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.

Signed: _____

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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)