Laboratory Detection and Gene Cassette Stability of the Novel Extended-Spectrum Beta-Lactamase, GES-2 from *Pseudomonas aeruginosa*.

By

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THIS THESIS IS DEDICATED TO A CLOSER UNDERSTANDING OF OUR NATURAL WORLD.

A SPECIAL WORD OF GRATITUDE GOES OUT TO MY WIFE MARLI WELDHAGEN, FOR HER ENDURING SUPPORT AND UNDERSTANDING DURING THE TIME IT TOOK TO CONDUCT THIS STUDY.

DECLARATION:

To my knowledge the work contained in this thesis is original, was undertaken by myself with assistance as indicated in the acknowledgements. The interpretation and analysis of data were my primary responsibilities.

It is being submitted for the degree Doctor of Philosophy (Medical Microbiology) at the University of Pretoria. It has not been submitted before for any degree or examination at any other university.

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CONTENTS

SUMMARY:

Extended-spectrum beta-lactamases (ESBLs) in *Pseudomonas aeruginosa* tend to be geographically scattered, such as GES-2, which partially compromises the efficacy of imipenem. The G170N mutation, ascribed to a CC to AA base pair substitution on positions 493-494 of the bla_{GES-2} coding region, distinguishes this ESBL from bla_{GES-1} and the bla_{IBC}-type genes, making it an ideal target for developing a novel sequence-specific, peptide nucleic acid (PNA)-based, multiplex-PCR detection method. Utilizing two primer pairs in conjunction with a PNA probe, this novel method delivered accurate identification of bla_{GES-2} compared to standard PCR and gene sequencing techniques, when tested against one hundred (n = 100) *P. aeruginosa* clinical isolates as well as previously published, well-described control strains. This method has the potential to be used in large-scale, cost-effective screening programmes for specific or geographically restricted ESBLs.

To date, in addition to being only described in South Africa, GES-2 is notoriously difficult to identify in *P. aeruginosa*, using standard methodology. A real-time PCR method using the LightCycler™ was compared to a two-step nested-PCR assay for the detection of bla_{GES} and bla_{IBC} genes from one hundred *P. aeruginosa* clinical isolates collected over a four-year period from two teaching hospitals in Pretoria, South Africa. Real-time PCR amplification was monitored through hybridisation of fluorescently labelled probes followed by melting curve analysis to detect the relevant G170N mutation occurring in the omega loop region of bla_{GES-2}. Nested-PCR products were subjected to automated DNA sequencing and compared to melting point (*Tm*) analyses results obtained from the LightCycler assay. Real time and nested-PCR assays detected a $bla_{GES/IBC}$ gene product from 83 and 88 clinical isolates respectively, with the LightCycler thus exhibiting a sensitivity of 94.3% compared to the nested-PCR assay. Comparison of *Tm* and gene sequencing data however revealed 100% specificity for sequence

specific detection of *bla*$_{GES-2}$ with the LightCycler. One clinical isolate was found to harbour a *bla*$_{GES-1}$ gene, making this the first report of this specific ESBL from South Africa.

Selective antibiotic pressure has recently been implicated as a possible driving force behind point mutations observed in *bla*$_{GES}$–type genes. This part of the study subjected two well-characterized clinical isolates with class 1 integron-borne *bla*$_{GES}$-type genes to five days incubation in the presence of sub-inhibitory concentrations of 15 different antibiotics, including beta-lactams, aminoglycosides and quinolones. Restriction enzyme analysis and DNA sequencing of *bla*$_{GES-1}$, *bla*$_{GES-2}$ and their immediate upstream genetic environments failed to demonstrate any changes compared to non-exposed controls. Short-term exposure to a sub-inhibitory level of a single antimicrobial agent is thus unlikely to select significant mutations in these beta-lactamase genes or their regulatory mechanisms.

Word count = 426.

Keywords: *Pseudomonas aeruginosa*, *bla*$_{GES}$, peptide nucleic acid, LightCycler, genetic stability, antibiotic selective pressure.

SAMEVATTING:

Uitgebreide-spektrum beta-laktamases (ESBLs) in *Pseudomonas aeruginosa* neig om geografies versprei te wees, soos GES-2 wat die doeltreffendheid van imipenem benadeel. Die G170N mutasie, toegeskryf aan `n CC na AA basis paar verandering op posisies 493-494 van *bla*$_{GES-2}$, onderskei hierdie ESBL van *bla*$_{GES-1}$ en die *bla*$_{IBC}$-tipe gene. Die mutasie is dan ook `n ideale teiken vir die ontwikkeling van `n nuwe, volgorde-spesifieke, peptied-nukleiënsuur (PNA) gebasseerde, multiplex-PKR diagnostiese metode. Deur die gebruik van twee pare oligonukleotied voorlopers in samewerking met `n PNA merker, het hierdie metode *bla*$_{GES-2}$ akkuraat geidentifiseer vanuit 100 *P. aeruginosa* kliniese isolate, asook vanuit goed-beskryfde verwysings isolate. Die metode het verder die potensiaal om in grootskaalse, koste-effektiewe, siftings programme gebruik te word vir die opsporing van spesifieke of geografies beperkte ESBLs.

GES-2, wat huidiglik slegs in Suid Afrika beskryf is, is ook besonder moeilik om te diagnoseer vanuit *P. aeruginosa* met standaard metodologie. Die LightCycler was vergelyk met `n dubbel-stap PKR metode, vir die identifikasie van *bla*$_{GES}$ en *bla*$_{IBC}$-tipe gene vanuit 100 *P. aeruginosa* kliniese isolate versamel vanaf twee akademiese hospitale in Pretoria, Suid Afrika, oor `n vier jaar periode. PKR amplifikasie met die LightCycler was gemoniteer dmv hibridisasie van fluoreserende merkers en smeltpunt analise (*Tm*), om sodoende die G170N mutasie in die omegalus area van *bla*$_{GES-2}$ op te spoor. Produkte verkry vanaf die dubbel-stap PKR metode is onderwerp aan outomatiese DNS volgorde bepaling asook vergelyk met *Tm* resultate verkry vanaf die LightCycler metode. Die LightCycler en dubbel-stap PKR metodes het *bla*$_{GES/IBC}$-tipe gene geidentifiseer vanuit 83 en 88 kliniese isolate onderskeidelik. Vir die identifisering van *bla*$_{GES-2}$ het die LightCycler metode dus `n sensitiwiteit van 94.3% en `n spesifisiteit van 100% behaal, vergeleke met die dubbel-stap PKR en DNS volgorde bepaling metodes onderskeidelik. Die eerste *bla*$_{GES-1}$ geen in Suid Afrika is in een kliniese isolaat aangetoon.

Selektiewe antibiotiese druk is onlangs geimpliseer om `n rol te speel in die formasie van punt mutasies wat in *bla*$_{GES}$-tipe gene waargeneem word. Hierdie deel van die studie het twee goed beskryfde kliniese isolate met klas 1 integron gedraagde *bla*$_{GES}$-tipe gene, onderwerp aan sub-inhibitoriese antibiotika konsentrasies vir `n tydperk van vyf dae. Vyftien verskillende antibiotika is gebruik, insluitende beta-laktams, aminoglikosiede en kinolone. Restriksie ensiem analise en DNS volgorde bepaling van *bla*$_{GES-1}$, *bla*$_{GES-2}$ en hul direkte stroomop genetiese omgewing, kon geen genetiese veranderinge aantoon nie. Kort-termyn blootstelling van hierdie genetiese strukture aan sub-inhibitoriese vlakke van enkel antimikrobiese middels is dus onwaarskynlik om enige noemenswaardige mutasies te veroorsaak.

Aantal woorde = 402.

Sleutel woorde: *Pseudomonas aeruginosa*, *bla*$_{GES}$, peptied nukleiënsuur, LightCycler, genetiese stabiliteit, selektiewe antibiotiese druk.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems Inc.
AIDS	acquired immunodeficiency syndrome
AMK	amikacin
AMP	ampicillin
AmpC	chromosomal located cephalosporinase
API	analytical profile index
ATCC	American Type Culture Collection
be	base element
Bla	beta-lactamase
BLAST	basic local alignment search tool
Bp	base pair(s)
C	centigrade
ca	circa
CARB	carbenicillinase
CDC	Centers for Disease Control (and Prevention) (USA)
CFZ	cefazolin
CAZ	ceftazidime
CXM	cefuroxime
CIP	ciprofloxacin
Co	company
COL	colistin sulphate
Corp	corporation
CSF	cerebrospinal fluid
CTX-M	cefotaximase
DNA	deoxyribonucleic acid, complementary DNA
DNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ESBL	extended-spectrum beta-lactamase
F	fluorescence
FIGE	field inversion gel electrophoresis
FITC	fluorescein isothiocyanate
FL	fluorescein

FRET	fluorescence resonance energy transfer
g	acceleration due to gravity
GEN	gentamicin
GES	Guiana extended spectrum beta-lactamase
h	hour(s)
HIV	human immunodeficiency virus
IBC	integron-borne cephalosporinase
IC_{50}	50% inhibitory concentration
ICU	intensive care unit
IEF	iso-electric focussing
IMP	beta-lactamase named after preferred substrate (imipenem)
In	integron
IPM	imipenem
IS	insertion sequence
IU	international unit(s)
Kcat	catalytic kinetic constant
Kg	kilogram(s)
KLUA	beta-lactamase named after *Kluyvera ascorbata*
Km	Michaelis-Menten kinetic constant
L	litre(s)
LC	LightCycler
LCR	LightCycler red
Ltd	limited
M	molecular weight
MBC	minimum bactericidal concentration
MEM	meropenem
MIC	minimum inhibitory concentration
Min	minute(s)
MW	molecular weight
NAL	nalidixic acid
NCCLS	National Committee for Clinical Laboratory Standards
NE	non-enteric
NIT	nitrofurantoin

NMR	nuclear magnetic resonance
Nt	nucleotide(s)
OFX	ofloxacin
OprD	operon D / porin D
ORF	open reading frame
OXA	oxacillinase
PAGE	polyacrylamide gel electrophoresis
PAH	Pretoria Academic Hospital
PCR	polymerase chain reaction
PER	beta-lactamase named after original authors (P. Nordmann, E. Ronco R. Labia)
PFGE	pulsed-field gel electrophoresis
PH	phosphorylation
pI	iso-electric point
PNA	peptide nucleic acid
PSE	beta-lactamase specifically named after *P. aeruginosa*
Pty	property
R	purine (A or G)
RNA	ribonucleic acid
Rpm	revolutions per min
s	second(s)
SD	standard deviation
SDS	sodium dodecyl sulphate
SET	Salt-EDTA-Tris buffer
SHV	sulfhydryl variable beta-lactamase
sp.	species (singular)
spp.	species (plural)
SXT	trimethoprim/sulfamethoxazole
T	temperature
$t_{½}$	half-life
Taq	polymerase named after *Thermus aquaticus*
TBE	tris – borate - EDTA buffer
TE	tris-EDTA buffer
TEM	beta-lactamase named after first patient isolated from (Temarina)

Tm	melting point
Tn	transposon
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
TZP	piperacillin/tazobactam
U	unit(s)
UK	United Kingdom
URL	unique resource location
UV	ultraviolet
VEB	Vietnamese extended spectrum beta-lactamase
VIM	Veronese integron-borne metallo beta-lactamase
V_{max}	maximum rate of metabolism
WHO	World Health Organization
Wt	wild type
Y	pyrimidine (C or T)

CHAPTER 1: INTRODUCTION.

1.1 General introduction

Extended-spectrum beta-lactamases (ESBLs) that are inhibited by clavulanic acid, belong mostly to class A of the Ambler classification scheme, conferring resistance to a wide variety of expanded-spectrum cephalosporins. To date however, a bewildering variety of enzymes have been described that can be classified as set out in Table 1-1. After being widely reported in *Enterobacteriaceae* isolates from the early 1980`s, ESBLs have been described in *Pseudomonas aeruginosa* only more recently (1, 2, 3). These enzymes described in *P. aeruginosa*, are either of the TEM- and SHV-types that are also well known in *Enterobacteriaceae*, or of the PER-type mostly originating from Turkey, or of the VEB-type from Southeast Asia and more recently, of the GES / IBC types originally reported from French Guinea, France, Greece and South Africa, respectively (4, 5, 6, 7). These five types of enzymes are remotely related, both from a genetic point of view and with respect to similarities in hydrolytic profiles. To date, CTX-M-type enzymes have not yet been described in *P. aeruginosa*.

Recent studies indicated that these enzymes may play an important role in the dissemination of antibiotic resistant bacterial isolates and may condition future choices of antibiotic regimens for treating life-threatening infections due to ESBL-producing *P. aeruginosa* (8, 9). Recent work further indicated the propensity of ESBL producing *P. aeruginosa* to establish long-term residence in the nosocomial environment, making re-infection an imminent danger (10). After the discovery of the novel ESBL, GES-2 from *P. aeruginosa*, in the Pretoria Academic Hospital (PAH) in May of 2000 (7), it was clear that class 1 integron borne ESBLs were established in the South African nosocomial setting (Discussed in detail in Chapter 3). The same *P. aeruginosa* strain subsequently caused a nosocomial outbreak in the PAH, exhibiting a 62.5% mortality

rate (11). The *P. aeruginosa* strain described during that outbreak exhibited a tendency to widely colonise and infect mostly debilitated patients, significantly increasing both their length of stay in the ICU and cost of treatment (11).

Table 1-1: Beta-lactamase classification schemes referred to in this study.

Bush group	Subgroup	Ambler molecular class	Characteristics
1		C	Mainly chromosomal located in Gram-negative bacteria but may be plasmid mediated. Confer resistance to beta-lactams (except carbapenems). Not inhibited by clavulanate.
2		A, D	Most enzymes inhibited by clavulanate (unless otherwise stated).
	2a	A	Penicillinases (narrow hydrolysis spectrum) conferring resistance to penicillins.
	2b	A	Broad-spectrum penicillinases (TEM-1, SHV-1) primarily from Gram-negative bacteria.
	2be	A	Extended-spectrum beta-lactamases conferring resistance to oxyimino-cephalosporins and monobactams.
	2br	A	Inhibitor resistant beta-lactamases (mostly TEM-types and to a lesser extent SHV derived enzymes).
	2c	A	Carbenicillinases.
	2d	D	Oxacillinases, modestly inhibited by clavulanate.
	2e	A	Cephalosporinases inhibited by clavulanate.
	2f	A	Serine active site carbapenemases, inhibited by clavulanate.
3	3a, b, c	B	Metallo-beta-lactamases conferring resistance to beta-lactams (except monobactams), not inhibited by clavulanic acid.
4			Miscellaneous unsequenced beta-lactamases that do not conform to other groups.

Data adapted from references 16 and 17.

Previous analysis of bla_{GES} genes suggested that these genetic structures did not primarily evolve from *P. aeruginosa* as the G+C content of bla_{GES-2} was 51.5%, a value which is not within the range of G+C content of *P. aeruginosa* genes (60.1 – 69.5%)

(7), this value corresponds with genes originating from the *Enterobacteriaceae*. This fact then raises the question about the origin of the GES-type genes and the possibility that they may have developed in *Enterobacteriaceae* strains including *Klebsiella pneumoniae* isolates, as was previously described in a GES-1 producing *K. pneumoniae* isolate originating from French Guinea (12). Recent developments in Japan with the finding of *bla*$_{GES-3}$ and *bla*$_{GES-4}$ (13, 14) in clinical isolates of *K. pneumoniae* strongly support this theory. The integron genetic support that these novel enzymes enjoy, not only confers resistance towards broad-spectrum beta-lactam antibiotics, but also towards unrelated classes of antibiotics such as aminoglycosides and sulphonamides as well as to several hospital disinfectants (15). Due to the composition of class 1 integrons and the simultaneous expression of all the gene cassettes comprising the integron structure (9), it is in theory possible that non-beta-lactam antibiotics may actually co-select ESBL producing *P. aeruginosa* and certain *Enterobacteriaceae*. This selection phenomenon may cause widespread nosocomial dissemination of bacterial strains harbouring these genetic structures, making treatment and control exceptionally difficult.

1.2 OBJECTIVES:

- To develop improved molecular screening and detection methods for the novel beta-lactamase GES-2 in *P. aeruginosa* isolates.
- To determine the genetic stability of *bla*$_{GES}$-type gene cassettes in class 1 integrons under antibiotic pressure in vitro.

1.3 HYPOTHESIS:

- Class 1 integron located *bla*$_{GES}$ gene cassette stability is expected to change under selective antibiotic pressure.

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CHAPTER 2: AMBLER CLASS A EXTENDED-SPECTRUM BETA-LACTAMASES IN *PSEUDOMONAS AERUGINOSA* – NOVEL DEVELOPMENTS AND CLINICAL IMPACT.

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2.1 Introduction and epidemiology.

The so-called clavulanic-acid inhibited extended-spectrum beta-lactamases (ESBLs) belong mostly to class A of the Ambler classification scheme (1) and confer resistance to at least several expanded-spectrum cephalosporins (21, 22, 34) (Table 1-1, 1.1). They have been extensively reported in *Enterobacteriaceae* from the early 1980`s whereas they have been described in *Pseudomonas aeruginosa* only more recently (22, 34, 40). These enzymes described in *P. aeruginosa*, are either of the TEM- and SHV-types that are also well known in *Enterobacteriaceae*, of the PER-type mostly originating from Turkish isolates, or of the VEB-type from Southeast Asia and, more recently, of the GES /IBC types reported from France, Greece and South Africa, respectively (14, 33, 37-39, 41, 48, 52). These five types of enzymes are remotely related, both from a genetic point of view and similarities in hydrolytic profiles. Recent studies indicated that these enzymes may play an important role in the dissemination of antibiotic resistant isolates and may condition future choices of antibiotic regimens for treating life-threatening infections due to ESBL-producing *P. aeruginosa* (12, 17, 51).

As summarized in Table 2-1, these enzymes have been found so far in a limited number of geographical areas, suggesting that some of these beta-lactamase genes may at least in several cases represent a specific local selection.

Table 2-1. Ambler Class A extended-spectrum beta-lactamases occurring in *P. aeruginosa.*

Beta-lactamase	Genetic support	Country of first isolation	Year of first isolation	Other countries of isolation	Reference
VEB-1	C/P/I	France	1998	Thailand, India,* China*	6, 18, 38, 60
VEB-1a	C/I	Kuwait	1999	-	50
VEB-1b	C/I	Kuwait	1999	-	50
VEB-2	C/I	Thailand	1999		18
PER-1	C	France	1991	Turkey, Italy, Belgium	8, 12, 30, 39, 41, 61, 63
SHV-2a	C/P	France	1995	Thailand, Poland*	37
SHV-5	P	Thailand	1994-1996	Greece *	6
SHV-12	C	Thailand	1994-1996	-	6
TEM-4	P/C	France	1996	-	42
TEM-21	C	France	1997	-	13
TEM-24	P	France	1998	-	31
TEM-42	P	France	1992	-	36
GES-1	C/I	France	1999	-	14
GES-2	P/I	South Africa	2000	_	51, 52
IBC-2	C/I	Greece	1998	_	33

C, chromosomal location; P, plasmid borne; I, integron-borne.
* P. Nordmann, personal communication.

The SHV-type ESBLs have been identified in very rare isolates of *P. aeruginosa*, SHV-2a from France, whereas SHV-5 and SHV-12 were from Thailand (6, 37). These isolates were nosocomial strains except the SHV-12 producer, isolated from a clinical sample from an outpatient of a Thai hospital (6).

The TEM enzymes described in *P. aeruginosa* namely, TEM-4, TEM-21, TEM-24 and TEM-42 have been reported in rare isolates from France (3, 13, 31, 36, 42, 49). A French survey indicated that only 10% of ticarcillin-resistant *P. aeruginosa* produce a TEM-type beta-lactamase, whereas other narrow-spectrum beta-lactamases (OXA, CARB) constitute a higher proportion in that species (3). Conversely, the TEM-type

enzymes are widely distributed in *Enterobacteriaceae*, whereas OXA-type and CARB-type beta-lactamases are rare (27). The few reports of *P. aeruginosa* strains harbouring TEM and SHV-type genes may have several explanations. Firstly, the rarity of narrow-spectrum TEM and SHV-type enzymes may limit antibiotic selection of these enzymes with an expanded-spectrum hydrolysis. Secondly, a higher proportion of acquired oxacillinase and carbenicillinase genes (most of them being chromosome-encoded) may fulfil the function of genes encoding narrow-spectrum enzymes such as the TEM and SHV types. Indeed, several oxacillinases (OXA-2 and OXA-10 derivatives, and OXA-18) have been reported in *P. aeruginosa* that have extended substrate profiles including extended-spectrum cephalosporins (7, 40, 45). Thirdly, expression of the chromosome-encoded cephalosporinase of *P. aeruginosa* may be up regulated (derepression) and may thereby be a convenient way for acquisition of resistance to expanded-spectrum cephalosporins (23), without the need for expanding its genetic repertoire. It is likely that the origin of TEM- and SHV-type ESBLs in *P. aeruginosa* may result from gene transfer from *Enterobacteriaceae* (27). This has been shown for TEM-24 (31) and the downstream-located DNA sequences of the chromosome of *P. aeruginosa* RP-1 that produces SHV-2a, which were found to be identical to those reported as plasmid-encoded in a *Klebsiella pneumoniae* isolate (37, 46). Differences in replication origins of plasmids from *Enterobacteriaceae* and *P. aeruginosa* may however limit such intergeneric transfers. Additionally, difficulty of detection of TEM- and SHV-type ESBLs in the clinical laboratory may underestimate their true prevalence in *P. aeruginosa*.

Beta-lactamase PER-1 was the first ESBL identified and fully characterized in *P. aeruginosa* in 1993 (39, 41). It was found from a *P. aeruginosa* isolate of a Turkish patient hospitalised in the Paris area in 1991 (39). A subsequent study on the

distribution of the *bla*$_{PER-1}$ gene revealed that it is widespread in Turkey, with PER-1 being identified in up to 46% of *Acinetobacter* strains and 11% of *P. aeruginosa* analysed in a nation-based survey performed over a three-month period in 1999 (61). PER-1 was identified in up to 38% of ceftazidime-resistant *P. aeruginosa* isolates, with ribotyping results indicating spread of different clones (61). Since screening for the *bla*$_{PER-1}$ gene has not been performed in *P. aeruginosa* isolates originating from countries located to the south and east of Turkey such as Syria, Iran and Iraq, no current data are available on the prevalence of PER-1 in the Middle East.

It is possible that the spread of PER-1 in Western Europe may be mostly related to the widespread immigration of Turkish nationals. Interestingly, although reported in several enterobacterial species including community-acquired pathogens such as *Salmonella* spp. (62), beta-lactamase PER-1 seems mostly expressed from *P. aeruginosa* and *Acinetobacter* spp. isolates in Turkey (61, 63). A large nosocomial outbreak of PER-1-producing *P. aeruginosa* has been documented in Varese, Italy, occurring over a 10-month period in a tertiary hospital (30). During that outbreak, a total of 108 clinical isolates were recovered from 18 patients, reflecting the propensity of *P. aeruginosa* to widely colonize hospitalised patients. In that case, apart from the beta-lactam resistance phenotype conferred by PER-1, epidemic strains were resistant to several disinfectants, including chlorhexidine, iodide povidone, and toluene-*p*-sulphochloramide (30). Control of the outbreak was achieved by implementing strict hygienic measures, carbapenem therapy and disinfection of decubitus ulcers and surgical wounds with mercurochrome or silver nitrate solutions (30). As a result of increased carbapenem consumption, selection of several carbapenem-resistant organisms occurred in the nosocomial environment including OprD-defective *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Pseudomonas putida* producing the

class B carbapenemase VIM-1 (30). The same group had reported a *P. aeruginosa* strain that produced the plasmid-mediated beta-lactamase VIM-2 together with beta-lactamase PER-1 (12) thus showing that the same *P. aeruginosa* strain may produce two unrelated beta-lactamases both with expanded-spectrum hydrolysis. Recently, another *P. aeruginosa* strain that produced PER-1 has been isolated from a patient hospitalised in Clermont-Ferrand in the central part of France (11). Indeed, this latter patient had been hospitalised previously in Strasbourg, in the eastern part of France where the patient might have been in contact with hospitalised Turkish patients (P. Nordmann, personal communication). A pseudo-outbreak (false positive culture results due to specimen contamination) has also been reported from Belgium (8), revealing the obstacles that face investigators when searching for the source of multiresistant *P. aeruginosa* isolates. Although no mention is made about the antibiotic regimen used for treating the infected patient, the pseudo-outbreak was successfully terminated by decontamination of a side-room urine densitometer (8).

Another unrelated ESBL from *P. aeruginosa*, i. e. beta-lactamase VEB-1 was originally identified in *E. coli* and *Klebsiella* isolates from a 4-month old Vietnamese child transferred from Vietnam and hospitalized in France (48). Subsequent isolation of VEB-1 from *P. aeruginosa* was documented from two patients hospitalised in France and transferred from Thailand (38). A study conducted in a university hospital in Thailand (17), revealed that *bla*$_{VEB}$-like genes were present in up to 93% of the ceftazidime-resistant isolates whereas ceftazidime resistance occurred in 24% of *P. aeruginosa* isolates. As this is an isolated study, the clinical significance of VEB-type enzymes in the region is still uncertain. Another *bla*$_{VEB-1}$-like gene, *bla*$_{VEB-2}$ had been identified during this study, with VEB-2 differing from VEB-1 by only one amino acid change located outside the active site of the enzyme (17). The latest development in

analysis of *bla*$_{VEB-1}$-like genes was the isolation of *P. aeruginosa* strains from an intensive care unit of a Kuwaiti hospital harboring *bla*$_{VEB}$–like genes, *bla*$_{VEB-1a}$ and *bla*$_{VEB-1b}$, that differ from the *bla*$_{VEB-1}$ gene by nucleotide substitutions in the DNA sequence encoding the leader peptide (50) (Table 2-1). Unpublished data have also identified VEB-1 from *P. aeruginosa* in India and China (P. Nordmann, personal communication). It is likely that VEB-type enzymes may be isolated mostly from patients coming from or hospitalised in Southeast Asia.

Another ESBL, beta-lactamase GES-1, was first identified from a French Guiana *K. pneumoniae* strain isolated in Paris (47). Subsequently, *bla*$_{GES-1}$ was identified from a *P. aeruginosa* isolate in France (14) as well as the structurally related *bla*$_{IBC-2}$ gene from a Greek isolate in Thessaloniki (33). These results suggested that these ESBL genes might have a wider scattered distribution than VEB and PER enzymes. One of the most interesting developments in research in ESBLs in *P. aeruginosa* is the identification of GES-2 that differs from GES-1 by a single amino acid change located in the active site of these enzymes (52). GES-2 hydrolyses not only extended-spectrum cephalosporins but also imipenem to a minor extent. This enzyme was identified in *P. aeruginosa* from a university hospital in Pretoria, South Africa, and was associated with an outbreak occurring in the same hospital from March to July 2000 (51). Seventy-two ceftazidime-resistant isolates were isolated from nine patients, whereas only eight of these patients were found to harbour seventy clonally related isolates carrying the same *bla*$_{GES-2}$ gene. Presence of the *bla*$_{GES/IBC}$ genes in *P. aeruginosa* and in other gram-negative rods in different countries might indicate a yet undiscovered potential spread of these enzymes.

The only clavulanic-acid inhibited oxacillinase from *P. aeruginosa* with extended-spectrum profile is OXA-18, that has been reported only once from a *P. aeruginosa* strain isolated in Paris, from a patient previously hospitalized in Italy (45). Another

putative but not yet fully characterised ESBL identified in Tunisia (2, 54), again focuses attention on Mediterranean countries as possible reservoirs of ESBL-producing *P. aeruginosa* isolates. Additionally, other non-characterized ESBLs have been described from *P. aeruginosa* isolates in Brazil (44) and in Poland (64).

2.2 Substrate profile.

Concerning the hydrolytic properties of TEM-type ESBLs found in *P. aeruginosa* (13, 31, 36), they are similar to those of classical TEM-type ESBLs hydrolyzing narrow-spectrum penicillins, extended-spectrum cephalosporins and the monobactam aztreonam (4, 34). TEM-4 has a substrate profile that includes mostly cefotaxime (Table 2-2), whereas TEM-42 exhibits a low K_m value for ceftazidime (36), which is widely regarded as an excellent indicator for ESBL activity. The high relative rate of hydrolysis (V_{max}) measured against several extended-spectrum cephalosporins and the overproduction of the TEM enzymes in *P. aeruginosa* may make these enzymes powerful tools for beta-lactam resistance in *P. aeruginosa* (36).

Beta-lactamase SHV-2a mirrors the affinities of TEM-4 to some extent (Table 2-2) with high affinity for latest developed cephalosporins such as cefpirome (37, 46). The kinetic constants of SHV-5 reveal a subtle difference in substrate profile when compared to that of the TEM enzymes, most notably that of TEM-42 (36). Although rare in *P. aeruginosa* and only described in France and Greece (P. Nordmann, personal communication), SHV-5 tends to hydrolyse ceftazidime and cephaloridine most efficiently. Other less favourable substrates include aztreonam, the narrow-spectrum cephalosporins and other extended-spectrum cephalosporins (40). SHV-12 confers resistance towards the extended-spectrum cephalosporins and aztreonam with a marked inhibitory effect of clavulanic acid (6).

Table 2-2. Comparative kinetic parameters for extended-spectrum beta-lactamases found in *P. aeruginosa* [a]

Antibiotics	Kinetic parameters																	
	GES-1			GES-2			PER-1			VEB-1			TEM-4			SHV-2a		
	kcat	*Km*	*Vmax / Km*	*kcat*	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*
Benzylpenicillin	2.8	40	70	0.4	4	96	100	2.5	40	100	2.8	100	NA	NA	NA	33	17	1.9
Amoxicillin	13	200	65	0.7	25.8	26	174	54	3	110	6.0	50	50	NA	NA	NA	NA	NA
Ampicillin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	47	28	1.7
Ticarcillin	0.3	400	0.7	0.06	13.3	4.5	7	1.7	4	8	1	22	NA	NA	NA	NA	NA	NA
Piperacillin	8	900	13	0.3	22.8	23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cephalothin	179	3400	52	0.3	3	112	473	7	68	700	6.0	325	NA	NA	NA	NA	NA	NA
Cephaloridin	53	2000	26	0.5	7.7	65	356	16	22	2300	12	533	232	NA	NA	100	30	3.2
Cefoxitin	0.9	30	33	NH	NH	NH	< 0.5	0.04	NA	NA	NA	NA	< 1	NA	NA	NA	NA	NA
Cefuroxime	NA	NA	NA	NA	NA	NA	668	18	37	2000	24	230	NA	NA	NA	NA	NA	NA
Ceftazidime	380	2000	188	ND	> 3000	ND	2470	148	17	NA	NA	NA	10	NA	NA	NA	NA	NA
Cefotaxime	68	4600	15	2.2	890	2.5	1510	26	58	4300	38	314	300	NA	NA	7	26	0.2
Cefepime	2.8	1800	1.6	1.1	1900	0.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cefpirome	NA	NA	NA	NA	NA	NA	772	35	22	NA	NA	NA	NA	NA	NA	20	151	0.14
Imipenem	0.003	45	0.07	0.004	0.45	9	< 0.5	0.07	NA	NA	NA	NA	< 1	NA	NA	NA	NA	NA
Meropenem	NH	NH	NH	NH	NH	NH	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Aztreonam	NH	NH	NH	NH	NH	NH	1	23	0.04	400	500	2	< 1	NA	NA	NA	NA	NA

Data were adapted from references 39, 42, 46-48, and 52.
[a] *kcat* (s^{-1}) [only available for GES-1 and GES-2]; *Km* (μM); *kcat* / *Km* ratio ($mM^{-1} . s^{-1}$)
NA – Data not available
NH – Not hydrolyzed (Initial rate of hydrolysis reported lower than 0.001 $\mu M^{-1}. s^{-1}$)
ND – Not determinable due to high *Km* values.

Table 2-3: MICs (µg/ml) of beta-lactams for several representative non-isogenic class A extended-spectrum beta-lactamase producing *P. aeruginosa* isolates.

	Extended-spectrum beta-lactamase producers [b]						
Beta-lactams [a]	**VEB1 (JES-1)**	**PER-1 (RNL-1)**	**GES-1 (695)**	**GES-2 (GW-1)**	**SHV2a (RP-1)**	**TEM-4 (Stel)**	**IBC-2 (555)**
Amoxicillin	> 512	> 512	> 512	> 512	>512	>512	NA
Amoxicillin +CLA	>512	> 512	64	> 512	>512	>512	NA
Ticarcillin	> 512	512	> 512	> 512	>512	>512	> 256
Ticarcillin + CLA	256	256	64	> 512	64	32	> 256
Piperacillin	128	32	512	128	256	32	> 256
Piperacillin + TZB	NA	NA	64	128	16	8	> 256
Cephalothin	>512	> 512	64	> 512	>512	>512	NA
Cefoxitin	NA	> 512	512	> 512	>512	>512	> 256
Ceftazidime	512	128	32	32	32	8	> 256
Ceftazidime + CLA	128	4	32	16	8	4	NA
Cefotaxime	NA	64	NA	128	>512	128	> 256
Cefepime	128	NA	16	32	NA	8	NA
Imipenem	32	0.5	1	16	2	4	> 128
Meropenem	8	NA	NA	16	NA	1	> 128
Aztreonam	>256	256	4	16	32	16	32

[a] CLA, clavulanic acid concentration fixed at 2 µg/ml; TZB, tazobactam concentration fixed at 4 µg/ml.
[b] Well-characterised strain designations appear in brackets.
NA – data not available.
Data were adapted from references 14, 33, 37-39, 49, and 52.

The non-TEM, non-SHV ESBLs from *P. aeruginosa* also tend to exhibit a fairly broad range of substrate specificities (Tables 2-2, 2-3). VEB-1 and PER-1 exhibit the typical substrate profile of classical ESBLs, i.e. high affinities for narrow-spectrum penicillins, narrow- and expanded-spectrum cephalosporins (Table 2-2). PER-1 in particular, exhibits high affinity towards ceftazidime and aztreonam (39), whereas VEB-1 hydrolyses cefotaxime more efficiently than ceftazidime (48). These ESBLs have low-level affinities for the carbapenems and are moderately inhibited by clavulanic acid and imipenem. Beta-lactamases VEB-1a and VEB-1b have the same substrate specificities as VEB-1 since the distinctive mutations were located in the mature protein sequences

outside the putative active site (50). In addition VEB-1 and PER-1 are well inhibited by cefoxitin.

Beta-lactamase GES-1 exhibits a low catalytic activity and low affinity for most substrates (47) and an inhibition profile that includes clavulanic acid and imipenem (Table 2-3). The more recent addition in the GES lineage (GES-2), tends to swing its substrate affinities towards the narrow-spectrum penicillins and carbapenems (52), noticeably against imipenem (Table 2-3). GES-2 has higher affinity for imipenem compared to that of GES-1 (Table 2-3) (52). Although the rate of hydrolysis of imipenem by GES-2 is marginal as compared to that of class B enzymes, GES-2 may confer resistance towards imipenem, most likely when associated with a membrane impermeability or efflux mediated resistance mechanism (52). Inhibition studies of GES-2 revealed a marked increase in its IC_{50} value for imipenem compared to GES-1 (8 ± 2 µM and 0.1 µM, respectively) (52). The IC_{50} value of GES-1 for clavulanic acid compared to that of GES-2 reveals a *ca.* 10^3 factor difference (15 nM and 1 ± 0.5 µM, respectively) (52), that may indicate future selection of a bla_{GES} derivatives with resistance to enzyme inhibitors.

The latest characterized non-TEM non-SHV ESBL in *P. aeruginosa* is IBC-2, reported from a Greek isolate (33). IBC-2 confers resistance to ceftazidime and to other oxyimino-cephalosporins and is inhibited by imipenem, tazobactam and clavulanic acid (33). IBC-2 differs from IBC-1 by one amino acid change occurring outside of the omega loop in Ambler position 104 (Glu to Lys substitution) (16, 33), both enzymes being highly related to the GES-1/GES-2 enzymes (33).

The ability of any beta-lactamase to confer resistance in Gram-negative rods depends on a complex interplay of diffusion of beta-lactam molecules in the periplasmic space, hydrolysis parameters, and produced quantity of the enzyme (gene copy number and

promoter strength) (27). The MIC of a beta-lactam is raised in case of rapid hydrolysis (high *Vmax* or high *kcat*) when the beta-lactamase has high affinity for the substrate (low *Km*) and/or when the diffusion coefficient of the beta-lactam molecule is low (27). Indeed, MICs of beta-lactams for several ESBL producers mirror combinations of these parameters (Table 2-3). In this regard, the permeability diffusion coefficient is especially important for *P. aeruginosa,* in as much that it is a hundred times lower than that of *E. coli* (40). It must be noted however that unlike for *E. coli*, a simple permeability co-efficient for *P. aeruginosa* cannot be calculated (29). When this very low diffusion coefficient is coupled with any other membrane permeability or efflux-related resistance mechanism, the organism may be well protected against broad-spectrum beta-lactams by relatively restricted-spectrum beta-lactamases. This phenomenon is well illustrated by the protection afforded to *P. aeruginosa* against imipenem by beta-lactamase GES-2, whereas the same enzyme fails to induce resistance to imipenem in an *E. coli* clone (51, 52).

Beta-lactamase OXA-18 is the only oxacillinase that is very well inhibited by clavulanic acid (45). Its hydrolytic profile includes extended-spectrum cephalosporins as well as aztreonam. Compared to other OXA-type enzymes in *P. aeruginosa*, its kinetic parameters include both low affinity and high catalytic activity against several cephalosporins. Additionally, like GES-1, its activity is strongly inhibited by imipenem (45).

In addition to acquired beta-lactamases, *P. aeruginosa* has an inducible class C cephalosporinase (AmpC) that confers low-level resistance towards aminopenicillins, narrow-spectrum cephalosporins and cephamycins such as cephalothin and cefoxitin respectively (4, 23). In addition to resistance mediated by efflux and impermeability, resistance to extended-spectrum cephalosporins may arise from i) derepression of

biosynthesis of the chromosomal cephalosporinase (23), ii) acquired secondary beta-lactamases or iii) both mechanisms in tandem (7), making detection of ESBLs very difficult in this species.

2.3 Genetic determinants

The genes encoding the TEM and SHV-type enzymes are usually plasmid-located in *Enterobacteriaceae* (27). Spread of these plasmids may be limited by species-related plasmid replication. Plasmid location of genes encoding ESBLs of the TEM and SHV series has been reported for *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-24}}$, and *bla*$_{\text{TEM-42}}$ in *P. aeruginosa* and simultaneously in enterobacterial isolates from the same patients (6, 31, 36). Whereas *bla*$_{\text{VEB-like}}$ genes are mostly plasmid-encoded in *Enterobacteriaceae*, they are mostly chromosome-encoded in *P. aeruginosa* (17, 18). The same is true for the *bla*$_{\text{PER}}$ -like genes (61-63) whereas the *bla*$_{\text{GES}}$ genes have been found to be either plasmid- or chromosome-encoded in *P. aeruginosa* (14, 33, 51). However, in the latter case, epidemiological surveys are not yet available.

Along with a plasmid location, many antibiotic resistance genes have been identified as a form of gene cassettes and as part of class 1 integrons in *P. aeruginosa*. These genetic structures are vectors of co-localization and of co-expression of antibiotic resistance genes (9, 19, 56). They are associated with typical sequences (59-bp elements) recognized as target sites by the integrase, and that are essential for their mobility (20, 57, 58). The 5' conserved segment of integrons contains the integrase gene (*intI*) and the recombination site *attI1* (53) whereas the 3' conserved sequence carries the antiseptic-resistance determinant *qacEΔ1*, and the *sul1* gene that confers resistance to sulfonamides (25, 43). Whereas genes encoding beta-lactamases of class B (metallo-enzymes) and of class D (oxacillinases) are usually located in class 1 integrons, genes

encoding VEB- and GES-type enzymes are the only genes encoding class A ESBLs that are associated with these genetic structures. Conversely, the *bla*$_{PER-1}$ gene is not integron-associated (40, 41). In several cases, *bla*$_{GES}$ and *bla*$_{VEB}$ genes have been associated in integrons with other beta-lactamase genes *bla*$_{OXA-5}$ and *bla*$_{OXA-10,}$ respectively (18, 51). The gene-associated sequences are almost identical for the *bla*$_{GES}$ genes (the same for the *bla*$_{VEB}$ genes) thus underlining in those cases, epidemic spread of gene cassettes.

Since several integrons have been reported as transposon-located, these structures may provide an additional means of mobility for these antibiotic resistance genes and may explain plasmid and chromosomal locations of the same ESBL gene in *P. aeruginosa* (14, 17, 50-52). Future work should be directed to the identification of the transposon structures that contain these integrons in *P. aeruginosa*. The *bla*$_{VEB-1}$ gene has been identified in *E. coli* within a composite transposon (17, 18) whereas known class 1 integrons are located onto Tn*21* derivatives that are transposons commonly found in *Pseudomonas* spp. (26). Since *bla*$_{VEB}$-like and *bla*$_{GES}$-like genes are integron-located, it is possible that their presence in *P. aeruginosa* may result from horizontal transfer from Gram-negative aerobes (other than *Enterobacteriaceae*) that are known as a source of integrons (56) and that may be present in the same ecological niche.

Recently, the *bla*$_{TEM-21}$ gene was identified as part of a chromosome-located Tn*801* transposon disrupted by insertion of an IS*6100* element (13). The *bla*$_{OXA-18}$ gene was found to be chromosome-encoded, and surprisingly for an oxacillinase gene not integron-associated (45).

2.4 Current detection methods.

The presence of ESBLs in *P. aeruginosa* may be suspected in the face of an antibiotic resistance phenotype combining resistance to ticarcillin and ceftazidime and susceptibility to ticarcillin plus clavulanic acid. Detection of ESBLs using double-disk synergy tests with clavulanate and any extended-spectrum cephalosporins are sensitive and specific in *Enterobacteriaceae* (5, 15, 28). However, the same test may not be as useful for detection of ESBLs in *P. aeruginosa* (8, 40). These difficulties stem from several factors: i) false negative results due to naturally-occurring beta-lactamases such as the chromosome-encoded AmpCs that may be overexpressed ii) simultaneous presence of metallo-enzymes with carbapenem-hydrolyzing activity (IMP and VIM series [12, 32]) or with extended-spectrum oxacillinases (OXA-2 and OXA-10 derivatives) (17), iii) relative resistance to inhibition by clavulanate as examplified by GES-2 (52), and iv) combined mechanisms of resistance such as impermeability and efflux.

Experience indicates that positive results with the double disk synergy test are quite easily obtained with VEB-1- and PER-1-positive strains whereas synergy patterns may be more difficult to detect with GES-type enzymes. In several cases, the synergy image with TEM- and SHV-type ESBLs may be hardly visible in *P. aeruginosa*. Synergy between imipenem and ceftazidime may be observed with *bla*$_{GES}$-like and *bla*$_{PER-1}$ enzymes (14). This synergy may be obscured in some cases by the induction effect of imipenem on the expression of the chromosomal cephalosporinase, exhibiting a concomitant antagonism line between ceftazidime- and imipenem-containing disks. This effect can be overcome to some extent by performing the double-disk synergy test with oxacillin-containing agar plates, since oxacillin inhibits the activity of Ambler class C enzymes (4). The antibiotic resistance phenotype induced by OXA-18 is

identical to that reported for class A clavulanic-acid inhibited ESBLs, making its phenotypic-based differentiation impossible (45).

When an ESBL is suspected in *P. aeruginosa*, PCR-based molecular techniques may help to identify the gene. Standard PCR conditions with a series of primers designed for detection of class A beta-lactamase genes bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, and $bla_{GES/IBC}$ could be used (Table 2-4).

Table 2-4. Oligonucleotide primers used for detection of genes encoding class A ESBLs in *P. aeruginosa.*

Primer names	Sequence 5' to 3'	Detection of gene	Reference
VEB-1A	CGACTTCCATTTCCCGATGC	bla_{VEB}	48, 51
VEB-1B	GGACTCTGCAACAAATACGC		
PER-A	ATGAATGTCATTATAAAAGC	bla_{PER}	8, 51
PER-B	AATTTGGGCTTAGGGCAGAA		
GES-1A	ATGCGCTTCATTCACGCAC	$bla_{GES\ /\ IBC}$	51
GES-1B	CTATTTGTCCGTGCTCAGG		
TEM-A	GAGTATTCAACATTTCCGTGTC	bla_{TEM}	51
TEM-B	TAATCAGTGAGGCACCTATCTC		
SWSHV-A	AAGATCCACTATCGCCAGCAG	bla_{SHV}	37
SWSHV-B	ATTCAGTTCCGTTTCCCAGCGG		

However, PCR experiments without further sequencing of the PCR products cannot differentiate between narrow-spectrum and extended-spectrum enzymes of the TEM and SHV series. Other methods such as isoelectric focusing analysis (IEF) may just indicate the presence of acquired ß-lactamases rather than to identify an ESBL precisely. For example, PER-1, and narrow-spectrum TEM-1 enzymes share very similar pI values (39, http://www.lahey.org/studies). Primers designed for annealing to the ends of class 1 integrons may also help for obtaining PCR products that may contain ESBL encoding genes. Nucleotide sequence analysis of PCR products, whether or not combined with other methods (24), is still the only acceptable way to accurately discriminate between ESBL genes of the same family.

2.5 Clinical consequences.

The most appropriate antibiotic regimen for treating infections due to ESBL-positive *P. aeruginosa* strains remains to be determined due the lack of clinical studies in this research field. Three reports detail antibiotic therapy and outcome of patients infected with ESBL-positive *P. aeruginosa* isolates (17, 30, 51). Experimental pneumonia conducted in rats with a PER-1-producing *P. aeruginosa* strain (35), indicated that a combination of amikacin and imipenem was synergistic against an imipenem- and amikacin-susceptible strain. As predicted by results of in-vitro susceptibility testing, cefepime and piperacillin-tazobactam exhibited marked inoculum effects in vivo (35) and as previously documented for ESBL-producing *Enterobacteriaceae* (55), these results indicate that PER-1-producing *P. aeruginosa* would not be treated safely with piperacillin-tazobactam or cefepime alone (35). A population-based cohort study conducted with PER-1-producing *P. aeruginosa* in Turkey (61), identified the following factors as independent predictors of poor clinical outcome namely: i)

impaired consciousness, ii) male sex, and iii) urinary tract infection. Other clinically significant variables in that study were the presence of a central venous catheter, the acquisition of the infection in ICU, and hypotension. Unfortunately, the authors did not comment on the antibiotic regimen used in the study (61). Clinical experience with VEB-1-positive *P. aeruginosa* strains may indicate the efficacy of imipenem-containing antibiotic regimens as target therapy (17).

In a documented outbreak of a GES-2-producing *P. aeruginosa* strain that had occurred in South Africa, seventy-two ceftazidime-resistant isolates originating from nine patients were collected carrying $bla_{\text{GES-2}}$ and $bla_{\text{OXA-5}}$ genes (51). Out of these nine patients, only one had been hospitalised in a general gynaecology ward. The patient had a short stay in hospital after successful treatment of a wound infection with topical application of an acetic acid solution. The other patients had chronic underlying conditions, associated with extended periods in ICU and multiple courses of broad-spectrum antibiotics. Eight clonally related isolates tested resistant to most common beta-lactams (except aztreonam), aminoglycosides and ciprofloxacin. A total mortality of 62.5% (5/8 patients) was recorded, and the most successful treatment regimens consisted of combinations of imipenem with ciprofloxacin or aztreonam with amikacin, respectively (51). Piperacillin-tazobactam proved to be ineffective, even in combination with amikacin, against GES-2-producing *P. aeruginosa* (51). Retrospectively, this could be due to the relative resistance of this enzyme to beta-lactamase inhibitors (Table 2-3). Proof of invasive infections during this outbreak could be established by culturing GES-2-producing isolates from: i) blood cultures, ii) tissue biopsies and iii) arterial catheter tips (51). The outbreak was effectively terminated by: i) increasing the hygiene and housekeeping measures in ICUs, ii) restricting movement of patients infected or colonised with multiple-resistant *P. aeruginosa* isolates, and iii) increasing

the turn-over of patients hospitalised in these ICUs (G. F. Weldhagen, personal data). Use of topical non-absorbable antibiotics given orally such as colistin (59), for controlling enteric reservoirs of ESBL-positive enterobacterial isolates, has not been evaluated in the case of ESBL-positive *P. aeruginosa* isolates.

MIC results may help in choosing the optimal antibiotic regimen, but in-vitro susceptibility does not always guarantee in-vivo success. If the ESBL-positive isolate remains susceptible to carbapenems, a carbapenem should be proposed in combination with an antibiotic molecule of another non-beta-lactam class. Meropenem unlike imipenem remains stable to the hydrolytic activities of all class A ESBLs found in *P.aeruginosa*, including beta-lactamase GES-2 (52). Colonized skin wounds should not be treated with systemic antibiotics but rather dressed with topical applications of antiseptics. Increased general hospital hygiene measures as reported for controlling outbreaks of ESBL-positive enterobacterial isolates is a crucial point in controlling outbreaks due to ESBL-positive *P. aeruginosa.*

2.6 Conclusion.

Some ESBL-producing *P. aeruginosa* strains seem to be widely scattered in certain geographic locations such as VEB and PER enzymes in Southeast Asia and Turkey, respectively. Detection of other ESBLs in other countries may however reflect selective laboratory research interests rather than true distribution of these enzymes in *P. aeruginosa*.

High levels of frequency of detection of ESBLs in *P. aeruginosa* are from countries that have not yet implemented molecular-based surveillance programs for antibiotic resistance detection. Difficulties in laboratory detection of ESBLs and thus underreporting may likely increase the incidence and the prevalence of these enzymes

worldwide, especially in developing countries. In several cases, the current high prevalence of ESBLs in *P. aeruginosa* in those countries may be the source of transfer of ESBL-producing *P. aeruginosa* to developed Western countries as well as a hidden reservoir for ESBL genes transferred to other gram-negative aerobes. Since *P. aeruginosa* is known to be a formidable pathogen for acquiring additional resistance mechanisms, one should be aware that a multi-drug resistance trend would be very difficult to reverse in this species. The report of beta-lactamase GES-2 that is a weak carbapenem-hydrolyzing beta-lactamase raises an additional threat of selection of beta-lactamases with a very broad substrate profile and increased inhibitor resistance from ESBLs.

Reports of structurally related, integron-located ESBL genes in *P. aeruginosa* from different parts of the world, add novel steps in the saga of evolutive transfer of beta-lactamase-mediated antibiotic resistance, with questions arising on the origin of these genes. Additionally, co-resistance and co-expression of resistance determinants as the result of their integron location may further stabilize non-related antibiotic resistance genes. In other words, antibiotic regimens that may contain rifampin (60) or aminoglycosides for example may enhance prevalence of genes encoding resistance to structurally unrelated antibiotic molecules including expanded-spectrum cephalosporins and carbapenems (beta-lactamase GES-2). Thus, changes in antibiotic policy may include not only extended-spectrum cephalosporins, but also non-beta-lactam antibiotics and should involve discussions that include representatives of various clinical disciplines. Since the natural reservoir of many ESBL genes found in *P. aeruginosa* is unknown as well as the origin of the integron-located beta-lactamase genes, further research should be directed to search for these natural ESBL producers.

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CHAPTER 3: INTEGRONS AND BETA-LACTAMASES – A NOVEL PERSPECTIVE ON RESISTANCE.

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3.1 Introduction.

The horizontal transfer of genetic material within and between microbial genera has been instrumental in the emergence of novel antibiotic resistance traits observed worldwide. Rapid and widespread emergence of resistance and more importantly similar patterns of resistance have been encountered in phylogenetically diverse Gram-negative clinical isolates on an increasing scale (1). Apart from well-known bacterial methods of mutation, antibiotic genes are frequently carried in integron-borne cassettes, which provide an efficient means for capturing and exchanging various resistance genes (2). To date four classes of integrons, each with distinct *int* genes, have been described in Gram-negative bacterial isolates (3, 4, 5), with class 1 integrons being most prevalent in clinical isolates, carrying single or multiple gene cassettes. Integron inserted genes encode for various antibiotic resistance mechanisms, including over 40 distinct genes, conferring resistance towards aminoglycosides, beta-lactams, chloramphenicol, macrolides, sulphonamides, antiseptics and disinfectants (3, 5, 6). Concerning beta-lactamases, integron-borne gene cassettes have been found mainly in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and various *Enterobacteriaceae* species encompassing Ambler classes A (except TEM and SHV types), B and D beta-lactamase enzymes, giving rise to widespread beta-lactam resistance (7, 8, 9, 10). Only eight discrete families of beta-lactamases have so far been reported from classes 1 and 3 integron structures

originating from geographically distinct locations, with some rare exceptions (Tables 3-1 to 3-3).

Table 3-1: Ambler class A, integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
VEB-1	*Klebsiella pneumoniae*	Vietnam	9
	Escherichia coli	Vietnam	9
	Pseudomonas aeruginosa	France	12
	Citrobacter freundii	Thailand	13
VEB-1a	*Pseudomonas aeruginosa*	Kuwait	14
VEB-1b	*Pseudomonas aeruginosa*	Kuwait	14
VEB-2	*Pseudomonas aeruginosa*	Thailand	15
GES-1	*Klebsiella pneumoniae*	French Guiana	18
	Pseudomonas aeruginosa	France	56
	*Klebsiella pneumoniae**	Portugal	60
GES-2	*Pseudomonas aeruginosa*	South Africa	10, 11
IBC-1	*Enterobacter cloacae*	Greece	16
IBC-2	*Pseudomonas aeruginosa*	Greece	17
CTX-M-2	*Salmonella enterica*	Argentina	19
	Proteus mirabilis	Argentina	20
CTX-M-9	*Escherichia coli*	Spain	21
PSE-1	*Vibrio cholerae*	Thailand	22

All genes listed were found on Class 1 integron structures with notable exceptions.
* - Class 3 integron location.

Table 3-2: Ambler class B, integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
IMP-1	*Serratia marcescens**	Japan	23, 24, 25
	Pseudomonas aeruginosa	Japan	44, 45
IMP-2	*Acinetobacter baumannii*	Italy	26
IMP-3	*Shigella flexneri*	Japan	47, 48
IMP-4	*Acinetobacter baumannii*	Hong Kong	8
	Citrobacter youngae	China	49
IMP-6	*Serratia marcescens*	Japan	50
IMP-7	*Pseudomonas aeruginosa*	Canada	7, 51
IMP-8	*Klebsiella pneumoniae*	Taiwan	27
IMP-12	*Pseudomonas putida*	Italy	28
VIM-1	*Acinetobacter baumannii*	Italy	7
	Pseudomonas aeruginosa	Italy	46
	Achromobacter xylosoxydans	Italy	29
	Pseudomonas aeruginosa	Greece	52
VIM-2	*Pseudomonas aeruginosa*	France	53, 54
	Pseudomonas aeruginosa	Italy	55
	Pseudomonas aeruginosa	Spain	56

All genes listed were found on Class 1 integron structures with notable exceptions.

* - Class 3 integron location.

Table 3-3: Ambler class D, class 1 integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
OXA-1	*Salmonella enterica*	Italy	32
OXA-5	*Pseudomonas aeruginosa*	South Africa	10, 11
OXA-9	*Enterobacter aerogenes*	France	33
OXA-10	*Pseudomonas aeruginosa*	Vietnam	9
OXA-11	*Pseudomonas aeruginosa*	Turkey	31
OXA-14	*Pseudomonas aeruginosa*	Turkey	31
OXA-16	*Pseudomonas aeruginosa*	Turkey	31
OXA-19	*Pseudomonas aeruginosa*	France	30
OXA-20	*Pseudomonas aeruginosa*	France	57
OXA-28	*Pseudomonas aeruginosa*	France	31
OXA-30	*Escherichia coli*	France	59

This apparent restriction of geographical range regarding enzyme families can be ascribed to various factors including genetic incompatibility, geographic location, antibiotic selective pressure and selective research interests (6, 7, 10, 11). Although complex, understanding the underlying genetic mechanisms responsible for the acquisition and spread of these unique beta-lactamase mediated antibiotic resistance mechanisms, could eventually facilitate development of effective prevention and control strategies.

3.2 Epidemiology.

Beta-lactamase VEB-1 was the first class A enzyme found to be encoded by an integron-located gene cassette (9), demonstrating horizontal transfer in the same patient, between different genera belonging to the *Enterobacteriaceae*. The same gene was subsequently found in *P.aeruginosa* and *Escherichia coli* strains in France, originating from patients previously hospitalised in South-East Asia (12). A study conducted in Thailand found $bla_{\text{VEB-1}}$ and $bla_{\text{VEB-1-like}}$ genes in *P. aeruginosa*, *Citrobacter freundii* and *Pseudomonas putida* (13), with clonal dissemination of $bla_{\text{VEB-1,}}$ demonstrated by pulsed field gel electrophoresis (PFGE) in *P. aeruginosa*. Integron-borne VEB-1a and VEB-1b type enzymes were found in *P. aeruginosa* isolates originating from Kuwait (14), while $bla_{\text{VEB-2}}$ was found on a class 1 integron structure in *P. aeruginosa* from Thailand (15).

The closely related class A beta-lactamases belonging to the GES / IBC group exhibit a widely scattered distribution with isolates in France, Greece, French Guinea and South Africa (10, 11, 16, 17). GES-1, the first described enzyme from this group was found on a class 1 integron element In52, in a *Klebsiella pneumoniae* isolate originating from French Guinea (18). Virtual simultaneous description of the closely related integron-borne cephalosporinase IBC-1 followed, the $bla_{\text{IBC-1}}$ gene originating from an *Enterobacter cloacae* strain found in Greece (16). The simultaneous detection of closely related enzymes from geographically distinct locations most likely signifies the hidden potential of the GES / IBC type enzymes among strains belonging to the *Enterobacteriaceae*. Both GES-1 and IBC-1 were found on highly mobile gene cassettes located in class 1 integron structures, with typical GC content suggestive of an enterobacterial origin (16, 18). Further developments revealed the possible horizontal transfer of bla_{GES} and bla_{IBC} genes from *Enterobacteriaceae* to *P.*

aeruginosa (10, 17), with GC content analysis clearly revealing the incompatibility of bla_{GES-2} with *P. aeruginosa* DNA (10). A small outbreak caused by GES-2-producing *P. aeruginosa* in a South African tertiary hospital, revealed clonal dissemination of this resistance threat amongst clinical isolates (11). A recent development in the GES-1 evolutive saga came in the form of bla_{GES-1} located on a class 3 integron, isolated from a *K. pneumoniae* isolate in Portugal (60). The clinical significance of this specific genetic localization is however still largely uncertain.

A novel class 1 integron (InS21) carrying the class A $bla_{CTX-M-2}$ gene, was found in a *Salmonella enterica* serovar infantis isolate, originating from Argentina (19). The dissemination of $bla_{CTX-M-2}$ between bacterial genera via class 1 integron structures is evident from previous findings, locating CTX-M-2 in the variable region of a class 1 integron present in a high molecular weight plasmid originating from a *Morganella morganii* isolate (19). Analysis of a *Proteus mirabilis* strain originating from Argentina revealed $bla_{CTX-M-2}$ on an unusual class 1 integron (In35) (20). Sequence analysis of the $bla_{CTX-M-2}$ gene and surrounding DNA revealed 99% homology with the chromosomally borne, class A bla_{KLUA-1} gene, originating from *Kluyvera ascorbata* (20). Data from that study suggests plasmid acquisition of $bla_{CTX-M-2}$ through an uncharacterised recombinational event incorporating open reading frames of unknown function. The latest integron-borne CTX-M-type enzyme was found in Spain, carried on a complex *sul1*-type, class 1 integron originating from *E. coli* (21). This enzyme was subsequently identified as CTX-M-9. Another rare find was made when bla_{PSE-1} was discovered on a class 1 integron, originating from *Vibrio cholerae* in Thailand (22).

Regarding beta-lactamases from Ambler class B, to date only two distinct families have made their appearance on integrons. IMP type enzymes found on class 1

integron structures include IMP-1 – 4, IMP-6 – 8 and IMP- 12 (Table 3-2). These integron-borne genes were reported from geographic locations as diverse as Japan, Hong Kong, Taiwan, China, Italy and Canada (Table 3-2). IMP-1 however, made its appearance on a class 3 integron structure, isolated from a high molecular weight transferable plasmid originating from a *Serratia marcescens* isolate in Japan (23, 24, 25). Of interest, the $bla_{\text{IMP-2}}$ gene found in an *A. baumannii* isolate from Italy directed the attention on spontaneous development of this gene in an isolate unrelated to the Far East (26). This finding suggests that the reservoir for IMP-type genes could be widespread and could pose a serious threat for global dissemination. Finally, the isolation of integron-borne $bla_{\text{IMP-8}}$ in *K. pneumoniae* from Taiwan (27) and $bla_{\text{IMP-12}}$ in *P. putida* from Italy (28) respectively, concludes the current integron based evolution of this enzyme family.

The second class B enzyme type found on class 1 integron structures are the enzymes belonging to the VIM family, namely VIM-1 and VIM-2. They were found mainly on integron structures in *A. baumanii* and *P. aeruginosa* isolates, mostly originating from European locations such as Italy, France, Greece and Spain (Table 3-2). Integron-mediated spread to other non-fermenting Gram-negative and *Enterobacteriaceae* species has been documented in Italy and Greece, with $bla_{\text{VIM-1}}$ genes detected in clinical isolates of *Achromobacter xylosoxidans* (29) and *E. coli* respectively (Unpublished data [Genbank accession # AY152821]). Despite their predominant integron genetic support, the VIM-type genes tend to be currently located in the Mediterranean region and France, the exception being however the chromosome located, non-integron mediated, metallo-beta-lactamase gene $bla_{\text{VIM-3}}$ discovered in Taiwan (7).

Class D beta-lactamases found on integron structures, belong to the OXA type family (Table 3-3). They tend to occur mainly on class 1 integrons isolated from *P. aeruginosa* (9, 10, 30, 31), but in isolated instances have been found to occur in integron-borne form isolated from *Salmonella enterica* serotype typhimurium and *Enterobacter aerogenes* respectively (32, 33). OXA-type genes found on class 1 integrons from *Enterobacteriaceae* tend to be carried as the sole *bla*-gene cassette on the integron, in conjunction with numerous other co-resistance genes (32, 33). In contrast, OXA-type integron-borne gene cassettes in *P. aeruginosa* tend to be the secondary *bla*-gene cassette on the integron, with class A-type *bla*-genes mostly functioning as the primary integron-borne beta-lactamase (9, 10, 31).

3.3 Genetic determinants.

Integrons are genetic elements able to capture individual antibiotic resistance genes including those encoding various beta-lactamases and in the process promote their transcription and expression (4, 34, 35). Integrons include a receptor site, *attI*, where captured genes are integrated, together with an adjacent sequence coding for a recombinase, IntI (35). Uncaptured gene cassettes exist in their free form as circular molecules consisting only of one open reading frame (ORF) and a 59-base element (59-be) situated downstream (3, 35, 36). Integration of these gene cassettes involves IntI-catalysed site-specific recombination between the integron associated *attI* site and the 59-be recombination site, associated with the incoming gene cassette (35). Each gene cassette has a unique 59-be, implying that 59-be associated with specific gene cassettes may differ both in sequence and length (35, 37). The 59-be family exhibits a common inverted repeat structure and consensus sequences at each end that consists of approximately 25 bases (35). The outer boundaries of the 59-be in addition contain

the conserved seven base pair core site GTTRRRY at the recombinant cross-over point, and an inverse core site RYYYAAC at the 3' end of the inserted gene cassette (10, 35, 38). Integron cassettes identified to date are in the same orientation, the core site of an integrated cassette is complementary to the inverse core site of the next cassette downstream (6).

Although integrons are considered to be natural expression vectors, great variability of the expression level of integrated gene cassettes are exhibited. This variability is linked to the intrinsic structures of both gene cassettes and integrons (34). Gene expression in an integron is dependant on various factors including promoter strength, gene copy number, the relative distance of the gene cassette from the promoter, and the presence of additional internal promoters (3, 4). Expression is usually mediated via a common promoter situated upstream (5` end) of gene cassettes, rather than through individual promoters. Higher levels of gene expression can be achieved if a second promoter is included adjacent to the first, or if the gene in question is included as multiple copies (3). The relative distance between a gene cassette and the promoter plays a significant role regarding expression, proximal genes tend to be expressed more effectively than distal genes. As a result distal genes may be poorly expressed and have very little effect on the susceptibility of the host bacterium to relevant antibiotics (3, 4).

Integron carriage of resistance gene cassettes by the host bacterium was found to be dependent on the environment that the host organism found itself in, with loss of integron-borne resistance genes in the absence of antibiotic selective pressure (5). Stability of integron gene cassette arrangement and therefore gene expression, during or after an antibiotic onslaught on the organism remains undetermined and may prove

helpful in understanding the role that antibiotics play in the evolution and possible future control of these genetic structures in the nosocomial environment.

3.4 Expression of co-resistance.

The presence of co-resistance gene cassettes (set of resistance genes, conferring resistance to different antimicrobial classes) on integrons, make these genetic elements extremely useful to bacteria, as resistance to a variety of non-related substances can be conferred simultaneously. Additionally, integron-mediated multi-drug resistance tends to favour co-selection of isolates, thereby facilitating widespread dissemination through patients from a wide variety of clinical disciplines (11). Most integron-borne beta-lactamase genes are situated on class 1 integrons, as is evident from the typical genetic structure and the co-resistance genes to quaternary ammonium compounds (*qacΔE1*) and sulphonamides (*sul1*) that classically occur at the distal 3`-end (10, 18, 39). Gene cassettes encoding aminoglycoside resistance tend to co-occur commonly with beta-lactamase gene cassettes on integron structures, with *aac*-type (aminoglycoside acetyltransferase) and *aad*-type (aminoglycoside adenyltransferase) genes occurring most often (Tables 3-4 and 3-5).

Fusion of two aminoglycoside resistance gene cassettes co-occurring with bla_{GES-1} on a class 1 integron, has been reported from France with fusion of an *aac(3)-I* to an *aac(6`)* cassette (39). In that study it was proved that the fused gene product, as well as the two separate cloned genes conferred resistance to various aminoglycosides. The fused gene product however exhibited a wider resistance repertoire towards aminoglycosides than the two separate genes; this may be an indication towards future development trends in integron-borne aminoglycoside resistance (39).

Table 3-4: Integron-borne co-resistance genes reported to occur with class A, beta-lactamase genes.

Beta-lactamase	Co-resistance genes located on integron	Host species	Reference
	5`-end 3`-end		
VEB-1	*bla*$_{OXA-10-like}$	*C. freundii, P. aeruginosa P. putida*	13
	bla$_{OXA-10-like}$, *arr*-2-like	*E. coli, Enterobacter spp, K. pneumoniae*	40
	arr-2, *cml*A-5, *bla*$_{OXA-10-like}$, *aad*B, *aad*A1	*P. aeruginosa*	15
VEB-2	*arr*-2, *cml*A-5, *bla*$_{OXA-10-like}$, *aad*B, *aad*A1	*P. aeruginosa*	15
GES-1	*aac(6`)-Ib`*, *dfr*XVb, *cml*A4, *aad*A2, *qac*EΔ1, *sul1*	*K. pneumoniae*	18
	aac(3)-Ib / aac(6`)-Ib`, *qac*EΔ1, *sul1*	*P. aeruginosa*	39
	bla$_{OXA}$, *aac(6`)-Ib*	*K. pneumoniae*	60
GES-2	*bla*$_{OXA-5}$, *aac(3)-I*, *qac*EΔ1, *sul1*	*P. aeruginosa*	10, 11
IBC-1	*aac(6`)-Ib*, *qac*EΔ1, *sul1*	*Enterobacter cloacae*	16
IBC-2	None found.	*P. aeruginosa*	17
CTX-M-2	*aac(6`)-Ib*, *bla*$_{OXA-2}$, *qac*EΔ1, *sul1*	*Salmonella enterica* serovar infantis	19
	aac(6`)-Ib, *bla*$_{OXA-2}$, *qac*EΔ1, *sul1*	*Proteus mirabilis*	20
CTX-M-9	*dfr*A16, *aad*A2, *qac*EΔ1, *sul1*	*Escherichia coli*	21
PSE-1	*aad*B, *qac*EΔ1, *sul1*	*Vibrio cholerae*	22

Table 3-5: Integron-borne co-resistance genes reported to occur with class B, beta-lactamases.

Beta-lactamase	Co-resistance genes located on integron 5`-end 3`end	Host species	Reference
IMP-1	*aac(6`)-Ib, bla*$_{TEM-1}$	*Serratia marcescens*	*
	aacA4, catB6, qacG, qacEΔ1, sul1	*P. aeruginosa*	7
	aac(6`)-Ib	*P. aeruginosa*	44
IMP-2	*aacA4, aadA1*	*Acinetobacter baumannii*	26
IMP-3	No data available		
IMP-4	*qacG, aacA4, catB3*	*Acinetobacter* spp.	8
IMP-6	No data available		
IMP-7	*aacC4, aacC1*	*P. aeruginosa*	51
IMP-8	*aac(6`)-Ib, catB4, qacEΔ1, sul1.*	*Klebsiella pneumoniae*	27
VIM-1	*aacA4, aphA15, aadA1, qacEΔ1, sul1*	*Achromobacter xylosoxidans*	29 46
	aacA4	*P. aeruginosa*	
VIM-2	*qacEΔ1, sul1*	*P. aeruginosa*	53
	aacA7, aacC1, qacEΔ1, sul1	*P. aeruginosa*	7, 54
	aacA29a, aacA29b, qacEΔ1, sul1	*P. aeruginosa*	7, 54
	aacA4, qacEΔ1, sul1	*P. aeruginosa*	55
	aac(3)-Ic, cmlA7	*P. aeruginosa*	58

* Unpublished data (Genbank Accession # AB070224)

Sequential trimethoprim and sulphonamide resistance is in some cases encoded for on two different gene cassettes within the same integron; the *sul*-type genes

(dihydropterorate synthase) and *dhfr* / *dfr*-type genes (dihydrofolate reductase) commonly occur together on class 1 integrons in conjunction with class A *bla* gene cassettes (Table 3-4). *Sul*-type gene cassettes commonly occur in the 3`-conserved regions of class 1 integrons (10). In most cases the *dhfr / dfr* – type gene cassettes are situated closer to the promoter region (5`- end) of the integron and should therefore be expressed more efficiently than the *sul* – type cassettes (Table 3-4). The clinical significance of this phenomenon is however still uncertain. Resistance towards rifampin encoded by the *arr*-type genes (rifampin ADP-ribosylating transferase) was only found in isolated cases, co-occurring on class 1 integrons, with bla_{VEB-1} and bla_{VEB-2} genes originating from Thailand and Vietnam respectively (15, 40). Two different integron-borne genes conferring resistance towards chloramphenicol have been described to date. Gene cassettes of the *cml*–type (chloramphenicol resistance protein) and *cat*–type (chloramphenicol acetyltransferase) were found to co-occur on class 1 integrons with class A and class B beta-lactamase gene cassettes, respectively (8, 15, 18, 27).

Considering beta-lactamase genes as co-resistance factors co-occurring with primary *bla* gene cassettes on integrons, mainly the class D oxacillinases or bla_{OXA}-type genes have been reported to occur significantly on both class 1 and 3 integrons (Table 3-4). These gene cassettes are most frequently reported from integrons originating from non-fermenting bacterial species such as *P. aeruginosa* (10, 15), and rarely made an appearance as secondary beta-lactamase gene cassettes on integrons originating from *Enterobacteriaceae* (13, 19, 20). Although none of the class A or D type beta-lactamase genes have been described previously to co-occur together with integron-borne class B type genes, a rare exception was found in the form of bla_{TEM-1}, co-occurring with bla_{IMP-1} originating from a *S. marcescens* clinical isolate (Unpublished

data. [Genbank accession # AB070224]). To date, this is the only description of an integron-borne *bla*$_{TEM}$ gene. Secondary beta-lactamase gene cassettes are most commonly recruited into integron structures to fill very specific niches in the organism's resistance profile against beta-lactam antibiotics (10, 11), possibly reflecting on regional selective pressures.

3.5 Detection.

Detection of integron-borne resistance gene cassettes has traditionally been relegated to dedicated research institutions rather than the clinical microbiology laboratory. The most likely explanation for this can mostly be related to the costs, personnel time involved and slow turn around time of the diagnostic process. However, even with modern molecular techniques, the specific identification of all the cassettes inserted into an integron structure may take from several days to weeks or longer, to unravel. This slow identification process is clearly of very little clinical benefit to individual patients in the ICU. Developments in the field of real-time PCR detection of class 1 integrons (41) from *Enterobacteriaceae* and non-fermenting Gram-negative bacteria may prove to revolutionise this particular field of research. Utilisation of a primer pair specific for a 300-base pair (bp) conserved region at the 5`-end of class 1 integrons, was shown to be both highly sensitive and specific to detect these genetic structures from various bacterial species (41). That study utilised the LightCycler instrument and results obtained, compared favourable with conventional PCR detection of class 1 integrons (41). Primers designed for the 5'- and 3'- conserved ends of class 1 integrons may prove to be a valuable screening tool to further detect beta-lactamase and other co-resistance genes that form part of class 1 integron structures (42, 43),

especially when used in conjunction with the phenotypic exhibition of co-resistance factors revealing the likely presence of integron-mediated resistance.

When an integron is detected, standard PCR conditions with a series of primers designed for detection of the more prevalent classes A and B, beta-lactamase genes, such as *bla*$_{VEB}$, *bla*$_{GES/IBC,}$ *bla*$_{IMP,}$ and *bla*$_{VIM}$ could be used to identify specific genes (9, 18, 44, 45, 46, 61). A thorough knowledge of circulating local strains and resistance patterns (the place to place phenomenon) are extremely helpful in targeting a molecular approach in order to conduct cost-effective screening. Sequencing of PCR products is unfortunately still the preferred method of differentiation between beta-lactamase genes and could substantially add to the cost of a screening program, a factor most developing countries can hardly afford. Future research should focus on quick, simplified and cost effective molecular methods that could bring integron identification into the realm of the clinical microbiology laboratory.

3.6 Conclusion.

Reports of structurally related, integron-located ESBL and carbapenemase genes in mainly *P. aeruginosa, A.baumannii* and the *Enterobacteriaceae* from different parts of the world, paint a bleak picture in terms of evolutive transfer of beta-lactamase-mediated antibiotic resistance, with questions arising concerning the origin of these genes. Further research gaining knowledge about integron-related antibiotic selective pressure, is absolutely crucial in order to stem the tide of bacterial resistance in the nosocomial environment.

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CHAPTER 4: SEQUENCE-SELECTIVE RECOGNITION OF EXTENDED-SPECTRUM BETA-LACTAMASE GES-2, BY A COMPETITIVE, PEPTIDE NUCLEIC ACID BASED, MULTIPLEX-PCR ASSAY.

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4.1 Introduction

After the discovery of the extended-spectrum beta-lactamase (ESBL), GES-2 from *Pseudomonas aeruginosa*, in Pretoria, South Africa in the year 2000 (24), it became clear that class 1 integron borne ESBLs were established in the South African nosocomial setting. The same strain subsequently caused a nosocomial outbreak with diverse clinical presentations in three Intensive Care Units (ICUs) in a teaching hospital, during which several patients succumbed (25). The *P. aeruginosa* strain described during that outbreak exhibited a tendency to colonise and infect mostly debilitated patients, including human immunodeficiency virus (HIV)-positive patients, significantly increasing both their length of stay in the ICU and cost of treatment (25). Detection of GES-2 and other ESBL-producing *P. aeruginosa* isolates in the clinical microbiology laboratory utilising ordinary methods is notoriously difficult due to various reasons including: i) false negative results due to naturally occurring beta-lactamases such as the chromosome-encoded AmpCs that may be over-expressed, ii) simultaneous presence of metallo-enzymes with carbapenem-hydrolysing activity (IMP and VIM series), or with extended-spectrum oxacillinases (OXA-2 and OXA-10 derivatives), iii) relative resistance to inhibition by clavulanate as exemplified by GES-2, and iv) combined mechanisms of resistance such as impermeability and efflux (29). When an ESBL is suspected in *P. aeruginosa*, polymerase chain reaction (PCR)-based molecular techniques may help to detect the gene with a series of primers designed for recognition of class A beta-lactamase

genes in this species (29). However, PCR experiments without further sequencing of the amplification products could not accurately differentiate between all the genes in a beta-lactamase family, substantially adding to the costs of such an exercise. Other methods such as isoelectric focusing analysis (IEF) may just indicate the presence of acquired beta-lactamases rather than to identify an ESBL precisely (18, 29). Descriptions of bla_{GES-1} (5, 26) and the bla_{IBC}-type genes (8, 13) from different geographical locations further complicates the laboratory identification of bla_{GES-2}, the more potent and currently geographically restricted gene in this family (24, 29). Since the initial description of peptide nucleic acid (PNA) in 1991 (17), interest in this synthetic polymer as a molecular device has steadily increased. The hallmark of PNA that stimulated this interest is its intrinsic high affinity and specificity for complementary nucleic acids (17), making it the ideal nucleic acid molecule to recognise point mutations without the need for highly specialised equipment. The fact that the GES / IBC beta-lactamase family is still relatively small and with only a few point mutations separating the genes in question (29), makes it the ideal model for the development of a novel sequence-specific, PNA-based identification method for geographically restricted ESBLs, suited to large scale, low-cost screening programmes.

4.2 Materials and methods

4.2.1 Bacterial strains. One hundred clinical isolates of *P. aeruginosa*, collected and cryo-preserved by the Department of Medical Microbiology, University of Pretoria, South Africa, were included in this study. Isolates were identified with the API-20NE system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Control bacterial strains used in this study to standardize reactions are listed in Table 4-1.

Table 4-1: Well-characterized bacterial strains used in this study.

Strain	Relevant properties	Source or reference
P. aeruginosa GW-1	bla_{GES-2} producing isolate	24
P. aeruginosa Pu21	bla_{GES-2} transconjugant isolate	24
K. pneumoniae ORI-1	bla_{GES-1}-producing isolate	26
E.cloacae HT-9	bla_{IBC-1}-producing isolate	8
E. coli brcpHT-8	bla_{IBC-1} transconjugant isolate	8
E.coli DH5α	bla_{IBC-2} transconjugant isolate	13
E. coli ATCC 25922	bla_{GES} / bla_{IBC} negative strain	ATCC[a]

[a] ATCC, American Type Culture Collection, Manassas, VA.

4.2.2 Susceptibility testing. Antibiotic-containing disks (Mast Diagnostics, Merseyside, UK) were used for routine laboratory antibiograms by the disk diffusion assay as described and interpreted, according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (15). Resistance to ceftazidime (MIC ≥ 32 µg/ml) (15), was an absolute requirement for inclusion in the study.

4.2.3 DNA extraction. Extraction of whole-cell DNA was performed with a precipitation-based method as described previously (23, Appendix A) combined with an added double ethanol final wash step to improve template purity (9). DNA pellets were dried in a DNA Speed Vac 110 (Savant Instruments Inc., Farmingdale, NY) and resuspended in 1 ml TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 7.4). DNA extraction from each isolate was verified by means of electrophoresis at 2 V/cm for 1 hour in 0.8% agarose gel containing ethidium bromide 0.5 µg / ml in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 7.4) and visualised under UV light.

4.2.4 Standard PCR amplification.

i) Standard PCR primer construction. A primer pair annealing internally to the binding sites of previously published primers GES-1A and GES-1B (24), was constructed from the gene sequence of *bla*$_{GES-2}$ [GenBank accession number AF 326 355, (24)], by utilising Primer3 software (Whitehead Institute for Biomedical Research) available from URL: http://www.inqaba.com. The following primer sequences were generated: GES-C, 5`-GTT TTG CAA TGT GCT CAA CG-3` (positions 176 – 195 of the coding region) (*Tm* = 54.6ºC) and GES-D, 5`-TGC CAT AGC AAT AGG CGT AG-3` (positions 527 – 546) (*Tm* = 55.6ºC)(Table 4-2), targeting a 371 base pair (bp) region, incorporating all currently known point mutations of *bla*$_{GES}$ and *bla*$_{IBC}$ genes (5, 8, 13, 24, 26). Primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, Iowa.

ii) Standard PCR amplification and detection. GES-C and GES-D primers were utilized under standard PCR conditions (28), to amplify a 371 bp product. The PCR reaction mix comprised 0.32 µM of each primer, 5 µL *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 1.25 U *Taq* DNA polymerase, (Promega Corp., Madison, WI), 2 µL DNA template and distilled water to a final reaction volume of 50 µL. Amplification was conducted on a GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA) and 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 sec, followed by an annealing step at 50°C for 1 min, and an extension step at 72°C for 1 min. Completion of 35 amplification cycles was finally followed by an extension step at 72°C for 5 min. PCR products were visualized by electrophoresis at 4 V/cm for 45 min in 0.8% agarose gel containing ethidium bromide 0.5 µg/ml. Amplicon size was verified with a 100 bp

DNA ladder (Promega Corp.). DNA templates obtained from *P. aeruginosa* GW-1, bla_{GES-2} transconjugant *P. aeruginosa* Pu21, *K. pneumoniae* ORI-1, *Enterobacter cloacae* HT-9, bla_{IBC-1} transconjugant *E. coli* brcpHT-8 and bla_{IBC-2} transconjugant *E. coli* DH5α (Table 4-1), served as PCR positive controls. *E. coli* ATCC 25922 was used as a $bla_{GES/IBC}$ PCR negative control. All PCR experiments were performed in duplicate. Reaction preparation was done in separate rooms equipped with laminar flow cabinets to avoid cross-contamination with amplification products.

Table 4-2: Oligonucleotide sequences used in this study.

Primer / probe	Sequence [a]	Function	Source or reference
Standard PCR and DNA sequencing			
GES-C	GTT TTG CAA TGT GCT CAA CG	Forward primer	This study
GES-D	TGC CAT AGC AAT AGG CGT AG	Reverse primer	This study
PNA-based multiplex PCR			
GES-1A	ATG CGC TTC ATT CAC GCA C	Forward primer	24, 29
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse primer	24, 29
GES-E	GTG TGT TGT CGT TCA TCT C	Reverse primer – complementary to bla_{GES-2}	This study
GES-F	CCT GGC GAC CTC AGA GAT AC	Forward primer	This study
PNA probe	GTT GTC GCC CAT CTC	Complementary to bla_{GES-1} / bla_{IBC}	This study

[a] – Sequences are delineated in 5` - 3` order.
Relevant point mutations are depicted in shaded boxes.

4.2.5 Competitive PNA-based multiplex PCR. This novel method to detect point mutations in ESBL genes by PCR relies on two features of PNA: i) its intrinsically

high affinity for DNA (17) and ii) its inability to serve as a primer for DNA polymerases (21). This method utilizes a forward primer in conjunction with a reverse primer that is complementary to the desired mutant sequence, as well as a PNA probe similar to the reverse primer, but complementary to the wild type (wt) sequence. When the reaction contains a wt DNA template, the PNA probe out competes the reverse primer for binding and no amplification occurs, conversely the opposite happens when a mutant sequence is present during the reaction (20). A second set of primers targets an area downstream from the mutation site in both wt and mutant genes; this then serves as an internal amplification control measure.

i) Multiplex PCR primer construction. In addition to primers GES-1A and GES-1B (24), primers for multiplex PCR analysis were constructed in a similar fashion as described for the standard PCR method. The following primer sequences were generated: GES-E, 5`-GTGT GTT GTC GTT CAT CTC-3` (positions 487 – 505 of the *bla*$_{GES-2}$ coding region) (*Tm* = 51.8ºC) and GES-F, 5`- CCT GGC GAC CTC AGA GAT AC-3` (positions 505 – 524) (*Tm* = 57.4ºC) (Table 4-2).

ii) Peptide nucleic acid probe construction. A mixed sequence PNA probe was designed to be complementary to positions 487 – 501 of the coding regions of *bla*$_{GES-1}$, *bla*$_{IBC1}$ and *bla*$_{IBC2}$ [GenBank accession numbers AF 156486, AF 208529 and AF 329699, (8, 13, 26)] utilizing Custom PNA Probe Designer software (Applied Biosystems, Rotkreutz, Switzerland) and available from URL: http://www.appliedbiosystems.com. The 3` L-lysine-labeled probe sequence in antiparallel binding mode (20), 5`-GTT GTC GCC CAT CTC-3` (*Tm* = 70ºC), was synthesized and purified by Boston Probes, Bedford, MA.

iii) Reaction preparation and conditions. In a multiplex-PCR assay, primers GES-1A with GES-E and GES-F with GES-1B were used to amplify 505 and 360 bp

products respectively. The PCR reaction mix comprised 0.32 µM of each primer, 5 µL *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide, 1.25 U *Taq* DNA polymerase, (Promega Corp.), 2 µL DNA template and distilled water to a final reaction volume of 50 µL. Amplification and detection was performed as described for the standard PCR procedure previously. Once the reaction conditions were standardized with well-described bacterial isolates (Table 4-1), PNA probe to a final concentration of 0.32 µM were added to subsequent multiplex PCR reactions to compete with the reverse primer GES-E.

4.2.6 DNA sequencing analysis. Sequencing was performed on standard PCR products on both the forward and reverse strands, on a SpectruMedix model SCE 2410 automated sequencer (Spectru Medix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Electropherograms of generated sequences were inspected with Chromas software version 1.45 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Obtained DNA sequences were analyzed with the BLAST program (1), available from URL: http://www.ncbi.nlm.nih.gov. A PCR product obtained from *P. aeruginosa* GW-1 (Table 4-1), was used as a control.

4.3 Results

i) Detection of *bla*$_{GES/IBC}$ with standard PCR. Clinical isolates of *P. aeruginosa* collected and cryo-preserved from 1998 – 2001 by the Department of Medical Microbiology, University of Pretoria, South Africa, originated from a variety of specimens including endotracheal aspirates, urine samples, pus swabs, blood cultures, tissue biopsies, and intravenous catheters. Specimens originated mainly from ICUs, serving Internal Medicine, Surgery, Pediatrics and Neurosurgery, while the remainder

of specimens originated from general hospital wards including orthopedic and gynaecology patients. (For the purposes of this study, clonal relatedness of isolates was not determined.) Although varying zone size diameters were observed, all isolates (n = 100) were resistant to ceftazidime (MIC ≥ 32 µg/ml) in the disc-diffusion assay according to NCCLS guidelines (15) (data not shown). Repeated standard PCR amplification resulted in 51 of the isolates testing positive for a *bla*$_{\text{GES / IBC}}$ gene product comprising 371 bp in length. A 371 bp PCR product was obtained from each control strain (Table 4-1), except from the *bla* $_{\text{GES / IBC}}$ negative control, *E. coli* ATCC 25922.

ii) DNA sequencing of standard PCR products. By sequence analysis of the standard PCR products (n = 51) on both the forward and reverse strands, all products corresponded to *bla*$_{\text{GES-2}}$. Each sequence was thoroughly inspected for signature point mutations, such as the 5`-GCT-3` motif (positions 358 – 360 of the coding region), differentiating *bla*$_{\text{GES}}$– from *bla*$_{\text{IBC}}$-type genes (26) and the 5`-GAA-3` motif (positions 492 – 494), differentiating *bla*$_{\text{GES-2}}$ from *bla*$_{\text{GES-1}}$ and *bla*$_{\text{IBC}}$-type genes (24). In addition to these specific motifs as mentioned above, a stemloop in the secondary DNA structure (positions 466 – 470 and 482 – 486 of the coding region) was noted.

iii) PNA-based, sequence specific detection of *bla*$_{\text{GES-2}}$. Subjecting the 100 clinical isolates to sequence-specific multiplex-PCR amplification resulted in 50 of the 51 standard PCR positive isolates, testing positive for *bla*$_{\text{GES-2}}$. A repeat multiplex-PCR reaction with DNA template volume increased from 2 – 5 µl, yielded a positive result for the outstanding isolate. Detection of amplification products by gel electrophoresis exhibited two distinctive patterns, GES-2-producing clinical isolates and control strains producing two distinct bands of 505 and 360 bp respectively, while GES-1-

and IBC-producing control strains only produced the 360 bp internal amplification control band, clearly distinguishing them from GES-2 producing isolates (Figure 4-1).

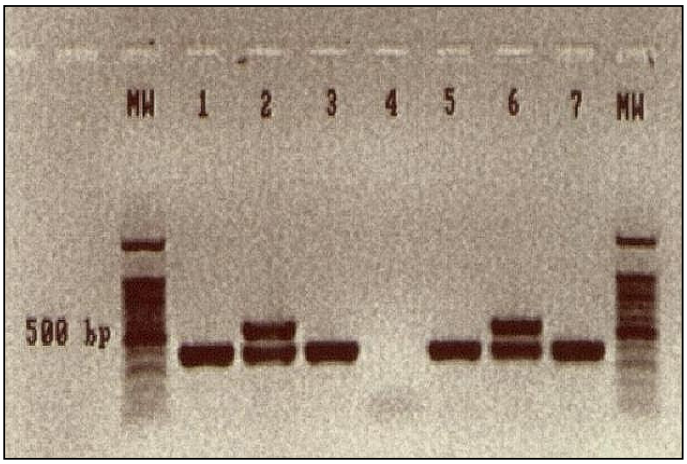

Figure 4-1: Gel electrophoresis depicting PNA-based, sequence-specific PCR amplification products obtained from well-characterized bacterial isolates listed in Table 4-1. MW: 100 bp marker (Promega Corp.), 500 bp segment indicated; lane 1: *E. cloacae* HT-9; lane 2: *P. aeruginosa* GW-1; lane 3: *E. coli* brcpHT-8; lane 4: *E. coli* ATCC 25922; lane 5: *K. pneumoniae* ORI-1, lane 6: *P. aeruginosa* Pu21; lane 7: IBC-2 transconjugant *E. coli* DH5α.

Control strains *P. aeruginosa* GW-1 and *P. aeruginosa* Pu21 (24) both tested positive for $bla_{\text{GES-2}}$ in repeated reactions, while repetitive results for $bla_{\text{GES-1}}$ and bla_{IBC}-producing strains (Table 4-1) were obtained. The *E. coli* ATCC 25922 isolate tested negative in all reactions. These results corresponded with the results obtained for the standard PCR amplification and sequencing reactions.

4.4 Discussion

Despite considerable effort from various investigators, detection of ESBLs in *P. aeruginosa* still remains a problem due to the notoriously low sensitivities of easy-to-perform susceptibility tests or to the laborious and often expensive molecular

detection methods (29). Apart from standard PCR and gene sequencing, molecular detection methods for ESBLs in general developed a wide array of applications most suited for the TEM- and SHV-type of ESBL families (4). These methods may include oligotyping (12), PCR-restriction fragment length polymorphism analysis (2, 19), single-strand conformation polymorphism analysis (14), real-time PCR with melting curve analysis (27), and ligase chain reaction-based tests (10, 16). In addition to these aforementioned methods, this study describes the application of PNA as a novel method to detect point mutations in ESBL genes of the GES / IBC family.

Data from studies with nuclear magnetic resonance (NMR) (11) and X-ray crystallography (3) have shown that mixed sequence PNA forms duplexes with complementary DNA sequences by way of base specific hydrogen bonds, with a 1:1 stoichiometry, obeying Watson-Crick hydrogen bonding rules (7). Hybridization properties of mixed sequence PNA and consequently the high affinity of the interaction of PNA with DNA can be attributed to the lack of negative charge in the PNA backbone as demonstrated by melting point analysis at high ionic concentrations (> 1 M Na^+) (7). Previous analysis of PNA base mismatch recognition in DNA templates have shown that from the 12 possible single base mismatches, all but one (G_{PNA} and T_{DNA}) had a larger destabilizing effect on the PNA-DNA duplexes than on the corresponding DNA-DNA duplexes, regardless of the ionic concentration of the medium (22). The two bp mismatch targeted in this study (CC_{PNA} and AA_{DNA}), provided sufficient instability of the PNA-DNA duplex in the presence of *bla*$_{GES-2}$ (mutant) templates to facilitate replacement of the PNA probe by the reverse primer GES-E, in order for amplification to occur (Figure 4-2). In the presence of *bla*$_{GES-1}$ (wild type) or *bla*$_{IBC}$-type templates, the resulting PNA-DNA duplex experienced no mismatch and was therefore stable enough to prevent binding of the reverse primer,

henceforth disabling PCR amplification in a sequence specific manner. These data coupled with the ability of PNA to be sequence specific regardless of ionic concentration during the PCR reaction (7, 22), makes it a very robust molecular tool, well suited for screening large numbers of isolates for very specific point mutations.

Sensitivity and specificity of this novel method to accurately detect the GG to AA point mutation in *bla*$_{GES-2}$, matched those generated by the "gold standard" (PCR and DNA sequencing) perfectly. As published previously, high quality of the DNA template is however imperative to avoid false negative results (29), as was noted with one of the clinical isolates when subjected to the sequence-specific multiplex-PCR method. Large-scale epidemiological use of this novel PNA-PCR method would possibly require the use of a commercially available DNA extraction process, to standardize DNA template yield and input. The addition of a second primer pair, targeting a 360 bp region downstream from the mutation site has therefore been incorporated into the reaction to serve as an internal amplification control. The presence of a stemloop in the secondary structure of the *bla*$_{GES}$ and *bla*$_{IBC}$ DNA templates used in this study was noted (CGGCT and AGCCG motifs situated at positions 466 – 470 and 482 – 486 of the coding region)(*Tm* = 82ºC), creating a possible steric hindrance during annealing of the reverse primer GES-E to the closely situated target site. This was overcome by a thorough initial denaturation step in the amplification protocol and permitting the PNA probe to bind earlier to the target sequence relative to the competing reverse primer, due to the rapid formation of antiparallel PNA-DNA duplexes in comparison with duplexes formed in parallel binding mode (20).

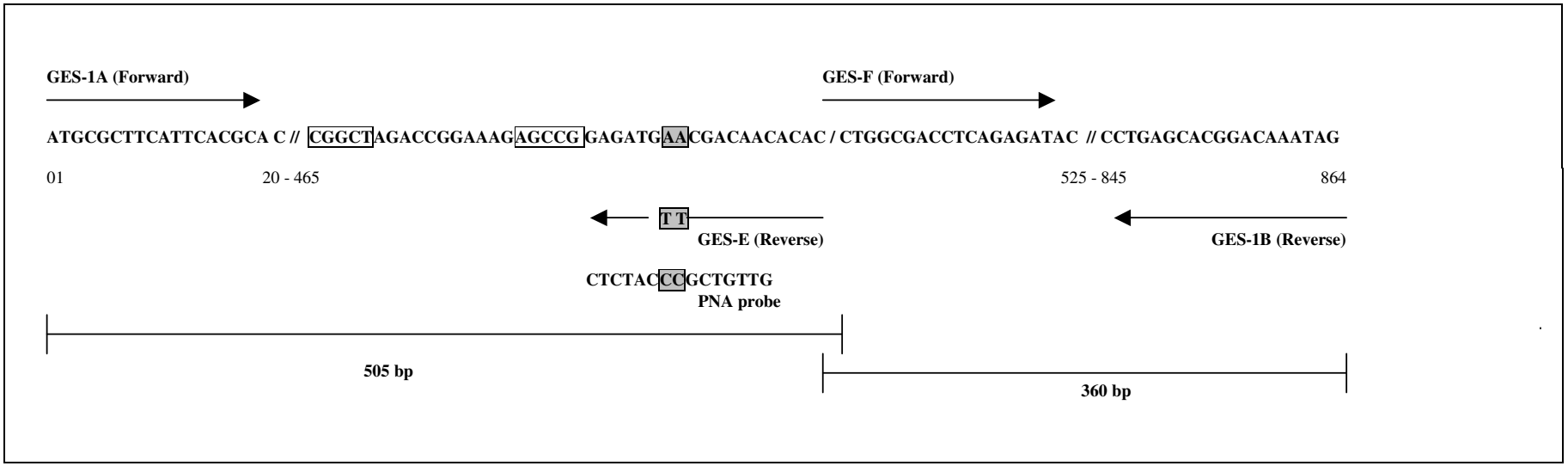

Figure 4-2: Schematic diagram depicting the placement of amplification primers and the competitive PNA probe relative to the gene sequence of *bla*$_{GES-2}$, (nt 1 – 864). Relevant point mutations are exhibited in shaded boxes, whereas stemloop sequences are depicted in open boxes. Note the CC_{PNA} - AA_{DNA} nucleotide mismatch, causing instability of the PNA-DNA duplex, thereby facilitating replacement of the PNA probe with the reverse amplification primer GES-E. Amplification products (505 and 360 bp respectively) are depicted by straight lines. Sequence direction is delineated as 5` - 3`.

Additionally, by constructing the PNA probe to bind in a highly thermostable, antiparallel mode to the complementary DNA target (20), the resulting PNA-DNA duplex formation impaired the amplicon interstrand reassociation (6), thereby further stabilizing the reverse primer-binding site on the DNA template.

Novel methods to detect and identify genes encoding ESBLs will continue to develop as our understanding of molecular methods increase. However, the most powerful driving mechanism behind this movement will most probably be to stay within budgetary restraints. The remarkable ability of PNA to hybridize in a sequence-specific manner with high affinity to complementary nucleic acids and thereby acting as a PCR controller, makes it the ideal molecule to use in screening programmes for well-characterized, specific or geographically restricted ESBLs. This method will however not detect point mutations outside of the chosen mutation site and therefore only offers a partial alternative to gene sequencing. Proper modification of this method to suit individual needs may prove to be highly cost effective in resource poor settings, not only from savings incurred by direct detection of epidemiologically relevant point mutations, but also through not having to procure expensive equipment.

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CHAPTER 5: RAPID DETECTION AND SEQUENCE-SPECIFIC DIFFERENTIATION OF EXTENDED-SPECTRUM BETA-LACTAMASE GES-2 FROM *PSEUDOMONAS AERUGINOSA*, WITH A REAL-TIME PCR ASSAY.

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5.1 Introduction

Detection of GES-2 and other extended-spectrum beta-lactamase (ESBL)-producing *Pseudomonas aeruginosa* isolates in the clinical microbiology laboratory utilizing ordinary methods is notoriously difficult and laborious (20). In addition, the integron genetic structures that support these ESBLs such as GES-2, not only confers resistance towards broad-spectrum beta-lactam antibiotics, but also towards other unrelated classes of antibiotics such as aminoglycosides as well as several non-related chemical compounds (20). These isolates are therefore very difficult indeed, to treat and control successfully. Apart from standard PCR and gene sequencing, molecular detection methods for ESBLs in general developed a wide array of applications most suited for the TEM- and SHV-type of ESBL families (4). These methods may include oligotyping (10), PCR-restriction fragment length polymorphism analysis (2), single-strand conformation polymorphism analysis (12), real-time PCR with melting curve analysis (18), and ligase chain reaction-based tests (7, 14). This chapter describes the application of the LightCycler to rapidly and specifically detect the more potent gene, bla_{GES-2}, in the GES / IBC ESBL family.

5.2 Materials and methods

5.2.1 Bacterial strains. One hundred *P. aeruginosa* clinical isolates, as described in Chapter 4, were included in this study (section 4.2.1). Well-characterized bacterial isolates used to standardize the molecular detection methods are listed in Table 4-1.

5.2.2 Susceptibility testing. A disk diffusion assay as described in Chapter 4 was performed and results interpreted (section 4.2.2, 13).

5.2.3 DNA extraction. Extraction of whole-cell DNA was accomplished with an ethanol precipitation-based method as described previously (section 4.2.3, 6,15).

5.2.4 LightCycler fluorescence resonance energy transfer (FRET) mediated mutation assay. The mutation detection assay was performed as described previously (18), with reaction specific modifications as necessary.

i) Amplification primer design. A forward primer GES-C, 5`-GTT TTG CAA TGT GCT CAA CG-3` (nt 176 – 195) (*Tm* = 54.6°C), was designed from the gene sequence of *bla*$_{GES-2}$ [GenBank accession number AF 326355] (17), by utilizing Primer3 software (Whitehead Institute for Biomedical Research). The previously described primer GES-1B (20-mer, *Tm* = 56.4°C) (17, 20), was used as the reverse primer, amplifying a 689 bp product. Both primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, IO.

ii) Fluorogenic probe design. Since the GG to AA mutation defining *bla*$_{GES-2}$ from *bla*$_{GES-1}$ and *bla*$_{IBC1/2}$ respectively, resides on nt 493 – 494 of the coding region (17), a 20-mer, 3`-FITC labeled sensor probe, 5`-AGA TGG GCG ACA ACA CAC CT-3` (*Tm* = 59.8°C), was designed from the gene sequence of *bla*$_{GES-1}$ [GenBank accession number AF 156486] (16). The anchor probe consisted of a 33-mer, 5`-LC Red640 labeled and 3`-phosphorylated, 5`- GCG ACC TCA GAG ATA CAA CTA CGC CTA TTG CTA-3` (*Tm* = 68.4°C) oligonucleotide, binding at a distance of one nucleotide, directly downstream of the sensor probe (Table 5-1).

Table 5-1: Oligonucleotide sequences for PCR analysis used in this study.

Primer / probe	Sequence [a]	Function	Source or reference
Nested PCR			
GES-1A	ATG CGC TTC ATT CAC GCA C	Forward primer	17, 20
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse primer	17, 20
GES-C	GTT TTG CAA TGT GCT CAA CG	Forward primer	This study
GES-D	TGC CAT AGC AAT AGG CGT AG	Reverse primer	This study
Real-time PCR			
GES-C	GTT TTG CAA TGT GCT CAA CG	Forward primer	This study
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse primer	17, 20
Probe 1	AGA TGG GCG ACA ACA CAC CT - FL	Sensor probe complementary to bla_{GES-1} / bla_{IBC}	This study
Probe 2	LC Red 640- GCG ACC TCA GAG ATA CAA CTA CGC CTA TTG CTA - PH	Anchor probe	This study

[a] – Sequences are delineated in 5` - 3` order. Shaded box depict relevant point mutation. FL – fluorescein, PH – phosphorylation, LC Red 640 – LightCycler Red 640.

Table 5-2: LightCycler amplification and melting curve protocol followed in this study.

Program	Number of cycles	Target temperature (ºC)	Hold time (s)	Temperature transition rate (ºC/s)	Fluorescence acquisition mode [a]
Polymerase activation	01	95	600	20	None
Three-step PCR	40				
a) Denaturation cycle		96	01	20	None
b) Amplification cycle		52	10	20	Single
c) Extension cycle		72	15	20	None
Three step melting curve	01				
a) Denaturation cycle		95	10	20	None
b) Holding cycle		45	30	20	None
c) Melting cycle		95	0	0.2	Continuous

[a] Fluorimeter gains settings were: F1 equal to 1, F2 equal to 15 and F3 equal to 30.

Both fluorophore–labeled probes were synthesized and purified by reversed-phase high-pressure liquid chromatography by TIB Mol Biol (Berlin, Germany). Primer and probe placement on the DNA template is depicted in Figure 5-1.

iii) Preparation of PCR reaction. The PCR reaction mix comprised 0.5 µM of each primer, 0.2 µM of the sensor and anchor probes respectively, 4 µl LightCycler FastStart DNA Master^{PLUS} reaction mix (Roche Diagnostics, Penzberg, Germany), 5 µl DNA template and nuclease free water to a final reaction volume of 20 µl. This reaction mix was loaded into glass capillary cuvettes and after brief centrifugation (20 rpm for 30 s), the sealed capillaries were loaded into the LightCycler (Idaho Technology Inc., Salt Lake City, UT) for rapid PCR analysis. Controls included DNA templates from well-characterized isolates (Table 4-1) as well as distilled water as a DNA negative control.

iv) LightCycler program and melting curve analysis. Programming of the LightCycler (Table 5-2), consisted of four distinct steps namely: a) one cycle for polymerase activation and template denaturation (10 min at 95°C), b) 40 cycles each comprising of denaturation (one second (s) at 96°C), annealing (10 s at 52°C) with single fluorescence acquisition mode and extension (15 s at 72°C) with a temperature transition rate of 20°C/s, c) a melting curve comprising of one denaturation cycle (10 s at 95°C), one holding cycle (30 s at 45°C) and one melting cycle with a target temperature of 95°C (temperature transition rate 0.2°C/s) with continuous fluorescence acquisition mode, and finally d) one cooling cycle to 40°C (temperature transition rate 20°C/s). Optimum automated fluorimeter gains settings as determined by LightCycler Software version 3.5 (Idaho Technology Inc., Salt Lake City, UT) comprised of: F1 equal to 1, F2 equal to 15 and F3 equal to 30. Continuous fluorescence monitoring during the melting cycle measured the decrease of FRET at

fluorescence F2, as the FITC-labeled sensor probe dissociated from the LC Red640-labelled anchor probe during the slow temperature rise. Fluorescence signals from F2 were plotted automatically in real-time against temperature (*T*) to produce a melting curve for the mutation occurring at nt 493 - 494. Automated conversion of this data to melting peaks was obtained by plotting the negative derivative of fluorescence (-*dF2/dT*) versus *T*.

v) Analysis of amplification products. The entire contents of capillaries containing amplification products from well-characterized isolates (Table 4-1), were analyzed by means of agarose gel electrophoresis as previously described (18).

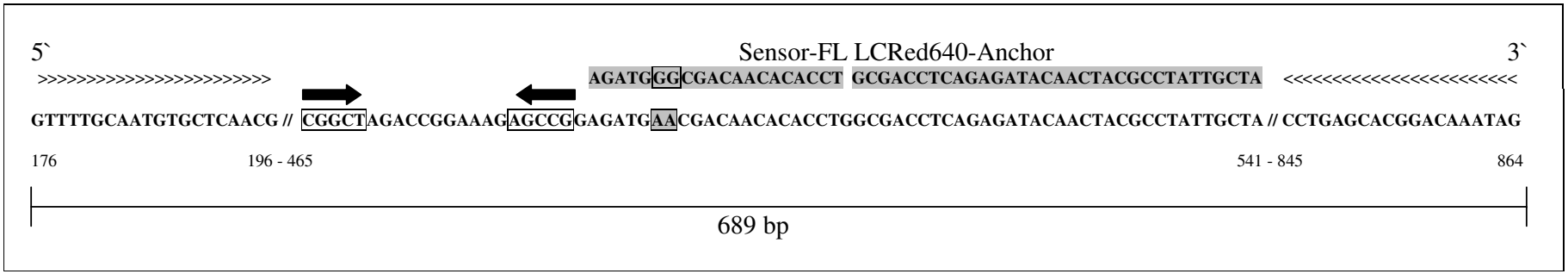

Figure 5-1: Alignment of real-time PCR primer and fluorogenic probe sequences with *bla*$_{GES-2}$. Stem loop sequences appear in boxes, arrows indicate the direction of stem loop formation. The relevant nucleotide mismatch (nt 493 – 494) is depicted as a shaded box. Note the close approximation of the sensor probe to the stem loop structure in addition to the one nt distance between the sensor and anchor probes respectively. [>>> Sense and <<< Antisense primers] [nt 488 – 507 sensor probe 3` marked with fluorescein (FL) and nt 509 – 541 anchor probe 5` marked with LightCycler Red 640 (LCR640)].

5.2.5 Nested-PCR amplification.

i) Nested-PCR primer design. Previously published primers GES-1A and GES-1B (17, 20), were used to amplify the entire *bla*$_{GES\ /\ IBC}$ coding region during the first round of amplification. For a second round of amplification, an alternative reverse primer was constructed from the gene sequence of *bla*$_{GES-2}$ [GenBank accession number AF 326355] (17), utilizing Primer3 software (Whitehead Institute). The following primer sequence was generated: GES-D, 5`-TGC CAT AGC AAT AGG CGT AG-3` (nt 527 – 546), targeting a 371 bp region in conjunction with forward primer GES-C (Table 5-1), spanning all currently published point mutations of *bla*$_{GES}$ and *bla*$_{IBC}$ genes (5, 11, 16, 17).

ii) Nested PCR reaction conditions. Primers GES-1A and GES-1B were first utilized under standard PCR conditions (19), to amplify an 864 bp product. The first round PCR reaction mix comprised 0.32 µM of each primer, 5 µl *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 U *Taq* DNA polymerase, (Promega Corporation, Madison, WI), 5 µl DNA template and distilled water to a final reaction volume of 50 µl. Amplification was performed with a GeneAmp PCR System 9600 (Perkin Elmer Cetus, Emeryville, CA) and consisted of an initial denaturation step (2 min at 95°C), followed by 35 amplification cycles each comprising a denaturation step (30 s at 95°C), followed by annealing (1 min at 50°C), and extension (1 min at 72°C). Completion of 35 amplification cycles was finally followed by an extension step of 5 min at 72°C. Second round amplification conditions were exactly similar to the first round, primers GES-C and GES-D were used together with 2 µl of DNA product obtained from first round amplification as a reaction template. All reaction preparations were performed in separate rooms

equipped with laminar flow cabinets to avoid cross-contamination with amplification products. PCR products obtained from both amplification rounds were electrophoresed as described previously (4.2.4). DNA templates from well-characterized isolates (Table 4-1), as well as distilled water, served as PCR controls.

5.2.6 DNA sequencing analysis. Sequencing was performed on 371 bp PCR products on both the forward and reverse strands, on a SpectruMedix model SCE 2410 (Spectru Medix, State College, PA) automated sequencer, incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Electropherograms of generated sequences were inspected with Chromas software version 1.45 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Obtained DNA sequences were analyzed with BLAST software (1). A PCR product obtained from *P. aeruginosa* GW-1 was used as a sequencing control.

5.3 Results.

i) Real-time PCR and melting curve analysis. *P. aeruginosa* clinical isolates retrieved from departmental cryo-archives from 1998 – 2001, originated from a variety of specimens including endotracheal aspirates, urine samples, pus swabs, blood cultures, tissue biopsies, and intravenous catheters. Specimens originated from teaching hospitals associated with the Department of Medical Microbiology, University of Pretoria, South Africa, serving mainly the clinical disciplines of Internal Medicine, Surgery, Pediatrics, Neurosurgery, Orthopedics and Gynecology. Although varying zone size diameters were observed, all clinical isolates (n = 100) were resistant to ceftazidime in the disc-diffusion assay (data not shown). Rapid PCR revealed 83 clinical isolates to exhibit DNA template amplification in real-time with

continuous fluorescence monitoring in channel F2. Melting curve analysis revealed 82 of the 83 clinical isolates to exhibit a mean *Tm* of 55.68 ± 0.33 ºC, whilst one clinical isolate exhibited a *Tm* of 66.83 ºC (Table 5-3). Mean *Tm* values of 55.25 ± 0.30 ºC, 64.07 ± 0.72 ºC and 64.78 ± 0.59 ºC, were obtained by analysis of amplification products from bla_{GES-2}, bla_{GES-1} and bla_{IBC} control isolates respectively. Electrophoresis of amplicons obtained from well-characterized isolates (Table 4-1), all exhibited a 689 bp $bla_{GES/IBC}$ gene product except the *E. coli* ATCC 25922 isolate (data not shown). No DNA template amplification was observed in real-time with distilled water controls.

ii) Nested-PCR detection of $bla_{GES/IBC}$. First round PCR amplification resulted in 51 of the clinical isolates exhibiting an 864 bp amplicon. These isolates corresponded with the bla_{GES-2} producing organisms described previously (section 4.3). Second round nested-PCR amplification detected an additional 37 isolates, exhibiting a 371 bp product. Thus from a 100 *P. aeruginosa* clinical isolates, 88 tested positive for a $bla_{GES/IBC}$ gene product (Table 5-3). Amplicons 864 and 371 bp in size were obtained from each control strain during first and second amplification rounds respectively, except from the negative control, *E. coli* ATCC 25922.

iii) Sequence analysis of nested-PCR products. Analysis of 371 bp amplification products obtained from clinical isolates resulted in 87 sequences corresponding to that of bla_{GES-2} and one sequence being homologous with bla_{GES-1}. These results corresponded with results obtained from melting curve analysis on the LightCycler, with bla_{GES-2} products exhibiting a mean *Tm* of 55.68 ± 0.33 ºC. The bla_{GES-1} product corresponded with a *Tm* of 66.83 ºC. In addition to these findings, a stem loop in the secondary DNA structure (nt 466 – 470 and 482 – 486 of the coding region) was noted.

Table 5-3: Results obtained with nested-PCR, real-time PCR and DNA sequencing methods.

Method	Isolate type	*bla*$_{GES/IBC}$ amplification positive	*bla*$_{GES/IBC}$ amplification negative	Number of amplicons analyzed	Tm (°C) ± SD	Sequencing result
Nested-PCR	Clinical isolates [a]	88	12	87	-	*bla*$_{GES-2}$
				1	-	*bla*$_{GES-1}$
Real-time PCR	Clinical isolates [a]	83	17			
Melting curve analyses	Clinical isolates			82	55.63 ± 0.33	*bla*$_{GES-2}$
	Clinical isolate			1	66.83	*bla*$_{GES-1}$
	GES-2 control isolates [b]				55.25 ± 0.3	
	GES-1 control isolate [b]				64.07 ± 0.72	
	IBC control isolates [b]				64.78 ± 0.59	

[a] All isolates tested resistant to ceftazidime.
[b] Control isolates are shown in Table 4-1.

5.4 Discussion.

Multi-drug resistant *P. aeruginosa* has become a major burden in the care of long-term debilitated intensive care unit patients, affecting a wide spectrum of clinical disciplines both in the public and private sector hospitals in the Pretoria region, South Africa. Although routine detection of ESBL production in *Enterobacteriaceae* is well described (9), it is not easily accomplished with *P. aeruginosa.* In the case of GES-2 - producing *P. aeruginosa* isolates, it can be extremely difficult and time consuming in the clinical microbiology laboratory (20). The aim of this study was to develop a real-time PCR assay to reliably detect the ESBL GES-2, partially compromising the efficacy of imipenem in South African *P. aeruginosa* clinical isolates. For this, the ultra rapid LightCycler, based on real-time fluorimetric measurement of amplification products, proved ideal to analyze the "hot spot" area in the bla_{GES} gene for resistance mutations as previously described with *Candida albicans* isolates (8). Previous real-time PCR work conducted on the detection of SHV-type ESBLs from *Enterobacteriaceae* clinical isolates (18), reported a 100 % sensitivity and specificity for the described LightCycler assay. During this study, the LightCycler and nested-PCR assays detected a $bla_{GES/IBC}$ product from 83 and 88 clinical isolates respectively, exhibiting a sensitivity of 94.3% for the LightCycler compared to nested-PCR. Comparison of real-time amplicon *Tm* determinations with sequencing data however revealed 100% specificity, therefore concurring with the previous study (18), with regard to the usefulness of the LightCycler for accurate identification of point mutations in ESBL genes. The noted decrease in sensitivity may be influenced by a number of factors including the quality of DNA template used in experiments (20) and the choice of both DNA polymerase and polymerase buffer systems utilized in the LightCycler assay (21).

Design and placement of the fluorogenic probes proved to be problematic due to the presence of the stem loop structure situated in close proximity upstream from the mutation site (Figure 5-1). This was overcome by placing the shorter sensor probe directly alongside the stem loop structure and designing a reverse primer that binds well away downstream from the mutation site, while still keeping the resulting amplicon short. A thorough initial denaturation step in the amplification protocol further facilitated probe and template binding prior to primer extension and amplification. Based on a previous study which demonstrated a *ca.* 6ºC temperature shift per nt mismatch (18), and due to the two nt mismatch between bla_{GES-2} and the sensor probe, a *ca.* 10 – 12 ºC difference in *Tm* was expected between bla_{GES-2} and $bla_{GES-1\ /\ IBC}$ amplification products respectively. However, *Tm* analyses of bla_{GES-2} and bla_{GES-1} products obtained from control isolates demonstrated a difference of 7.8 – 9.84 ºC, whereas bla_{GES-2} and bla_{IBC}-type products exhibited a temperature difference of 8.64 – 10.42 ºC. Melting point analysis of 83 bla_{GES-2} clinical isolates exhibited a *Tm* of 55.63 ± 0.33 ºC, clearly distinguishing the two nt mismatch from bla_{GES-1} type amplification products (Figure 5-2).

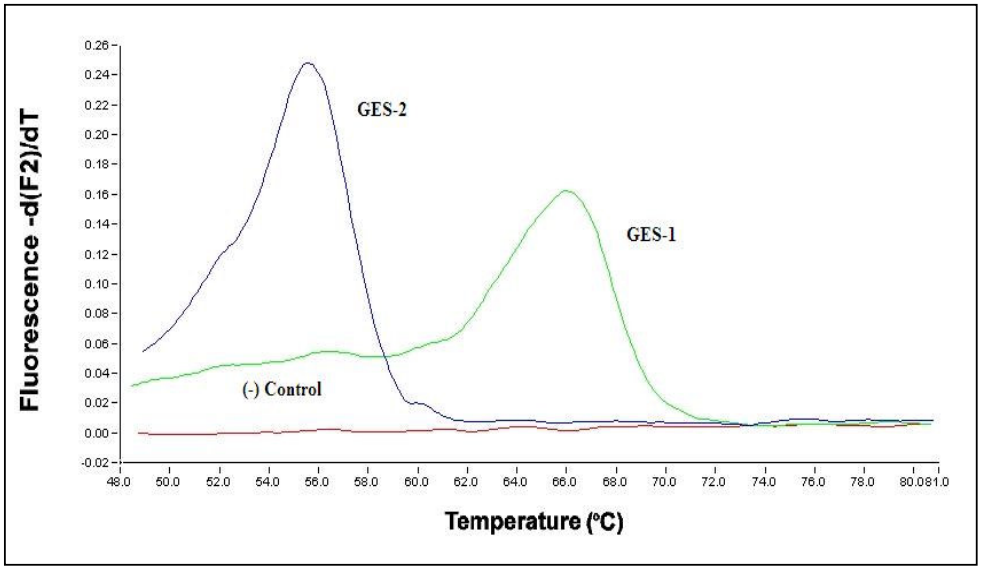

Figure 5-2: Melting peaks of *bla*$_{GES-1}$, *bla*$_{GES-2}$ and *E. coli* ATCC 25922 (*bla*$_{GES/IBC}$ template negative control), amplification products plotted as the negative derivative of fluorescence F2 [*-d(F2)/dt)*] versus temperature [T]. The *Tm* difference between an exact sensor probe match (GES-1) and a two-nucleotide mismatch (GES-2) is clearly visible. No melting peak was generated with the negative control isolate.

Where there was an exact match between the sensor probe and the template sequence, a *Tm* of 64.07 ± 0.72 °C (*bla*$_{GES-1}$) and 64.78 ± 0.59 °C (*bla*$_{IBC}$) was found, in comparison with 66 °C reported previously (18). These differences in *Tm* could most probably be ascribed to the genetic divergence between *bla*$_{SHV}$- and *bla*$_{GES\ /\ IBC}$-type genes (20) as well as the GC content differences evident between *bla*$_{GES-2}$ and the *bla*$_{GES-1\ /\ IBC}$ mutation areas examined by this study (17). Future research incorporating real-time PCR detection of the *bla*$_{GES}$ genes should investigate the efficacy of silica based DNA extraction to improve template purity (3), as well as the development of fluorogenic probes that can cost-effectively categorize this growing family of ESBL genes into clinically relevant subgroups (18).

One clinical isolate with a *Tm* of 66.83 °C, proved to be a bla_{GES-1} product on sequence analysis, making this the first report of beta-lactamase GES-1 from a South African clinical isolate of *P. aeruginosa*. This isolate was collected in 1999 originating from a blood culture from a 38 year old, male patient in a surgical ICU after being treated for multi-trauma complicated by a nosocomial pneumonia. On treatment with parenteral imipenem he made a subsequent full recovery. The collection of this GES-1-producing isolate almost coincides with the original description of bla_{GES-1} from a *K. pneumoniae* clinical isolate originating from French Guiana in South America (16). This data finally proves that both GES-1 and GES-2 ESBL-producing *P. aeruginosa* circulate in Pretoria, South Africa, with GES-2 being possibly more advantageous to, and prevalent in, this species.

Until such time as the NCCLS and other laboratory standards organizations formulate criteria for the detection of ESBLs from *P. aeruginosa* and other non-fermentative bacterial species, this phenomenon will go by mostly undetected in the routine laboratory with possible dire clinical consequences. Currently, molecular detection of ESBL encoding genes from these species are the only reliable method available and as such, the LightCycler has proved to be sensitive and highly specific in the detection of ESBL genes of the GES / IBC family from *P. aeruginosa.* Further developments in ESBL production from non-fermenting Gram-negative bacteria will certainly necessitate the use of a fast and dependable molecular diagnostic method, to possibly enhance the diagnostic capabilities of the clinical microbiology laboratory in this regard.

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CHAPTER 6: GENETIC STABILITY OF CLASS 1 INTEGRON-BORNE, *BLA*$_{GES}$-TYPE GENES UNDER SHORT-TERM, SELECTIVE, IN-VITRO ANTIBIOTIC PRESSURE.

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6.1 Introduction

The emergence of the novel GES family of extended-spectrum beta-lactamases (ESBLs) belonging to Ambler class A, has been demonstrated in South America, Europe, South Africa and Japan recently (1, 6, 15, 16, 20, 21, 22). In addition to hydrolyzing various beta-lactam antibiotics, including several expanded-spectrum cephalosporins, weak carbapenem hydrolysis and relative resistance to beta-lactamase inhibitors have been demonstrated previously in GES-2 (15). This expansion of substrate profile, compared to beta-lactamase GES-1, was ascribed to the G170N substitution in the omega loop of the mature protein (15, 16). Recent reports from Japan (21) and Greece (20) respectively, describe amino acid substitutions in Ambler position 170 of the omega loop region of novel enzymes belonging to the GES family. A single G170S substitution as reported for GES-4 is most likely associated with more efficient hydrolysis of carbapenems and cephamycins as well as exhibiting a decreased affinity for beta-lactamase inhibitors (21). Structural changes to the omega loop, as hypothesized previously, may be involved in increased binding of the hydroxy-ethyl moieties of carbapenems (12). In recent reports describing novel point mutations in GES-type genes, selective antibiotic pressure is thought to be a major factor, facilitating mutation and possible dissemination in the nosocomial environment (20, 21). In this study, the short-term, continuous exposure, of two well-described *bla*$_{GES}$–producing isolates to sub-inhibitory levels of single antimicrobial

agents, was examined to determine the possible effects on these genes and their immediate upstream regulatory environments.

6.2 Materials and methods

6.2.1 Bacterial strains. Bacterial isolates *Klebsiella pneumoniae* ORI-1 (16) and *Pseudomonas aeruginosa* GW-1 (15) were used in this study. Both were initially described as clinical isolates and natural carriers of class 1 integron-borne bla_{GES-1} and bla_{GES-2} respectively. In addition, both were found to be resistant to various antibiotics as reported previously (15, 16), and were suitable to imitate local clinical conditions closely. Isolates were cryo-preserved at -75˚C prior to this study (17).

6.2.2 Antibiotic challenge assay. Fifteen different antibiotic containing solutions were made up in sterile Mueller-Hinton broth (Becton Dickinson Co, Cockeysville, MD) with concentrations corresponding to sensitive breakpoints for each species as specified by the National Committee for Clinical Laboratory Standards (NCCLS) or as previously published (7, 14) (Table 6-1). Antimicrobial agents were obtained from Abtek Biologicals Ltd., Liverpool, UK, Merck Sharpe & Dohme (Pty) Ltd, Halfway House, South Africa (imipenem) and AstraZeneca Pharmaceuticals (Pty) Ltd, Sunninghill, South Africa (meropenem) and reconstituted according to the recommendations of the respective manufacturers. Commonly used antibiotics in local training hospitals were chosen for evaluation during this study, based on data obtained during a recent departmental audit (Department of Medical Microbiology, University of Pretoria – 2004). Inoculae of *K. pneumoniae* ORI-1 and *P. aeruginosa* GW-1 were prepared by the growth method in broth medium as described elsewhere (14). On day one, 100 µl of this cell suspension was then inoculated into 10 ml of each antibiotic solution (Table 6-1) and incubated under constant shaking at 37˚C for

24 hours. On day two, 100 µl of the day one culture was passaged to a corresponding freshly prepared 10 ml antibiotic solution and incubated as described.

Table 6-1: Antibiotics and antibiotic concentrations used in this study.

Antibiotic	***K. pneumoniae* ORI-1 (µg/ml)**	***P. aeruginosa* GW-1 (µg/ml)**
Ampicillin	8	8[a]
Piperacillin-Tazobactam	16/4	64/4
Cephazolin	8	8[a]
Cefuroxime	8	8[a]
Ceftazidime	8	8
Meropenem	4[c]	4
Imipenem	4[c]	4
Nalidixic acid	16	16[a]
Ofloxacin	2	2
Ciprofloxacin	1	1
Amikacin	16	16
Gentamicin	4	4
Trimethoprim - sulfamethoxazole	2/38	2/38
Colistin sulphate	1[b]	1[b, c]
Nitrofurantoin	32	32[a]

[a] – Antimicrobial breakpoints for *Enterobacteriaceae* were used for *P. aeruginosa* isolates where none existed (14).
[b] – Colistin sulphate concentrations were reconstituted as previously published (15).
[c] – Isolates did not survive beyond 24 hours of incubation and were excluded from further analyses.

This process was repeated for each isolate until five days of incubation (120 hours) was reached. The same process was followed with controls, which were inoculated

into sterile, antibiotic-free Mueller-Hinton broth (Becton Dickinson Co). Cells were then harvested from each solution by centrifugation (4500 x g, 10 min, 4˚C) and the cell pellet was washed twice in 1 ml distilled water by vortexing (2500 rpm, 10 s) and centrifugation (4500 x g, 10 min, 4˚C). Washed cell pellets were resuspended in sterile liquid broth [1ml Brain-heart infusion broth (Oxoid Ltd., Hampshire, UK) with 10% v/v glycerol added (Merck, Darmstadt, Germany)] and stored at -75˚C (17), until further analysis. Cryopreserved cells were revived on a non-selective, antibiotic free, nutrient agar medium (Oxoid Ltd.) for 24 hours, incubated at 37˚C in ambient atmosphere, prior to further analysis.

6.2.3 DNA extraction. Extraction of whole-cell DNA was performed with an ethanol precipitation-based method as described previously (23, Appendix A). DNA pellets were dried in a DNA Speed Vac 110 (Savant Instruments Inc., Farmingdale, NY), resuspended in 1ml TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 7.4) and stored at 4˚C until further analysis.

6.2.4 PCR amplification and detection. A primer pair annealing to both the *IntI* and bla_{GES} genes of class 1 integrons from *K. pneumoniae* ORI-1 and *P aeruginosa* GW-1 respectively (15, 16), was designed with Primer3 software (Whitehead Institute for Biomedical Research) as described previously (23). Primers Int-E and Int-F targeted a 814 bp region from both isolates including the –10 region of promoter P_1 (3, 15), the –35 and –10 regions of P_2 (15), the *attI1* site and the core site motif GTTAGAC, situated upstream of the first gene cassette, bla_{GES} (15, 16). Primer Int-E and previously published reverse primer GES-1B (15) targeted the aforementioned structures as well as the complete sequence of the first gene cassette bla_{GES}. Primer

sequences appear in Table 6-2. Primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, Iowa. Whole-cell DNA obtained from isolates subjected to the antibiotic challenge assay served as experimental templates in a standard PCR amplification assay. PCR cycling conditions and detection of amplicons by electrophoresis are described elsewhere (23). DNA from non-exposed isolates served as controls, distilled water served as a DNA negative PCR control.

Table 6-2: Oligonucleotide sequences used in this study.

Primer name	Sequence 5` – 3`	Function	Origin or reference
Int-E	AGG ATG CGA ACC ACT TCA TC	Forward	This study
Int-F	CGG TGC CTG AGT CAA TTC TT	Reverse	This study
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse	8

6.2.5 Restriction enzyme analysis: PCR products produced with primers Int-E and Int-F (Table 6-2) were analyzed for cassette insertion into the *attI1* site (5, 10) with restriction enzyme *Ava*I (Promega Corporation, Madison, WI). *Ava*I was chosen based upon virtual restriction digests of expected template sequences performed with Restriction Enzyme Site Mapper version 3 software available from URL: http://www.restrictionmapper.org. Restriction enzyme reactions were carried out according to the directions of the manufacturer. Detection of restriction fragments was performed with field inversion gel electrophoresis (FIGE) (4V/cm, reorientation angle 180˚, pulse frequency $1s^{-1}$) in a 1% small fragment agarose gel (Promega Corp.) containing ethidium bromide (0.5 µg/ml) with TBE running buffer (45 mM Tris-

borate, 1 mM EDTA, pH 7.4) and visualized under UV light. A 100 bp DNA marker (Promega Corp.) was used for sizing of fragments.

6.2.6 DNA sequencing. Automated sequencing of PCR products obtained with primers Int-E and GES-1B (Table 6-2) was performed and analyzed as described previously (23).

6.3 Results

Standard PCR amplification. Amplicons obtained with primers Int-E and Int-F (Table 2) from *P. aeruginosa* GW-1 and *K. pneumoniae* ORI-1 isolates subjected to 5 days incubation in the presence of sub-inhibitory concentrations of antibiotics, were all ca. 800 bp in size. This correlated with PCR products obtained from non-exposed controls (Figure 6-1). Amplicons obtained with primer pair Int-E and GES-1B (Table 6-2) were all ca. 1500 bp in size, again correlating with results obtained with non-exposed controls (data not shown). No amplification product was obtained with the DNA negative control. *K. pneumoniae* ORI-1 and *P. aeruginosa* GW-1 isolates could not sustain growth beyond 24 hours of incubation in the presence of carbapenems and colistin sulphate respectively (Table 6-1), and were excluded from further analysis.

***Ava*I restriction enzyme analysis.** PCR products produced with primers Int-E and Int-F (Table 6-2) were cut with *Ava*I, 100 bp upstream of the core site motif GTTAGAC, yielding two restriction fragments of 394 and 416 bp respectively (Figures 6-1, 6-2). Cassette insertion at the *attI1* site would have significantly increased the size of the 394 bp fragment making it relatively simple to detect by electrophoresis, none of which could be demonstrated when compared to non-exposed controls.

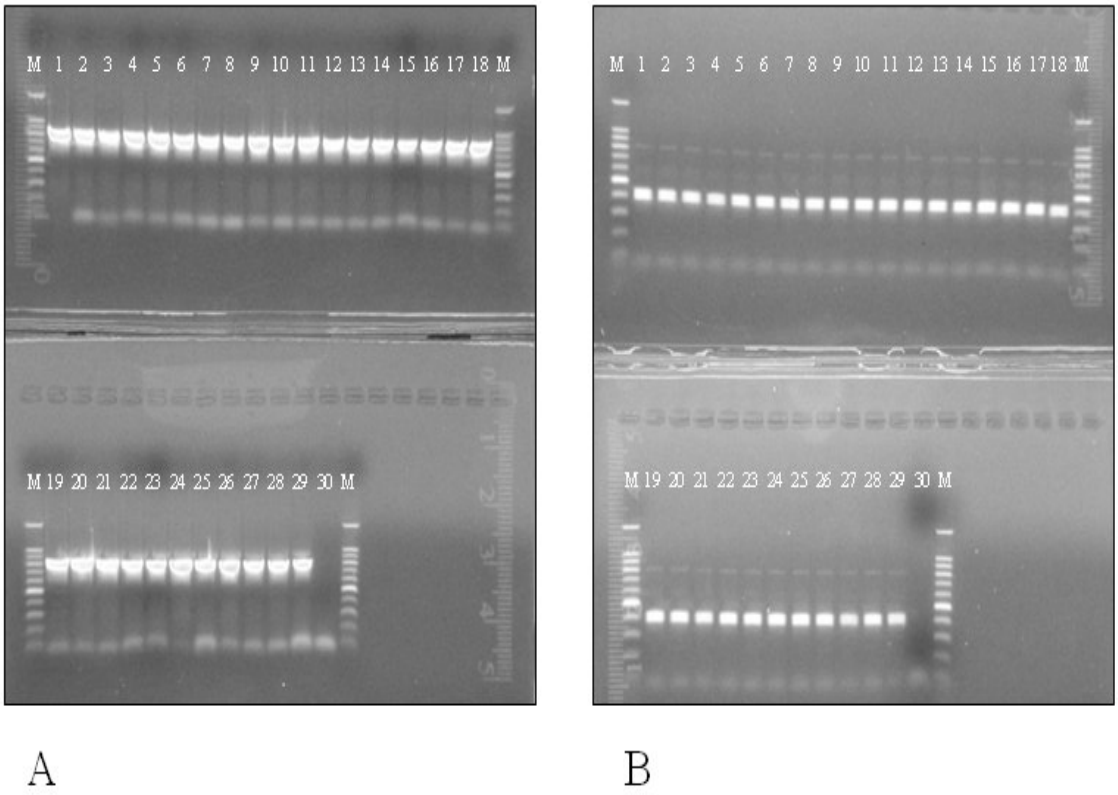

Figure 6-1: Gel electrophoresis depicting Int-EF PCR products (Panel A) and *Ava*I restriction of Int-EF PCR products (Panel B). M – 100 bp molecular marker (Promega Corp.). Lanes 1-13 *K. pneumoniae* ORI-1 exposed isolates: [1-SXT, 2-AMP, 3-COL, 4-CXM, 5-AMK, 6-NIT, 7-OFX, 8-CAZ, 9-CIP, 10-GEN, 11-NAL, 12-CFZ, 13-TZP], 14-*K. pneumoniae* ORI-1 non-exposed control. Lanes 15 – 28 *P. aeruginosa* exposed isolates: [15-NIT, 16-CFZ, 17-CAZ, 18-GEN, 19-AMP, 20-TZP, 21-CXM, 22-NAL, 23-CIP, 24-OFX, 25-AMK, 26-SXT, 27-MEM, 28-IPM], 29-*P. aeruginosa* GW-1 non-exposed control, 30-DNA negative control.

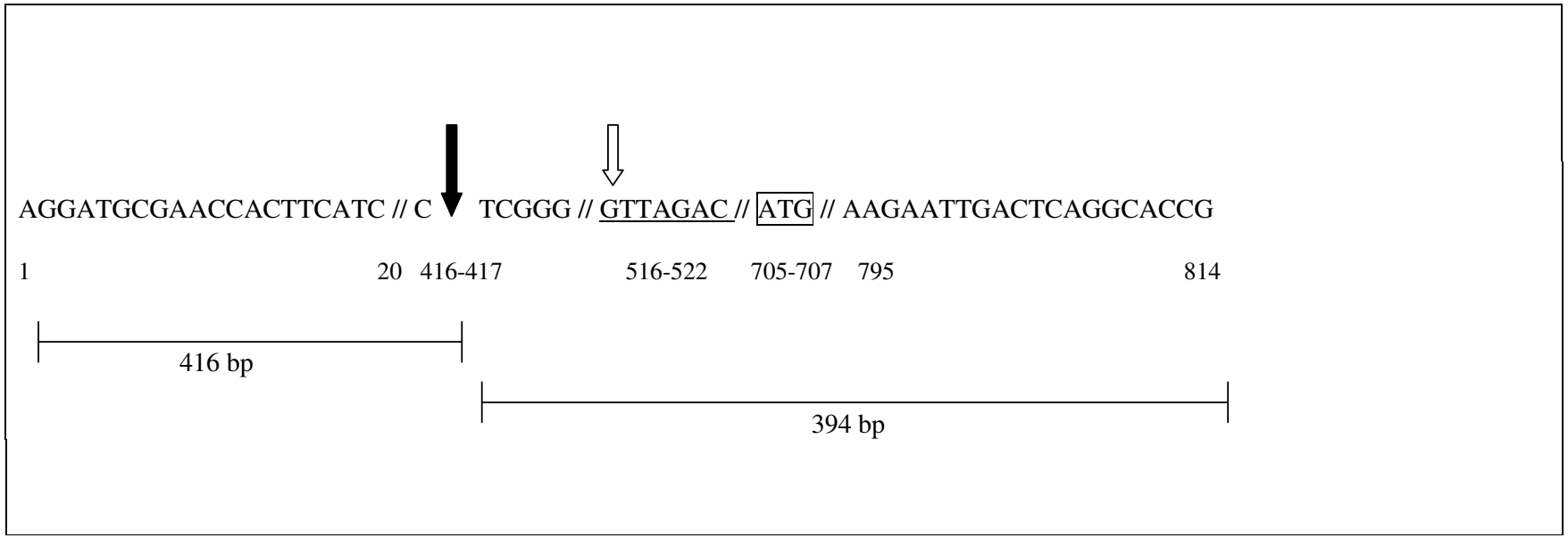

Figure 6-2: Schematic representation of *Ava*I restriction of PCR products obtained with primers Int-E and Int-F. The restriction site is marked with a black arrow (nt 416-417), the core site is underlined (nt 516-522), a white arrow indicates the recombinant crossover point (nt 516-517) and the bla_{GES} start codon (nt 705-707) is boxed. Horizontal lines depict the restriction fragment sizes of 416 and 394 bp respectively (not drawn to scale). Primers Int-E (forward) and Int-F (reverse) are depicted by nt 1-20 and 795-814 respectively.

DNA sequencing. Automated sequencing of amplicons obtained with primers Int-E and GES1-B (Table 6-2), demonstrated no difference between antibiotic exposed isolates and non-exposed controls. No differences could be detected in the promoter sequences of P_1 and P_2 compared to the previously published sequences (15, 16). The core site motif GTTAGAC upstream from *bla*$_{GES}$ remained stable, as did the nucleotide sequences of the omega loop regions of both *bla*$_{GES-1}$ and *bla*$_{GES-2}$ respectively.

6.4 Discussion

Rapid developments in the GES-type family of extended-spectrum beta-lactamases were reported lately, focusing mainly on novel point mutations leading to expansion of substrate profiles to include carbapenems (20, 21, 22). Reports originated from geographically diverse locations, suggesting that local antibiotic usage and practices may play an active role in promoting the selection of point mutations in *bla*$_{GES}$-type genes (20, 21). All GES-type genes described to date, mainly exist as gene cassettes on class 1 integrons [a notable exception was reported from Portugal (6)], with initial descriptions of *bla*$_{GES}$- type genes being situated closest to the 5` conserved segment (5`CS), containing the dual promoters P_1 and P_2 (15, 16). Very similar descriptions of the genetic environments of *bla*$_{GES-3}$ and *bla*$_{GES-4}$ originated from Japan, with GES-type genes situated closest to the promoter region (21, 22). A recent report from Greece however, details the gene cassette arrangement of *bla*$_{GES-3}$ and *bla*$_{GES-4}$ being preceded by an *aacA4* gene cassette (20), imitating integron In111 in which *bla*$_{IBC-1}$ is preceded by *aac(6`)Ib* (8, 19). Consequently, the expression level of an integron-borne antibiotic resistance gene is influenced by its position in the variable region and thus its relative distance from the common promoter region (2, 3). Major changes in

class 1 integrons, such as novel cassette integration events in the variable region, preferentially take place at the *attI1* recombination site, located at the 5`-end of the residing cassettes (3, 5, 9, 10, 11). Since *attI* sites lack the inverse core site motif RYYAAC, it cannot form palindromic structures like *attC* sites (10), therefore making it the preferred site to examine for cassette integration events. However, as demonstrated previously, where several cassettes are already present in the integron, recombinational events may occur between two 59-be sites, depending on the efficiencies of the various possible recombination reactions (4). In addition, gene cassette arrangement in the variable region can be altered by excision or reassortment, through the action of the integron integrase (2, 3). Gene cassette reassortment could possibly place certain genes closer to the promoter area, thus enhancing expression and resistance (2). All this data suggests that integrons are extremely pliable genetic structures, able to evolve rapidly in order to ensure the survival of the host bacterial species.

To date the role of single antimicrobials, employed at sub-inhibitory levels (as would occur during inappropriate antibiotic therapy), has not been examined in terms of their possible selective effect upon *bla*$_{GES}$-type genes. This study examined well-characterized bacterial isolates (15, 16) with class 1 integron-located *bla*$_{GES}$-type genes for three possible genetic scenarios: i) major events such as cassette integration into the *attI1* site upstream from *bla*$_{GES}$, ii) mutation of the regulatory environment upstream from *bla*$_{GES}$ and iii) spontaneous point mutations occurring within the respective *bla*$_{GES}$ genes. Despite the continuous exposure of isolates to sub-inhibitory levels of antimicrobials over 120 hours of incubation (the average local antibiotic course duration), none of these three events could be demonstrated. In fact, the DNA regions examined, were indistinguishable from those obtained from non-exposed

isolates in this study. Future research should focus on possible recombinational events occurring away from the *attI* site (4), while the organism is under antibiotic stress. From the data obtained, it thus appears unlikely that short-term exposure to a single antimicrobial agent will select for spontaneous mutations in *bla*$_{GES}$-type genes or their immediate upstream regulatory environments. As suggested by a previous study (18), integrons in bacteria originating from a natural habitat appear to preferentially exist without any antibiotic resistance gene cassettes, in the absence of antibiotic selective pressure. Thus, the precise role of prolonged, multi-antibiotic stress in the nosocomial environment as reported from Japan and Greece (20, 21, 22), on the genetic stability of *bla*$_{GES}$-type genes and the integrons that support them, is however still largely uncertain. Only further research conducted over longer time periods and possibly with various combinations of antimicrobials and drug concentrations, will possibly shed some light on the driving force behind spontaneous mutations observed in these genetic structures.

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CHAPTER 7: GENERAL DISCUSSION AND FINAL CONCLUSIONS.

"Reviews on extended spectrum beta-lactamases (ESBLs) are numerous, and cynics argue that there is nothing left besides cataloguing minor variants." (David M. Livermore – 3rd International Symposium on Antimicrobial Agents and Resistance, Seoul, Korea, 2001). This view cannot be further from the truth, as rapid development occurs in the bacterial genetic environment, bringing with it an accumulation of enzymes unrelated to the "traditional" TEM- and SHV-type beta-lactamases. Another problematic aspect is the emergence of integron-borne beta-lactamases with their respective armamentariums of co-resistance factors (1, Chapter 3). Recent developments in the class A beta-lactamases of *Pseudomonas aeruginosa* demonstrated exactly this phenomenon with great clarity (2, 3, Chapter 2).

A novel extended-spectrum beta-lactamase enzyme was discovered in the hospital-associated pathogen, *P. aeruginosa* in the PAH during May 2000, in Pretoria, South Africa (4). After chemical and molecular characterization of the enzyme was completed, it became clear that beta-lactamase GES-2 (Guiana Extended-Spectrum) was unique in its composition and antibiotic substrate profile (4), making it the first identification of this specific bacterial enzyme worldwide. What made this enzyme so different from all other known ESBLs was the fact that it was able to confer partial resistance towards the broad-spectrum beta-lactam antibiotic, imipenem (4). To compound matters, the detection of this resistance threat in the routine clinical microbiology laboratory is notoriously slow and laborious. False negative test results, utilizing routine detection methods are fairly common, making diagnosis and infection control extremely difficult (5). Phase 1 research (Chapters 4 and 5) conducted during this study, focused on improving the molecular laboratory detection of bla_{GES-2}. This research produced two highly specific molecular methods of detection. Firstly, the

development of a novel screening technique for this specific resistance mechanism (*bla*$_{GES-2}$) utilizing a peptide nucleic acid-mediated, competitive multiplex PCR method (6, Chapter 4), opened this specific research field to cost effective epidemiological surveys on a national scale. At the time of writing, the PNA-mediated PCR method (6) is already in use to detect *bla*$_{GES-2}$ from *P. aeruginosa* isolates obtained from several South African laboratories (G. Weldhagen – unpublished data). This method, being the first of its kind in the field of ESBL research, certainly through personal adaptation, has the ability to be used in the detection of a variety of very specific ESBL encoding genes. To illustrate the current impetus of PNA in the field of infectious disease research, a similar method targeting point mutations in *rpoB* genes of *Mycobacterium tuberculosis,* was published shortly (1 month) after the ESBL detection work (6), by a Japanese research group (7).

Secondly, to improve the rapid identification and diagnosis of this important resistance threat (GES-2) in the clinical microbiology laboratory, a highly specific and rapid detection process utilizing real-time PCR methodology was developed (8, Chapter 5). In ESBL research, only once before has the process of real-time PCR been used to detect ESBL genes of the SHV family by a German research group (9). Previous work proved that the LightCycler could be used to detect ESBL genes from *Enterobacteriaceae* with great sensitivity and specificity (9). Although still relatively novel in ESBL research, the application of real-time PCR may possibly yield the best results when applied to the division of large ESBL families into genetically similar groups (9). The clinical benefits of such an approach is however still largely uncertain. The sensitive LightCycler method in this study detected *bla*$_{GES-1}$ from one clinical isolate (1 % of isolates tested) (8), proving once and for all that both *bla*$_{GES-1}$ and *bla*$_{GES-2}$ circulate in South African isolates of *P. aeruginosa*. GES-2 however,

with a broader substrate profile, seems to be more prevalent in, and advantageous to, this species. This finding correlates well with those of other studies where *bla*$_{GES-1}$ occurred only in isolated specimens of *P. aeruginosa* (10, 11). Recent reports detailing novel point mutations in *bla*$_{GES}$ genes, is typically conspicuous in the absence of *P. aeruginosa* isolates either carrying or producing these genetic structures (12, 13, 14). Although too early to come to a final conclusion, the relatively low (51.5%) G+C-content of GES-type genes (4) and the propensity of *P. aeruginosa* genes to utilise cytosine and guanine in the wobble position (15) may possibly explain the incompatibility of these genetic structures with this species. Although highly sensitive and specific, the detection methods described in this thesis however, do not give one any insight into areas outside of the chosen target site and are therefore unsuitable to detect new GES-type variants without prior adaptation.

Phase 2 research of this thesis (Chapter 6), focused on determining the genetic stability of *bla*$_{GES}$–type genes in their integron-borne genetic environment, whilst under selective antibiotic pressure. Results obtained, showed that short-term exposure of GES-type-producing organisms to sub-inhibitory concentrations of single antimicrobial agents, are unlikely to select significant spontaneous mutations in these beta-lactamase genes. In addition to these findings, the regulatory mechanisms of *bla*$_{GES}$-type genes remained genetically stable during the experiment. Although these are in essence negative findings, it may still add some value to the research process and possibly offer the clinician some degree of reprieve from the short-term abuse of antibiotics. The GES family of ESBLs is still in its infancy and under no circumstances should one assume that evolution and dissemination of these genetic structures would slow down in any way. That is, unless we see a combined worldwide effort furthering research into understanding these genes (and similar

other ESBLs) and the mechanisms that control them. Intensive research leading to greater understanding is imperative, for tomorrow's resistance is nothing more than the outcome of today's actions (1).

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APPENDIX A: EXTRACTION OF WHOLE-CELL BACTERIAL DNA.

Method:

1. Inoculate one selected colony in 10 ml liquid broth and incubate overnight at 37°C while shaking continuously.
2. Aliquot 1 ml into a sterile centrifuge tube and centrifuge at 3000 x g for 10 min.
3. Remove the supernatant and resuspend the precipitate in 1 ml distilled water.
4. Centrifuge at 3000 x g for 10 min and remove the supernatant.
5. Resuspend the pellet in 500 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5).
6. Add 25 µl of a 20 % SDS solution and 1 µl of lysozyme (50 mg/ml).
7. Incubate the mixture for 1h at 37°C, then add 220 µl of NaCl (5M).
8. Add 700 µl chloroform/isoamylalcohol (24:1) and shake the tube vigorously.
9. Centrifuge at 5000 x g for 10 min, then remove the upper phase and transfer into a new tube.
10. Add 700 µl cold isopropanol (-20°C), then gently shake by inversion and let precipitate for 1h at -20°C.
11. Centrifuge at 5000 x g for 10 min, then remove the isopropanol, don't touch the pellet and add 800 µl 70% cold ethanol (-20°C).
12. Centrifuge at 5000 x g for 5 min, then remove the ethanol and add 800 µl cold 70% ethanol (-20°C) again.
13. Centrifuge at 5000 x g for 5 min, remove the ethanol, let dry and resuspend the visible (or not) pellet in 1ml TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 7.4).
14. Store extracted DNA at 4°C until further analysis.

APPENDIX B: PUBLICATIONS FROM THIS THESIS.

1. **Weldhagen G.F., Poirel L. and Nordmann P.** 2003. Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa* – Novel developments and clinical impact. Antimicrobial Agents and Chemotherapy. **47:** 2385 - 2392.

2. **Weldhagen G. F.** 2004. Integrons and beta-lactamases – A novel perspective on resistance. International Journal of Antimicrobial Agents **23(6):** 556 – 562.

3. **Weldhagen G. F.** 2004. Class A, extended –spectrum beta-lactamase production in *Pseudomonas aeruginosa* – Fact or Fiction? (Review article). The Southern African Journal of Epidemiology and Infection. **18(4):** 77 – 84.

4. **Weldhagen G. F. and Prinsloo A.** 2004. Molecular detection of GES-2 extended spectrum beta-lactamase producing *Pseudomonas aeruginosa* in Pretoria, South Africa. International Journal of Antimicrobial Agents. **24(1)**: 35 – 38.

5. **Weldhagen G. F.** 2004. Sequence-selective recognition of beta-lactamase GES-2 by a competitive, peptide nucleic acid based, multiplex PCR assay. Antimicrobial Agents and Chemotherapy. **48(9):** 3402 – 3406.

6. **Weldhagen G. F.** 2004. Rapid detection and sequence-specific discrimination of extended-spectrum beta-lactamase GES-2, from *Pseudomonas aeruginosa* with a real-time PCR assay. Antimicrobial Agents and Chemotherapy. **48(10):** 4059 – 4062.