1.

6.3.2 Whole-cell DNA extraction

Extraction of whole-cell DNA was performed by a precipitation based method in combination with a repeated final washing step with 70% ethanol to improve template purity as described previously (Chapter 3.3.) (Philippon *et al*., 1997). Isolated DNA was stored in 500 µl aliquots in TE buffer (pH 7.4) at -20°C and -70°C.

6.3.3 Real-time competitive PNA-based multiplex PCR

The reaction mixture comprised of a 0.4 µM concentration of each primer (GES-1A, GES-1B, GES-E, GES-F), 0.4 µM concentration of the PNA-probe, 4 µl of LightCycler FastStart DNA Master^{PLUS} reaction mix (Roche Diagnostics, Pensberg, Germany), 5 µl of DNA template and distilled water to a final reaction volume of 20 µl. Deoxyribonucleic acid (DNA) templates from control isolates (Table 6.1) and molecular grade water (Promega, Madison, WI) served as positive and negative controls, respectively. The primer and probe characteristics were tabulated in Table 6.2. The amplification and melting curve analysis protocol was given in Table 6.3.

6.4 Results and Discussion

The poor performance of double-disk synergy tests in *P. aeruginosa* stem from several factors: i) over-expression of naturally occurring β-lactamases such as chromosome-encoded AmpC, which may give false-negative results; ii) relative resistance to β-lactamase inhibitors such as clavulanate, as exemplified by GES-2; iii) simultaneous occurrence with metallo-enzymes with carbapenem-hydrolysing activities (e.g. IMP and VIM enzymes) or extended-spectrum oxacillinases (e.g. OXA-2, OXA-10 and OXA-18); iv) other resistance mechanisms combined, e.g. impermeability and efflux; and v) false-negative results due to the inoculum effect (Bradford, 2001; Stürenburg and Mack, 2003; Weldhagen *et al*., 2003). Molecular techniques targeting genetic determinants may offer more sensitive alternatives for ESBL detection compared to sensitivity testing.

Initial results for applying the competitive PNA-based multiplex PCR in real-time utilising SYBR Green with primer and probe concentrations at 0.4 µM were not optimal. No difference in melting temperature (T_m) could be observed for the amplification products obtained for the different GES variants as depicted in Figure 6.1. The melting temperatures were recorded as 89.96°C for all the GES variants. Since amplification seemed sufficient, probe concentration was adjusted to increase the probe influence on the observed melting temperatures. The melting temperatures obtained for different probe concentrations were tabulated in Table 6.4. Due to the high concentration of PNA-probe used these reactions were only performed for *bla*$_{GES-1}$, *bla*$_{GES-2}$, *bla*$_{GES-3}$ and *bla*$_{GES-4}$. The PNA-probe was designed to be complementary to *bla*$_{GES-1}$ and should have increased *bla*$_{GES-1}$ templates T_m values above

other GES variants that contained mismatches to the PNA-probe. At a probe concentration of 2 µM, this was the case with GES-1 (89.32°C) melting 0.19°C above GES-2 (89.17°C). The GES-3 (89.67°C) and GES-4 (89.48°C) amplicons however produced T_m values 0.32°C and 0.16°C above that of GES-1 respectively. At PNA-probe concentrations ranging from 4 to 20 µM, GES-1 had lower T_m values compared to GES-2, GES-3 and GES-4. The largest difference in T_m value was observed at a probe concentration of 14 µM. The GES-2 (89.51°C) amplicons melted 1.24°C above that of GES-1 (88.27°C), while GES-3 (89.72°C) melted 0.16°C above GES-4 (89.56°C). A probe concentration of 14 µM was thus chosen for the following experimental work.

The PNA-based multiplex PCR used a forward primer (GES-1A) and reverse primer (GES-E) that was complementary to *bla*$_{GES-2}$, as well as a PNA-probe similar to the reverse primer, but complementary to *bla*$_{GES-1}$ (Weldhagen, 2004a). In the case of a GES-1 DNA template present in the PCR reaction, the PNA-probe out-competed the reverse primer for binding to the template blocking amplification between forward primer GES-1A and reverse primer GES-E (Weldhagen, 2004a). The opposite occurred when a mutant sequence was present during the reaction, the primer out-competed the PNA-probe resulting in amplification of a 505 base pair (bp) product between primers GES-1A and GES-E (Weldhagen, 2004a). An internal control was amplified in all GES-positive PCR reactions between forward primer GES-F and reverse primer GES-1B amplifying a 360 bp product. The same was true when applying the PNA-based multiplex PCR in real-time PCR utilising SYBR Green. The SYBR Green dye binds double-stranded DNA and fluoresces at 360 nano meters (nm) enabling the detection and monitoring of amplification during the PNA-based multiplex PCR in real-time.

The phenomenon of GES variants with mismatches compared to the PNA-probe exhibiting higher T_m values compared to GES-1 with no mismatches to the PNA-probe may be explained by the competitive nature of the PNA-based multiplex PCR. With a GES-1 template present in the PCR reaction the PNA-probe should out compete the reverse primer blocking the amplification of the 505 bp amplification product. Only the 360 bp internal control was amplified in the presence of a GES-1 template. The T_m value of a particular amplicon is influenced by GC content, product length, sequence structure and homology to the PNA-probe (Marziliano *et al.*, 2000). The lower T_m value of GES-1 may be due to the shorter length (360 bp) of the amplicon compared to mismatch templates producing longer templates (505 bp). However, running the real-time PCR products on a 1% agarose gel

revealed the presence of a 864 bp by-product for all templates. This amplicon was the result of the whole *bla*$_{GES}$ gene being amplified between forward primer GES-1A and reverse primer GES-1B as discussed in chapter 3. Thus the T_m value differences observed for the different GES-types were not the result of amplicon length and similarity to the PNA-probe alone. Repeating the real-time PCR with only forward primer GES-1A and reverse primer GES-1B resulted in indistinguishable T_m values for the different GES-types, which indicated that the competitive exclusion between reverse primer GES-E and the PNA-probe was necessary for differentiation between the GES-types. These results indicated that a more complex hybridisation dynamic was responsible for the T_m value differences observed.

It has been reported that PNA-DNA hybrids that contain a single mismatch typically melt at a temperature 10°C-18°C lower than the same pair lacking the mismatch (Pellestor and Paulasova, 2004). This was not the case in this study. The possibility exists that the larger amplification product (864 bp) influenced the observable T_m value difference. Shorter amplicon size has been reported as strongly preferred above long amplicon size since it results in more reliable T_m value calling (Marziliano *et al*., 2000). The formation of PNA-DNA duplexes and PNA_2-DNA triplexes may play a role in the hybridisation dynamics of the assay. It has been noted that PNA_2-DNA triplexes possess stronger binding than PNA-DNA duplexes and could thus result in higher T_m values of such structures (Pellestor and Paulasova, 2004). The stability of PNA_2-DNA triplexes is due to an increase in stacking interactions and the formation of hydrogen bonds between the oxygens of the phosphates in the DNA backbone and the amides in the Hoogsteen molecule of the PNA. A higher T_m value observed for mismatch templates may be due to the formations of PNA_2-DNA triplexes. Perhaps the mismatch allowed better steric accessibility to the DNA and promoted triplex formation.

The T_m values obtained in subsequent experiments using a probe concentration of 14 µM were tabulated in Table 6.5. Figures 6.3, 6.4, 6.5, 6.6, and 6.7 depicted the melting curves obtained for the different GES variants. In two separate reactions using GES-1 as template, T_m values of 88.37°C and 87.99°C were observed. The average T_m value observed for GES-2 was 88.9°C with the highest T_m value at 89.51°C and the lowest T_m value at 88.60°C. The T_m values obtained for GES-3 and GES-4 were 89.13°C and 89.06°C respectively. The small temperature difference observed between GES-3 (G) and GES-4 (A) was due to *bla*$_{GES-3}$ and *bla*$_{GES-4}$ differing by a single nucleotide at position 492. The larger temperature difference

observed between GES-1 (GG) and GES-2 (AA) was due to the amplicons differing by two nucleotides at position 492 and 493.

Although GES-1 and GES-8 have identical sequences at the PNA-probe binding site, GES-1 (88.37°C) had a T_m 0.64°C higher than the temperature recorded for GES-8 (87.73°C). The T_m difference observed was a result of nucleotide differences outside the probe target site between GES-1 and GES-8. The nucleotide differences between the different GES-type sequences were tabulated in Table 3.2. Subsequent experiments included a mixture of GES-1 and GES-2 template DNA. The T_m value observed (88.66°C) was between that obtained for GES-1 (88.37°C) and GES-2 (89.04°C) separately, indicating that the real-time PNA-based method is not suitable for identifying mixed genotypes.

The basic T_m values for all amplification products were calculated using the formula T_m = 64.9°C + 41°C x (number of G's and C's in the primer – 16.4)/N (where N was the number of bases in the amplicon) (Rychlik and Rhoads, 1989) and were presented in Table 6.6. The T_m values were between 85°C and 86°C. The amplicon length and GC content made little difference to the T_m value of the amplicons. Thus the T_m values observed with the PNA-based multiplex real-time PCR assay were mostly due to the influence of the PNA-probe. Binding of the PNA-probe however caused a T_m value difference between GES-1 and GES-8 which share the same sequence in the PNA-probe target site. Thus the T_m values observed were a consequence of the PNA-probe binding, but not a direct reflection of homology to the PNA-probe.

Although the assay produced different T_m values for the different GES variants due to binding of the PNA-probe and differences in the overall nucleotide sequences, the temperatures recorded differed by a fraction of a degree. The difference in temperature was not suitable to accurately distinguish between the different GES variants on the basis of mutations. The T_m values obtained for the GES variants using a probe concentration of 14 µM were graphically depicted in Figure 6.8. In this figure it is clearly indicated that the real-time PCR assay produced overlapping temperatures for the GES variants. The competitive PNA-based multiplex real-time PCR appeared unsuitable for accurately differentiating between the different GES variants.

6.5 Conclusions

The extension of the competitive PNA-based multiple PCR into real-time PCR was not suitable for the accurate differentiation of GES ESBL variants. Although different T_m values were recorded for the various GES variants, the temperature differences were small and not suitable to accurately distinguish between the variants since T_m values obtained for different variants overlapped. The high cost of PNA synthesis together with the high concentrations used in the study made this method unsuitable for low resource settings.

The phenomenon of GES variants with mismatches to the PNA-probe exhibiting higher T_m values than GES-1 (fully complementary to the probe) is as yet unknown. The ability of PNA to form PNA-DNA duplexes and triplexes may play a role in this phenomenon. Further research into the binding dynamic between the PNA-probe and the different amplicons (360 bp, 505 bp and 864 bp) formed during the real-time assay is needed to better understand this phenomenon. Redesign of the assay to produce a single shorter amplicon with the PNA-probe spanning the mutational region might be more successful in differentiating the *bla*$_{GES}$ variants in real-time PCR.

The spread of ESBLs in the South African clinical setting is a problem with new enzymes such as GES-5 threatening the efficacy of currently used antimicrobial regimes. An assay capable of rapid and sensitive identification and differentiation of ESBLs in bacteria where sensitivity testing is ineffective (e.g. *P. aeruginosa*) is needed. The sensitivity information may be used to adjust antimicrobial therapy and prevent treatment with ineffective antimicrobials. Pyrosequencing may offer an alternative, although such a method may prove too expensive for routine diagnostics. Simple probes targeted to mutational sites in ESBL genes may be a worthwhile real-time PCR avenue to explore in future research.

6.6 References

Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*. **14:** 993-951.

Jeong SH, Hong SG, Song JS, Lee JH, Lee SH (2005) First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. *Antimicrob. Agents Chemother.* **49:** 4809-4810.

Livermore DM (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: Our worst nightmare? *Clin. Infect. Dis.* **34:** 634-640.

Marziliano N, Pelo E, Minuti B, Passerini I, Torricelli F, Da Prato L (2000) T_m assay for a *UGT1A* gene variant in Gilbert syndrome. *Clin. Chem.* **46:** 423-425.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Navon-Venezia S, Ben-Ami R, Carmeli Y (2005) Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Curr. Opin. Infect. Dis.* **18:** 306-313.

Pellestor F, Paulasova P (2004) The peptide nucleic acids, efficient tools for molecular diagnosis (Review). *Int. J. Mol. Med.* **13:** 521-525.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Poole K (2004) Resistance to beta-lactam antibiotics. *Cell Mol. Life Sci.* **61:** 2200-2223.

Rychlik W, Rhoads RE (1989) A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. *Nucl. Acids Res.* **17:** 8543-8551.

Schweizer HP (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: Unanswered questions. *Genet. Mol. Res.* **1:** 48-62.

Stürenburg E, Mack D (2003) Extended-spectrum beta-lactamases: Implications for the clinical microbiology laboratory, therapy, and infection control. *J. Infect.* **47:** 273-295.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Weldhagen GF (2004a) Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based, multiplex PCR assay. *Antimicrob. Agents Chemother.* **48:** 3402-3406.

Weldhagen GF (2004b) Rapid detection and sequence-specific differentiation of extended-spectrum beta-lactamase GES-2 from *Pseudomonas aeruginosa* by use of a real-time PCR assay. *Antimicrob. Agents Chemother.* **48:** 4059-4062.

Table 6.1: Characterised bacterial strains used for standardisation of molecular methods

Strain	Relevant property	References
Pseudomonas aeruginosa GW-1	bla_{GES-2} producing isolate	Poirel *et al.*, 2001
Klebsiella pneumoniae ORI-1	bla_{GES-1} producing isolate	Poirel *et al.*, 2000
Escherichia coli DH5α	bla_{IBC-2} transconjugate isolate	Mavroidi *et al.*, 2001
Escherichia coli 365-02	bla_{GES-3} producing isolate	Vourli *et al.*, 2004
Klebsiella pneumoniae 78-01	bla_{GES-4} producing isolate	Vourli *et al.*, 2004

Table 6.2: Characteristics of the primers and PNA-probe used for the competitive PNA-based multiplex real-time PCR

Primer or Probe	Sequence	T_m (°C)	Position (nt) on *bla_{GES}*	Product size	Reference
GES-1A Forward primer	5'ATGCGCTTCA TTCACGCAC3'	56.6	01-19	505 bp	Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
GES-E Reverse primer complementary to bla_{GES-2}	5'GTGTGTTGTC GTTCATCTC3'	51.8	487-505		Weldhagen, 2004a
GES-F Forward primer	5'CCTGGCGACC TCAGAGATAC3'	57.4	505-524	360 bp	Weldhagen, 2004a
GES-1B Reverse primer	5'CTATTTGTCC GTGCTCAGG3'	56.4	846-864		Poirel *et al.*, 2001; Weldhagen *et al.*, 2003
PNA-probe Complimentary to bla_{GES-1} and bla_{IBC}	5'GTTGTCGCCC ATCTC3'	70	487-501	na*	Weldhagen, 2004a

*not applicable

Table 6.3: LightCycler amplification and melting curve protocol followed in this study

Program	No. of cycles	Target temp (°C)	Hold time (s)	Ramp rate (°C/s)	Fluorescence acquisition mode [a]
Polymerase activation	1	95	600	20	None
Four-step PCR	45				
Denaturation cycle		95	1	20	None
PNA-probe binding cycle		76	7	20	None
Amplification cycle		52	10	20	None
Extension cycle		72	15	20	Single
Three-step melting curve	1				
Denaturation cycle		95	10	20	None
Holding cycle		40	30	20	None
Melting cycle		95	0	0.05	Continuous
Cooling cycle	1	40	30	20	None

[a]Fluorescence was measured at 530 nm.

Table 6.4: Melting temperatures observed for *bla*$_{GES-1}$, *bla*$_{GES-2}$, *bla*$_{GES-3}$ and *bla*$_{GES-4}$ for different PNA-probe concentrations

bla$_{GES}$ gene	PNA-probe concentration (µM)	Melting temperature (T_m value) (°C)	Difference in melting temperature (°C)
GES-1	2	89.32	0.15
GES-2	2	89.17	
GES-3	2	89.67	0.19
GES-4	2	89.48	
GES-1	4	89.01	0.09
GES-2	4	89.10	
GES-3	4	89.48	0.11
GES-4	4	89.37	
GES-1	6	88.18	0.63
GES-2	6	88.81	
GES-3	6	89.31	0.13
GES-4	6	89.18	
GES-1	8	88.78	0.12
GES-2	8	88.90	
GES-3	8	89.34	0.12
GES-4	8	89.22	
GES-1	10	88.46	0.2
GES-2	10	88.66	
GES-3	10	89.03	0.15
GES-4	10	88.88	
GES-1	12	87.85	0.87
GES-2	12	88.72	
GES-3	12	89.01	0.00
GES-4	12	89.01	
GES-1	14	88.27	1.24
GES-2	14	89.51	
GES-3	14	89.72	0.16
GES-4	14	89.56	
GES-1	16	88.02	1.19
GES-2	16	89.21	
GES-3	16	89.41	0.56
GES-4	16	88.85	
GES-1	18	87.90	1.13
GES-2	18	89.03	
GES-3	18	89.27	0.1
GES-4	18	89.17	
GES-1	20	88.17	0.1
GES-2	20	89.27	
GES-3	20	89.55	0.73
GES-4	20	88.82	

Table 6.5: Melting temperature obtained with 14 µM PNA-probe concentration during real-time PCR reactions

bla$_{GES}$	Melting temperature (T_m value) (°C)	Difference in meting temperature compared to GES-1 (°C)
GW-1 (GES-2)	89.04	0.67
ORI-1 (GES-1)	88.37	na*
GES-4	89.06	0.69
GES-3	89.13	0.76
DH5-α (GES-8)	87.73	0.64
Db 1 (GES-2)	88.74	0.37
Db 26 (GES-2)	88.72	0.35
Db 36 (GES-2)	88.59	0.22
Db 24 (GES-5)	87.98	0.39
Db 18 (GES-2)	88.60	0.23
Db 50 (GES-2)	89.20	0.83
P 48 (GES-5)	87.88	0.49
P 28 (GES-2 + GES-5)	87.73	0.64
GW-1 + ORI-1 (GES-1 + GES-2)	88.66	0.29
GW-1 (GES-2)	88.80	0.43
ORI-1 (GES-1)	87.99	0.38

*not applicable

Table 6.6: Expected basic melting temperatures for the amplicons formed during the competitive PNA-based multiplex PCR

Template	Amplicon length	% GC	Basic T_m (°C)
GES-1	864 bp	53.24	86
	360 bp	52.5	85
GES-2	864 bp	53.01	86
	505 bp	53.47	85
	360 bp	52.5	85
GES-3	864 bp	53.36	86
	505 bp	54.06	86
	360 bp	52.5	85
GES-4	864 bp	53.24	86
	505 bp	53.86	86
	360 bp	52.5	85
GES-5	864 bp	53.01	86
	505 bp	53.47	85
	360 bp	52.5	85
GES-8	864 bp	53.13	86
	360 bp	52.5	85

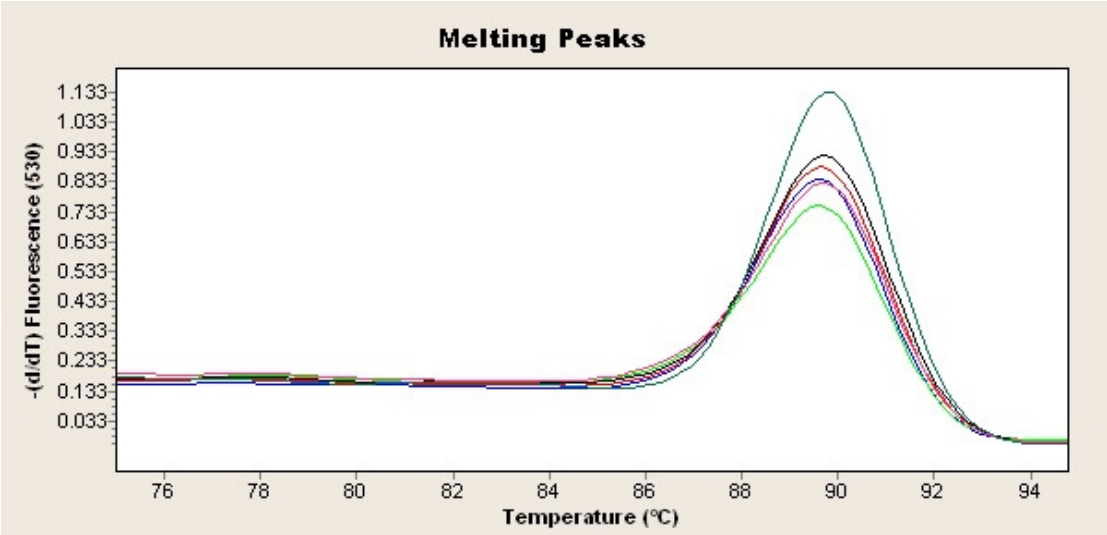

Figure 6.1: Melting curve analysis depicting the melting curves for GES-1, GES-2, GES-3, GES-4, GES-5 and GES-8 at a PNA-probe concentration of 0.4 µM

PNA-Probe	5'-GTTGTCG**CC**CATCTC-3'
GES-1	5'-GTTGTCG**CC**CATCTC-3'
GES-2	5'-GTTGTCG**TT**CATCTC-3'
GES-3	5'-GTTGTCG**CC**CATCTC-3'
GES-4	5'-GTTGTCG**TC**CATCTC-3'
GES-5	5'-GTTGTCG**TC**CATCTC-3'
GES-8	5'-GTTGTCG**CC**CATCTC-3'

Figure 6.2: Schematic representation of the PNA-probe sequence compared to the GES variants used the in study. The nucleotides of interest are underlined and in bold face

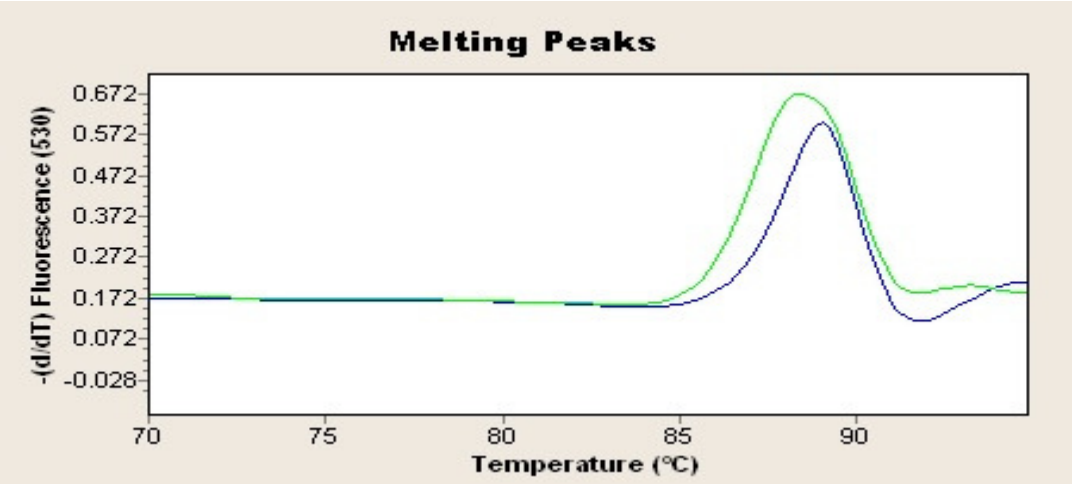

Figure 6.3: Diagram depicting the melting temperature peaks obtained with a PNA-probe concentration of 14 µM for GES-1 (88.27°C) and GES-2 89.51°C)

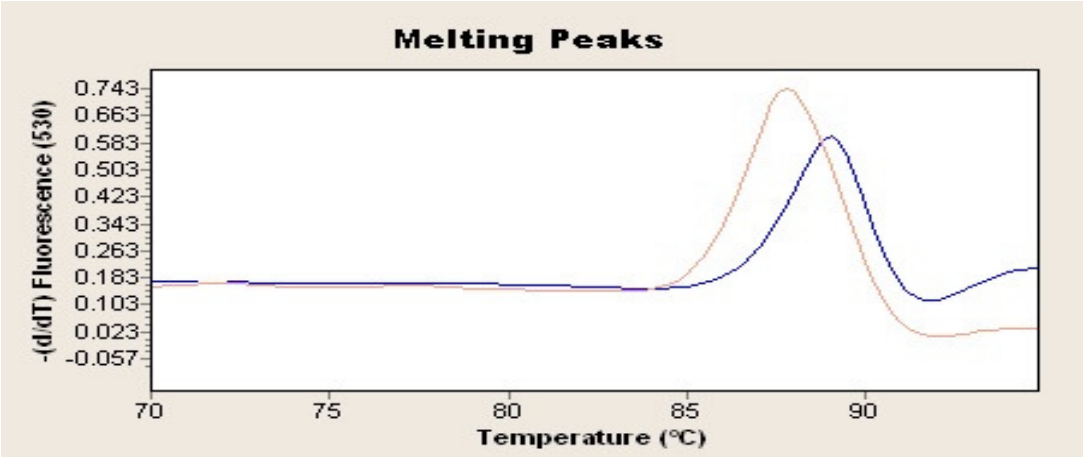

Figure 6.4: Diagram depicting the melting temperature peaks obtained for GES-2 and GES-5

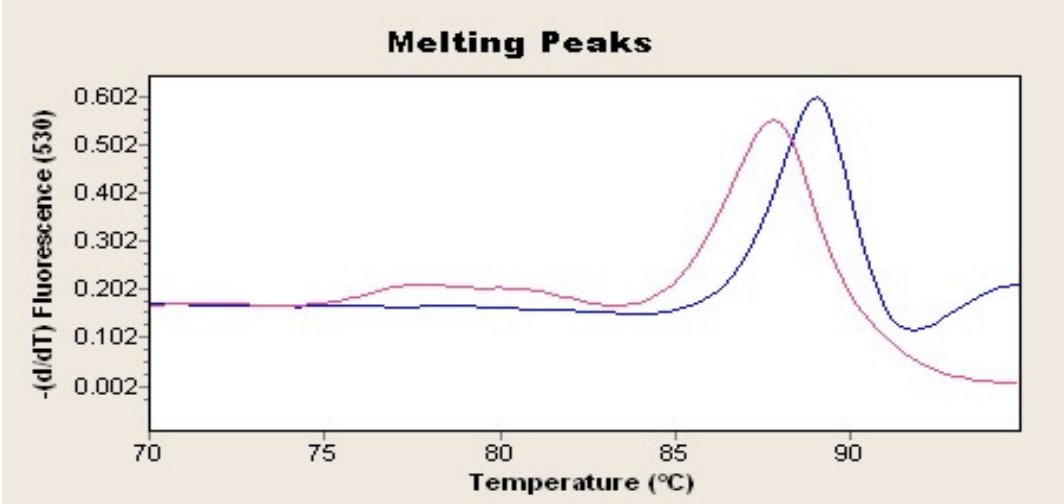

Figure 6.5: Diagram depicting the melting temperature peaks obtained for GES-2 and GES-8

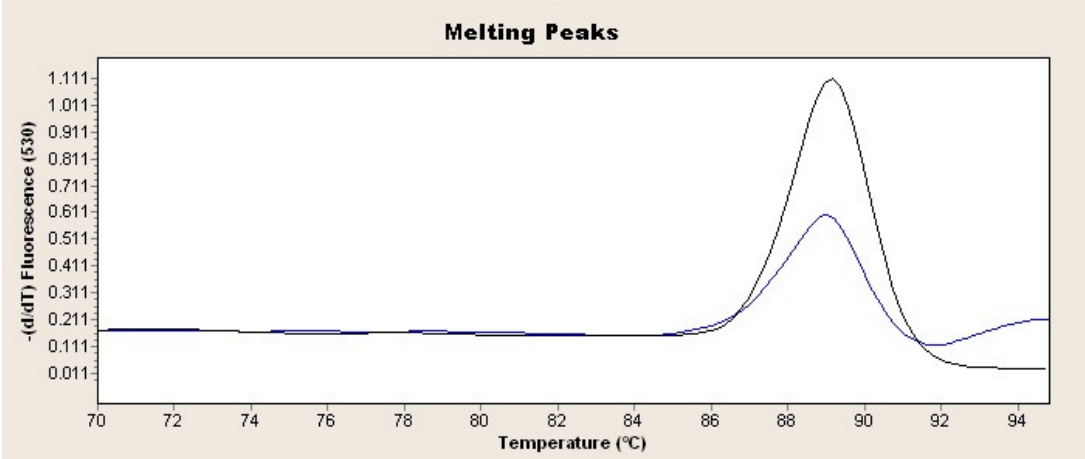

Figure 6.6: Diagram depicting the melting temperature peaks obtained for GES-2 and GES-3

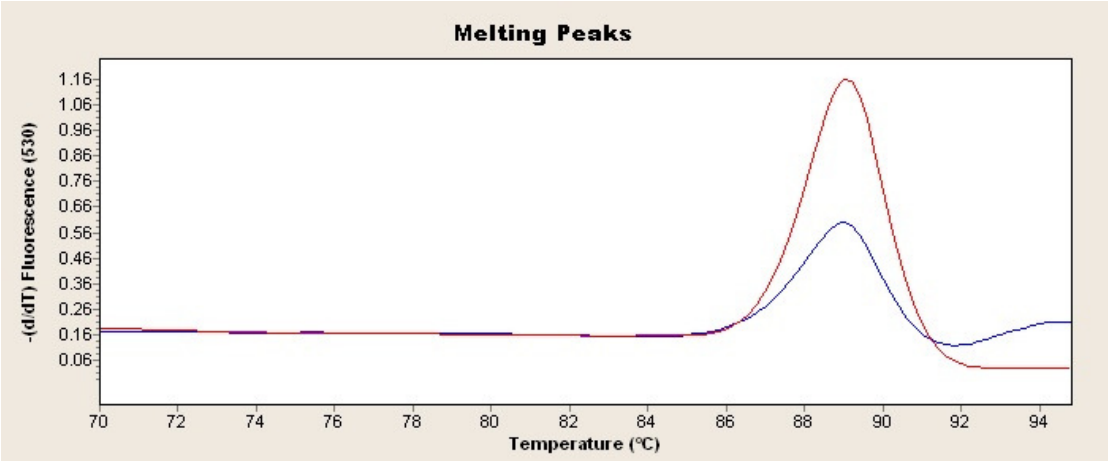

Figure 6.7: Diagram depicting the melting temperature peaks obtained for GES-2 and GES-4

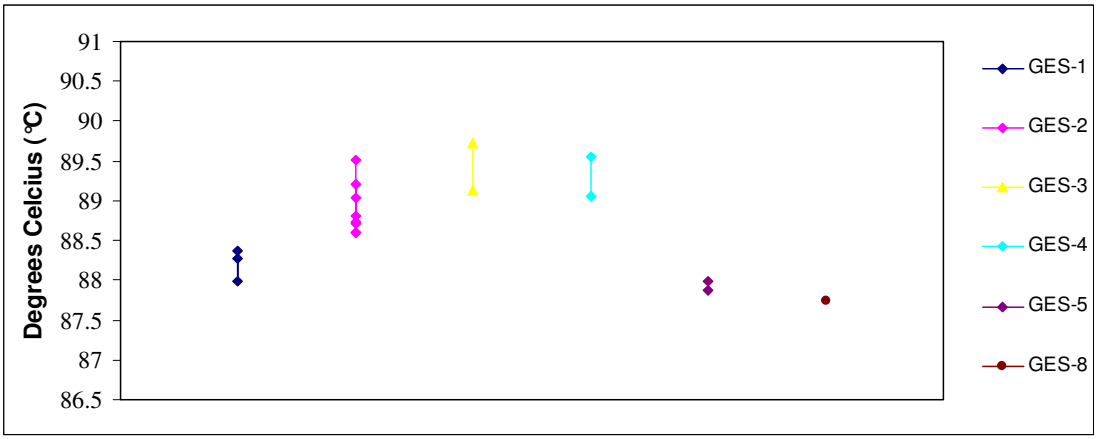

Figure 6.8: X Y scatter plot depicting the T_m values obtained for GES-1, GES-2, GES-3, GES-4, GES-5 and GES-8 with the real-time competitive PNA-based multiplex PCR at a PNA-probe concentration of 14 µM

Chapter 7

Random amplified polymorphic DNA typing of ESBL-positive *Pseudomonas aeruginosa* isolates recovered in the Gauteng region of South Africa

The editorial style of the Journal of Clinical Microbiology was used in this chapter.

7.1 Abstract

Pseudomonas aeruginosa is a common nosocomial pathogen, especially in intensive care units. The occurrence of extended-spectrum β-lactamases (ESBLs) in *P. aeruginosa* complicates the treatment of infections caused by these aerobic rods. The dissemination of ESBLs in *P. aeruginosa* may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections. Genomic fingerprinting methods are regarded as accurate methods for the typing of microorganisms for epidemiological purposes and include random amplified polymorphic DNA (RAPD) typing. The aim of the study was to investigate the genetic relatedness of 53 GES ESBL-positive *P. aeruginosa* clinical isolates collected in the Gauteng region of South Africa. The banding patterns obtained were similar to previously published patterns. The RAPD fingerprints obtained proved reproducible when repeated on three isolates for which whole-cell DNA was isolated on two separate occasions. Random amplified polymorphic DNA fingerprints that possessed more than 75% similarity were considered identical and assigned a RAPD type. Twenty-four unique RAPD fingerprints were found. Results revealed isolates collected from the same hospital to be identical or highly similar which indicated clonal relatedness. These research findings showed a single acquisition of a specific bla_{GES} rather than separate mutational events. This is typical of a nosocomial spread or outbreak. The occurrence of similar *P. aeruginosa* isolates producing GES ESBLs in a single hospital setting emphasizes the importance of constant surveillance to determine the prevalence of antibiotic resistance genes and monitor the efficacy of current infection control measures.

7.2 Introduction

Despite advances in hospital care and the introduction of a wide variety of antimicrobial agents, *Pseudomonas aeruginosa* remains a common nosocomial pathogen, often a problem in intensive care units and in patients who are immunosuppressed (Speijer *et al*., 1999; Matar *et al*., 2005). The occurrence of ESBLs in *P. aeruginosa* complicates the treatment of infections caused by these aerobic rods (Weldhagen *et al*., 2003). Extended-spectrum β-lactamases have been reported for *P. aeruginosa* and belong to various families including the TEM- and SHV-types which are common among *Enterobacteriaceae*, the PER-type from Turkey, the VEB-type from Southeast Asia and more recently, the GES-types which have been reported from various parts of the world including Brazil, France, Greece, Korea and South Africa (Wachino *et al*., 2004; Jeong *et al*., 2005; Poirel *et al*., 2005). The GES-type ESBL enzymes characteristically hydrolyse ceftazidime with higher efficiency than other oxyimino β-lactams (Vourli *et al*., 2004). Currently, nine GES-types have been identified, named GES-1 to GES-9 (Jeong *et al*., 2005; Poirel *et al*., 2005).

Infection control measures have led to the need to develop adequate techniques for strain typing since antibiotic susceptibility and other phenotypic methods such as serotyping are not reliable for epidemiological studies (Da Silva Filho *et al*., 2001). Molecular typing methods have emerged as the most efficient tools for strain discrimination and include PFGE, ribotyping and PCR-based techniques, such as RAPD typing and RFLP analysis (Speijer *et al*., 1999). These methods are important epidemiologically for recognising outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the infection, recognising particularly virulent strains of bacteria and monitoring vaccination programmes (Olive and Bean, 1999). Techniques such as DNA macro-restriction and pulse-field or field inversion gel electrophoresis have been used in many studies for the identification of cross-infection (Da Silva Filho *et al*., 2001). These techniques have high discriminatory power, but are expensive, difficult and generally require a hybridisation step (Da Silva Filho *et al*., 2001). Random amplified polymorphic DNA analysis has emerged as a fast and simple PCR-derived technique with high discriminatory power, suitable for application in epidemiological studies (Da Silva Filho *et al*., 2001).

The dissemination of ESBLs may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections due to *P. aeruginosa* producing ESBLs (Weldhagen *et al.*, 2003). The monitoring of new antibiotic resistance genes (e.g. GES-type ESBLs) in bacterial species such as *P. aeruginosa* is essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings (Sardelic *et al.*, 2003). This study investigated the genetic relatedness of GES-type ESBL-positive *P. aeruginosa* clinical isolates collected in the Gauteng region of South Africa.

7.3 Materials and Methods

7.3.1 Bacterial isolates

A total of 53 isolates of *P. aeruginosa*, obtained previously to determine GES-type ESBL prevalence (Chapter 3), were analysed. The source study included 100 clinical *P. aeruginosa* isolates of which the 53 included in this study were shown to be bla_{GES} positive (Chapter 3).

7.3.2 Whole-cell DNA extraction

Whole-cell DNA was extracted with a precipitation based method as previously described (Chapter 3.3) (Philippon *et al.*, 1997). Extracted DNA was stored at 4°C and -20°C until further analysis.

7.3.3 Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA analysis was performed on whole-cell DNA as template to determine clonal relationships between isolates according to the modified method of Mahenthiralingam and colleagues (1996) as previously described (Chapter 4). The reaction mixture consisted of 0.375 µl primer (20 mM), 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI), 4 µl whole-cell DNA as template and molecular grade sterile water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The following cycle programme was utilised and run on a GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA): 4 cycles of denaturation at 94°C for 5 min, annealing at 36°C for 5 min and extension at 72°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 1 min. The programme was completed by a final extension step at 72°C for 10 min. The isolates were typed using three different

primers (primer 277, primer 272 and primer 275) to obtain banding patterns representative of the whole genome. The primers used were described in Table 4.1.

7.3.4 Analysis of RAPD fingerprints

Banding patterns were obtained by electrophoresis at 80 V (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 3 hrs in a 1.5% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (0.5 $\mu g.ml^{-1}$) (Promega, Madison, WI) and run in a 1 X TBE running buffer pH 8.3 (Promega, Madison, WI). Molecular size standards (100 bp - 10 000 bp) were included on all gels (O'GeneRuler DNA Ladder Mix, Fermentas). The agarose gels were visualized under UV illumination (TFM-26 Ultraviolet Transilluminator, UVP, Upland, CA) and the images captured using the DigiDoc-It imaging system (UVP, Upland, CA). The gel images were analysed with the GelCompar II programme (Applied Maths, Kortrijk, Belgium). Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA).

7.4 Results and Discussion

A total of 53 GES ESBL-positive *P. aeruginosa* isolates obtained from a previous study were typed by RAPD analysis utilising primer 272, primer 275 and primer 277 to determine the genetic relatedness of the strains. The *P. aeruginosa* clinical isolates were obtained from the Gauteng region, South Africa. The RAPD typing method applied arbitrary 10-nucleotide primers identified specifically for their ability to discriminate between *P. aeruginosa* strains (Mahenthiralingam *et al*., 1996).

The banding patterns obtained (Figure 7.1; Figure 7.2; Figure 7.3) were similar to those obtained by Mahenthiralingam and colleagues (1996) and proved reproducible when repeated on three isolates for which whole-cell DNA was isolated on two separate occasions. Random amplified polymorphic DNA fingerprints which possessed more than 75% similarity were considered identical and were assigned a RAPD type. On this basis twenty-four unique RAPD types were assigned (Figure 7.4).

Banding patterns ranged from 1 to 2 bands for RAPD type 18 and as many as 11 bands for RAPD type 19. The isolates analysed were collected from 20 different hospitals located throughout the Gauteng region of South Africa. The number of GES-positive *P. aeruginosa* isolates collected per hospital ranged from one to eight. The isolates, the hospitals they were collected from and the specimen types from which isolates were obtained were shown in Table 7.1.

Only one GES-positive *P. aeruginosa* isolate was recovered from hospitals A, B, C, E, G, I, L, N, O, S and T. Hospitals A, B, C, G, I, L, N, S and T yielded a single unique RAPD type per hospital indicating that the *P. aeruginosa* isolates collected from these hospitals were unique to the hospitals. However, hospitals E and O yielded RAPD type 5 and 20 respectively. *Pseudomonas aeruginosa* isolates of RAPD type 5 were also isolated from hospital F, which indicated the presence of the same *P. aeruginosa* isolate at both hospital E and F.

Pseudomonas aeruginosa isolates of RAPD type 20 were isolated from 7 different hospitals (D, H, K, P, Q, R and O). Spread of a single *P. aeruginosa* isolate between 7 different hospitals is unlikely. The wide distribution of such an isolate may rather indicate spread of the isolate from an external source (e.g. a water source) to the different hospitals. In addition to mechanical spread by personnel, *P. aeruginosa* clones of RAPD type 20 may carry a number of adhesins that enhance its colonisation in the hospital environment and render it more accessible to patients (Matar *et al*., 2005). Hospitals where more than one GES-positive *P. aeruginosa* isolate were collected tended to have one predominant RAPD type together with a few unique RAPD types. The predominant RAPD type may be due to nosocomial spread of a *P. aeruginosa* clone, while the unique RAPD types in the same hospital may be due to environmental introduction. Hospital D was the exception with seven isolates resulting in five different RAPD types and two unique RAPD types.

The high occurrence of a single RAPD type and highly related RAPD types indicated the spread of a single *P. aeruginosa* isolate in a hospital instead of the introduction of different isolates from outside the hospital setting. Several studies have demonstrated that cross-acquisition may play a significant role in nosocomial outbreaks involving *P. aeruginosa* and that colonised patients represent a potential reservoir of (epidemic) strains from which other patients can be colonised (Aktaş *et al*., 2005). The spread of a single clone throughout a

clinical setting was particularly true in hospital M where 8 *P. aeruginosa* isolates were obtained, of which 7 were RAPD type 15. The eighth isolate was closely related and yielded RAPD type 16, which may be a result of mutation in the clone of RAPD type 15. The spread of a single clone was seen in hospitals K and R, which yielded 5 isolates each of RAPD type 20. The high occurrence of RAPD type 20 in both hospitals K and R may be due to separate introductions of the clone into these hospitals as well as nosocomial spread through the hospitals.

The RAPD typing PCR-based methodology has been shown to be as discriminatory as PFGE for the typing of *P. aeruginosa* (Mahenthiralingam *et al*., 1996). Kersulyte and co-workers (1995) reported that RAPD analysis with 10-nucleotide primers lacked reproducibility and did not recommend these primers for typing of *P. aeruginosa*. However, the 10-nucleotide primers evaluated in their study had previously been used for other microbial species and were not screened specifically for the ability to generate strain-specific polymorphisms for *P. aeruginosa* (Kersulyte *et al*., 1995; Mahenthiralingam *et al*., 1996). Mahenthiralingam and co-workers (1996) demonstrated primer 277, primer 275 and primer 272 to be the best suited for the amplification of reproducible discriminatory polymorphisms for *P. aeruginosa* isolates. Campbell and co-workers (2000) evaluated primer 272 for RAPD analysis of *P. aeruginosa* on 600 samples and reported RAPD analysis using this primer as being robust, simple and highly reproducible.

Results indicated that although the GES ESBL genes may spread among isolates of different geographical origins these genes are mostly spread by similar nosocomial *P. aeruginosa* clones (Llane *et al*., 2006). The observation that genotypically and geographically diverse *P. aeruginosa* strains have acquired the GES ESBL genes since the initial isolation of *P. aeruginosa* GW-1 in 2000 harbouring the $bla_{\text{GES-2}}$ gene, suggested that contact with still unknown bacterial reservoirs contribute to the evolution of multidrug-resistance in *P. aeruginosa* in South Africa. The clonal diversity and relatively high prevalence of the *P. aeruginosa* isolates studied (considering the number of hospitals and amount of RAPD types) indicated a considerable potential for spread amongst patients (Aktaş *et al*., 2005).

7.5 Conclusions

The spread of single *P. aeruginosa* clones through a particular hospital indicate a need for stringent infection control measures in these and other hospitals. The occurrence of a single clone in more than one hospital may indicate an external reservoir of highly resistant *P. aeruginosa* warranting further research into the spread of *P. aeruginosa* from external reservoirs such as contaminated water sources in hospitals. Genotypically and geographically diverse *P. aeruginosa* strains have acquired the GES ESBL genes as reflected in this study, which suggested contact with still unknown bacterial reservoirs that contribute to the spread of bla_{GES} among *P. aeruginosa* isolates in the South African clinical setting. It seems that bla_{GES} determinants have rapidly established a high-level of endemicity in the Gauteng province of South Africa and possibly the rest of South Africa as indicated by the bla_{GES-5} gene identified from another province. Should there be further reports in the future of GES ESBLs in Gram-negative bacteria, this may reflect an endemic situations rather than the development of novel outbreaks (Aubron *et al*., 2005).

These findings are of concern since they demonstrated that acquired GES ESBLs can rapidly emerge and become a major cause of broad-spectrum β-lactam resistance among nosocomial pathogens (Lagatolla *et al*., 2004). The possibility that spreading transferable GES ESBL genes among nosocomial Gram-negative pathogens could emerge as a major problem in the clinical setting, underscores the need for systematic surveillance of these resistance determinants. Since *P. aeruginosa* clones of the same RAPD type were identified in different hospitals, surveillance should not be restricted to nosocomial isolates, but should also include isolates from community-acquired infections (Matar *et al*., 2005). Future efforts should be directed towards the identification and elimination of ESBL-producing *P. aeruginosa* transmission routes inside hospitals and into hospitals from external sources. The identification of bacterial reservoirs that contribute to the spread of bla_{GES} and other ESBL genes among *P. aeruginosa* isolates in the South African clinical setting may play an important role in the prevention of ESBL producing *P. aeruginosa* in the future.

7.6 References

Aktaş Z, Poirel L, Şalcioğlu M, Özcan PE, Midilli K, Bal Ç, Anğ Ö, Nordmann P (2005) PER-1- and OXA-10-like beta-lactamases in ceftazidime-resistant *Pseudomonas aeruginosa* isolates from intensive care unit patients in Istanbul, Turkey. *Clin. Microbiol. Infect.* **11:** 193-198.

Aubron C, Poirel L, Fortineau N, Nicolas P, Collet L, Nordmann P (2005) Nosocomial spread of *Pseudomonas aeruginosa* isolates expressing the metallo-beta-lactamase VIM-2 in a haematology unit of a French hospital. *Microb. Drug Resist.* **11:** 254-259.

Campbell M, Mahenthiralingam E, Speert D (2000) Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **38:** 4614-4615.

Da Silva Filho LVF, Levi JE, Bento CNO, Rodrigues JC, Da Silva Ramos SRT (2001) Molecular epidemiology of *Pseudomonas aeruginosa* infections in a cystic fibrosis outpatient clinic. *J. Med. Microbiol.* **50:** 261-267.

Jeong SH, Hong SG, Song JS, Lee JH, Lee SH (2005) First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. *Antimicrob. Agents Chemother.* **49:** 4809-4810.

Kersulyte D, Struelens MJ, Deplano A, Berg DE (1995) Comparison of arbitrary primed PCR and macro-restriction (pulse-field gel electrophoresis) typing of *Pseudomonas aeruginosa* strains from cystic fibrosis patients. *J. Clin. Microbiol.* **33:** 2216-2219.

Lagatolla C, Tonin EA, Monti-Bragadin C, Dolzani L, Gombac F, Bearzi C, Edalucci E, Gionechetti F, Rossolini GM (2004) Endemic carbapenem-resistant *Pseudomonas aeruginosa* with acquired metallo-beta-lactamase determinants in European hospital. *Emerg. Infect. Dis.* **10:** 535-538.

Llanes C, Neuwirth C, Garch FE, Hocquet D, Plesiant P (2006) Genetic analysis of a multiresistance strain of *Pseudomonas aeruginosa* producing PER-1 β-lactamase. *Clin. Microbiol. Infect.* **12:** 270-278.

Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Spreet DP (1996) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34:** 1129-1135.

Matar GM, Chaar MH, Araj GF, Srour Z, Jamaleddine G, Hadi U (2005) Detection of a highly prevalent and potentially virulent strain of *Pseudomonas aeruginosa* from nosocomial infections in a medical center. *BMC Microbiol.* **5:** 29-35.

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:** 1661-1669.

Philippon LN, Naas T, Bouthors T, Barakett V, Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **41:** 2188-2195.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Sardelic S, Pallecchi L, Punda-Polic V, Rossolini GM (2003) Carbapenem-Resistant *Pseudomonas aeruginosa*-carrying VIM-2 metallo-beta-lactamase determinants, *Croatia. Emerg. Infect. Dis.* **9:** 1022-1023.

Speijer H, Savelkoul PHM, Bonten MJ, Stobberingh EE, Tjhie JHT (1999) Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. *J. Clin. Microbiol.* **37:** 3654-3661.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Table 7.1: Random Amplified Polymorphic DNA types, corresponding isolates and isolation sites

Hospital	Isolate	RAPD Type	Specimen type
A	Db2	1	Endotracheal aspirate
B	Db3	2	Sputum
C	Db5	3	Sputum
D	Db32	4	Catheter
	Db28	8	Endotracheal aspirate
	Db4	11	Sputum
	Db41	20	Endotracheal aspirate
	Db34	20	CVP
	Db26	21	Bronchial lavage
	Db27	21	Bronchial lavage
E	Db16	5	Endotracheal aspirate
F	Db19	5	Tissue
	Db15	5	CVP
	Db23	5	Pus swab foot
G	Db24*	6	Urine
H	Db29	8	Knee wound
	Db51	20	Endotracheal aspirate
I	Db30	9	Sputum
J	Db1	10	CSF
	Db7	19	Bronchial lavage
	Db12	19	Pus swab skin face
K	Db11	12	Sputum
	Db9	18	Sputum
	Db10	20	Sputum
	Db18	20	Endotracheal aspirate
	Db31	20	Catheter
	Db42	20	Sputum
	Db40	20	Pus swab
	Db6	22	Pus swab
L	Db21	14	Leg pus swab
M	P28	15	Irrigation fluid
	P15	15	Irrigation fluid
	P27	15	Irrigation fluid
	P29*	15	Irrigation fluid
	P16	15	Irrigation fluid
	P25	15	Irrigation fluid
	P40	15	Irrigation fluid
	P48*	16	Irrigation fluid
O	Db33	20	Pus swab
N	Db22	17	Sputum
P	Db20	20	Sputum
	Db36	20	Wound swab
	Db49	13	Blood culture
Q	Db25	20	Pus swab tracheostomy
	Db17	23	Colostomy
R	Db35	20	Sputum
	Db8	22	Pus swab leg
	Db48	20	Endotracheal aspirate
	Db59	20	Endotracheal aspirate
	Db57	20	Blood culture
	Db50	20	Blood culture
S	P23	24	Urine
T	Db47	7	Blood culture

*bla_{GES-5} positive isolates, P28 was both bla_{GES-2} and bla_{GES-5} positive, all other isolates were bla_{GES-2} positive

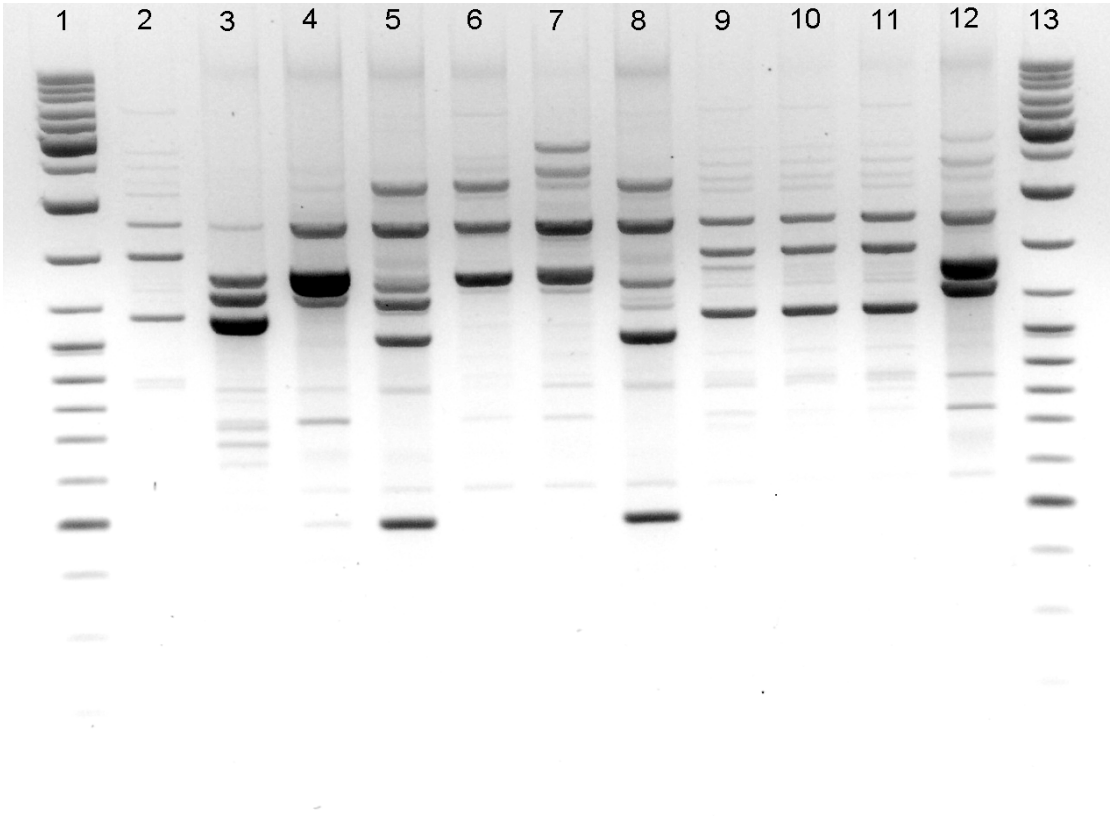

Figure 7.1: Gel electrophoresis depicting the RAPD banding pattern obtained with primer 277. Lane 1: Marker (100 bp to 10 000 bp). Lane 2: Db24. Lane 3: Db32. Lane 4: Db3. Lane 5: Db7. Lane 6: Db9. Lane 7: Db11. Lane 8: Db12. Lane 9: Db15. Lane 10: Db16. Lane 11: Db19. Lane 12: Db21. Lane 13: Marker (100 bp - 10 000 bp)

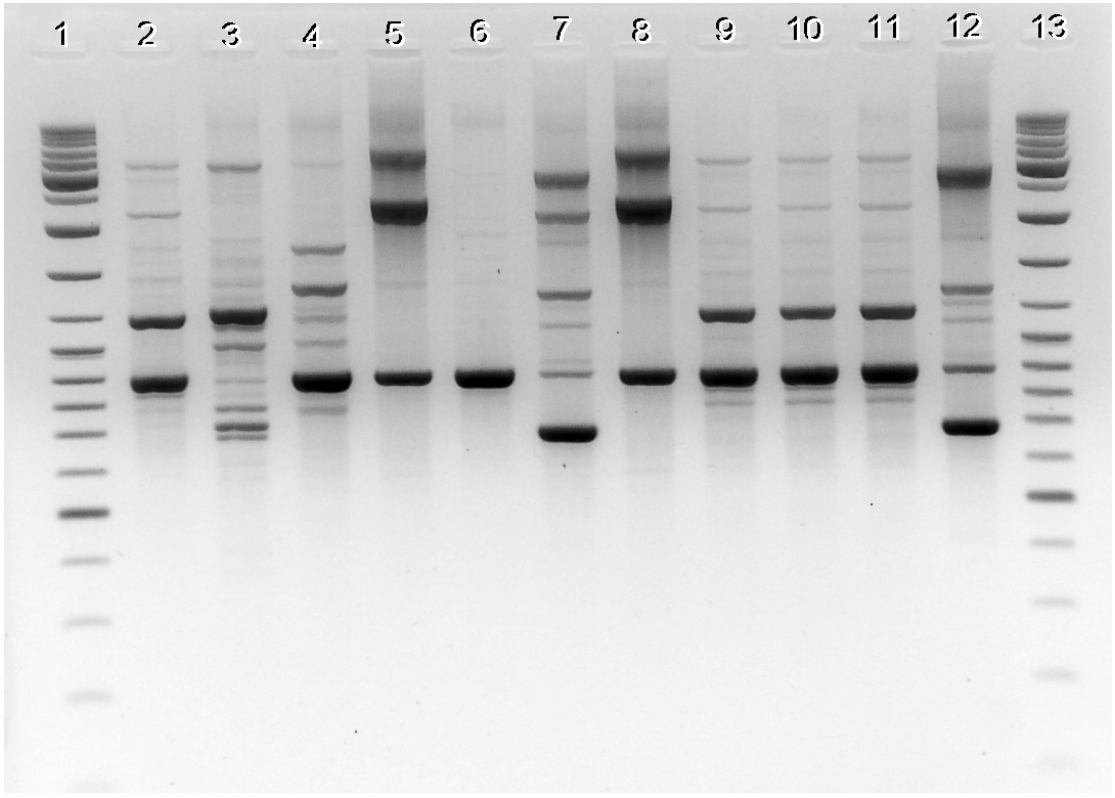

Figure 7.2: Gel electrophoresis depicting the RAPD banding pattern obtained with primer 272. Lane 1: Marker (100 bp to 10 000 bp). Lane 2: Db24. Lane 3: Db32. Lane 4: Db3. Lane 5: Db7. Lane 6: Db9. Lane 7: Db11. Lane 8: Db12. Lane 9: Db15. Lane 10: Db16. Lane 11: Db19. Lane 12: Db21. Lane 13: Marker (100 bp - 10 000 bp)

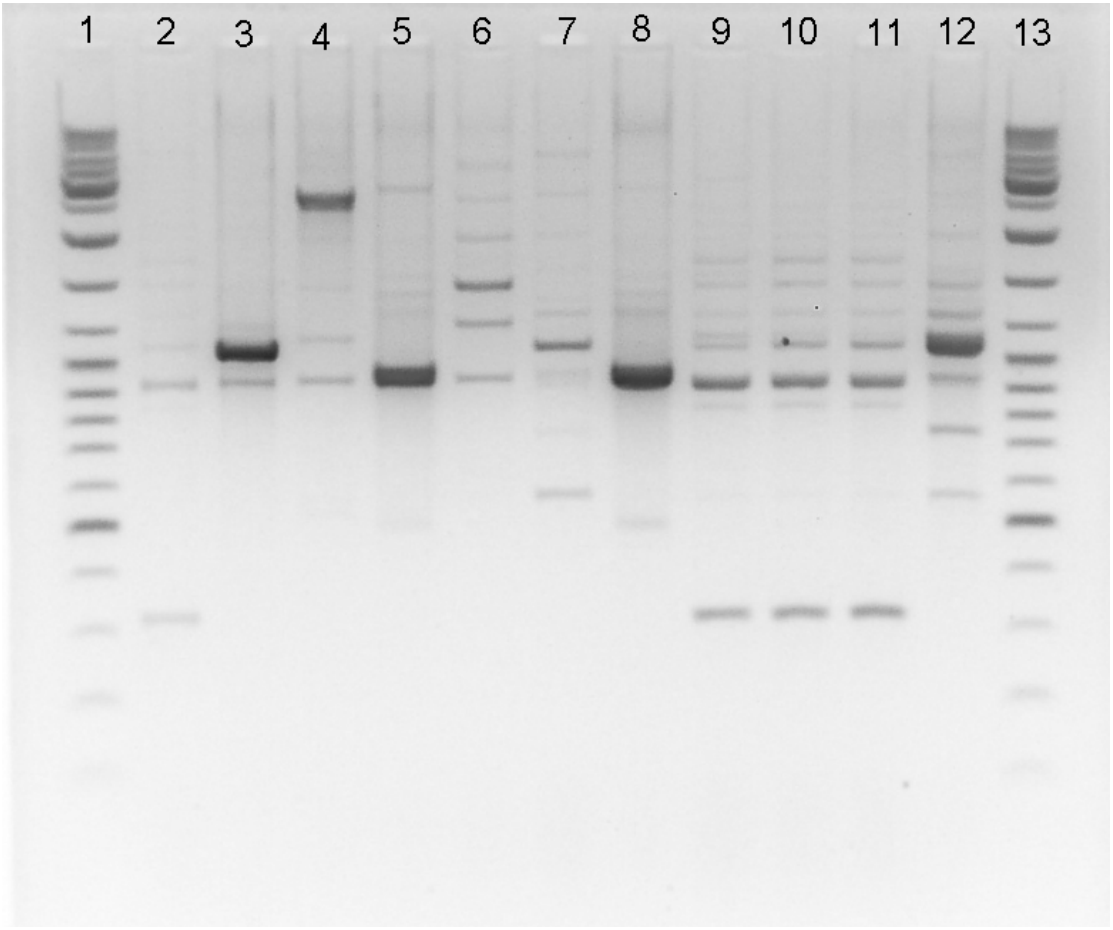

Figure 7.3: Gel electrophoresis depicting the RAPD banding pattern obtained with primer 275. Lane 1: Marker (100 bp to 10 000 bp). Lane 2: Db24. Lane 3: Db32. Lane 4: Db3. Lane 5: Db7. Lane 6: Db9. Lane 7: Db11. Lane 8: Db12. Lane 9: Db15. Lane 10: Db16. Lane 11: Db19. Lane 12: Db21. Lane 13: Marker (100 bp - 10 000 bp)

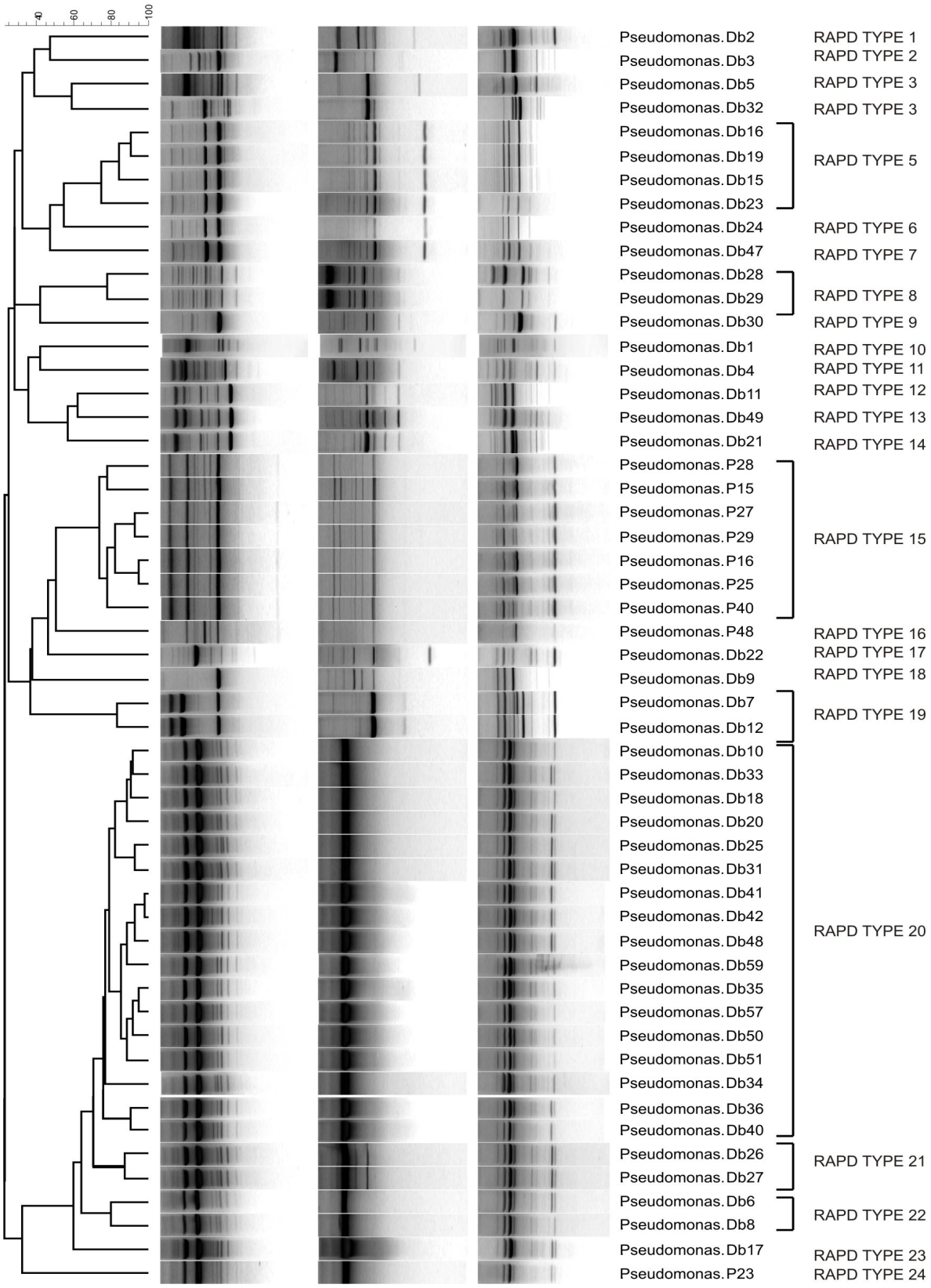

Figure 7.4: Dendogram obtained through RAPD typing with primers 277, 272 and 275 depicting the clonal relationship between GES-positive *P. aeruginosa* clinical isolates obtained from the Gauteng region of South Africa

Chapter 8

Concluding remarks

Pseudomonas aeruginosa continues to be a major nosocomial pathogen despite the advances in hospital care and the introduction of a wide variety of antimicrobial agents (Matar *et al*., 2005). Extended-spectrum β-lactamases (ESBLs) of the Guiana extended-spectrum (GES)-type have been reported increasingly in Gram-negative bacilli, including *P. aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (Giakkoupi *et al*., 2000; Poirel *et al*., 2000; Weldhagen *et al*., 2003; Poirel *et al*., 2005). Currently nine GES enzymes have been described (Poirel *et al*., 2005). Beta-lactamase GES-1 and GES-9 from France (Poirel *et al*., 2000; Poirel *et al*., 2005), GES-2 from South Africa (Poirel *et al*., 2001), GES-3 and GES-4 from Japan (Wachino *et al*., 2004a; Wachino *et al*., 2004b) and GES-5, GES-6, GES-7 and GES-8 from Greece (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001; Vourli *et al*., 2004). In addition, GES-5 has also recently been reported in Korea and China (Jeong *et al*., 2005; Wang *et al*., 2006). Clinical laboratory detection (e.g. double disk synergy test and Etest ESBL strips) of ESBLs was difficult and there were no tests recommended by the Clinical and Laboratory Standards Institute (CLSI) for the detection of ESBLs in *P. aeruginosa* (Jacoby and Munoz-Price, 2005).

In this study conventional PCR with *bla*$_{GES}$ and class 1 integron specific primers together with DNA sequencing, competitive PNA-based multiplex PCR and RAPD analysis were used to determine the prevalence of GES-type ESBLs, characterise their genetic determinants and determine their clonal relatedness. The study further investigated sequence-selective, competitive PNA-based multiplex PCR in real-time for the identification and differentiation of GES-type ESBL enzymes.

The prevalence of GES-type ESBLs was determined successfully in 100 clinical *P. aeruginosa* isolates by means of DNA sequencing. The PNA-based multiplex PCR was not as sensitive as a *bla*$_{GES}$ specific primer set in an optimised master mix and could not accurately differentiate between the GES variants due to new GES variants containing mutations outside and inside the PNA-probe target site. The noted increase in GES-2 prevalence since 2000 emphasised the importance of constant surveillance to monitor

antibiotic determinants, their spread and overall prevalence. Knowledge of GES prevalence may be used in turn to monitor the efficacy of infection control measures and antibiotic therapy regimens.

The *bla*$_{GES-5}$ genes identified during the prevalence study were successfully described through DNA sequencing of the gene itself and the class 1 integron preceding the gene. The GES-5 gene described was similar to the *bla*$_{GES-5}$ described by Vourli and co-workers (2004) with 99% homology to this gene (GenBank accession number AY494717). This study was the first to describe GES-5 in South Africa and was the second description of this ESBL occurring in *P. aeruginosa*. GES-5 was the third GES-type ESBL enzyme to be described in South Africa. The emergence of GES-type ESBLs indicates that antibiotic regimens currently in-use may facilitate the spread of *bla*$_{GES}$ genes and their establishment in pathogenic bacteria such as *P. aeruginosa* due to antimicrobial selective pressure.

The genetic locations of selected *bla*$_{GES-2}$ and *bla*$_{GES-5}$ genes were successfully determined by applying the PNA-based multiplex PCR to plasmid and chromosomal DNA as template. Results showed that most *bla*$_{GES-2}$ genes were present on both plasmids and the chromosome in the majority of isolates. As far as could be established this study described the first occurrence of *bla*$_{GES-2}$ genes on both plasmids and the chromosome in a single *P. aeruginosa* isolate. The multiple genetic location of the *bla*$_{GES-2}$ gene may be the result of class 1 integron association with transposons. The *bla*$_{GES-5}$ genes investigated were located on plasmids exclusively. The maintenance of the *bla*$_{GES-2}$ gene on both plasmids and the chromosome further indicated a selective pressure for these β-lactamases in the South African clinical setting.

Application of the PNA-based multiplex PCR in real-time utilising SYBR Green did not yield satisfactory results and was not suitable for the identification and differentiation of *bla*$_{GES}$ genes. The method was not appropriate since melting temperatures obtained for different GES variants showed little difference and overlapped in some cases (Figure 6.8). Furthermore, the PNA-probe was not entirely competitive as indicated by the amplification of the entire *bla*$_{GES}$ gene resulting in a 864 bp product. As a result the T_m values observed did not reflect the competitive amplification of a 360 bp product or a 360 bp product together with a 505 bp amplification product as was expected. It was more likely that the T_m values obtained reflected homology to the PNA-probe together with amplicon length and GC

content. However, the expected basic melting temperatures were between 85°C and 86°C irrespective of GC content or length. The T_m values observed were thus the result of the PNA-probe, but not a direct reflection of homology to the probe. Although the PNA-based multiplex PCR did not offer a suitable assay, such a method could still be potentially viable. Utilising the high temperature shifts caused by PNA binding instead of its competitive nature may result in a more reliable assay. Repositioning of the PNA-probe and primers could result in larger and more accurate temperature differences between the GES variants.

Random amplified polymorphic DNA analysis was successfully applied to determine the clonal relatedness of the *bla*$_{GES}$ positive *P. aeruginosa* isolates studied. The method proved simple, effective and rapid as described by Mahenthiralingam and co-workers, (1996). The use of a PCR master mix further simplified the method and may have improved reproducibility by standardising the amplification conditions. The results indicated nosocomial spread of isolates in hospitals, stressing the importance of more stringent infection control measures. The study further showed spread of a single *P. aeruginosa* clone between hospitals, indicating spread from a common external reservoir into these hospitals.

The occurrence of highly drug-resistant *P. aeruginosa* isolates in the environment has serious implications in a country with an ever increasing immune-compromised population. It was observed that genotypically and geographically diverse *P. aeruginosa* isolates have acquired the GES ESBL genes since the initial isolation of *P. aeruginosa* GW-1 in 2000 harbouring the *bla*$_{GES-2}$ gene. This suggests that contact with still unknown bacterial reservoirs may contribute to the evolution of *P. aeruginosa* towards multidrug-resistance in South Africa. The above findings were of concern since it demonstrated that acquired GES ESBLs can rapidly emerge and become a major cause of broad-spectrum β-lactam resistance among nosocomial pathogens as also pointed out by Lagatolla and co-workers, (2004).

Future Research

Future efforts should be directed towards the establishment of surveillance programmes to monitor the prevalence and spread of these antibiotic resistance determinants in South Africa. Data obtained would be useful in determining the efficacy of current infection control measures and antibiotic regimens. The rapid, accurate and economical identification of these determinants would be necessary in such a programme. Real-time PCR is still a viable avenue to explore. The introduction of simple probes (Roche) may offer a suitable chemistry able to differentiate the GES variants. Alternatives may include pyrosequencing (Poirel *et al*., 2006), but the cost effectiveness of such a technique remains to be established.

Concerted efforts should be directed towards creating better awareness of the ESBL threat and employing more stringent infection control measures. Strategies such as the use of antibiotic administration protocols, antibiotic rotation, de-escalation therapy in certain patients or restrictions in the hospital formulary should be employed to optimise the correct use of antibiotics (Chlebicki and Oh, 2004). These strategies may help to balance the need for providing adequate initial antibiotic cover to high-risk patients, with avoidance of unnecessary antibiotic utilisation which may promote resistance (Chlebicki and Oh, 2004).

References

Chlebicki MP, Oh HML (2004) Extended-spectrum beta-lactamases in clinical isolates of *Esherichia coli* and *Klebsiella spp.* in a Singapore Hospital: Clinical spectrum. *Ann. Acad. Med. Singapore.* **33:** 302-306.

Giakkoupi P, Tzouvelekis LS, Tsakris A, Loukova V, Sofianou D, Tzelepi E (2000) IBC-1, a novel integron-associated class A beta-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. *Antimicrob. Agents Chemother.* **44:** 2247-2253.

Jacoby GA, Munoz-Price LS (2005) Mechanisms of disease: The new beta-lactamases. *N. Engl. J. Med.* **352:** 380-391.

Jeong SH, Hong SG, Song JS, Lee JH, Lee SH (2005) First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. *Antimicrob. Agents Chemother.* **49:** 4809-4810.

Lagatolla C, Tonin EA, Monti-Bragadin C, Dolzani L, Gombac F, Bearzi C, Edalucci E, Gionechetti F, Rossolini GM (2004) Endemic carbapenem-resistant *Pseudomonas aeruginosa* with acquired metallo-beta-lactamase determinants in European hospital. *Emerg. Infect. Dis.* **10:** 535-538.

Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Spreet DP (1996) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34:** 1129-1135.

Matar GM, Chaar MH, Araj GF, Srour Z, Jamaleddine G, Hadi U (2005) Detection of a highly prevalent and potentially virulent strain of *Pseudomonas aeruginosa* from nosocomial infections in a medical center. *BMC Microbiol.* **5:** 29-35.

Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, Tzouvelekis LS (2001) An integron-associated beta-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum beta-lactamase IBC-1. *J. Antimicrob. Chemother.* **48:** 627-630.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analysis of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44:** 622-632.

Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P (2001) GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrob. Agents Chemother.* **45:** 2598-2603.

Poirel L, Brinas L, Fortineau N, Nordmann P (2005) Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity towards aztreonam in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49:** 3593-3597.

Poirel L, Naas T, Nordmann P (2006) Pyrosequencing as a rapid tool for identification of GES-type extended-spectrum beta-lactamases. *J. Clin. Microbiol.* **44:** 3008-3011.

Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004) Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol. Lett.* **234:** 209-213.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004a) Nosocomial spread of ceftazidime resistant *Klebsiella pneumoniae* strains producing a novel class A beta-lactamase, GES-3, in a neonatal intensive care unit in Japan. *Antimicrob. Agents Chemother.* **48:** 1960-1967.

Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Ito H, Arakawa Y (2004b) Molecular characterization of a cephamycin-hydrolyzing and inhibitor resistant class A beta-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. *Antimicrob. Agents Chemother.* **48:** 2905-2910.

Wang C, Cai P, Chang D, Mi Z (2006) A *Pseudomonas aeruginosa* isolate producing the GES-5 extended-spectrum beta-lactamase. *J. Antimicrob. Chemother.* **57:** 1261-1262.

Weldhagen GF, Poirel L, Nordmann P (2003) Amber class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. *Antimicrob. Agents Chemother.* **47:** 2385-2392.

Appendix A: *bla*$_{GES}$ Sequences

P48 Forward Primer GES-1A

CGTTTTGCATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAG
CGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAG
CATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCT
CTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGC
TAGACCGGAAAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTA
TGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTG
GCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAA
AACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTA
GCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGT*

P48 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

P48 Reverse Primer GES-1B

TCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGA
TCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAA
GTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATAT
GGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCG
AGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACC
TGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATG
AGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCC
TCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGA
CGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGC
CGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAAC
TATCGGCCGTAGAACGTGACGAATTA*

Db24 Forward Primer GES-1A

CGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCA
GCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTG
CAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGG
GGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCG
GGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTAC
TGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGA
CCGGAAAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGC
ACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTG
ATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACT
GGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGG
TGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAG*

P29 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG

GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCG*

P29 Reverse Primer GES-1B

CGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGC
GATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTC
AAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCAT
ATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCT
CGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGA
CCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGA
TGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGT
CCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGA
GACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGG
GCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAA
ACTATCGGCCGTAGAACGTGACGA*

P48 Forward Primer GES-1A

AAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTC
GATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTC
CGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCC
GGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCA
GCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTG
CAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAGCGA
CAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATG
GCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGA
CACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAA
CGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCG
GCCGTAGAACGTGACGAATTAGTTGCCTCTGTC*

P48 Forward Primer GES-1A

AGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAG
AGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTG
GTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCG
AATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGT
GCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTAT
TTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAGCGACAACACACCTGGCG
ACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGAC
GTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGG
TTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTT
TTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGA
CGAATTAGTTGCCTCTGTC*

P48 Reverse Primer GES-1B

GCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCG
GTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTC
AACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAA
CTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGAC
GGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATT
GGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGC
CGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGC
TAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAA
ACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCA
ACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGC
CCCGAAACTATCGGCCGTAGAACGTG*

P48 Reverse Primer GES-1B

AAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTC
GATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTC
CGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCC
GGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCA
GCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTG

CAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAGCGA
CAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATG
GCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGA
CACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAA
CGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCG
GCCGTAGAACGTGACGAATTAGTTGC*

Db24 Forward Primer GES-1A

TTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATC
CCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCT
AGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGAC
ATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGC
AAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAAT
GACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAGCGACAAC
ACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCG
GCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACAC
TACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGA
CATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCC
GTAGAACGTGACGAATTAG*

Db24 Reverse Primer GES-1B

GCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCG
GTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTC
AACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAA
CTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGAC
GGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATT
GGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGC
CGGAGATGAGCGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGC
TAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAA
ACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCA
ACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGC
CCCGAAACTATCGGCCGTAGAACG*

Db1 Forward Primer GES-1A

GACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGA
GATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGG
TCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGA
ATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTG
CAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATT
TTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCG
ACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGAC
GTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGG
TTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTT
TTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGA
CGAATTAG*

Db2 Forward Primer GES-1A

CTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCG
CGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAA
AGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTC
CTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAG
CGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAA
ATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGA
GATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTC
GACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAA
AGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCC
CAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTG*

Db5 Forward Primer GES-1A

TTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATC
CCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCT
AGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGAC

ATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGC
AAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAAT
GACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAAC
ACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCG
GCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACAC
TACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGA
CATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCC
GTAGAACGTGACGAATTAGTTGCCT*

Db6 Forward Primer GES-1A

GATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTC
AAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCAT
ATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCT
CGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGA
CCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGA
TGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGT
CCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGA
GACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGG
GCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAA
ACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

Db8 Forward Primer GES-1A

ATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCA
AGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATA
TGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTC
GAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGAC
CTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGAT
GAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTC
CTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAG
ACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGG
CCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAA
CTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

Db10 Forward Primer GES-1A

CGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTT
CAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCA
TATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCT
CGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGA
CCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGA
TGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGT
CCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGA
GACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGG
GCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAA
ACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTC*

Db18 Forward Primer GES-1A

GCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGT
TCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTC
ATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTT
CTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCG
GACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGA
GATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAA
GTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGG
GAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGG
GGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCG
AAACTATCGGCCGTAGAACGTGACGAATTAGTTGCC*

Db20 Forward Primer GES-1A

TCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGG
AGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCG
CTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCG
TCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGC

GGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAG
TATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTG
GCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACT
GACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGC
GGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGT
TTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAAC
G*

Db25 Forward Primer GES-1A

ATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGT
TGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACG
TTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTT
CATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGT
TCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGC
GGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGG
AGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAA
AGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACG
GGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACG
GGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCC
GAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCC*

Db26 Forward Primer GES-1A

TCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGA
TCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAA
GTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATAT
GGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCG
AGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACC
TGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATG
AACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCC
TCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGA
CGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGC
CGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAAC
TATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCG*

Db27 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

Db31 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db33 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db34 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTC*

Db35 Forward Primer GES-1A

GACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGA
GATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGG
TCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGA
ATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTG
CAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATT
TTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCG
ACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGAC
GTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGG
TTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTT
TTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGA
CGAATTAGTTGCCTCTGTCG*

Db36 Forward Primer GES-1A

AAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTC
GATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTC
CGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCC
GGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCA
GCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTG
CAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGA
CAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATG
GCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGA
CACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAA
CGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCG
GCCGTAGAACGTGACGAATTAGTTGCCTCTGTCG*

Db40 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA

ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCG*

Db41 Forward Primer GES-1A

TTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAG
GAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGC
GCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATC
GTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTG
CGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCA
GTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCT
GGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCAC
TGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAG
CGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGG
TTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAA
CGTGACGAATTAGTTGCCTCTGTC*

Db42 Forward Primer GES-1A

AACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCC
CAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAG
CCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACAT
GATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAA
GCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGA
CGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACAC
ACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGC
GCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTA
CGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACA
TTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGT
AGAACGTGACGAATTAGTTGCCTCTG*

Db48 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db50 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTGTC*

Db51 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG

ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

Db52 Forward Primer GES-1A

AAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGT
CGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTT
CCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGC
CGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGC
AGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCT
GCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACG
ACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTAT
GGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCG
ACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGA
ACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATC
GGCCGTAGAACGTGACGAATTAGTTGCC*

Db57 Forward Primer GES-1A

GGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATC
GTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGT
TTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGG
GCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAG
GCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTG
CTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAA
CGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTC
TATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGAC
GCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCC
GGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACT
ATCGGCCGTAGAACGTGACGAATTAG*

Db59 Forward Primer GES-1A

ATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGT
TGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACG
TTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTT
CATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGT
TCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGC
GGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGG
AGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAA
AGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACG
GGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACG
GGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCC
GAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTG*

P23 Forward Primer GES-1A

GGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATC
GTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGT
TTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGG
GCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAG
GCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTG
CTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAA
CGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTC
TATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGAC
GCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCC
GGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACT
ATCGGCCGTAGAACGTGACGAATTAGTTGCCTC*

P28 Forward Primer GES-1A

TTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATG
GGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGA
GGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCT

GCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGA
ACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCT
CTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGAC
GCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCC
GGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACT
ATCGGCCGTA*

Db32 Forward Primer GES-1A

ATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGG
ATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGG
ACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTG
AGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACC
GGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCAC
GTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGAT
CGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGG
TACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTG
TATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTACACA
*

Db3 Forward Primer GES-1A

CGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCA
GCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTG
CAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGG
GGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCG
GGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTAC
TGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGA
CCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGC
ACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTG
ATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACT
GGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGG
TGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTAC*

Db7 Forward Primer GES-1A

CGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCA
GCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTG
CAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGG
GGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCG
GGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTAC
TGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGA
CCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGC
ACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTG
ATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACT
GGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGG
TGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTA*

Db9 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTACACAA
CTCTC*

Db11 Forward Primer GES-1A

GTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTT
TCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGG

TTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGG
CGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCG
GATATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTA
AAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAAC
GGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAAC
GGGGGCCGGTAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCC
CCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db12 Forward Primer GES-1A

CGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGAT
CGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAG
TTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATG
GGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGA
GGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCT
GCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGA
ACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCT
CTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGAC
GCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCC
GGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACT
ATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTAT*

Db15 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db16 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTACACAA
CTC*

Db19 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAG*

Db21 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTT*

Db22 Forward Primer GES-1A

CGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTT
TGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGG
TCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGC
TTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGT
AAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTC
AGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCA
CCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCC
TAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAA
GCCCAGGAGAGATATACGCTGTAGCGGTGTATACAACGGCCCCTAAACTATCGGCCGTAGAACGTGACGAATT
AGTTGCCTCTGTCGG*

Db23 Forward Primer GES-1A

CGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCT
TTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATG
GTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAG
CTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCG
TAAAATTGGCGACTCTGTGAGCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTC
AGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCA
CCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCC
TAAAGATTGGGTGTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGC
CCAGGAGAGAGATTACGCTGTAGCGGCGATTACAACGGCCCCGAAATATCGGCG*

Db28 Forward Primer GES-1A

ATCCCCAAGGAGAGATACGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTC
CGCTAGCCGCGCTGGCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCG
GACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAG
CGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGC
AATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGAC
AACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATG
GCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGA
CACTACCAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTCCTGCGCCAACGGGGGCCGGAAC
GACATTGGTTTTTTTAAAGCCCAGGAGAGA*

Db29 Forward Primer GES-1A

CGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGC
TCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAA
AACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATG
ACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAA
TTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGA
GCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTG
GCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACC
AAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGC
CAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACG
GCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db30 Forward Primer GES-1A

CTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACC
GAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGAC
TCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGG
AGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGG
GGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACT
CTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTA
CGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACC
ATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTG
TTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGA
TTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCG
GTCAAGT*

Db47 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTACACAA
CTCATCGAG*

Db49 Forward Primer GES-1A

AGAGTACGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCG
CTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACATGATCG
TCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGC
GGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAG
TATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACACCTG
GCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACT
GACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTCGAGCG
GGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACTTGGTTT
TTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCGAAACTATCGGCCGAGAACGTG
ACGAATAGTTGCCTCTGTCGGCAAGTTTTACACAATCATCC*

Db17 Forward Primer GES-1A Chromosomal DNA

CGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCA
GCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTG
CAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGG
GGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCG
GGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTAC
TGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGA
CCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGC
ACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTG
ATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACT
GGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGG
TGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTAC*

Db17 Forward Primer GES-1A Plasmid DNA

CGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCA
GCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTG
CAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGG
GGATCGAAAACTTTCATATGGGCCGGACATGATCGTCAAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCG
GGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTAC
TGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGA
CCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGC
ACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTG
ATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACT

GGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGG
TGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGG*

Db4 Forward Primer GES-1A Chromosomal DNA

TGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCTCAGATCGGTGTT
GCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGT
TCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTC
ATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTT
CTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCG
GACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGA
GATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAA
GTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGG
GAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGG
GGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTATACAACGGCCCCG
AAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTACA*

Db4 Forward Primer GES-1A Plasmid DNA

GATCGCTCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAA
GCAGCTCAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTT
TTGCAATGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGG
GGGGATCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCAT
CGGGACACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTT
ACTGAGAGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTA
GACCGGAAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATG
GCACGTACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGC
TGATCGGAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAA
CTGGTACCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGC
GGTGTATACAACGGCCCCGAAACTATCGGCCGTAGAACGTGACGAATTAGTTGCCTCTGTCGGTCAAGTTATTA
CAC*

P15 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGG*

P16 Forward Primer GES-1A

TCACTCTGCATATGCGTCGGAAAAATTAACCTTCAAGACCGATCTTGAGAAGCTAGAGCGCGAAAAAGCAGCT
CAGATCGGTGTTGCGATCGTCGATCCCCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAA
TGTGCTCAACGTTCAAGTTTCCGCTAGCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGA
TCGAAAACTTTCATATGGGCCGGACATGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGAC
ACATGACGGTTCTCGAGGCAGCGCAAGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAG
AGAAATTGGCGGACCTGCTGCAATGACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGG
AAAGAGCCGGAGATGAACGACAACACACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGT
ACTGTGGCTAAAGTCCTCTATGGCGGCGCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCG
GAAACCAAACGGGAGACGCGACACTACGAGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTA
CCTGCGCCAACGGGGGCCGGAACGACATTGGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGTGTA
TACAACGGCCCCGAAACTATCGGCCGTAGAAC*

P25 Forward Primer GES-1A

CCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCAACGTTCAAGTTTCCGCTA
GCCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACA
TGATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCA
AGCTGCGGTGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATG
ACGCAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACACAC

ACCTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGTCCTCTATGGCGGC
GCACTGACGTCCACCTCGACCCACACCATTGAGAGGTGGCTGATCGGAAACCAATACGGGAGACGCGACACTA
CGAGCGGGTTTTCCTAAAGATTGGGGGGAGAGAAAACTGGACCTGCGCCAACGGGGGCCGGAACGACATTGG
TTTTTTTAAAGCCCAGGAGAGAGATTACGCTGTAGCGGGTATACAACGGCCCCGAAACTATCGGCCGAGAACG
TGA*

P40 Forward Primer GES-1A

CCAAGGAGAGATCGTCGCGGGCCACCGAATGGCGCAGCGTTTTGCAATGTGCTCACGTTCAAGTTTCCGCTAG
CCGCGCTGGTCTTTGAAAGAATTGACTCAGGCACCGAGCGGGGGGATCGAAAACTTTCATATGGGCCGGACAT
GATCGTCGAATGGTCTCCTGCCACGGAGCGGTTTCTAGCATCGGGACACATGACGGTTCTCGAGGCAGCGCAG
CTGCGGGCAGCTTAGCGACAATGGGGCTACTAACCTCTTACTGAGAGAAATTGGCGGACCTGCTGCAATGACG
CAGTATTTTCGTAAAATTGGCGACTCTGTGAGTCGGCTAGACCGGAAAGAGCCGGAGATGAACGACAACACAC
CTGGCGACCTCAGAGATACAACTACGCCTATTGCTATGGCACGTACTGTGGCTAAAGCCTCTATGGCGGCGCA
CTGACGTCCACCTCGAACCCACACCATTGAGAGGTGGCTGATCGGAAACCAAACGGGAGACGCGACACTACG
AGCGGGTTTTCCTAAAGATTGGGTTGTTGGAGAGAAAACTGGTACCTGCGCCAACGGGGGCCGGAACGACATT
GGTTTTTTTAAAGCCCAGGAGAGAGATTACGCTGT*