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: __________________________

Date: __________________________
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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*<sub>GES-2</sub> coding region, distinguishes this ESBL from *bla*<sub>GES-1</sub> and the *bla*<sub>IBC</sub>-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*<sub>GES-2</sub> 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*<sub>GES</sub> and *bla*<sub>IBC</sub> 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*<sub>GES-2</sub>. 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*<sub>GESIBC</sub> 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 blaGES-2 with the LightCycler. One clinical isolate was found to harbour a 
blaGES-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 blaGES-type genes. This part of the study subjected two well-characterized 
clinical isolates with class 1 integron-borne blaGES-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 blaGES-1, 
blaGES-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, blaGES, 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 geidentificeer 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 analysé (*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 automatiëse 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 geidentificeer 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

GEN  gentamicin

GES  Guiana extended spectrum beta-lactamase

h  hour(s)

HIV  human immunodeficiency virus

IBC  integron-borne cephalosporinase

IC₅₀  50% inhibitory concentration

ICU  intensive care unit

IEF  iso-electric focussing

IMP  beta-lactamase named after preferred substrate (imipenem)

ln  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  sulphydryl variable beta-lactamase
sp.  species (singular)
spp.  species (plural)
SXT  trimethoprim/sulfamethoxazole
T  temperature
\( t_{1/2} \)  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_{\text{max}}  maximum rate of metabolism
WHO  World Health Organization
Wt  wild type
Y  pyrimidine (C or T)