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Molecular characterization of cholera outbreak isolates in South Africa, 2008-2009

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Molecular characterization of cholera outbreak isolates in South Africa, 2008-2009

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A thesis submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, South Africa, in fulfillment of the requirements for the degree, Doctorate of Philosophy in Medicine, 2009-2015

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Declaration

I, Husna Ismail, student number 0201785A declare that this thesis is my own independent work. It is being submitted for the degree of Doctorate of Philosophy in Medicine at the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 2009-2015. It has not been submitted before for any degree or examination purpose at this or any other university.



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Day 11th of october 2015

Publications

Ismail H, Smith AM, Sooka A, Keddy KH (2011) Genetic characterization of multidrug-resistant, extended-spectrum-β-lactamase-producing *Vibrio cholerae* O1 outbreak strains, Mpumalanga, South Africa, 2008. *Journal of Clinical Microbiology* 49: 2976-2979.

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Presentations

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Poster

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48th US-Japan cooperative medical sciences program conference on cholera and other bacterial enteric Infections. Hosted by icddr,b, Dhaka, Bangladesh. 11-13 February 2014.

Abstract

Background

Cholera is an acute diarrhoeal disease that generally presents as abrupt watery diarrhoea and vomiting. For the years 2008 to 2009, South Africa experienced two major outbreaks of cholera. The first outbreak was reported from May to July 2008 (Chapter Three) and the second outbreak from November 2008 to April 2009 (Chapter Four). Within both events, *Vibrio cholerae* (*V. cholerae*) O1 identified at peripheral laboratories displayed resistance to three or more routinely tested antimicrobial agents. The molecular epidemiology and mechanism of antimicrobial resistance of *V. cholerae* O1 isolates was investigated. This was achieved by using various molecular techniques, which included pulsed-field gel electrophoresis (PFGE) analysis, polymerase chain reaction (PCR), nucleotide sequencing, identification of plasmid DNA and Southern blot hybridization analysis.

Methods

As part of routine characterization of *V. cholerae* isolates at the Centre for Enteric Diseases (CED), isolates underwent serological and biochemical confirmatory identification as well as antimicrobial susceptibility testing using the Etest method. PFGE analysis was performed on *V. cholerae* O1 isolates digested with *Not*I restriction enzyme. One-hundred *V. cholerae* O1 isolates, ten isolates characterized in Chapter Three and 90 isolates characterized in Chapter Four were selected for further analysis to ensure that all PFGE banding patterns were represented. Three probable mechanisms of antimicrobial resistance were investigated. Firstly, PCR was used to detect for the presence of class 1 integrons (3'-CS and 5'-CS), class 2 integrons (*intI2*), plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qnrC* and *qepA*), quinolone resistance determinant (*qnrVC3*), ESBL producing genes (bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$), genes coding for the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*), SXT element-integrase gene (SXT*int*) and associated SXT resistance genes (*floR*, *sul2*, *dfrA1*, *dfr18*, *strA* and *strB*) and the class A

tetracycline resistance determinant (*tetA*). The presence of resistance plasmids was investigated by isolation of intact bacterial plasmid DNA. Southern blotting and DNA probing was used to investigate the location of resistance genes on the plasmids. Secondly, nucleotide sequencing was used to detect amino acid mutations in the QRDR of DNA gyrase and topoisomerase IV respectively. Thirdly, to determine the role of an active efflux pump in quinolone resistance, susceptibility testing to nalidixic acid was investigated in ten *V. cholerae* O1 isolates characterised in Chapter Three using agar dilution in the presence and absence of two efflux pump inhibitors, reserpine and phenylalanyl arginine-β-naphthylamide. PCR analysis was used to detect for virulence determinants, which included the enzymatic A subunit of the cholera toxin (CT), *ctxA* and the gene encoding for the toxin co-regulated pilus (TCP), *tcpA* respectively. In addition, the complete coding region of the *ctxAB* gene was amplified and sequenced from four *V. cholerae* O1 isolates, two isolates characterized in Chapter Three and two isolates characterized in Chapter Four as several *V. cholerae* O1 atypical El Tor isolates have been described in Africa. Minimum inhibitory concentration (MIC) values for azithromycin were determined for all 100 *V. cholerae* O1 isolates using both the Etest and agar dilution methods (Chapter Five). PCR-analysis was used to determine the presence of seven macrolide resistance determinants (*mefA*, *ereA*, *ereB*, *ermB*, *mphA*, *mphB* and *mphD*) in all 100 *V. cholerae* O1 isolates.

Results

For both cholera outbreaks, a total of 751 isolates were received and available for analysis. All 31 isolates recovered from the first outbreak (Chapter Three) were characterized as *V. cholerae* O1 serotype Ogawa. For the second outbreak (Chapter Four) 708 isolates were characterized as serotype Ogawa, while the remaining 12 isolates were characterized as serotype Inaba. All isolates analyzed from both outbreaks were susceptible to ciprofloxacin and imipenem, but resistant to six or more antimicrobial agents tested for surveillance purposes. All *V. cholerae* O1 isolates were shown to be resistant to nalidixic acid, co-trimoxazole, trimethoprim, sulfamethoxazoleand streptomycin. Extended-spectrum β-lactamase (ESBL) activity was observed in *V. cholerae* O1 isolates (MIC 64 µg/ml) from both outbreaks. In the second outbreak

reduced susceptibility to ampicillin, tetracycline, kanamycin, chloramphenicol, erythromycin and furazolidone were observed. Dendrogram analysis produced two main PFGE clusters. PFGE fingerprint patterns from *V. cholerae* O1 isolates recovered from the first outbreak clustered away from *V. cholerae* O1 isolates recovered from the second outbreak (data not shown in this study). Class 1 integrons, class 2 integrons and PMQR genes were not detected by PCR.All 100 *V. cholerae* O1 isolates were PCR-positive for the SXT*int* gene and five of the six associated SXT resistance genes encoding for chloramphenicol (*floR*), sulfamethoxazole (*sul2*), trimethoprim (*dfrA1*) and streptomycin (*strA* and *strB*). Seventeen *V. cholerae* O1 isolates (ten isolates characterized in Chapter Three and seven isolates characterized in Chapter Four) were PCR-positive for the *tetA* resistance determinant. Nucleotide sequencing of the QRDR, showed that all nalidixic acid-resistant isolates harboured the same mutations in GyrA (S83-I) and ParC (S85-L) but none were observed in GyrB and ParE.There was no involvement of an active efflux pump in quinolone resistance in ten isolates characterised in Chapter Three. Sixteen *V. cholerae* O1 isolates (ten isolates characterized in Chapter Three and six isolates characterized in Chapter Four) harboured a single plasmid of approximately 140 kilobase pairs in size and showed to harbour the bla_{TEM} gene, which produced the TEM-63 β-lactamase.PCR analysis showed that all 100 *V. cholerae* O1 isolates were positive for the CT, and all were PCR-positive for the El Tor variant of the TCP. Nucleotide sequencing of the *ctxAB* gene of the four selected isolates showed that all four isolates expressed the encoded *ctxB* allele for the CT of the classical biotype and were defined as “altered El Tor”. A mobilome is characterized by related genome sequences that differ by combinations of genomic islands, prophages and integrative conjugative elements. All four isolates contained an identical mobilome profile pattern, profile B. Comparative analysis using both the Etest and agar dilution methods (Chapter Five) showed that all *V. cholerae* O1 isolates were susceptible to azithromycin provided that the tentative breakpoint of ≤ 16µg/ml is applied. All 100 isolates were PCR-negative for all seven macrolide resistance determinants, which are commonly associated in the family *Enterobacteriaceae* respectively.

Conclusion

This is the first incidence of TEM-63 β-lactamase-producing, antimicrobial-resistant, toxigenic *V. cholerae* O1 altered El Tor isolates in South Africa. This study highlights the need to further analyze antimicrobial resistance and track emerging epidemic isolates of *V. cholerae* O1. The MIC values and PCR results reported in this study for azithromycin provides a foundation for the surveillance of azithromycin susceptibility and to determine MIC breakpoints in *V. cholerae* O1 isolates circulating in South Africa.

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Ethics clearance certificates

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Husna Ismail

CLEARANCE CERTIFICATE	**M090817**
PROJECT	Molecular Characterization of Cholera Outbreaks Isolates in South Africa, 2008-2009
INVESTIGATORS	Miss Husna Ismail.
DEPARTMENT	National Institutue for Communicable Diseases
DATE CONSIDERED	09.08.28
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 30.08.09 **CHAIRPERSON** .....
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*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr A Marius

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INVESTIGATORS	Dr Vanessa Quan
DEPARTMENT	NICD
DATE CONSIDERED	26/11/2011
DECISION OF THE COMMITTEE* December 2012	Recertification Approved (1 year) November 2011 -

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List of Figures

Figure 1 A negatively-stained whole mount of *V. cholerae* illustrating curved-shaped bacilli containing single polar, sheathed flagella.

Figure 2a Classification of *V. cholerae* according to serogroup and serotype.

Figure 2b Classification of *V. cholerae* serogroup O1 according to biotype.

Figure 3 A flow diagram illustrating pathogenesis of *V. cholerae* O1.

Figure 4 Schematic representation of the *V. cholerae* O1 El Tor biotype strain N16961 genome.

Figure 5 The CTX genetic element of the filamentous bacteriophage CTXΦ composed of two functional domains including the core, which is flanked by 1 or more repetitive sequence (RS). The Illustration was taken from Waldor and Mekalanos, 1996 [35].

Figure 6 Regulation of the cholera toxin and toxin co-regulated pilus.

Figure 7 Preparation of agarose plugs for pulsed-field gel electrophoresis (PFGE) analysis.

Figure 8 Restriction enzyme digestion of total bacterial DNA contained in agarose plugs.

Figure 9 Pulsed-field gel electrophoresis (PFGE) image of ten *V. cholerae* O1 isolates digested with *Not*I restriction enzyme using the PulseNet standardized protocol for *V. cholerae*. Lane S *Salmonella* Braenderup strain CDC-H9812 digested with *Xba*I restriction enzyme.

Figure 10 An "In-house" Southern blot apparatus.

Figure 11 Agar dilution susceptibility testing.

Figure 12 (A) Plasmid DNA isolation from the ten selected *V. cholerae* O1 isolates. (B) Southern blot hybridization of plasmid DNA probed with DIG-bla_{TEM} from ten selected *V. cholerae* O1 isolates.

Figure 13 Alignment report showing the amino acid sequences of DNA gyrase subunit A (GyrA) for *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and isolate, TCD273377.

Figure 14 Alignment report showing the amino acid sequences of DNA topoisomerase IV subunit A (ParC) for *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and isolate, TCD273377.

Figure 15 Dendrogram of PFGE fingerprint patterns of *V. cholerae* O1 isolates (*Not*I-digestion) associated with the cholera outbreak in South Africa, November 2008 to April 2009. "*" indicates the most commonly identified pattern.

Figure 16 Alignment report showing the amino acid sequence of ctxB of four selected *V. cholerae* O1, serotype Ogawa, biotype El Tor isolates, TCD306373, TCD325765, TCD273214 and TCD273377; *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and *V. cholerae* O1, biotype classical, strain 569B accession number VCU25679. Amino acid residues that match the consensus sequence displayed on top are hidden. Amino acid residues that do not match the consensus sequence displayed on top are shown.

Figure 17 PCR amplification and detection of particular genetic markers in multiplex PCR 1 and multiplex PCR 2 for three *V. cholerae* O1 isolates. PCR-amplified products were separated on a 1.5 % (w/v) agarose gel.

Figure 18 Chemical structures of azithromycin and erythromycin A. Picture adapted from Retsema *et al*, 1987.

Figure 19 Schematic overview of creating internal positive control DNA for the detection of the macrolide resistance determinant, *mphA*.

Figure 20 PCR amplification and detection of the *eae* (~ 482 bp) gene in lane 1 and the internal positive control, *mphA-eae* (~ 522 bp) in lane 2. PCR-amplified products were separated on a 1.5 % (w/v) agarose gel. Lane M, HyperLadder™ IV (100 bp molecular weight marker).

List of Tables

Table 1 Phenotypic differences between *V. cholerae* O1 biotype El Tor and classical isolates.

Table 2 Proposed WHO clinical case definitions for cholera.

Table 3 Antimicrobial concentration range and minimum inhibitory concentration (MIC) breakpoints for antimicrobial resistance using ETest® strips tested for surveillance purposes at the Centre for Enteric Diseases.

Nomenclature

ACF	Accessory colonization factor
ADP	Adenosine diphosphate
ATP	Adenosine tri-phosphate
ABC	ATP-binding cassette
A	Alanine
AP	Alkaline phosphatase
AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
et al.	And others
D	Aspartic acid
~	Approximately
R	Arginine
N	Asparagine
att	Attachment
bp	Base pair
β	Beta
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CED	Centre for Enteric Diseases
CFR	Case-fatality-rate
cm	Centimetre
cm^2	Centimetre squared
CT	Cholera toxin
ChrI	Chromosome 1
ChrII	Chromosome 2
CLSI	Clinical and Laboratory Standards Institute
CFU/ml	Colony forming units per milliliter
CHEF	Contour-clamped homogenous electric field
$CTX^{class}\Phi$	CTX classical

$CTX^{ET}\Phi$	CTX El Tor
C	Cysteine
DMP	Diagnostic Media Products
º	Degree
ºC	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DIG	Digoxigenin
DGREA	Direct genomic restriction enzyme analysis
Dr.	Doctor
EDTA	Disodium ethylenediaminetetra-acetic acid
EPI	Efflux pump inhibitor
ERIC-PCR	Enterobacterial repetitive intergenic consensus-based PCR
=	Equal to
erm	Erythromycin ribosome methylase
ESBL	Extended spectrum β-lactamase
Etest	Episolimeter test
EtBr	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GI	Genomic island
E	Glutamic acid
Q	Glutamine
G	Glycine
g	gram
>	Greater than
≥	Greater than or equal to
G	Guanine
GERMS-SA	Group for Enteric Respiratory and Meningeal Surveillance-South Africa
H	Histidine
HIV	Human immunodeficiency virus

HCl	Hydrochloric acid
Inc	Incompatible
∞	Infinity
ICE	Integrating conjugative element
IV	Intravenous
I	Isoleucine
kb	Kilo base pair
kDa	Kilodalton
kg/cm^2	kilogram per centimeter squared
LGT	Lateral gene transfer
<	Less than
≤	Less than or equals to
L	Leucine
LPS	Lipopolysaccharide
l	Litre
K	Lysine
LB	Luria-Bertani
MLS	Macrolide, lincosamide and streptogramin
$MgCl_2$	Magnesium chloride
MFS	Major facilitator superfamily
MDGs	Millennium Development Goals
mRNA	Messenger ribonucleic acid
M	Methionine
µg	Microgram
µl	Microlitre
µl/ml	Microliter per millilitre
µg/ml	Microgram per millilitre
µm	Micrometer
µM	Micromolar
mg	Milligram

ml	Millilitre
mg/kg	Milligram per kilogram
mg/ml	Milligram per millilitre
mm	Millimetre
mM	millimolar
MIC	Minimum Inhibitory Concentration
-	Minus
M	Molar
>	More than
≥	More than or equal to
MATE	Multidrug and toxic compound extrusion
MLST	Multi locus sequence typing
MLVA	Multiple-locus variable-number of tandem repeat analysis
ng	Nanogram
ng/µl	Nanogram per microliter
NPET	Nascent peptide exit tunnel
NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NHLSRT	National Health Laboratory Service Research Trust
NICD	National Institute for Communicable Diseases
NDM-1	New Delhi metallo-β-lactamase
NBT	Nitro blue tetrazolium chloride
NTC	No template control
#	Number
( )	Open bracket close bracket / parenthesis
ORF	Open reading frame
[ ]	Open square bracket close square bracket
OCV	Oral cholera vaccine
ORS	Oral rehydration solution
PTC	Peptidyl transferase center

/	Per
%	Percent
pH	Percentage Hydrogen
F	Phenylalanine
+	Plus
PCR	Polymerase chain reaction
PMF	Proton-motive force
PFGE	Pulsed-field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance determining region
RAPD	Randomly amplified polymorphic DNA
REP-PCR	Repetitive extragenic palindromic PCR
RS	Repetitive sequence
RND	Resistance-nodulation-cell division
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Serine
STI	sexually transmitted infection
SMR	Small multidrug resistance
NaCl	Sodium chloride
SSC	Sodium chloride, Sodium citrate buffer
SDS	Sodium dodecyl sulphate
NaOH	Sodium hydroxide
TD	Traveller's diarrhoea
TCBS	Thiosulphate citrate bile salts sucrose agar
T	Threonine
X	Times
TCP	Toxin co-regulated pilus
tRNA	transfer ribonucleic acid

TD	Traveller's diarrhoea
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
W	Tryptophan
Y	Tyrosine
U	Unit
UPGMA	Unweighted pair group method with arithmetic averages
V	Valine
VNTR	Variable number of tandem repeats
V.	Vibrio
VPI	Vibrio pathogenicity island
V	Volt
V/cm	Volt per centimetre
w/v	Weight to volume
WHO	World Health Organization

Table of contents

Molecular characterization of cholera outbreak isolates in South Africa, 2008-2009 I
Advisory committee II
Declaration ..... **Error! Bookmark not defined.**
Publications IV
Presentations IV
Oral IV
Poster V
Conference attendance VI
Abstract VII
Acknowledgements XI
Ethics clearance certificates XII
List of Figures XIV
List of Tables XVI
Nomenclature XVII
Chapter One 1
Introduction 1
1.1 Bacteriology 1
1.1.1 Microbiology 1
1.1.2 Serology 2
1.1.3 Biotypes 3
1.2 Burden of disease 5
1.2.1 Pandemics of cholera 6
1.2.2 Cholera in Africa 8
1.3 Transmission of *Vibrio cholerae* O1 8
1.4 Clinical features 9
1.5 Pathogenesis of infection due to *Vibrio cholerae* O1 10
1.6 Prevention and treatment of cholera 11
1.6.1 Public health measures 12
1.6.2 Cholera vaccination strategies 12

1.6.3 Specific treatment for cholera..... 14
1.7 Molecular aspects of toxigenic *Vibrio cholerae* O1 15
1.7.1 Cholera toxin (CT) and the bacteriophage CTXΦ..... 16
1.7.2 Vibrio pathogenicity island (VPI) 18
1.7.3 Regulation of the Cholera Toxin (CT) and toxin co-regulated pilus (TCP)..... 18
1.8 Molecular epidemiology and genetic characterization of toxigenic *Vibrio cholerae* O1 19
1.9 Mechanisms of antimicrobial resistance in toxigenic *Vibrio cholerae* O1 21
1.9.1 Efflux systems 22
1.9.2 Spontaneous mutations in chromosomal DNA 23
1.9.3 Horizontal gene transfer through mobile genetic elements 23
1.10 Background and setting of my research project 25
1.11 Study objectives..... 25
1.12 References 27
Chapter Two..... 35
Materials and Methods 35
2.1 Collection, phenotypic identification, serological characterization and storage of bacterial isolates of *Vibrio cholerae* O1 35
2.2 Antimicrobial susceptibility testing..... 36
2.3 Genotypic characterization of *Vibrio cholerae* O1 by pulsed-field gel electrophoresis (PFGE) analysis..... 39
2.4 Crude extraction of genomic DNA..... 45
2.5 Polymerase chain reaction (PCR) amplification 46
2.5.1 Detection and analysis of the enzymatic A subunit of the cholera toxin (CT)..... 46
2.5.2 Detection and analysis of the toxin co-regulated pilus (TCP)..... 47
2.5.3 Detection and analysis for extended spectrum β-lactamases (ESBLs)..... 47
2.5.4 Detection and analysis for class 1 and class 2 integrons 48
2.5.5 Detection and analysis for the tetracycline resistance determinant, SXT element-integrase and SXT associated resistance genes 49
2.5.6 Detection and analysis for plasmid-mediated quinolone resistance (PMQR) genes and quinolone resistance determinants..... 50

2.5.7 Detection and analysis of genes encoding for the DNA gyrase and topoisomerase IV of the quinolone resistance-determining region (QRDR) 50
2.6 Detection and size analysis of PCR-positive products using conventional agarose gel electrophoresis 51
2.7 Nucleotide sequencing of PCR-positive amplicons 52
2.8 Identification and preparation of plasmid DNA 54
2.9 Southern hybridization analysis 56
2.10 References 62
Chapter Three 64
Molecular characterization of *Vibrio cholerae* O1 associated with an outbreak of cholera in the Mpumalanga Province, South Africa, May to July 2008 64
3.1 Introduction 64
3.2 Materials and Methods 64
3.2.1 Bacterial isolates and phenotypic characterization 64
3.2.1.1 Involvement of efflux pumps 655
3.2.2 Genotypic characterization 67
3.2.2.1 Pulsed-field gel electrophorsis (PFGE) analysis 67
3.2.2.2 Detection for virulence determinants 67
3.2.2.3 Detection for antimicrobial resistance determinants 67
3.2.2.4 Isolation of plasmid DNA and Southern blot hybridization analysis 68
3.3 Results 68
3.3.1 Bacterial isolates and antimicrobial susceptibility patterns 68
3.3.2 Genes encoding for virulence determinants 68
3.3.3 PFGE analysis 68
3.3.4 Genes conferring antimicrobial resistance 69
3.3.5 ESBL activity and identified ESBL genes 69
3.3.6 Chromosomal mutations in the QRDR of DNA gyrase and topoisomerase IV 69
3.4 Discussion 72
3.5 Conclusion 766
3.6 References 77

Chapter Four 81
Molecular characterization of *Vibrio cholerae* O1 isolates associated with a country wide outbreak of cholera in South Africa, 2008 to 2009 81
4.1 Introduction 81
4.2 Materials and Methods 822
4.2.1 Bacterial isolates and phenotypic characterization 822
4.2.2 Genotypic characterization 82
4.2.2.1 Pulsed-field gel electrophorsis (PFGE) analysis 82
4.2.2.2 Detection for virulence determinants 82
4.2.2.3 Detection for antimicrobial resistance determinants 833
4.2.2.4 Isolation of plasmid DNA and Southern blot hybridization analysis 83
4.2.2.5 Molecular characterization of *Vibrio cholerae* seventh pandemic variants 83
4.2.2.5.1 Cholera toxin 84
4.2.2.5.2 Toxin co-regulated pilus 84
4.2.2.5.3 Mobilome 84
4.3 Results 85
4.3.1 Bacterial isolates 85
4.3.2 Antimicrobial susceptibility patterns 85
4.3.3 PFGE analysis 86
4.3.4 Genes conferring antimicrobial resistance 86
4.3.5 ESBL activity and identified ESBL genes 86
4.3.6 Chromosomal mutations in the QRDR of DNA gyrase and topoisomerase IV 88
4.3.7 Molecular characterization of *Vibrio cholerae* seventh pandemic variants 88
4.4 Discussion 91
4.5 Conclusion 93
4.6 References 94
Chapter Five 97
Azithromycin susceptibility in recent antimicrobial-resistant *Vibrio cholerae* O1 altered El Tor variant isolates in South Africa. 97
5.1 Introduction 97

5.2 Materials and Methods 102
5.2.1 Bacterial isolates 102
5.2.2 Antimicrobial susceptibility testing 102
5.2.2.1 Episolimeter test (Etest) method 102
5.2.2.2 Doubling agar dilution method 102
5.2.3 Genotypic characterisation 103
5.2.3.1 Detection for macrolide resistance determinants 103
5.2.3.2 Internal control DNA constructs 104
5.2.4 Statistical analysis 106
5.3 Results 107
5.3.1 Azithromycin susceptibility testing 107
5.3.2 Detection for macrolide resistance determinants 107
5.3.3 Statistical analysis 107
5.4 Discussion 107
5.5 Conclusion 112
5.6 References 113
Chapter Six 119
Limitations 119
6.1 References 122
Chapter Seven 125
General Discussion and Conclusion 125
7.1 References 131
Appendices 1344
Appendix A: Pulsed-field gel electrophoresis (PFGE) 1344
Appendix B: Polymerase Chain Reaction (PCR) 1399
Appendix C: Detection of PCR products using conventional agarose gel electrophoresis 153
Appendix D: Nucleotide sequencing using the ABI Prism®BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) 154
Appendix E: Isolation and preparation of plasmid DNA 155
Appendix F: PCR DIG-labelling and Southern blot hybridization 157

Appendix G: Involvement of efflux pumps 161
Appendix H: Tracking seventh pandemic variants 162
Appendix I: Azithromycin susceptibility testing 168
Appendix J: Plagiarism declaration with Turnitin report 171

Chapter One
Introduction

Diarrhoeal illness is one of the contributing factors of mortality among children less than five years of age worldwide [1,2]. In South Africa, diarrhoeal illness contributes ~ 3 % of the total deaths and is the eighth largest cause of death nationally [3]. Unsafe drinking water together with the lack of sanitation and poor hygiene behaviours facilitate the transmission of enteric pathogens [3]. Cholera is a severe watery diarrhoeal disease caused by the toxin-producing bacterium, *Vibrio cholerae* (*V. cholerae*) [4]. Between 1849 and 1854, John Snow, a physician in London proposed that cholera was a communicable disease and that stool contained the infectious material [4]. *V. cholerae* was first described by Filippo Pacini in 1854 in Italy who observed a large number of curved bacteria in the gastrointestinal tract of patients [4]. This discovery was better described in cholera studies done by Robert Koch in 1883 in Egypt and continued in 1884 in Calcutta, India [4]. *V. cholerae* has since been well described based on biochemical testing and deoxyribonucleic acid (DNA) homology studies [4].

1.1 Bacteriology

1.1.1 Microbiology

V. cholerae is a non-invasive bacterium, which belongs to the family *Vibrionaceae* [4-6]. This organism is facultative anaerobic, non-spore forming, gram-negative, rod-shaped and highly motile with a single polar, sheathed flagellum [5-7]. *Vibrio* species are ~ 0.5 micrometre (μm) to 0.8 μm in diameter by 1.5 μm to 3.0 μm in length [5,7]. *V. cholerae* ferments glucose, sucrose, mannitol and produces lysine and ornithine decarboxylase [7]. The growth of the organism is stimulated by the addition of 1 % sodium chloride [7]. An important biochemical test for distinguishing *V. cholerae* from members of the family *Enterobacteriaceae* is the oxidase test [5,7]. *V. cholerae* is positive for oxidase [5-7].

The optimal growth temperature and percentage Hydrogen (pH) for *V. cholerae* is between plus (+) 30 Degree Celsius (°C) and + 40 °C and a pH 8.0 [7,8].

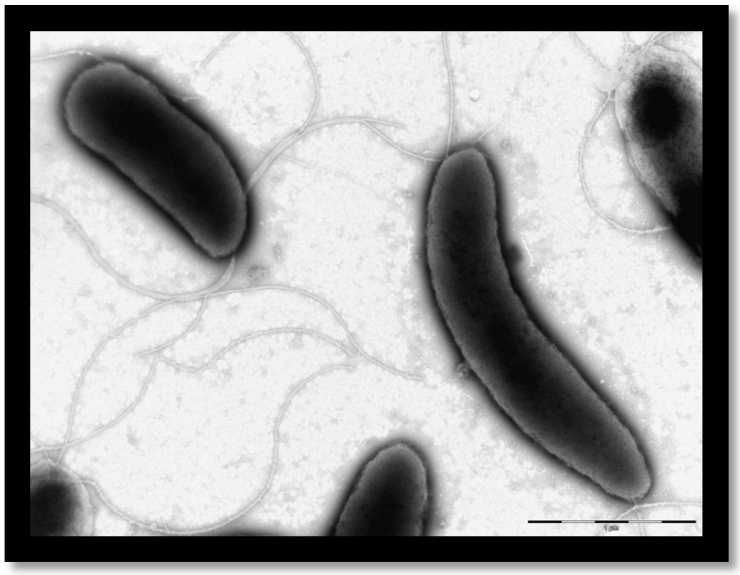

Figure 1 A negatively-stained whole mount of *V. cholerae* illustrating curved-shaped bacilli containing single polar, sheathed flagella.

1.1.2 Serology

V. cholerae is classified into serogroups based on antibody-antigen differences of the somatic "O"-antigen of the lipopolysaccharide (LPS) [4,6]. Based on these antigenic differences, 210"O"-antigen serogroups have been described [4,6]. Toxin-producing *V. cholerae* belonging to serogroups O1 and O139 Bengal are known to be associated with endemic and epidemic cholera [4,6]. Serogroups other than serogroups O1 and O139 are referred to as non-O1 and non-O139 however, they have been known to cause moderate to severe human gastroenteritis [9]. *V. cholerae* O1 is further characterized into three serotypes namely Inaba, Ogawa and Hikojima (Figure 2a) [4,6,9]. All three serotypes are clustered according to the structure of the "O" antigen on the LPS and have a common "A" antigenic determinant [9,10]. *V. cholerae* O1 serotype Ogawa isolates express the "B" antigenic determinant, while *V. cholerae* O1 serotype Inaba isolates express the "C" antigenic determinant [9,10]. *V. cholerae* O1 serotype Hikojima isolates

are rare and express both the "B" and "C" antigenic determinants [9,10]. To date, there have not been recent publications on the prevalence of *V. cholerae* O1 serotype Hikojima. Serotype switching has been described in *V. cholerae* isolates [10,11]. No serotype(s) exist for *V. cholerae* O139 Bengal [6].

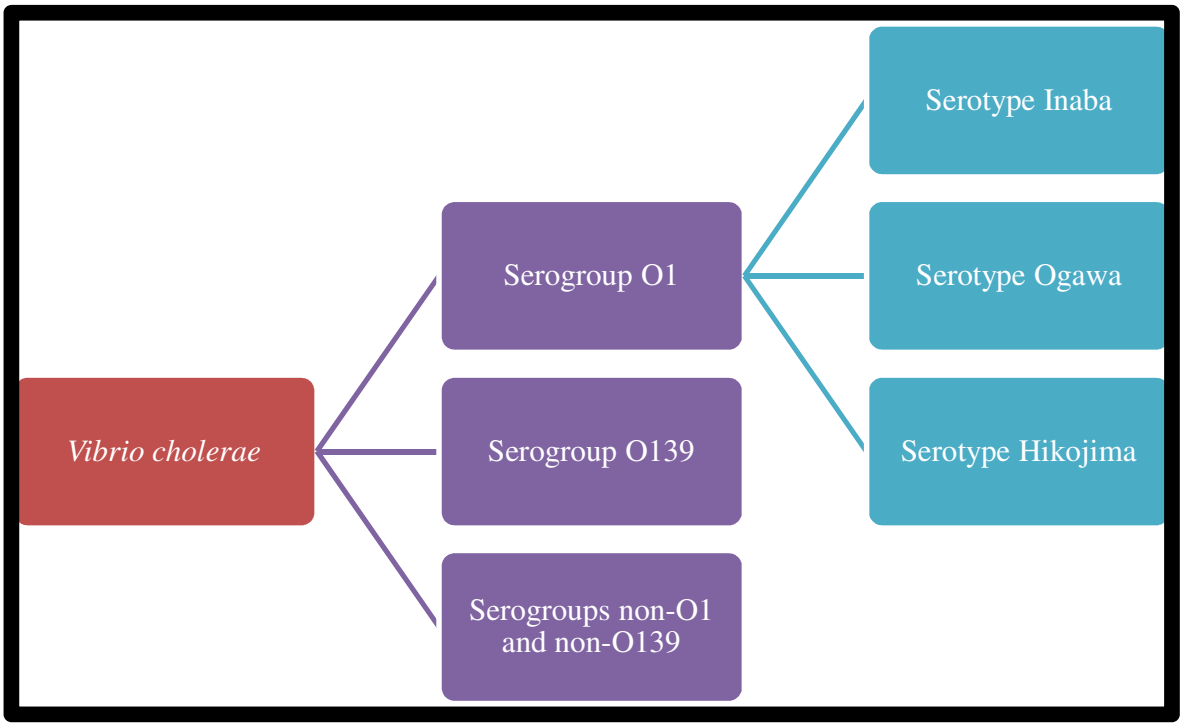

Figure 2a Classification of *V. cholerae* according to serogroup and serotype.

1.1.3 Biotypes

Cholera biotypes are discrete phenotypes that differ with respect to severity of infection, ability to survive outside of the human host and seasonality patterns [10]. *V. cholerae* O1 is characterized into two well established biotypes namely, El Tor and classical (Figure 2b) [6]. These biotypes are differentiated based on various phenotypic characteristics, which include susceptibility to polymyxin B, chicken cell agglutination, haemolysis of sheep erythrocytes, production of acetylmethylcarbinol by the Voges-Proskauer test and susceptibility to bacteriophages [12]. These differences are listed in Table 1 [12]. The majority of *V. cholerae* O1

biotype El Torisolates has become non-haemolytic worldwide [12]. However, this is an exception for *V. cholerae* O1 biotype El Torisolates from Australia and the United States Gulf Coast [12]. Differentiation between the two biotypes is now based on a Polymerase Chain Reaction (PCR) test, which exploits sequence differences in the toxin co-regulated pilus (TCP) gene [13].

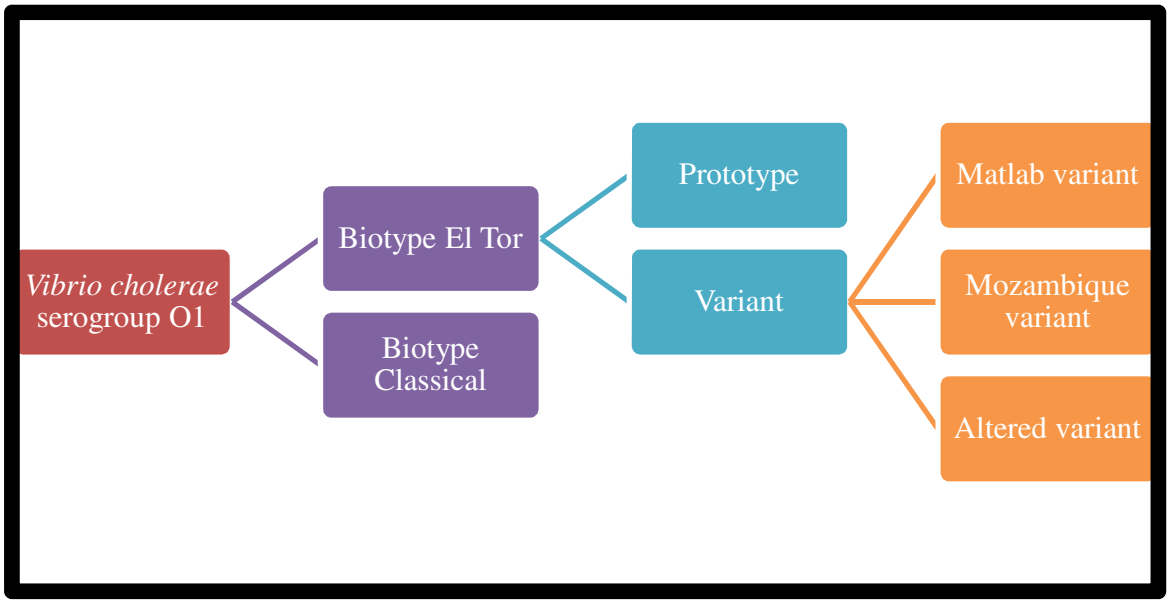

Figure 2b Classification of *V. cholerae* serogroup O1 according to biotype.

Table 1 Phenotypic differences between *V. cholerae* O1 biotype El Tor and classical isolates [12].

Phenotypic property	**Result for *V. cholerae* O1 biotype**	
	Classical	El Tor
Inhibition by polymyxin B	Positive	Negative
Voges-Proskauer test	Negative	Positive
Haemolysis of sheep erythrocytes	Negative	Positive or Negative
Agglutination of chicken cells	Negative	Positive
Bacterial cell lysis by classical IV bacteriophage	Positive	Negative
Bacterial cell lysis by El Tor V bacteriophage	Negative	Positive

1.2 Burden of disease

Cholera continues to be a global concern particularly in many developing countries [4]. Cholera is a notifiable diarrhoeal disease in many countries including South Africa (accessed from http://www.kznhealth.gov.za/pharmacy/edladult_2012.pdf; September 2014) with approximately one percent of cases being reported to the World Health Organization (WHO) [14]. Cases of cholera generally remain undetected for various reasons. For example, stool specimens are not always routinely cultured for isolation and identification of *V. cholerae* or health care advice is not sought after when mild symptoms are presented or limitations may exist in current surveillance and reporting systems [6,14,15].

Cholera in recent years has shown a 130 % increase in the number of cases from 2000 to 2010 and an overall increase of 43 % from 2009 to 2010 [6]. The WHO estimates more than 120 000 individuals die from cholera each yearand three to five million cholera cases occur worldwide (accessed from http://www.who.int/mediacentre/factsheets/fs107/en/; July 2015). Cholera is endemic (refer to Table 2 for proposed WHO clinical case definition [4,16]) in many regions of Asia, Africa, South and Central America [4,14]. It has been shown that *V. cholerae* O1 serotypes are likely to vary in dominance in geographical regions where cholera is endemic [10]. The reasons for these fluctuations have not been well described [10].

Table 2 Proposed WHO clinical case definitions for cholera.

Description	Case definition
Disease unknown in area	Severe dehydration or death from acute watery diarrhoea in a patient aged five or more.
Endemic cholera	Acute watery diarrhoea with or without vomiting in a patient aged five years and older.
Epidemic cholera	Acute watery diarrhoea with or without vomiting in any patient.

1.2.1 Pandemics of cholera

Seven pandemics of cholera have been described [4,7,17]. The first six pandemics of cholera were associated with human migration [7]. The first cholera pandemic began in 1817 in the Indian subcontinent along trade routes to the West [7,17]. The second pandemic began in 1826 and reached the British Isles and major European cities in the early 1830s [7,17]. In 1854 following the second cholera pandemic, John Snow conducted an archetypal investigation whereby he showed the association of cholera with contaminated drinking water [7,17]. During the 1850s, the United States of America was extensively affected by the third cholera pandemic [7,17]. Up to 1925, the fourth, fifth and sixth cholera pandemics, affected Australia, Europe, Africa and South America [7,17].

Compared to the first six cholera pandemics, which originated in Bangladesh, the ongoing seventh pandemic of cholera began in 1961 in Indonesia and has since, disseminated across continents [4,7,17,18]. The causative agent is *V. cholerae* O1 biotype El Tor and has therefore replaced the classical biotype [4,7,17,18]. This biotype was originally isolated in 1905, which was associated with Indonesian pilgrims travelling to Mecca through a village of El Tor, Egypt [4,7,17,18].

It is uncertain as to why the El Tor biotype became pandemic when it did as well as the replacement of the classical biotype worldwide [18]. A theory from evolutionary biology suggests that when there is improvement in sanitation, selection pressure acts against *V. cholerae* O1 biotype classical and in favour of the more benign El Tor biotype [18]. *V. cholerae* O1 biotype classical isolates are more virulent, as it kills the host in a shorter period of time and therefore reduces the opportunity of infection of other potential hosts [18]. *V. cholerae* O1 biotype El Tor isolates are less virulent and as a result, are more capable of infecting other potential hosts for a longer period of time [18].

Cholera spread across India in 1964 and re-emerged in Africa in 1970 and in South and Central America in 1991, which had been free of the disease for more than 100 years [4,14,17]. As a

result, cholera has now become endemic in many of these regions [1,10,14]. Both classical and El Tor biotypes behave differently from each other [18]. For example, the seventh cholera pandemic was more widespread with respect to covering larger geographical areas [18]. As a result more countries were affected [18]. This seventh pandemic has illustrated temporal differences with respect to how fast the disease has spread and duration of the pandemic [18]. The longest previous pandemic was the sixth, which lasted 24 years (1899 to 1923) [18]. As of 2014, the seventh pandemic lasted over 50 years and continues to show no signs of disappearing.

In 1992, in Bangladesh, a new serogroup *V. cholerae* O139 Bengal emerged [7,14,17,18]. Previously, only *V. cholerae* O1 was known to cause cholera epidemics [7,14,17,18]. Presently, *V. cholerae* serogroups O1 and O139 co-exist to cause a large number of cholera outbreaks in India and Bangladesh [7,14,17,18]. There were concerns that *V. cholerae* O139 Bengal could cause the eighth pandemic of cholera [7,14,17,18]. However, the number of cases caused by this serogroup remains a small proportion of the total number of cases of cholera [7,14,17,18].

Over recent years, new variants of *V. cholerae* O1 have been described namely, the Matlab, Mozambique and Altered El Tor (Figure 2b) [4,6,19]. These variants display characteristics of both El Tor and classical biotypes [4,6,19]. *V. cholerae* O1 El Tor variants were initially described by Nair *et al*. in clinical cholera isolates collected in Matlab, Bangladesh, for the period, 1991 to 1994 [19]. As a result, related variants have been isolated in other countries in Asia and Africa [4,6,19] with the first reports in Africa described in Mozambique [20].

1.2.2 Cholera in Africa

Before the seventh cholera pandemic reached Africa in 1970, cholera had an exclusively Asian focus [6,21]. In 2005, approximately 78 % of cholera cases that were reported to the WHO were from sub-Saharan Africa [6,22]. The reported annual incidence for cholera in 2005 was 95 times higher (166 cases per million population) than the reported annual incidence in Asia (1.74 cases per million population) and 16 600 times higher than Latin America (0.01 cases per million population) [6,22]. During the same year, the case-fatality-rate (CFR) was three times higher in sub-Saharan Africa (~ 1.8 %) than that in Asia (~ 0.6 %) [6,21].

In 2009, 45 countries reported a total of 221 226 cases of cholera including 4 946 deaths to the WHO [23]. Compared to 2008, the number of cholera cases increased by ~ 16 % with a CFR of ~ 2.24 % [23]. From the total number of cholera cases reported in 2009, 217 333 (~ 98 % of the global total) cases including 4 883 deaths were reported from Africa [23]. Compared to 2008 (179 323 cases), the number of cholera cases increased by ~ 20 % with a CFR of ~ 2.25 % [23]. From the total number of cholera cases reported from Africa (2009), Zimbabwe reported the highest number cases (68 153 cases), followed by Ethiopia (31 509 cases) and then the Democratic Republic of the Congo (22 899 cases) [23]. In 2009, a total of 10 520 (~ 4.76 %) cholera cases including 57 deaths with a CFR of ~ 0.54 % was reported from South Africa [23].

In 2010, Central Africa experienced a devastating wave of cholera. Countries that were affected included Cameroon, Chad, Niger, Nigeria and around the Lake Chad Basin [6,24].

1.3 Transmission of *Vibrio cholerae* O1

The main reservoir for the amplification and spread of *V. cholerae* O1 via the faecal-oral route is the human host [1,4]. A general definition for a host is “a living organism that temporarily harbours the pathogen, generally providing nourishment and shelter” [8]. However, environmental reservoirs have shown an important function in the persistence of *V. cholerae* O1 and its role in cholera [1,8]. An environmental reservoir is defined as “locations out of the human

body within the niche favouring bacterial persistence and replication in the environment, and pathogen transmission to susceptible hosts" [1]. *V. cholerae* O1 constitute part of the normal aquatic environment in estuarine and brackish waters [1,4,8]. Examples of these environmental reservoirs include chironomids and zooplankton [1,4,8].

The mode of transmission of *V. cholerae* O1 is the ingestion of contaminated water or food [1,4,8], although, human-to-human transmission has been described [1]. Symptomatic patients infected with either *V. cholerae* O1 or O139 usually shed the organism between two days and two weeks [1,4,25]. As *V. cholerae* O1 leaves the human body, the organism has a hyperinfectious phenotype [4,26]. This relates to the infectious dose, which is 10 to 100 times lower than that for non-human-shed organisms [4,26]. Recently shed *V. cholerae* O1 has shown to persist in water between five to 24 hours [1,4,26]. This suggests that human-to-human transmission might be more infectious than those who have adapted to the environment [4].

1.4 Clinical features

Bacterial infection with *V. cholerae* O1 leads to a clinical spectrum that ranges from asymptomatic colonization to cholera *gravis* (the most severe form of cholera) [1,4,14]. The death rate for untreated patients with severe cholera can exceed 70 % [4,27]. The incubation period for *V. cholerae* O1 to colonize the small intestine of the human host ranges between 12 hours to five days before symptoms appear [1,4,25].

Symptoms usually begin with abdominal cramps and vomiting (common feature) followed by diarrhoea, which is generally painless [1,4]. Stools acquire a characteristic rice-water appearance and harbour $1x10^{10}$ to $1x10^{12}$ vibrios per litre [1,4,7,28]. Dehydration and electrolyte imbalance are the most important complications of cholera [4,28]. Patients are generally described as being lethargic; they might have sunken eyes, a dry mouth, cold clammy skin, decreased skin turgor and kussmaul breathing can occur [4,28,29]. Urine output decreases with time [4]. Muscle cramps and weakness due to loss of electrolytes and ion shifts are common [4]. Children with depleted glycogen can lead to severe hypoglycaemia [4]. Another clinical feature described is

cholera sicca, which is an unusual form of the disease [4]. In cholera sicca, fluid accumulates in the intestinal lumen, which can result in circulatory collapse or even death before the passage of the first loose stool [4].

The presentation of cholera differs between endemic and epidemic settings [1,4]. In an endemic setting, children are more likely to be hospitalized with severe illness [1,4]. When *V. cholerae* O1 is introduced into an immunity naive population, all age groups are equally susceptible to symptoms of infection and this is usually associated with high CFRs [1,4].

The infectious dose of *V. cholerae* O1 required for pathogenesis in a human host varies with respect to the bacterial isolate and the host [1]. An infectious dose of $1x10^5$ to $1x10^{11}$ of bacterial cells is required to cause severe cholera in a healthy individual [1,4]. A lower infectious dose of $1x10^3$ to $1x10^8$ of bacterial cells is needed to cause cholera in an individual taking an antacid (Bicarbonate buffer) to neutralize stomach acid [1,4].

1.5 Pathogenesis of infection due to *Vibrio cholerae* O1

Pathogenic isolates of *V. cholerae* O1 harbour two essential virulence factors namely, the choleragen or cholera toxin (CT), which is primarily responsible for the excretion of profuse diarrhoea and the toxin co-regulated pilus (TCP), which is a colonization factor (Figure 3) [1,4,9]. Upon ingestion of contaminated water or food with *V. cholerae* O1, most of the organisms are killed by the gastric acid in the stomach (Figure 3) [1,4,9]. The surviving organisms pass through the acid barrier of the stomach and colonize the small intestine by means of the TCP (Figure 3) [1,4,9]. Colonization is an essential step in cholera pathogenesis [1]. Following adherence, the CT (secreted AB5-subunit toxin) and other proteins for example, Zot, Ace and haemolysin are produced [1,4,7]. The B subunit pentamer of the CT binds monosialotetrahexosyl-gangliosides on absorptive intestinal epithelial cells [1,4,7]. This initiates endocytosis of the enzymatic A subunit of the CT (Figure 3) [1,4,7]. This reaction results in the adenosine diphosphate (ADP) ribosylation of a subunit of the G protein (a heterotrimer that connects cell surface receptors to effector proteins at the plasma membrane), which regulates

adenylyl cyclase activity [1,4,7]. As a result ion transport by intestinal epithelial cells is disrupted and subsequent loss of water and electrolytes leads to the severe diarrhoea and dehydration [1,4,7].

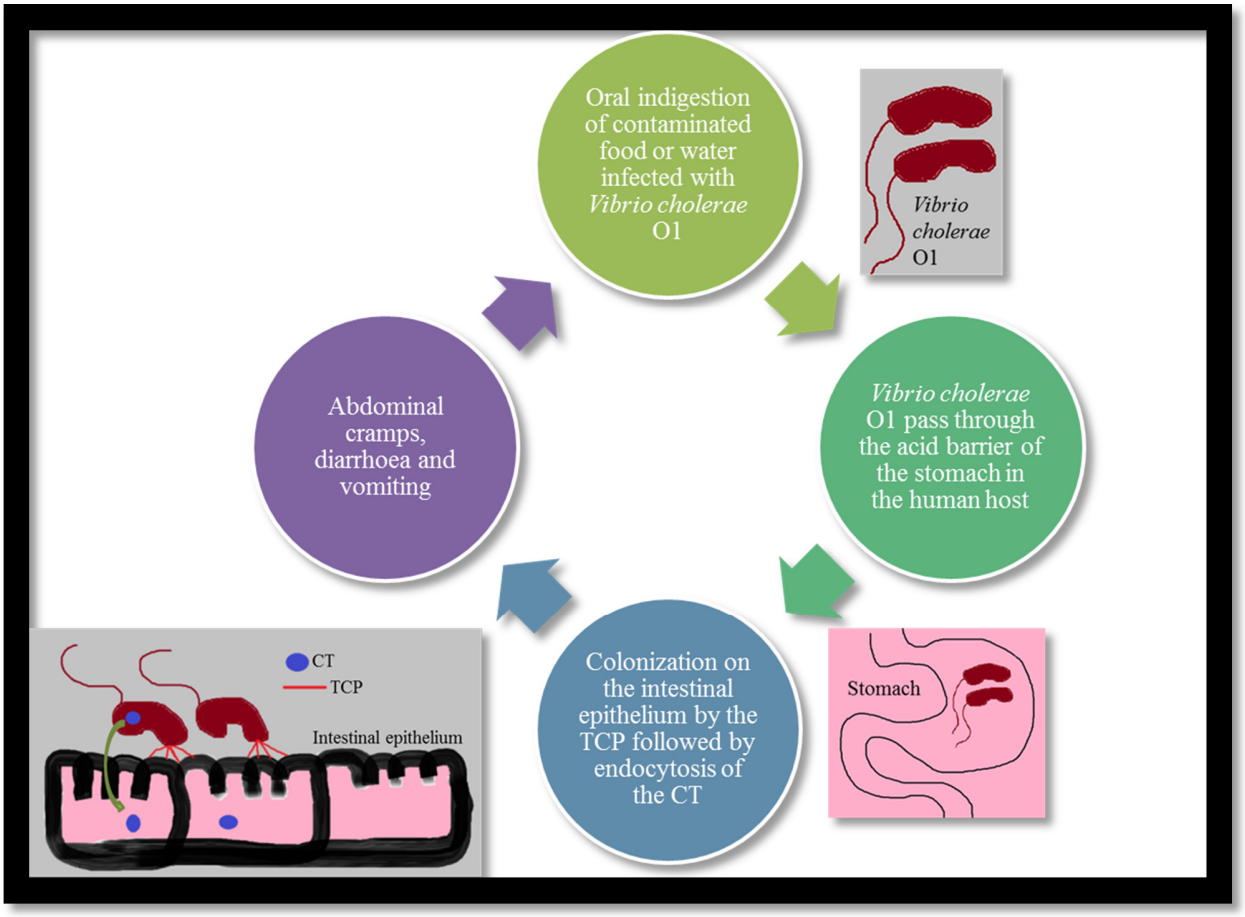

Figure 3 A flow diagram illustrating pathogenesis of *V. cholerae* O1.

1.6 Prevention and treatment of cholera

Decisions relating to prevention and control of cholera are generally based on surveillance reports [14]. Prevention strategies vary between indigenous populations and travellers as the majority of travellers do not reside in the same conditions as indigenous populations [14].

1.6.1 Public health measures

Cholera control measures have focused on safe drinking water, improved sanitation and effective food hygiene by appropriate preparation of high-risk foods [14,30].

1.6.2 Cholera vaccination strategies

Another preventative measure is the administration of oral cholera vaccines (OCVs) [14,30]. Cholera vaccines have evolved from injectable vaccines with side effects and questionable protective efficacy to state-of-the-art killed whole-cell OCVs, which have shown to be safe and guarantees a high level of protection for several years [30]. Since the early 1990s, OCVs have been licensed and pre-qualified for purchase by the United Nations agencies [30]. However, these OCVs have been overlooked for public health interventions and are now marketed for travellers from industrialized countries who perceive themselves at risk for cholera [30].

Up to the 1990s, the only cholera vaccines that were available were those made from phenol-killed whole cells of *V. cholerae* O1 organisms [30]. They were administered by injection as two doses given two weeks apart [30]. These vaccines offered ~ 50 % protection for a short duration and was associated with painful local inflammatory reactions [30]. These parenteral cholera vaccines are no longer in use and were replaced by orally administered vaccines, specifically killed whole-cell vaccines and genetically modified live attenuated vaccines [30]. Currently there are two WHO pre-qualified OCVs namely Dukoral and Shanchol [30].

Dukoral was the first OCV to be internationally licensed [30]. It is produced by Crucell and contains whole *V. cholerae* O1 organisms and the recombinant CT B subunit (WC/rBS) [30]. The minimum age for an individual to be vaccinated is two years and older [30]. Individuals aged five years or older require two doses while individuals aged less than five years require three doses [30]. This vaccine has shown a 60 % efficacy in individuals over two years of age [30]. Dukoral has been evaluated in several countries in Africa particularly in Beira, Mozambique [30]. The findings from Mozambique were of importance for two reasons [30]. The

first reason was the circulating isolates of *V. cholerae* O1 El Tor variants expressing the classical CT and second, the prevalence of human immunodeficiency virus (HIV) infection in Beira, which was very high, as shown by the 20 % to 30 % prevalence among women of child-bearing age [30]. Dukoral was shown to protect against *V. cholerae* O1 El Tor variants as well as in environments with a high HIV prevalence [30]. The findings from Indonesia and western Sudan showed that mass vaccination campaigns with Dukoral can be successful in complex emergencies [30].

Compared to Dukoral, which is relatively expensive, Shanchol, the next generation of killed whole-cell OVC, is significantly cheaper and much easier to administer [30]. Shanchol is a bivalent OCV and is produced by Shanta Biotechnics [30]. It contains whole *V. cholerae* O1 organisms together with *V. cholerae* O139 strain 4260B [30]. Therefore the difference between the two licensed OCVs is the presence of the recombinant CT B subunit in Dukoral [30]. The minimum age for an individual to be vaccinated is one year and older and only two doses are required [30]. This vaccine has shown a 67 % efficacy in individuals over three years of age [30]. Another WC/rBS OCV, which might have potential in an African setting, is OraVacs [30].

OraVacs is produced by Shanghai United Cell Biotechnology and is only licensed in China and Philippines [30]. Reports on the safety and efficacy of this OCV are still to be published in peer-reviewed international journals [30]. Compared to Dukoral, which needs to be reconstituted, OraVacs is formulated as an enteric coated capsule [30].

A second type of OCV described, is a genetically modified live attenuated OCV, CVD 103-HgR (Orachol or Mutachol) [30]. This OCV was licensed, administered as a single-dose with buffer and was shown to be safe in North American volunteers [30]. However it has not been in production since 2004 [30]. Studies conducted in Indonesia did not display convincing results and as a result the manufacturer ceased production [30]. Currently, PaxVax is preparing to reintroduce an improved, new generation version of CVD 103-HgR [30]. Aside from the licensed OCVs aforementioned, there are several vaccines, which are in development (current

phase 2, *V. cholerae* Peru 15) and several, which are undergoing clinical assessment (*V. cholerae* 638, VA 1.3, and IEM 108) [30].

1.6.3 Specific treatment for cholera

The mainstay of treatment of cholera is adequate rehydration and electrolyte replacement therapy [4,16,31]. Taking into consideration the present standard of care, the mortality rate of severe cholera can be reduced to less than 0.2 % even in resource-limited settings [4]. Patients presenting with mild dehydration can be treated effectively with oral rehydration solutions (ORSs) [4,16]. This ORS formula has been approved by the WHO since 2002 [4,16]. Patients presenting with severe dehydration require intravenous (IV) rehydration with a multi-electrolyte solution to replace fluid and electrolyte loss [4,16]. In conditions whereby there is limited access to rehydration treatment or in severe cases of dehydration, antimicrobial agents can be administered [4,16]. Appropriate antimicrobial agents can shorten the duration of diarrhoea and shorten the duration of excretion to reduce secondary transmission, mainly in settings where affected individuals are residing in close proximity to each other [4,14,16]. Antimicrobial agents can be administered once the initial fluid deficit is corrected and vomiting has stopped [4].

For adults, a single 300 milligram (mg) dose of doxycycline or 500 mg of tetracycline four times a day for three days should be given [4,16]. The prescribed regimen differs for children, for whom it is recommended that tetracycline in a dose of 12.50 milligram per kilogram (mg/kg) be administered four times daily for three days [4,16]. However, prophylaxis with tetracycline is not recommended due to the high incidence of antimicrobial resistance. Alternative antimicrobial regimens for the treatment of cholera include furazolidone, 100 mg four times daily for adults, 1.25 mg/kg four times daily for three days for children; or erythromycin, 250 mg four times daily for adults, 12.5 mg/kg four times daily for three days for children [4,16]. Ciprofloxacin may also be utilized as an alternative treatment for adults in a dose of 500mg twice daily for three days [4]. Single dose of azithromycin is the preferred therapy both in children (20 mg/kg) and in adults (1 g) and has been shown to be more effective than ciprofloxacin in randomized trials in regions where reduced susceptibility to fluoroquinolones are common [4]. In South Africa,

cholera vaccinations are not recommended [32]. The recommended treatment of cholera patients in South Africa is the equivalent to internationally accepted cornerstone of cholera treatment, which is timely and adequate rehydration therapy [32,33]. Antimicrobial resistance has been associated with antimicrobial usage and is further discussed in section 1.9.

1.7 Molecular aspects of toxigenic *Vibrio cholerae* O1

The *V. cholerae* genome is comprised of two circular chromosomes and belongs to the γ-subdivision of the family *Protobacteriaceae* [6,34]. This analysis is based on the complete genome sequence of *V. cholerae* O1 El Tor biotype strain N16961 [34]. The large chromosome is assigned as chromosome 1 or ChrI and consists of 2 961 146 base pairs (bp) in length, while the smaller chromosome is assigned chromosome 2 or ChrII and consists of 1 072 314 bp (Figure 4) [34]. Together they encode 3 885 open reading frames (ORFs). ChrI contains genes required for essential functions such as growth and virulence [34]. These genes encode for DNA replication, transcription, translation and cell-wall synthesis [34]. In addition, genes required for bacterial pathogenesis include surface antigens, toxins and adhesions [34]. ChrII is comprised of hypothetical genes and genes of unknown function [34]. It contains a gene capture system known as an integron island and contains "addiction" genes, which are usually associated on plasmids [34]. Both chromosomes contain several identical ORFs with the same function. Acquisition of genes may have been a result of horizontal gene transfer [34].

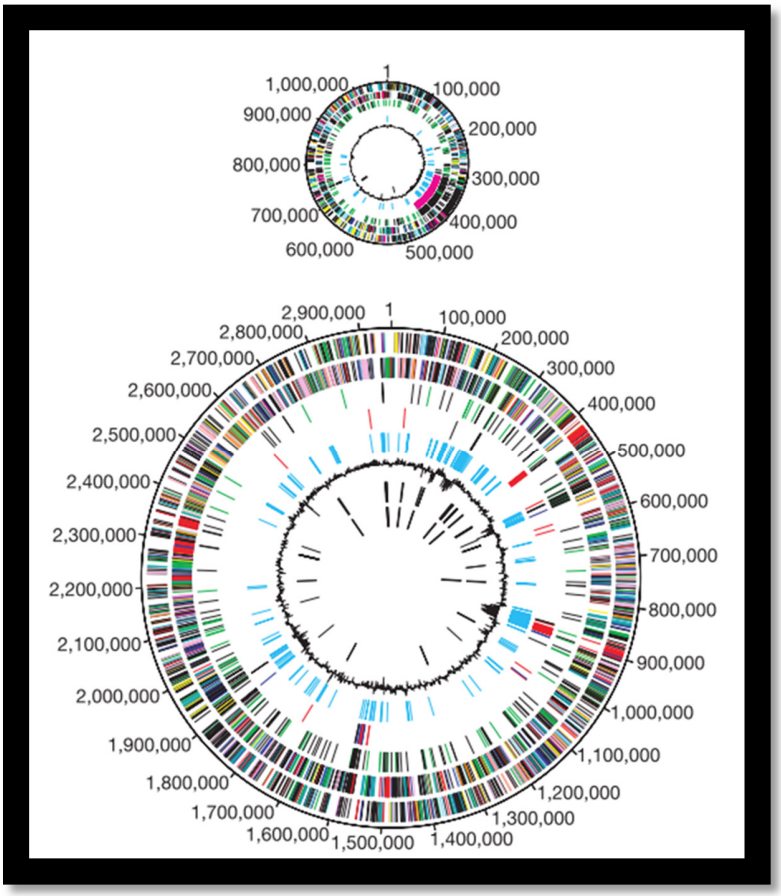

Figure 4 Schematic representation of the *V. cholerae* O1 El Tor biotype strain N16961 genome [34].

1.7.1 Cholera toxin (CT) and the bacteriophage CTXΦ

In *V. cholerae* O1, pathogenesis requires a number of essential virulence genes, which are located in clusters in regions on the chromosome [9,17,34-36]. The existence of these pathogenic gene clusters indicate that they have the potential to propagate laterally by means of horizontal gene transfer and disseminate to other bacteria [9,17,34-36]. Pathogenic *V. cholerae* O1 can be distinguished from non-pathogenic isolates by the presence of two genetic elements namely, the

CTX element, which is the genome of a filamentous bacteriophage, designated CTXΦ that encodes genes for the CT (Figure 5) and the vibrio pathogenicity island (VPI), which contains genes coding for the TCP [9,17,34-36].

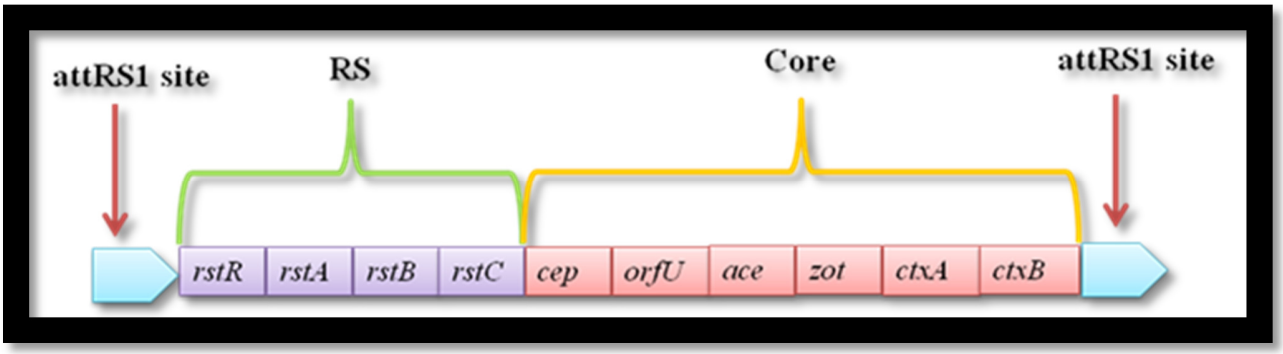

Figure 5 The CTX genetic element of the filamentous bacteriophage CTXΦ composed of two functional domains including the core, which is flanked by 1 or more repetitive sequence (RS). The Illustration was taken from Waldor and Mekalanos, 1996 [35].

Waldor and Mekalanos were the first to describe that the *ctxAB* genes coding for the A and B subunits of the CT were located on the filamentous CTXΦ [35]. The CTX genetic element is between seven and ten kilobase pairs (kb) in size and is composed of two functional domains namely, the core and the repetitive sequence (RS2) (Figure 5) [9,17,35]. The core is ~ 4.50 kb in length and contains six genes including *ctxAB*, *zot* (encodes for the zonula occludens toxin), *cep* (encodes for the core-encoded pilin), *ace* (encodes for the accessory cholera toxin) and *orfU* (encodes for a product of unknown function) respectively (Figure 5) [9,17,35]. The proteins responsible for bacteriophage packing and secretion are Zot, Ace, OrfU and Psh and the protein responsible for the assembly of CTXΦ is Zot [9,17,19,35]. The RS2 (2.7 kb) encodes for four open reading frames (ORF) namely, *rstA* (function in replication); *rstB* (function in integration); *rstC* (induce *ctxAB* expression) and *rstR* (function in regulation) (Figure 5), which together is responsible for site-specific recombination into the chromosome of non-toxigenic *V. cholerae* O1 isolates at a specific attachment (att) site, which is termed attRS [9,17,19,35]. CTX bacteriophage genomes are classified into two types based on nucleotide sequence variations of

the *rstR* gene, which includes the CTXΦ classical ($CTX^{class}\Phi$) and CTXΦ El Tor ($CTX^{ET}\Phi$) [19,37]. Several *V. cholerae* O1 El Tor variants have been described [19], which possess characteristics of both biotypes and this is further discussed in section 1.8.

1.7.2 Vibrio pathogenicity island (VPI)

The TCP acts as both a mediator for bacterial colonization to the intestinal epithelial cells and as a receptor for the bacteriophage (CTXΦ) entry into the bacterium [9]. The function of the TCP is augmented with a potential accessory colonization factor, ACF, which is coded by the *acf* gene [9,38]. The TCP-ACF gene cluster is located on the ~ 40 kb VPI and is characteristic of both epidemic and pandemic isolates of *V. cholerae* O1 [9,17,38,39]. The major subunit of the TCP is encoded by the *tcpA* gene [9,38,39]. The formation and function of the pilus assembly is the responsibility of at least 15 other genes located next to *tcpA* on the *tcp* gene cluster [9,39]. The *toxT*, *tcpP* and *tcpH* genes located on the pathogenicity island, encode for regulators of virulence genes, which may be necessary for the transfer and integration of the VPI (Figure 6) [9,38]. A study done by Karaolis *et al*. revealed that the VPI has a low guanine and cytosine (G + C) content of ~ 35 % suggesting acquisition of the pathogenicity island was from another source [9,38].

1.7.3 Regulation of the Cholera Toxin (CT) and toxin co-regulated pilus (TCP)

The regulation of the CT, TCP and ACF is mediated by the ToxR regulatory system, [9,17]. The ToxR is a 32 kilodalton (kDa) transmembrane protein that regulates the expression of the CT in response to appropriate environmental signals by means of a regulatory cascade [9,17]. ToxS is a sensory membranous protein encoded by the *toxS* gene, which activates ToxR [9]. ToxR is encoded by the *toxR* gene and mediates the expression of ToxT, which is encoded by the *toxT* gene and is present in the cytoplasm (Figure 6) [9]. The ToxR protein binds to a 7 bp tandem repeat DNA sequence upstream of the *ctxAB*, which results in the elevated expression of the CT [9].

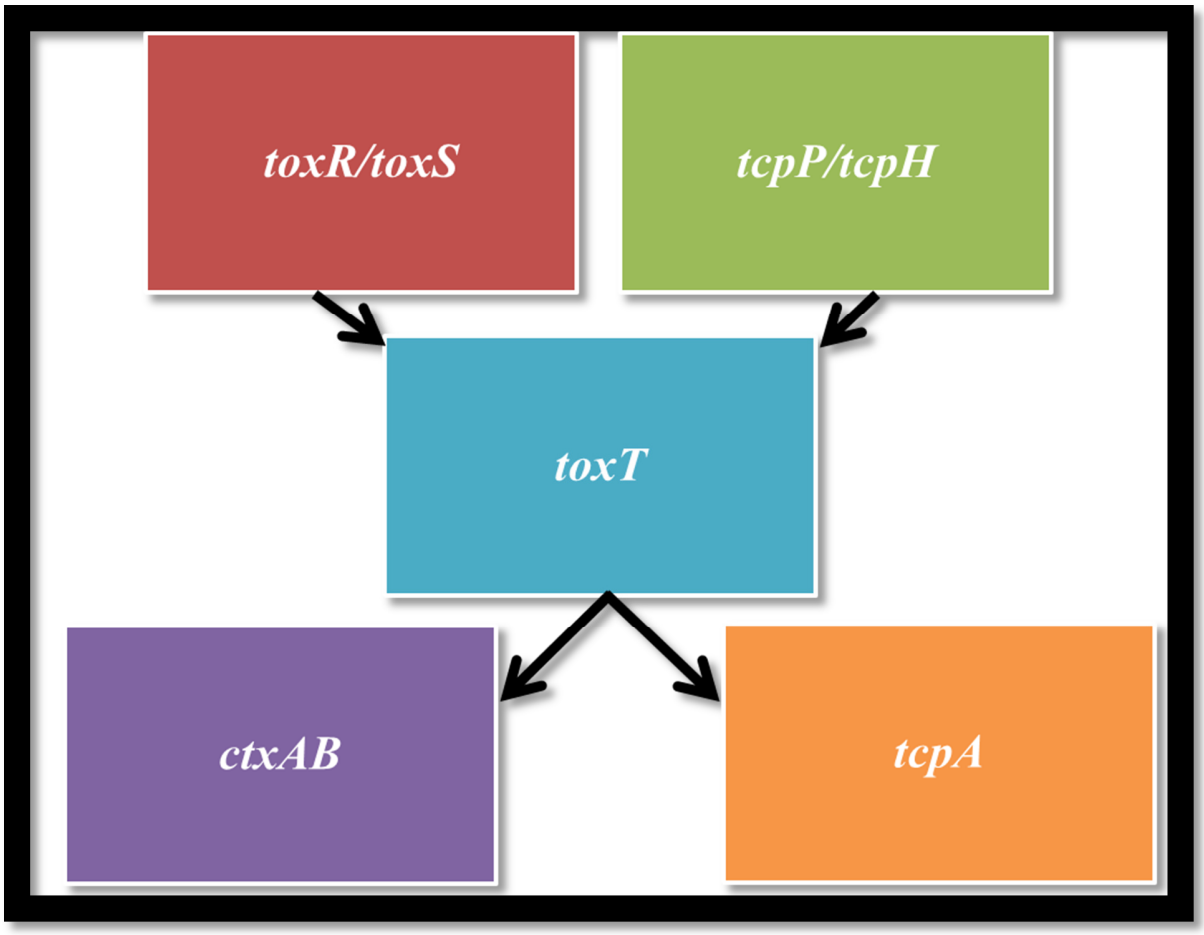

Figure 6 Regulation of the cholera toxin and toxin co-regulated pilus.

1.8 Molecular epidemiology and genetic characterization of toxigenic *Vibrio cholerae* O1

The epidemiological surveillance of cholera was limited due to the lack of suitable genotyping applications [6,9]. Recent developments of several DNA-based typing systems, have allowed scientists to investigate the epidemiology of toxigenic *V. cholerae* O1 [6,9,40,41]. Such advances have enabled the establishment of large databases of characterized organisms [6,9,40,41]. Genotyping systems have the potential to establish relatedness of isolates from disease epidemics and provide information on the geographical distribution, source or origin of infection [6,9,40,41].

Various DNA-based molecular techniques namely, pulsed-field gel electrophoresis (PFGE) analysis, direct genomic restriction enzyme analysis (DGREA), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic PCR (REP-PCR), ribotyping, multi locus sequence typing (MLST), enterobacterial repetitive intergenic consensus-based polymerase chain reaction (ERIC-PCR), microarray, plasmid fingerprinting and variable number of tandem repeats (VNTR), have been shown to be useful in the epidemiology, ecology and genetic exchange of *V. cholerae* [6,40,41]. Although many studies have shown these applications to be useful, one must consider both the rapidity and discriminatory power towards the species of interest [6,40,41]. The latest molecular applications such as whole genome sequencing has become the method of choice for the analysis and comparison of *V. cholerae* isolates [42].

PFGE analysis is recognized as the "gold standard" of genotyping applications [40]. The principle of PFGE is based on the digestion of genomic DNA with rare-cutting restriction enzymes followed by gel electrophoresis using pulsating polarized electric currents [6,40,41]. PFGE has been successfully applied for the analysis of clonal relatedness of *V. cholerae* (clinical and environmental sources) and has been evaluated as an epidemiological tool for outbreak investigations [6,40,41,43]. PFGE demonstrates both geographical and temporal stability and has shown to be more discriminatory than ribotyping [6,41].

V. cholerae O1 biotype classical and El Tor isolates display different phenotypic and genotypic properties [12,19]. Several *V. cholerae* O1 El Tor variants have been described and possess both characteristics of both biotypes [6,19]. These variants have been described in Bangladesh, Asia and Africa [19,44]. *V. cholerae* O1 altered El Tor variants produce the CT of the classical biotype, which is coded by the *ctxB1* gene and can be biotyped by means of conventional phenotypic assays [12,19]. *V. cholerae* O1 hybrid variants produce the CT of either biotype as they carry both $rstR^{Cla}$ and $rstR^{El}$ genes, but cannot be biotyped by means of conventional phenotypic assays [12,19]. For the characterization of *ctxAB* and *rstR* genes, nucleotide sequence analysis is required. This can be accomplished by PCR of the genes followed by Sanger-type DNA nucleotide sequencing. Alternatively, should whole genome sequencing data be available,

then the nucleotide sequences of genes could be obtained by extraction of gene sequences from whole genome sequencing data [37]. PCR was developed in 1985 and has since proved to be a powerful detection tool in various scientific areas [45,46]. Direct sequencing remains the "gold standard" for the identification of unknown products of PCR amplification [45,46].

1.9 Mechanisms of antimicrobial resistance in toxigenic *Vibrio cholerae* O1

V. cholerae O1 does not generally cause systemic infections and antimicrobial agents are not required in the treatment of cholera however, there are advantages of combining oral rehydration therapy with the use of antimicrobial agents [47]. Antimicrobial resistance has resulted in treatment complications of cholera [47,48]. Prior to the 1970s, *V. cholerae* O1 remained susceptible to antimicrobial agents [48]. This changed in 1976 during a worldwide survey that was conducted on randomly selected isolates [48]. This survey showed that ~ 3 % of the *V. cholerae* O1 isolates were antimicrobial-resistant to frequently used antimicrobial agents [48]. During the 1950s, the recommended antimicrobial agent furazolidone was widely used for the specific and symptomatic treatment of bacterial or protozoal diarrhoea in children [48]. However, during the late 1980s the majority of enteric pathogens have developed resistance to furazolidone and as a result, now has limited applications [48]. Analysis of antimicrobial susceptibility profiles of *V. cholerae* O1 from different regions of the world showed that for the period of 1938 to 1993, isolates were resistant to one to three antimicrobial agents and for the period of 1994 to 2005, isolates were resistant to three to eight antimicrobial agents including the fluoroquinolone, ciprofloxacin [48]. To emphasize how quickly antimicrobial resistance has developed in *V. cholerae* O1, a study done by Towner *et al*. in 1980 reported on isolates recovered during the fourth cholera epidemic in Tanzania that were susceptible to tetracycline [49]. However, after five months of extensive treatment with tetracycline, 76 % of *V. cholerae* O1 were shown to be resistant to tetracycline and other antimicrobial agents [49]. The increased minimum inhibitory concentrations (MICs) to ciprofloxacin coupled with resistance to older antimicrobial agents have forced clinicians to use a broad-spectrum macrolide, azithromycin for the treatment of cholera [50].

Bacteria possess various molecular mechanisms for antimicrobial resistance, which are either intrinsic (natural) or acquired [51-53]. Human and animal populations are potential reservoirs for antimicrobial resistance genes [52,54,55]. Antimicrobial resistance genes either are present in nature already or have the potential to emerge by mutation [53]. For example, rapid mutation was observed with the TEM β-lactamase, which was first reported in 1963 in Athens [53]. Several mechanisms of antimicrobial resistance have been well described for *V. cholerae* O1 [47,48,51]. Resistance mechanisms include, exporting antimicrobial agents before it can reach its target site by means of an efflux pump, spontaneous mutations in genetic material and through the exchange of conjugative plasmids, conjugative transposons, integrons or self-transmissible chromosomally integrating SXT elements [47,48,51].

1.9.1 Efflux systems

Bacterial efflux pumps are transport proteins, which are involved in the expulsion of toxic substrates within cells into the environment [56]. Efflux pumps might be specific for 1 substrate or a variety of compounds [56]. Two major groups of efflux pumps have been described for *V. cholerae*. These pumps are distinguished based energy sources namely, Adenosine tri-phosphate (ATP) hydrolysis and proton-motive force (PMF) [47,48,57]. Families belonging to the PMF include the multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), resistance-nodulation-cell division (RND) and small multidrug resistance (SMR) [47,48,57]. An example of a *V. cholerae* ATP-driven pump is VcaM, which is an ATP-binding cassette (ABC) multidrug resistance efflux pump [47]. VcaM confers antimicrobial resistance to tetracyclines, fluoroquinolones and anthracyclines [47]. In Gram-negative bacteria, the RND family is of particular interest because of its broad substrate specificity and mode of action [57]. *V. cholerae* encodes six RND efflux systems, which may suggest why this organism is highly adaptable in contaminated environments as well as survival in the host [57]. For example, the VexH RND efflux pump does not only function in the transport of antimicrobial agents out of the bacterial cell but also contributes in the production, regulation and expression of the CT and TCP. [57].

1.9.2 Spontaneous mutations in chromosomal DNA

Antimicrobial resistance by chromosomal mutation is natural to all bacteria [52,53]. These mutations occur spontaneously in replicating bacteria as a result of imperfect fidelity of DNA replication, termed vertical evolution [43,52]. It is proposed that antimicrobial agents that target multiple sites within the bacterial cell are more likely to be at lower risk of acquiring resistance via chromosomal mutation [52]. However this is limited as illustrated by quinolone resistance [52]. Fluoroquinolone resistance has been reported since early 2002 and has been the antimicrobial agent of choice in the treatment of various infectious diseases including cholera [48]. Quinolones target both DNA gyrase and topoisomerase IV, which are essential for DNA replication [52]. Quinolone resistance is usually associated with amino acid substitutions in the quinolone resistance-determining region (QRDR) of DNA gyrase (GyrA/GyrB) and topoisomerase IV (ParC/ParE) proteins [47,48]. Quinolone resistance due to chromosomal mutations detected in GyrA andParC has been reported in *V. cholerae* O1 [58-60].

1.9.3 Horizontal gene transfer through mobile genetic elements

Antimicrobial resistance by gene acquisition refers to prior existence of antimicrobial-resistant genetic determinants that are then acquired by other bacteria, termed horizontal evolution [43,51,52,55].

One of the common modes for the dissemination of antimicrobial resistance genes are conjugative resistance plasmids, which are able to promote cell to cell transfer of DNA [48,61]. Plasmids are “self-replicating circular pieces of DNA, smaller than the genome, which encode their transfer by replication into another bacterial strain or species” [53]. Plasmid-encoded antimicrobial resistance includes the majority of classes of antimicrobial agents currently in clinical use [61]. These include cephalosporins, fluoroquinolones and aminoglycosides. *V. cholerae* O1, prior to the 1970s was susceptible to tetracycline [47,48]. As a result of extensive use of this oral antimicrobial agent, *V. cholerae* O1 has now become resistant, particularly in many African countries [47,48]. For example, *V. cholerae* O1 isolates collected during the

Somali cholera epidemic for the period 1985 to 1986 were shown to harbour two incompatible (Inc) C groups plasmids conferring resistance to ampicillin, kanamycin, streptomycin sulfonamide and tetracycline [62].

An integrative conjugative element (ICE) is a self-transmissible, mobile genetic element, which possess both plasmid-like (transfer by means of conjugation) and bacteriophage-like (not self-replicating) characteristics [63,64]. The term ICE was introduced by Burrus *et al*. in 2002 and includes conjugative transposons [63,64]. The SXT element is an example of an ICE of *V. cholerae* [47,48,64,65]. The 99.5 kb ICE, SXT^{MO10} was first identified in clinical isolates of *V. cholerae* O139 from Madras, India and confers resistance to sulfamethoxazole, streptomycin, trimethoprim and chloramphenicol [47,48,64,65]. Several studies from Africa including South Africa have described *V. cholerae* O1 isolates harbouring the SXT element and its association in antimicrobial resistance [66-69].

An integron is a site-specific recombination gene capture system that consists of an integrase coded by the *intI* gene, a primary recombination site known as attI and an outward-orientated promoter Pc, at which short DNA sequences called gene cassettes are, inserted [48,61,70,71]. Integron-inserted gene cassettes usually consist of a single gene and an imperfect inverted repeat at the 3' end of the gene called the attC site [70,72]. Presently, there are five classes of integrons [70]. Class 1 integrons are commonly found in clinical isolates as most known classes of antimicrobial agents belong to this class [70]. Class 4 and class 5 integrons are associated with *V.* species [70]. To date, over 100 integron-inserted gene cassettes have been identified [48]. A superintegron was initially discovered in *V. cholerae* which harboured hundreds of genes but with an unknown function [47,48,73,74]. Epidemic *V. cholerae* isolates from Africa harbouring class 1 integrons and class 2 integrons have previously been described [67,68,75-77].

1.10 Background and setting of my research project

As early as 1971, South Africa was considered at risk for the introduction and facilitation of spread of cholera in and around the country [78]. Risk factors included hot and humid summer seasons, the presence of sea-ports and overcrowded communities living in areas with unsafe water, sanitation and hygiene [78]. The first cholera epidemic described in South Africa occurred between 1980 and 1987 [78]. The first case was confirmed in October 1980 in an open community at Shongwe Hospital in KaNgwane, Mpumalanga Province [78]. Common epidemiological characteristics of cases showed that patients lived on farms at Malelane and had consumed open river water from the Crocodile-Malelane irrigation canal [78]. Majority of the patients were black South Africans residing in rural or deep rural areas with a relatively high annual rainfall of 600 millimetres [78]. A total of 25 251 laboratory-confirmed cases were reported with a CFR of ~ 1.4 %. Cholera outbreak isolates were characterized as *V. cholerae* O1, serotype Inaba, biotype El Tor [78]. The second cholera epidemic described in South Africa occurred between 1997 and 2005 [11]. The worst affected Province was KwaZulu-Natal [11]. For the years 2000 to 2002, more than a 100 000 cases were reported (Based on clinical diagnoses) with a CFR of less than ~ 1 % (for the period of 2001 to 2002) [11]. Cholera outbreak isolates were characterized as *V. cholerae* O1, serotype Ogawa, biotype El Tor and *V. cholerae* O1, serotype Inaba, biotype El Tor [11]. For the period 1 January 2008 to 31 May 2009, South Africa experienced two major outbreaks of cholera [79-82]. Within both outbreaks, organisms isolated from probable cholera cases identified at peripheral laboratories displayed resistance to three or more routinely tested antimicrobial agents.

1.11 Study objectives

The objectives of this study were:

- To investigate South African outbreak isolates of *V. cholerae* O1, 1 January 2008 to 31 May 2009.

- To determine the genetic diversity and cluster analysis of toxin-producing *V. cholerae* O1 isolates by using PFGE analysis (1 January 2008 to 31 May 2009).
- To screen for presence of the enzymatic subunit A of the CT (*ctxA* gene) and the toxin co-regulated pilus (*tcpA* gene) using PCR assays.
- To determine the presence of amino acid mutations in the CT (ctxAB) by nucleotide sequencing.
- To screen for the presence of antimicrobial resistance determinants by PCR. This included class 1 integrons (3'-CS and 5'-CS), class 2 integrons (*intI2*), plasmid-mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrS, qnrC* and *qepA*), quinolone resistance determinant (*qnrVC3*), ESBL producing genes (bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$), genes coding for the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*), SXT element-integrase gene (SXT*int*) and associated SXT resistance genes (*floR, sul2, dfrA1, dfr18, strA* and *strB*) and the class A tetracycline resistance determinant (*tetA*).
- To determine the presence of amino acid mutations in the QRDR of DNA gyrase and topoisomerase IV by nucleotide sequencing.
- To characterize the sequence identity of PCR-positive ESBL genes by nucleotide sequencing.
- To investigate the presence of resistance plasmids by the isolation of intact plasmid DNA (bla_{TEM}).
- To determine the genomic location of antimicrobial resistance genes using Southern blot hybridization assays (bla_{TEM}).
- To identify and track major 7th pandemic *V. cholerae* O1 variants.
- To determine the MICs to azithromycin by both the Episolimeter test (Etest) and doubling agar dilution methods.
- To investigate the presence of seven macrolide resistance determinants by PCR. This included *mefA*, *ereA*, *ereB*, *ermB*, *mphA*, *mphB* and *mphD*.
- To characterize any of the seven macrolide resistance determinants found to be PCR-positive by nucleotide sequencing.

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Chapter Two
Materials and Methods

This chapter describes the general methods used in this study. Specific methods are described in the appropriate chapters.

2.1 Collection, phenotypic identification, serological characterization and storage of bacterial isolates of *Vibrio cholerae* O1

A probable case of cholera was defined by clinical signs and symptoms as discussed in Chapter one. At the Centre for Enteric Diseases (CED), a clinical laboratory-confirmed case of cholera was defined as a positive culture for *V. cholerae* O1, from stool specimens or rectal swabsfrom patients admitted to clinics or hospitals throughout South Africa for the years 2008 to 2009. Cultured *V. cholerae* isolates from environmental specimens such as Moore (sewer) pads or water were sent to the CED for confirmation and further characterization. Isolates sent from peripheral laboratories, were first cultured onto Dorset egg slopes (Diagnostic Media Products (DMP), NHLS, South Africa), before arriving at the CED.

As part of routine characterization of *V. cholerae* O1 isolates received by the CED, all isolates were sub-cultured and streaked for single colonies onto non-selective media, 5 % sheep blood agar (DMP) as well as selective media, thiosulphate citrate bile salts sucrose agar (TCBS) (DMP) and incubated overnight at + 37 °C for the following day. Aseptic techniques were implemented. Cultured isolates were serogrouped and serotyped by slide agglutination. Various Pipetman® models (Gilson Inc., Middleton, United States of America) were used to aspirate the set volume of different liquids.

A single colony for each cultured isolate was picked using a toothpick and emulsified on a glass slide (Thermo Fisher Scientific, Johannesburg (Pty), South Africa) containing ten microlitres (µl) of sterile 0.9 % saline (DMP). The turbid saline suspension was mixed by tilting the glass slide back and forth. The saline suspension was examined to ensure that no clumping formed as a

result of autoagglutination. Ten microlitres of polyvalent *V. cholerae* serogroup O1 antiserum (Mast Assure, Mast Group Ltd., Merseyside, United Kingdom) was added to this suspension and mixed as described above. If the emulsified suspension agglutinated, isolates were further characterized by means of serotyping.

A single colony was picked using a toothpick and emulsified on a glass slide (Thermo Fisher Scientific) containing 10 µl of sterile 0.9 % saline (DMP) on both ends of the glass slide. Ten microlitres of monovalent Inaba antiserum (Mast Assure, Mast Group Ltd.) and 10 µl of Ogawa antiserum (Mast Assure, Mast Group Ltd.) were independently added of each other to each suspension and mixed as described above. The suspension that showed the strongest agglutination was recorded as positive. *V. cholerae* O1 isolates were stored at – 70 °C in tryptic soy broth with 10 % glycerol (DMP).

2.2 Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) is defined as "The lowest concentration of antimicrobial agent required to inhibit growth of the bacteria". MIC-determination and disk diffusion testing for each isolate were performed, as per the Clinical and Laboratory Standards Institute (CLSI), 2008 guidelines[1].

Isolates were streaked for single colonies onto 5 % horse blood agar (DMP) and incubated overnight at + 37 °C for the following day. Three to four single colonies were picked using a flamed loop and resuspended in a glass tube containing five millilitres (ml) sterile 0.9 % saline (DMP) (one tube per a bacterial isolate). The turbidity for each isolate was measured against a blank tube containing 5 ml sterile 0.9 % saline (DMP). A microscan turbidity meter (DADE BEHRING, California, United States of America) was used to measure the absorbance and the turbidity was adjusted to ~ 0.05, which corresponds to a McFarland 0.50 or $1x10^8$ colony forming units per millilitre (CFU/ml). This is to ensure that the density of bacterial inoculum is standardized. A cotton swab stick was dipped into the inoculum suspension and applied to eightMuller-Hinton agar plates (DMP) with the use of a retro C80 plate spreader (bioMérieux,

Marcy-l'Etoile, France). Episolimetertests (Etest) were performed using antimicrobial agents in the form of ETest® strips (bioMérieux). Using a sterile forceps, two ETest® strips (bioMérieux) were appropriately applied to each plate. Aseptic techniques were implemented, whereby the forceps was flamed in between the application of each ETest® strip (bioMérieux). Antimicrobial agents together with the concentration range and MIC breakpoints for antimicrobial agents tested for surveillance purposes at the CED are described in Table 3. MIC interpretation for erythromycin was performed using a previously published method by Ng *et al*. [2]. The Mueller-Hinton agar plates (DMP) containing the ETest® strips (bioMérieux) were incubated overnight at + 37 °C for the following day. MIC results were recorded. Isolates resistant to third and fourth generation cephalosporins were further investigated for the presence of extended-spectrum β-lactamase (ESBL) activity.

ESBL activity was investigated by disk diffusion testing using MAST ID™ ESBL discs (Mast Assure, Mast Group Ltd.) as described by the CLSI 2008 guidelines. *Escherichia coli*, American Type Culture Collection (ATCC) 25922 (ESBL-negative) and *Klebsiella pneumoniae* ATCC700603 (ESBL-positive) were used for quality control purposes. The inoculum suspension for each isolate was prepared as previously described. Only one Mueller-Hinton agar plate (DMP) was required per a bacterial isolate.

All six MAST ID™ ESBL discs (Mast Assure, Mast Group Ltd.) were appropriately applied to each plate. MAST ID™ ESBL discs (Mast Assure, Mast Group Ltd.) included: ceftazidime (30 µg), ceftazidime (30 µg) with clavulanate (10 µg); cefotaxime (30 µg), cefotaxime (30 µg) with clavulanate (10 µg); cefpodoxime (30 µg) and cefpodoxime (30 µg) with clavulanate (10 µg). The muller-hinton agar plates (DMP) containing the MAST ID™ ESBL discs (Mast Assure, Mast Group Ltd.) were incubated overnight at + 37 °C for the following day. MIC results were recorded.ESBL-positive results were recorded with respect to an increase in ratio of 1.5 or greater in zone diameter of inhibition by the cephalosporin disc containing clavulanate compared to the cephalosporin disc by itself. To determine whether isolates were susceptible, intermediately resistant, or resistant to a particular antimicrobial agent, isolates were referred to as non-susceptible if they were intermediately resistant or resistant to an antimicrobial agent.

Table 3 Antimicrobial concentration range and minimum inhibitory concentration (MIC) breakpoints for antimicrobial resistance using ETest® strips tested for surveillance purposes at the Centre for Enteric Diseases.

Antimicrobial agent	Antimicrobial concentration range of ETest® strips	MIC breakpoint for resistance
Ampicillin	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Augmentin	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Co-trimoxazole	0.002 µg/ml – 32 µg/ml	MIC ≥ 16 µg/ml
Trimethoprim	0.002 µg/ml – 32 µg/ml	MIC ≥ 16 µg/ml
Sulfamethoxazole	0.064 µg/ml – 1 024 µg/ml	MIC ≥ 512 µg/ml
Chloramphenicol	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Nalidixic acid	0.016 µg/ml – 256 µg/ml	MIC ≥ 32 µg/ml
Ciprofloxacin	0.002 µg/ml – 32 µg/ml	MIC ≥ 2 µg/ml
Tetracycline	0.016 µg/ml – 256 µg/ml	MIC ≥ 8 µg/ml
Kanamycin	0.016 µg/ml – 256 µg/ml	MIC ≥ 32 µg/ml
Streptomycin	0.064 µg/ml – 1 024 µg/ml	MIC ≥ 64 µg/ml
Imipenem	0.002 µg/ml – 32 µg/ml	MIC ≥ 8 µg/ml
Ceftriaxone	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Ceftazidime	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Cefepime	0.016 µg/ml – 256 µg/ml	MIC ≥ 16 µg/ml
Furazolidone	0.016 µg/ml – 256 µg/ml	MIC ≥ 128 µg/ml
Erythromycin*	0.016 µg/ml – 256 µg/ml	MIC ≥ 3 µg/ml

Abbreviations: Microgram per millilitre (µg/ml), greater than or equal to (≥)

MIC interpretation was performed as per the Clinical and Laboratory Standards Institute (CLSI), 2008 guidelines.

*MIC interpretation was performed as per previously published method by Ng *et al.* in 2003.

2.3 Genotypic characterization of *Vibrio cholerae* O1 by pulsed-field gel electrophoresis (PFGE) analysis

PFGE analysis was used to investigate the clonal relationship amongst the *V. cholerae* O1 isolates. This method was performed according to an adaption from the PulseNet standardized protocol for *V. cholerae* incorporating single restriction enzyme digestion of the genomic DNA with *Not*I (Fermentas International Inc, Burlington, Canada) [3]. The reference strain, CDC-H9812 *Salmonella enterica* serotype Braenderup was included as an internal control of known molecular size[4].

Figure 7 illustrates the schematic overview for the preparation of agarose plugs for PFGE analysis.Isolates were cultured on 5 % horse blood agar (DMP) and incubated overnight at + 37 °C for the following day. Pure bacterial culture was collected using a cotton swab stick (Lasec) and resuspended in a 5 ml plastic tube (one tube per a bacterial isolate) containing 1 000 µl autoclaved cell suspension buffer (Appendix A1-A3). The turbidity for each isolate was measured against a blank 5 ml plastic tube containing 1 000 µl autoclaved cell suspension buffer. A microscan turbidity meter (DADE BEHRING) was used to measure the absorbance and the turbidity was adjusted to ~ 0.70. The tubes containing the inoculum suspension were kept on ice.

Three-hundred microlitres of the inoculum suspension was transferred from the 5 ml plastic tube to a clean 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) (one tube per a bacterial isolate). For CDC-H9812, 200 µl of inoculum suspension was transferred. For the isolates, 15 µl of 20 milligrams per milliliter (mg/ml) proteinase-K (Roche Diagnostics, Mannheim, Germany) was added each 1.5 ml Eppendorf tube containing the 300 µl inoculum suspension (Appendix A4. For CDC-H9812, 20 µl of 10 mg/ml proteinase-K (Roche) was added (Appendix A5). The 1.5 ml Eppendorf tubes containing both the inoculum suspension and proteinase-K were capped and incubated on a Stuart model SBH200D3 dry heating block (Barloworld Scientific Ltd, Staffordshire, United Kingdom) at + 37 °C for five minutes. Agarose plugs were then prepared. The SeaKem Gold® agarose (Lonza, Rockland, USA) used to prepare the agarose plugs was kept liquid at + 55 °C (Appendix A6-A8).

For each isolate, 300 µl of 1 % SeaKem Gold® agarose (Lonza) (Weight to volume (w/v)) was added to the 1.5 ml Eppendorf tube containing both the inoculum suspension and proteinase-K. The agarose suspension was mixed by pipetting up and down. Three-hundred µl of this agarose suspension was then immediately transferred into a reusable plug mould (Bio-Rad Laboratories, California, United States of America) and allowed to solidify for ten minutes. For CDC-H9812, 280 µl of 1 %SeaKem Gold® agarose (Lonza) : 1 % sodium dodecyl sulphate (SDS) (Merck KGaA, Darmstadt, Germany) (w/v) was added. The agarose suspension was mixed by pipetting. Two-hundred and eighty microlitres of this agarose suspension was then immediately transferred into a reusable plug mould (Bio-Rad) and allowed to solidify for ten minutes. This was followed by bacterial cell lysis to release the genomic DNA. The agarose plugs were transferred to Nunc 50 ml graduated centrifuge tubes (Thermo Fisher Scientific) (one tube per a bacterial isolate) using a spatula. Aseptic techniques were implemented, whereby the spatula was wiped down with 70 % alcohol (DMP) in between each isolate. Five mililitres of cell lysis buffer (Appendix A9-A10) was added to each 50 ml test tube containing the agarose plug. The tubes were incubated for two hours at + 55 °C in a Julabo SW22 shaking water bath (JULABO Labortechnik GmbH, Seelbach, Germany) at 70 rotations per minute (rpm). This allowed for the lysis of the agarose plugs. After two hours, the tubes were taken out of the shaking water bath (JULABO Labortechnik GmbH). The cell lysis buffer was discarded. Fifteen mililitres of pre-heated distilled water (+ 50 °C) was added to each tube. The tubes were incubated for 15 minutes at + 50 °C in the shaking water bath (JULABO Labortechnik GmbH) at 70 rpm. This allowed for the lyzed agarose plugs to be washed. The distilled water was discarded. This wash step was repeated twice. Fifteen mililitres of pre-heated Tris-disodium ethylenediaminetetra-acetic acid (TE) buffer was added to each tube (Appendix A11). The tubes were incubated for 15 minutes at + 50 °C in the shaking water bath (JULABO Labortechnik GmbH) at 70 rpm. The TE buffer was then discarded. This wash step was repeated four times.

Agarose plugs were stored away for further use in 14 ml polypropylene test tubes (Thermo Fisher Scientific) containing 3 ml TE buffer and kept refrigerated at + 2 ºC to + 8 ºC (one tube per a bacterial isolate). This was followed by restriction enzyme digestion of bacterial DNA.

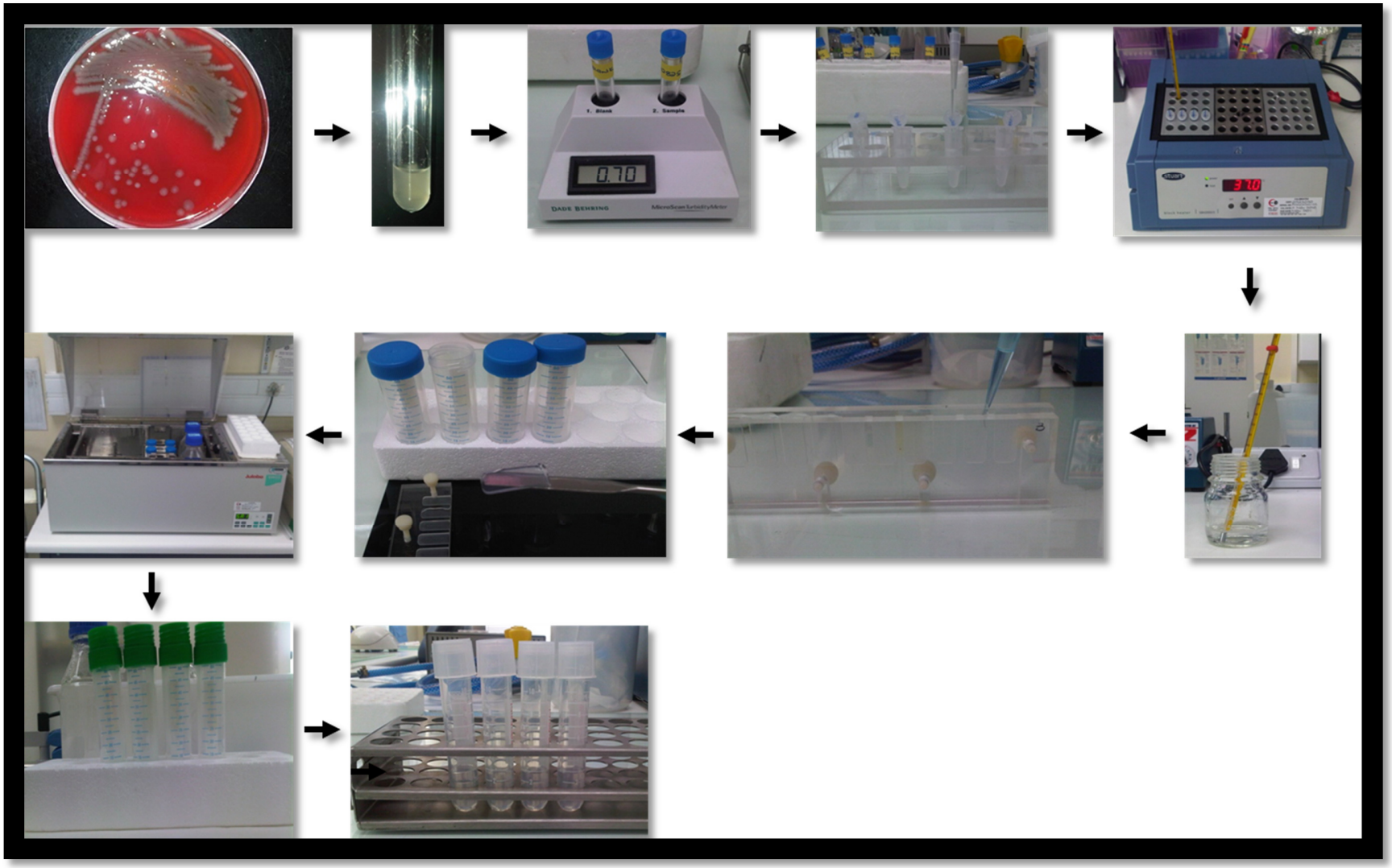

Figure 7 Preparation of agarose plugs for pulsed-field gel electrophoresis (PFGE) analysis.

Figure 8 illustrates the schematic overview for the restriction enzyme digestion of total bacterial DNA contained in agarose plugs. For bacterial DNA digestion, an agarose plug was transferred to a glass slide (Thermo Fisher Scientific) using a spatula. Aseptic techniques were implemented. A thin slice of agarose, ~1 millimetre (mm) in width was cut using a disposable scalpel blade number (#) 22 (All Pro Medical Apparatus and Instruments Co., Ltd, Qingdao, China). The agarose slice was transferred from the glass slide to a 1.5 ml Eppendorf tube (Eppendorf) using a spatula (one tube per a bacterial isolate). Approximately three agarose slices of CDC-H9812 were cut and prepared. One-hundred microlitres of restriction enzyme O-buffer mix (Appendix A12) was added to each 1.5 ml Eppendorf tube containing an agarose slice. For CDC-H9812, 100 µl of restriction enzyme H-buffer mix (Appendix A13) was added. The 1.5 ml Eppendorf tubes were capped and incubated on a dry heating block (Barloworld Scientific Ltd) for 15 minutes at + 37 °C. This step allowed for the agarose slice to adapt to the external environment. The 1.5 ml Eppendorf tubes (Eppendorf) were taken off the dry heating block (Barloworld Scientific Ltd), uncapped and the restriction enzyme buffer mix were aspirated out from each 1.5 ml Eppendorf tube (Eppendorf). One-hundred and fifty microlitres of fresh restriction enzyme O-buffer mix with *Not*I restriction enzyme (Appendix A14) was added to each 1.5 ml Eppendorf tube containing an agarose slice. For CDC-H9812, 150 µl of fresh restriction enzyme H-buffer mix with *Xba*I restriction enzyme (Appendix A15) was added. The 1.5 ml Eppendorf tubes were capped and incubated on a dry heating block (Barloworld Scientific Ltd) for 240 minutes at + 37 °C.

In order to halt the restriction enzyme reaction, the 1.5 ml Eppendorf tubes (Eppendorf) were taken off the dry heating block (Barloworld Scientific Ltd) and uncapped. The restriction enzyme buffer mix with restriction enzyme was aspirated out from each 1.5 ml Eppendorf tube (Eppendorf). Two-hundred microlitres of 0.5X Tris-Borate-EDTA (TBE) (Merck) buffer (Appendix A16-A17) was added to each 1.5 ml Eppendorf tube (Eppendorf). The 1.5 ml Eppendorf tubes (Eppendorf) were capped and incubated at room temperature (+ 25 °C) for ten minutes. Using a spatula, the agarose slices were removed from the 1.5 ml Eppendorf tube (Eppendorf) and loaded onto a 15 well gel comb (Bio-Rad) with gel casting tray (Bio-Rad). CDC-H9812 agarose slices were loaded on the first well, middle well and last well. A 1 %

SeaKem Gold® agarose (Lonza) gel (w/v) was prepared (Appendix A18). The molten agarose was cooled to + 50 °C and poured immediately into the gel casting tray with the gel comb containing the agarose slices. The gel was allowed to solidify for 25 minutesat room temperature. This was followed by electrophoresis. Approximately three litres (l) of 0.5X TBE (Merck) running buffer (Appendix A17) were prepared and poured into a contour-clamped homogenous electric field (CHEF-DR III) apparatus (Bio-Rad). The running buffer was cooled to + 14 °C. The gel comb was removed and the solidified gel was submerged in the electrophoresis tank. The electrophoresis running conditions was comprised of a two-block program at 200 volts (V). In the first block, the pulse times were set from an initial switch time of 2 seconds to a final switch time of 10 seconds with a run time of 14 hours at a gradient voltage of 6 volts per centimetre (V/cm). In the second block, the pulse times were set from an initial switch time of 20 seconds to a final switch time of 25 seconds with a run time of seven hours at a gradient voltage of 6 V/cm.

Upon completion of the electrophoresis run, the gel was removed from the gel chamber of the CHEF-DR III (Bio-Rad) and placed in a plastic tray along with of 250 ml of ethidium bromide (EtBr, 10 mg/ml) staining solution (Appendix A19-A20). The plastic tray was oscillated on a Stuart SSL4 see-saw rocker (Barloworld Scientific Ltd) for 20 minutes at room temperature. The EtBr staining solution was discarded and 250 ml of distilled water was added. The plastic tray was oscillated on a Stuart SSL4 see-saw rocker (Barloworld Scientific Ltd) for 30 minutesat room temperature. This step allowed for the gel to de-stain. The distilled water was discarded and the image of the gel was captured on the Molecular Imager® Gel DocTM XR System (Bio-Rad) using the Quantity One program software (Bio-Rad). The image of the gel as shown in Figure 9 was saved in a TIFF format (*.tif), which is required for further analysis using the BioNumerics™ (version 6) software program (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated and compared by using an unweighted pair group method with arithmetic averages (UPGMA) analysis. Cluster analysis was conducted using a dice co-efficient with both an optimization value and tolerance factor of 1.5 %. For this study, PFGE clusters were defined by groups of PFGE banding patterns, which have similarity values of greater than or equal to (≥) 95 %.

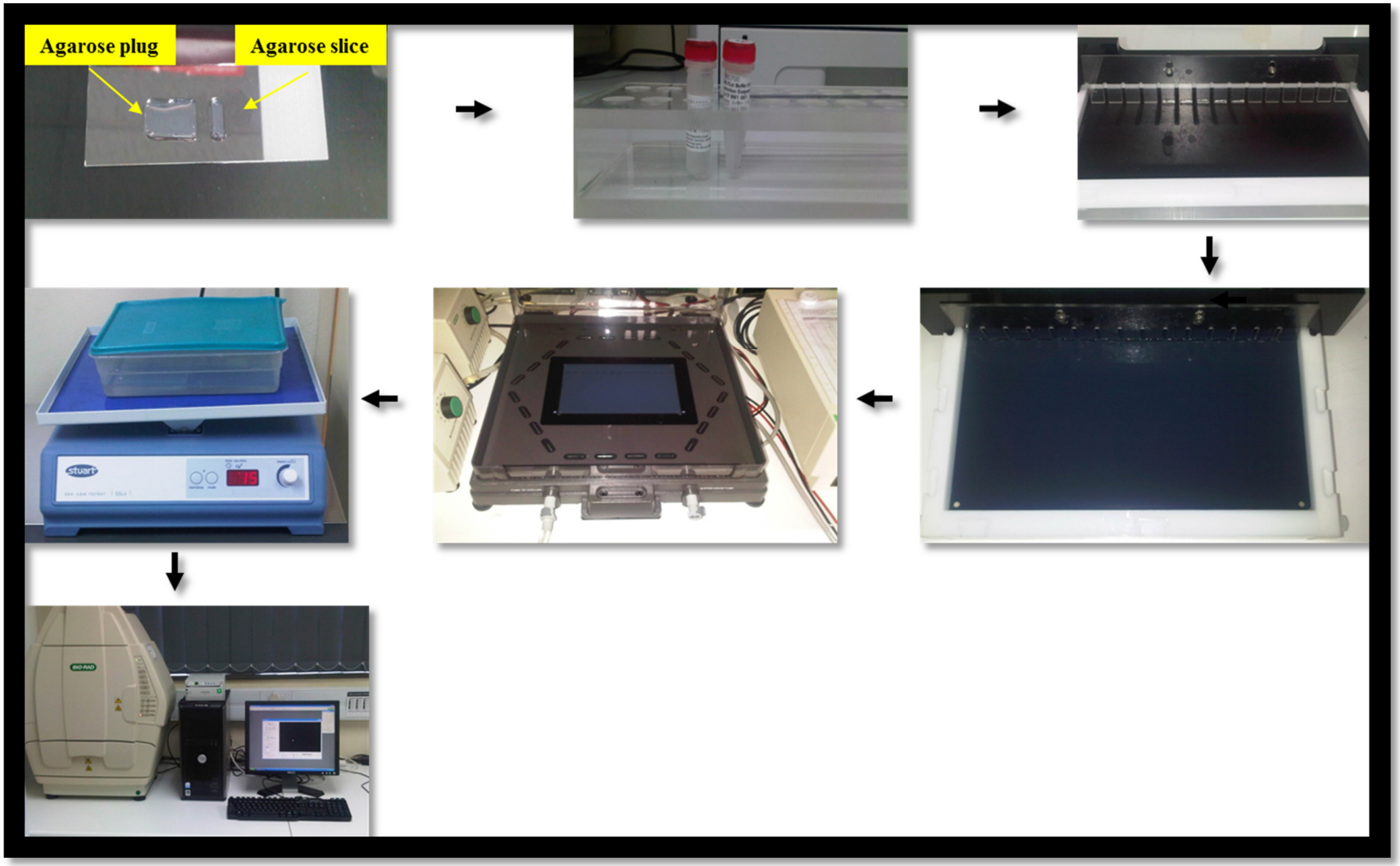

Figure 8 Restriction enzyme digestion of total bacterial DNA contained in agarose plugs.

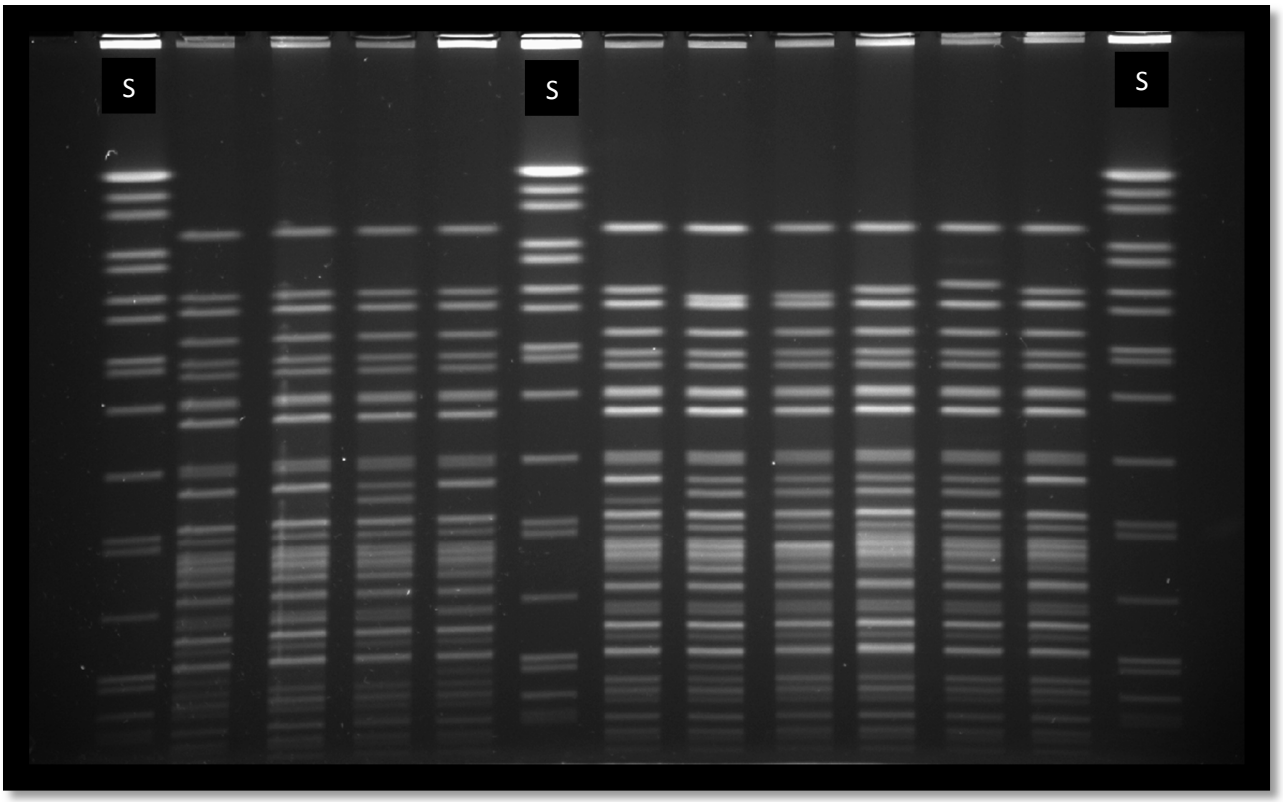

Figure 9 Pulsed-field gel electrophoresis (PFGE) image of ten *V. cholerae* O1 isolates digested with *Not*I restriction enzyme using the PulseNet standardized protocol for *V. cholerae*. Lane S *Salmonella* Braenderup strain CDC-H9812 digested with *Xba*I restriction enzyme.

2.4 Crude extraction of genomic DNA

V. cholerae O1 isolates and PCR-positive control isolates were cultured on 5 % sheep blood agar (DMP) and incubated overnight at + 37 °C for the following day. A half loopful of bacterial culture was resuspended in a clean 1.5 ml Eppendorf tube (Eppendorf) (One tube per a bacterial isolate) containing 400 µl autoclaved TE Buffer (Appendix A11). Aseptic techniques were implemented. Tubes were capped and incubated for 25 minutes at + 95 °C on a dry heating block (Barloworld Scientific Ltd). The 1.5 ml Eppendorf tubes (Eppendorf) were taken off the dry heating block (Barloworld Scientific Ltd) and allowed to cool at room temperature. The 1.5 ml Eppendorf tubes (Eppendorf) containing the boiled cell suspensions were vortexed using a

GENIE2 vortex (Scientific Industries, Inc., New York, United States of America). The tubes were centrifuged in a balanced Eppendorf 5415R table-top centrifuge (Eppendorf) at 12 000 rpm for three minutes. The tubes were uncapped and 50 µl of the supernatant was transferred into a clean 1.5 ml Eppendorf tube (Eppendorf) containing 450 µl autoclaved TE Buffer and this served as the template DNA for the PCR assays.

The 1.5 ml Eppendorf tubes (Eppendorf) containing the supernatant was stored for further use in the freezer at - 10 °C to - 20 °C.

2.5 Polymerase chain reaction (PCR) amplification

Polymerase chain reaction (PCR) was performed for the screening and amplification of genes coding for particular virulence determinants, antimicrobial resistance determinants and the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV. All PCR primers (100 micromolar (µM) stock concentrations) used in this study were synthesized by Inqaba Biotechnical Industries (Inqaba Biotechnical Industries, Hatfield, South Africa). The 16S rRNA gene was amplified and this served as an internal control as all bacteria contain this gene, which is essential for cell function. A no template control was included in all PCR assays to exclude contamination.

2.5.1 Detection and analysis of the enzymatic A subunit of the cholera toxin (CT)

A multiplex PCR assay was implemented to simultaneously detect for bacteria [5] and to identify CT producing *V. cholerae* O1 isolates [6] by the presence of the 16S rRNA gene and the CT enzymatic A subunit, *ctxA* gene. A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc., California, United States of America) for each study isolate and the positive control, *V. cholerae* O1 ATCC9458. A standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below (Appendix B1).

Target genes	Expected size (~ bp)
ctxA	301
16S rRNA	726

2.5.2 Detection and analysis of the toxin co-regulated pilus (TCP)

A multiplex PCR assay was implemented to simultaneously characterize CT producing *V. cholerae* O1 isolates as either classical or El Tor biotypes by the presence of the TCP, *tcpA* gene [6] (Appendix B2). A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and positive controls, *V. cholerae* O1 ATCC9458 and *V. cholerae* O139 VIBCH05. A standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	Expected size (~ bp)
tcpA-classical	617
tcpA-El Tor	471

2.5.3 Detection and analysis for extended spectrum β-lactamases (ESBLs)

V. cholerae O1 isolates that displayed extended spectrum β-lactamase (ESBL) activity against third generation cephalosporins; ceftazidime (MIC ≥ 16 µg /ml), ceftriaxone (MIC ≥ 16 µg/ml) and cefepime (MIC ≥ 16 µg/ml) were further characterized. Single PCR assays were implemented to detect for the presence of bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ [7,8] (Appendix B3). A 25 µl PCR reactionwas prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and positive controls, *Klebsiella pneumoniae* ATCC51503, *Klebsiella pneumoniae* ATCC700603 and *Klebsiella pneumoniae* D-17. Positive control isolates were kindly provided, courtesy of the Antimicrobial Resistance Reference Unit. A standard-3 PCR

assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	**Expected size (~ bp)**
bla$_{TEM}$	840
bla$_{SHV}$	846
bla$_{CTX-M}$	550

2.5.4 Detection and analysis for class 1 and class 2 integrons

Single PCR assays were implemented to detect for the presence of the 3'-CS and 5'-CS conserved segments of the class 1 integron and the conserved region of the integron-encoded integrase *intI2* gene of the class 2 integron [9-11] (Appendix B4). A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and the positive control, *Escherichia coli* 803Rif:p3iANG. *Escherichia coli* 803Rif:p3iANG, was kindly provided, courtesy of Doctor Daniela Ceccarelli. A standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	**Expected size (~ bp)**
3'-CS	800
5'-CS	870
intI2	450

2.5.5 Detection and analysis for the tetracycline resistance determinant, SXT element-integrase and SXT associated resistance genes

Single PCR assays were implemented to detect for the presence of the SXT element-integrase gene (SXT*int*), associated SXT resistance genes (*floR, sul2, dfrA1, dfr18, strA* and *strB*) [12]and the class A tetracycline resistance determinant (*tetA*) [13] (Appendix B5). A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and positive controls, *Escherichia coli* CAG18439:SXT^{MO10} and CED isolate TCD273377. *Escherichia coli* CAG18439:SXT^{MO10} was kindly provided, courtesy of Doctor Daniela Ceccarelli. A standard-3 PCR assay consisting of 35amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	**Expected size (~ bp)**
SXT*int*	592
floR	526
sul2	625
dfrA1	372
dfr18	389
strA	383
strB	470
tetA	950

2.5.6 Detection and analysis for plasmid-mediated quinolone resistance (PMQR) genes and quinolone resistance determinants

Single PCR assays were implemented to detect for the presence of the plasmid-mediated quinolone resistance (PMQR) genes *qnrA*, *qnrB*, *qnrS*, *qnrC* and *qepA* and the quinolone resistance determinant *qnrVC3* [14-16] (Appendix B6). A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and positive controls, *Escherichia coli* pMG252, *Escherichia coli* pMG298, *Escherichia coli* pMG306, *Escherichia coli* pAT851 and *Proteus mirabils* 06-498. Positive control isolates were kindly provided, courtesy of Doctor George A. Jacoby. A standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	**Expected size (~ bp)**
qnrA	657
qnrB	566
qnrS	585
qnrC	307
qepA	596
qnrVC3	521

2.5.7 Detection and analysis of genes encoding for the DNA gyrase and topoisomerase IV of the quinolone resistance-determining region (QRDR)

Single PCR assays were implemented to detect for the presence of genes coding for the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*) [14] (Appendix B7). A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each test isolate and positive control, CEDisolate TCD273377. PCR assays both consisting of 35 amplification cycles were set up

using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Expected sizes for the PCR amplicons are shown below.

Target genes	**Expected size (~ bp)**
gyrA	239
gyrB	309
parC	248
parE	268

2.6 Detection and size analysis of PCR-positive products using conventional agarose gel electrophoresis

The detection and size of the PCR product was determined using conventional agarose gel electrophoresis (Appendix C). Upon completion of the PCR, the 200 µl thin-walled PCR tubes (Axygen Inc.) were uncapped and 3 µl of loading buffer was added to each tube. The PCR products were mixed by pipetting up and down. A 1.5 % Seakem LE agarose (Lonza) gel (w/v) was prepared. The molten agarose was cooled to + 50 °C. Approximately 6 µl of the 10 mg/ml EtBr stock solution was added to the molten agarose and mixed before being poured into the UVTP gel tray (Bio-Rad) with the fixed height comb (Bio-Rad).

The gel was allowed to solidify for 25 minutes at room temperature. The comb was removed and the solidified gel was submerged in the Sub-Cell® GT base (Buffer chamber) (Bio-Rad) containing cold 1X Tris : acetate : EDTA (TAE) running buffer. Approximately 6 µl of the PCR product for each isolate was loaded in a separate well. Sizes were compared alongside either a HyperLadder™ IV or HyperLadder™ I molecular weight marker (Bioline, Boston, United States of America) or Marker III (Roche). Approximately 3 µl of the appropriate molecular weight marker was loaded in the first, middle and last well of the gel. Conventional gel electrophoresis was performed using a Thermo EC105 power supply (The Scientific Group, Midrand, South Africa) at 140 V for 45 minutes. The image of the gel was captured on the gel documentation system (Bio-Rad) using the Quantity One program software (Bio-Rad).

2.7 Nucleotide sequencing of PCR-positive amplicons

A 50 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.). The volumes required for a 25 µl PCR reaction were doubled and the cycle PCR conditions remained the same. To visualize the synthesis of the PCR-positive amplicons, 5 µl of the PCR product was loaded with 1 µl of loading buffer on a 1.5 % Seakem LE agarose gel (Lonza). Sizes were compared alongside the appropriate molecular weight marker, HyperLadder™ IV molecular weight marker (Bioline) or HyperLadder™ I molecular weight marker (Bioline). Agarose gels were prepared and PCR-products were resolved as described in section 2.6.

The resultant 45 µl PCR-positive product was purified as per the manufacturer's guidelines using the MSB® Spin PCRapace clean-up kit (Invitek, Berlin, Germany) and served as template DNA in the cycle sequencing PCR reaction using the ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an Applied Biosystems Model 3500 automated genetic analyzer.

The 45 µl PCR-positive product was transferred to a clean 1.5 ml Eppendorf tube (Eppendorf) (one tube per a bacterial isolate) containing 250 µl binding buffer 1 (Invitek). The PCR mixture was mixed by pipetting up and down. The mixed PCR mixture was transferred to a 2 ml receiver tube with a spin filter (Invitek) (one tube per a bacterial isolate). The tube was capped and centrifuged in a table-top centrifuge (Eppendorf) at 12 000 rpm for three minutes. The spin filter was transferred to a 1.5 ml receiver tube (Invitek) (one tube per a bacterial isolate). Approximately 10 µl of distilled water was added to the centre of the column of the spin filter. The tube was capped and incubated at room temperature for one minute. The capped tube was centrifuged in a table-top centrifuge (Eppendorf) at 10 000 rpm for one minute. The spin filter was discarded and the resultant cleaned-up PCR product served as template DNA for cycle sequencing PCR. The concentration and purity of the nucleic acid without dilution was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The concentration and purity of purified DNA required for each isolate was ~ 50 to 100 nanograms per microlitre (ng/µl).

Approximately 1 µl of undiluted purified DNA was pipetted onto the receiving optic fiber (pedestal) of the Nanodrop 1000 spectrophotometer. The sampling arm holding the source optic fiber was brought into contact with the liquid DNA creating a column. The spectral measurement was made using the operating software program, NanoDrop 1000 V3.7 (Thermo Fisher Scientific). Approximately 2 µl of undiluted purified DNA was used in each cycle sequencing PCR reaction.

A 15 µl cycle sequencing PCR reaction using the ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) (Appendix D). A standard-2 PCR assay consisting of 25 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad) (Appendix D).

The 15 µl cycle sequencing PCR products were purified as per the manufacturer's guidelines using the QIAGEN DyeEx® 2.0 Spin Kit (QIAGEN gmbH, Hilden, Germany). Capped DyeEx® 2.0 spin columns were vortexed using a GENIE2 vortex (Scientific Industries) and the bottom closure of the columns snapped off. The capped DyeEx® 2.0 spin columns were placed in 2 ml collection tubes and centrifuged in a balanced Eppendorf 5415R table-top centrifuge (Eppendorf) at a calculated speed of 3 000 rpm for three minutes. The capped DyeEx® 2.0 spin columns were removed and transferred to clean 1.5 ml Eppendorf tubes (Eppendorf) and uncapped. The 15 µl cycle sequencing PCR products were transferred onto the centre of the slanted gel-bed surface of the DyeEx® 2.0 spin column (one tube per a bacterial isolate) and capped. The 1.5 ml Eppendorf tubes containing the DyeEx® 2.0 spin columns were centrifuged in an Eppendorf 5415R table-top centrifuge (Eppendorf) at a calculated speed of 3 000 rpm for three minutes. The tubes were removed and the spin columns discarded.

The 1.5 ml Eppendorf tubes containing the gel-filtrated DNA products were uncapped and vacuum-dried in a balanced Speed Vac Concentrator vacuum centrifuge (Savant, GMI Incorporated, Minnesota, United States of America) for 60 minutes. Vacuum-dried DNA products were reconstituted in 15 µl of Hi-Di formamide (Applied Biosystems). The 1.5 ml Eppendorf tubes containing the 15 µl reconstituted DNA products were capped, vortexed using a

GENIE2 vortex (Scientific Industries) and incubated for three minutes at + 95 °C on a dry heating block (Barloworld Scientific Ltd). The tubes were immediately placed on ice. The tubes were vortexed using a GENIE2 vortex (Scientific Industries) and centrifuged for 10 seconds in a balanced Eppendorf 5415R- centrifuge (Eppendorf).

The 15 µl products were transferred to a MicroAmp optical 96-well reaction plate (Applied Biosystems) (one well per DNA sample) and placed in an Applied Biosystems Model 3500 automated genetic analyzer. The DNA samples were sequenced using the "3500 Data Collection Software V1.0" (Applied Biosystems) operating program. The run module "RapidSeq50-POP7" (Applied Biosystems) was applied. This run module was selected based on a read length of ~ 500 bp, which could be determined within 40 minutes.

DNASTAR Lasergene® software (version 8.1) (DNASTAR, Inc., Madison, Wisconsin) was used to analyze the nucleotide sequences, and sequence identity was determined at the DNA database of the National Center for Biotechnology Information (NCBI) using the nucleotide-nucleotide BLAST algorithm (Accessed from http://www.ncbi.nlm.nih.gov/BLAST.html; November 2010).

2.8 Identification and preparation of plasmid DNA

Characterization of bacterial isolates harboring resistance plasmids was investigated using a previously described method by Kado and Liu [17]. This protocol served as a screening tool to detect small and large plasmids.

Selected isolates were cultured and streaked for single colonies on Luria-Bertani (LB) agar plates. Selected isolates that displayed ESBL activity were cultured on LB agar plates (Appendix E1) containing 2 µg/ml ceftriaxone (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) (Appendix E2). Aseptic techniques were implemented. Inoculated LB agar plates were incubated overnight at + 37 °C for the following day. Single colonies were inoculated in Nunc 50 ml graduated centrifuge tubes (Thermo Fisher Scientific) (one tube per a bacterial isolate) containing 10 ml of LB broth (Appendix E3). Selected isolates that displayed ESBL activity

were inoculated in 10 ml LB broth containing 20 µl of 2 µg/ml ceftriaxone (Sigma). The inoculated 50 ml test tubes containing the LB broth with ceftriaxone were capped and incubated overnight at + 37 °C for the following day. Un-inoculated LB agar plates and LB broth were included as negative controls. This was essential to exclude contamination.

Approximately 1.5 ml of the cell culture was transferred from the 50 ml test tube to a clean 1.5 ml Eppendorf tube (Eppendorf) (one tube per a bacterial isolate). The 1.5 ml Eppendorf tubes (Eppendorf) containing the cell cultures were capped and centrifuged in a table-top centrifuge (Eppendorf) at 12 000 rpm for five minutes. The tubes were uncapped and the supernatant was removed from each tube. As a result the bacterial pellet was left behind in the tube. These tubes were kept on ice.

Each bacterial pellet was resuspended in 20 µl of cold autoclaved 1X E-Buffer (Appendix E4-E5). One-hundred microlitres of lysis solution (Appendix E6) was added to the 1.5 ml Eppendorf tube (Eppendorf) containing the resuspended bacterial pellet. The suspensions were mixed by inverting the capped tubes ten times. The 1.5 ml Eppendorf tubes (Eppendorf) containing the lyzed suspensions were incubated for 60 minutes at + 55 °C on a dry heating block (Barloworld Scientific Ltd).

The tubes were taken off the dry heating block and 100 µl of phenol : chloroform : isoamyl alcohol (Appendix E7) was added to each tube. The suspensions were mixed by inverting the capped tubes ten times. The tubes were centrifuged in a table-top centrifuge (Eppendorf) at 12 000 rpm for five minutes to release and separate the bacterial plasmid DNA in solution. The tubes were uncapped and 100 µl of the aqueous phase of the supernatant was transferred into a clean 1.5 ml Eppendorf tube (Eppendorf) (one tube per a bacterial isolate). The 1.5 ml Eppendorf tubes (Eppendorf) containing the plasmid DNA was placed on ice for 20 minutes. Plasmid DNA was stored away for further use in the freezer at - 10 °C to - 20 °C. To screen for resistance plasmids, extracted plasmid DNA was resolved by PFGE.

Approximately 40 µl of extracted plasmid DNA of each selected isolate was loaded together with 10 µl of loading buffer on a 1 % SeaKem Gold® agarose (Lonza) gel (w/v) (Appendix A18). This is followed by electrophoresis using a CHEF-DR III apparatus (Bio-Rad) containing 3 l of 0.5X TBE (Merck) running buffer (Appendix A17) cooled to a temperature of + 14 °C. The gel was prepared as previously described. The electrophoresis running conditions was comprised of a one-block program at 200 V. The pulse times were set from an initial switch time of 5 to a final switch time of 15 seconds with a run time of 16 hours at a gradient voltage of 6 V/cm. Sizes were compared alongside a supercoiled molecular size standard, BAC-TrackerTM (EPICENTRE® Biotechnologies, Madison, Wisconsin). The first, middle and last well of the gel each contained 15 µl of BAC-TrackerTM (EPICENTRE® Biotechnologies).

Upon completion of the electrophoresis run, the gel was stained (Appendix A20), de-stained and the image captured on the gel documentation system (BioRad) using the Quantity One program software (Bio-Rad) as previously described in section 2.3.

2.9 Southern hybridization analysis

Southern blot hybridization studies were performed on plasmid DNA separated by PFGE to determine whether ESBL resistance genes are located on plasmids. The sizes of plasmid DNA were analyzed using the Quantity One program software (Bio-Rad) and estimated by comparison alongside a supercoiled molecular size standard, BAC-TrackerTM (EPICENTRE® Biotechnologies). The protocol was carried out according to the Roche manufacturer's guidelines, which are readily available (Accessed from https://www.roche-applied-science.com/sis/lad/index.jsp; November 2010). Digoxigenin (DIG)-labeled probes and unlabelled controls were first generated with the PCR DIG Probe Synthesis Kit (Roche) (Appendix F1-F2). A 50 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.) for each hybridization target gene (DIG-Labelled) and unlabelled control. Compared to the unlabelled control PCR reaction, the DIG-labelled PCR reaction contained the PCR DIG mix. A standard-3 PCR assay consisting of 30 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad) (Appendix F3). To visualize the synthesis of the

DIG-labelled probes and unlabelled controls, 5 µl of the PCR product was loaded with 1 µl of loading buffer on a 1.5 % Seakem LE agarose gel (Lonza) (Appendix C). Sizes were compared alongside either with HyperLadder™ I molecular weight marker (Bioline) or Marker III (Roche). The gel was prepared and resolved as previously described in section 2.6.

An "In-house" Southern blot apparatus required for the capillary transfer of plasmid DNA to the nylon membrane was set up as shown in Figure 10. Following the completion of the PFGE analysis, a Southern blot was prepared. Before the Southern blot could be prepared, the separated DNA on the gel had to be denatured to single-stranded DNA. The hybridization target gene was less than 5 kb. Therefore, it was not necessary to depurinate the gel. The gel was first submerged in denaturation solution (Appendix F4) in a plastic tray. The plastic tray was oscillated on a see-saw rocker (Barloworld Scientific Ltd) for 15 minutes at room temperature. The denaturation solution was discarded and fresh denaturation solution was added. This was done twice. The gel was rinsed with distilled water. The gel was then submerged in neutralization solution (Appendix F5). The plastic tray was oscillated on a see-saw rocker (Barloworld Scientific Ltd) for 15 minutes. The neutralization solution was discarded and fresh neutralization solution was added. This was done twice. The gel was transferred to another plastic tray consisting of 20X Sodium chloride, Sodium citrate buffer (SSC) (Roche) (Appendix F6) and incubated for ten minutes at room temperature. This allowed for the gel to adjust to the external environment. The Southern blot was set up as follows. Firstly, a bridge was created in a plastic tray in order for the gel to be appropriately positioned. The plastic tray was filled to a shallow level (~ 300 ml) with 20X SSC buffer. A piece of pre-soaked (20X SSC buffer) Whatman® 3 MM paper (Merck) was placed over the bridge resting in 20X SSC buffer. The air bubbles were removed by gently rolling a 5 ml pipette across the bridge. The denatured gel was placed over the pre-soaked Whatman® 3 MM paper. A piece of positively charged nylon membrane (Roche) (cut to size) that has been pre-soaked in 2X SSC buffer (Appendix F7) was placed over the denatured gel.

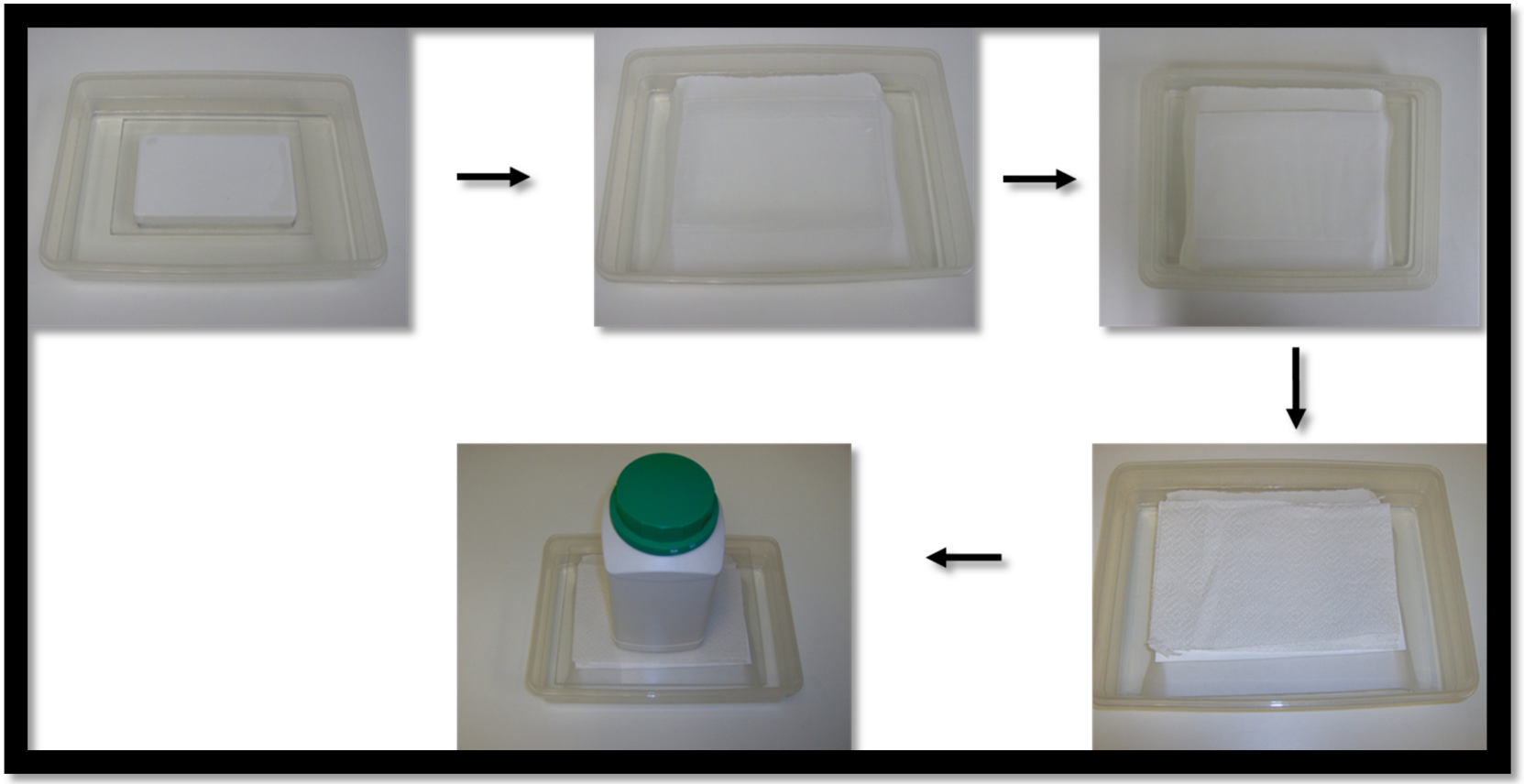

Figure 10 An “In-house” Southern blot apparatus.

The air bubbles were removed by gently rolling a 5 ml pipette across the positively charged nylon membrane. Dry Whatman® 3 MM paper (Merck) (cut to size) was placed on top of the positively charged nylon membrane. This was followed by a stack of paper towels. A Glass plate was placed on top of the stack of paper towels, followed by a weight piece of 500 g in mass. The Southern blot transfer was incubated overnight at room temperature for the following day. The next step involved fixing the denatured DNA to the positively charged nylon membrane.

The damp membrane was briefly rinsed with 2X SSC buffer and was baked in a drying oven incubator (Labex) for 120 minutes at + 80 °C. The dried membrane was taken out of the drying oven. The volume of hybridization buffer DIG Easy Hyb (Roche), was determined according to the surface area (centimetre squared (cm^2)) of the membrane. The surface area of the membrane was 294 cm^2. The membrane was placed in a borosilicate glass tube with sealing end cap (hybridization tube) (Techne, Cambridge, United Kingdom). Approximately 30 ml of DIG Easy Hyb (Roche) was added to the hybridization tube and was capped. The capped hybridization tube was placed in a rotating drive ring of the Techne Hybridiser HB-1D (Techne).

The capped hybridization tube was incubated at + 42 °C for 30 minutes. This pre-hybridization step allowed for the membrane to adapt to the external environment. In this protocol, the optimal hybridization temperature of + 42 °C was recommended on the basis that the hybrid type generated was DNA : DNA (DIG-probe : target gene). The hybridization tube was taken out of the Techne Hybridiser, the tube was uncapped and the excess hybridization buffer poured out. The next step involved the hybridization of the DIG-labelled probe to the target DNA.

The DIG-labelled probe was diluted in DIG Easy Hyb (Roche) to a final working concentration of 2 µl/ml. A Southern blot was performed for the hybridization target gene. The DIG-labelled probe was first denatured. Approximately 21 µl of DIG-labelled probe was transferred to a clean 1.5 ml Eppendorf tube (Eppendorf) along with 50 µl of distilled water. The tube was capped and incubated for five minutes at + 95 °C on a dry heating block (Labex). The capped tube was taken off the dry heating block and immediately place on ice. The denatured DIG-labelled probe was immediately transferred to a 14 ml polypropylene test tube (Lasec) containing 10.50 ml DIG

Easy Hyb (Roche). Once again, the volume of DIG Easy Hyb (Roche) was determined according to the surface area (cm^2) of the membrane. This suspension was immediately added to the hybridization tube containing the pre-hybridized membrane. The hybridization tube was capped and placed in a rotating drive ring of the Techne Hybridiser (Techne). The capped hybridization tube was incubated overnight at + 42 °C for the following day. The hybridization tube was taken out of the Techne Hybridiser, the tube was uncapped and the excess DIG-labelled hybridization buffer poured out. The next step involved stringent washing of the membrane.

The membrane was placed in a plastic tray along with 200 ml low stringency buffer (Appendix F9). The plastic tray was oscillated on a see-saw rocker (Merck) for five minutes at room temperature. The low stringency buffer was discarded and fresh low stringency buffer was added. This was done twice. Two-hundred mililitres of pre-heated high stringency buffer (Appendix F10) was added to the plastic tray. The plastic tray was incubated for 15 minutes at + 65 °C in the shaking water bath (Labotec) at 70 rpm. The high stringency buffer was discarded and fresh high stringency buffer was added. This was done twice. The A chromogenic method with anti-dioxigenin-alkaline phosphatase (AP) antibody (Roche) was used for the detection of the DIG-probe : target gene hybrid on the membrane.

Advantages of using this method include: chromogenic signals can be visualized without the use of exposure film and two or more colour substrates can be used in combination to detect more than one target. The AP colour substrate used in this protocol was a combination of nitro blue tetrazolium chloride (NBT) with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). As a result, a blue precipitate is formed and can be seen with the naked eye.

The membrane was first washed and blocked using the DIG Wash and Block Buffer Set (DNase and RNase-free) (Roche). The DIG Wash and Block Buffer Set stock solutions were diluted to produce a 1:10 final working solution. The membrane was transferred to a plastic tray containing 100 ml of 1X washing buffer (Roche). The plastic tray was oscillated on a see-saw rocker (Merck) for two minutes at room temperature. The 1X washing buffer was discarded and 100 ml of 1X blocking solution (Roche) was added. The plastic tray was oscillated on a see-saw rocker

(Merck) for 30 minutes at room temperature. The 1X blocking solution was discarded. The antibody solution was prepared.

The tube containing the anti-dioxigenin-AP antibody (Roche) was centrifuged in a table-top centrifuge (Merck) at 10 000 rpm for five minutes. Approximately 4 µl of the anti-dioxigenin-AP antibody (Roche) was added to a 50 ml test tube (Lasec) containing 16 ml of 1X blocking solution (Roche). The 20 ml of antibody solution was transferred to the plastic tray containing the blocked membrane. The plastic tray was oscillated on a see-saw rocker (Merck) for 30 minutes at room temperature. The antibody solution was discarded and 100 ml of fresh 1X washing buffer (Roche) was added. The plastic tray was oscillated on a see-saw rocker (Merck) for 15 minutes at room temperature. The 1X washing buffer was discarded. This step was done twice. Approximately 20 ml of 1X detection buffer (Roche) was added. The plastic tray was oscillated on a see-saw rocker (Merck) for three minutes at room temperature. The 1X detection buffer was discarded. This allowed for the membrane to adjust to the external environment.

The NBT/BCIP colour substrate solution was prepared as follows. Fifty microlitres of NBT (Roche) along with 37.5 µl of BCIP (Roche) was added to a clean 1.5 ml Eppendorf tube (Eppendorf) containing 112.5 µl of distilled water. The 200 µl NBT/BCIP solution was transferred to a 14 ml polypropylene test tube (Lasec) containing 10 ml of fresh 1X detection buffer (Roche). This colour substrate solution was poured over the membrane. The plastic tray was incubated overnight in a dark cupboard at room temperature for the following day. The colour reaction was stopped by adding 50 ml TE buffer to the membrane. The membrane was air-dried for further analysis.

2.10 References

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Chapter Three

Molecular characterization of *Vibrio cholerae* O1 associated with an outbreak of cholera in the Mpumalanga Province, South Africa, May to July 2008

This chapter has been published in 2011 in the Journal of Clinical Microbiology (volume 49, pages 2976-2979). The first author was responsible for the molecular characterization of the *V. cholerae* O1 outbreak isolates and preparation of the manuscript for publication. The second author was responsible for the supervision and oversight of methods necessary to complete this chapter. The third author was responsible for the serological characterization and the antimicrobial susceptibility testing of the *V. cholerae* O1 outbreak isolates. The last author was responsible for supervision and guidance on the content of this chapter.

3.1 Introduction

During May to July 2008, an outbreak of cholera associated with illegal miners on a gold mine in the Ehlanzeni district of the Mpumalanga Province, South Africa was identified [1,2]. Thirty-four laboratory-confirmed cases of cholera, including five deaths, were reported related to this outbreak [1,2]. Eighteen cases were confirmed from illegal miners (~ 53 %), six cases from close contacts (~ 18 %) and ten cases from other sources (~ 29 %) [1-3]. This study proposed to investigate the molecular epidemiology and mechanism of antimicrobial resistance of toxigenic *V. cholerae* O1 isolates using various molecular techniques.

3.2 Materials and Methods

3.2.1 Bacterial isolates and phenotypic characterization

Thirty-one isolates (~ 91 %) out of the 34 laboratory-confirmed clinical cases were received by the CED [3]. *V. cholerae* pathogens were isolated from both stool (~ 58 %) and rectal swab (~ 42 %) specimens[3]. Confirmatory identification was done using standard microbiological

techniques (section 2.1). MIC testing for each isolate was performed as per the CLSI 2008 guidelines (section 2.2).

3.2.1.1 Involvement of efflux pumps

To determine the role of an active efflux pump as a possible mechanism of quinolone resistance, susceptibility to nalidixic acid was investigated using agar dilution MIC testing [4] in the presence and absence of two efflux pump inhibitors (EPIs), reserpine and phenylalanyl arginine-β-naphthylamide (PAβN) (Sigma). Reserpine and PAβN were tested independently of each other and added to molten Mueller-Hinton agar (Appendix G1) at a final concentration of 20 µg/ml for reserpine [5] (Appendix G2) and 40 µg/ml for PaβN [6] (Appendix G3).

Antimicrobial-free control plates were included. A doubling dilution series of increased concentration for nalidixic acid (Sigma) starting from 0.015 µg/ml to 512 µg/ml was prepared [7] (Appendix G4). Twenty millilitres of molten Mueller-Hinton containing both the diluted nalidixic acid and either EPI were mixed by swirling the container and poured in 90 mm petri dishes. Mueller-Hinton agar plates were allowed to set at room temperature. Bacterial inoculum suspensions (one tube per bacterial isolate) were prepared as described in section 2.2. This initial inoculum (McFarland 0.50 or $1x10^8$ CFU/ml) was then diluted ten-fold. One-hundred microlitres of the initial inoculum was transferred to a 1.5 ml Eppendorf tube containing 900 µl of sterile 0.9 % saline. Four-hundred microlitres of the diluted inoculum suspensions were transferred to multi-point inoculator wells (Figure 11). The inoculum replicating apparatus was then placed on the specimen plate of a multipointelite™ inoculator (Mast Assure, Mast Group Ltd.) (Figure 11). Mueller-Hinton agar plates were inoculated starting from the control plate followed by the lowest concentration (0.015 µg/ml) to the highest concentration (512 µg/ml). Replicator pins transferred about 10 µl of the inoculum to the Mueller-Hinton agar plate (Figure 11) containing both nalidixic acid and EPI. Plates were air dried at room temperature to allow for the inoculum spots to dry. Plates were incubated overnight at + 37 °C for 18 hours. The MIC corresponded to the lowest concentration of nalidixic acid without visible bacterial growth.

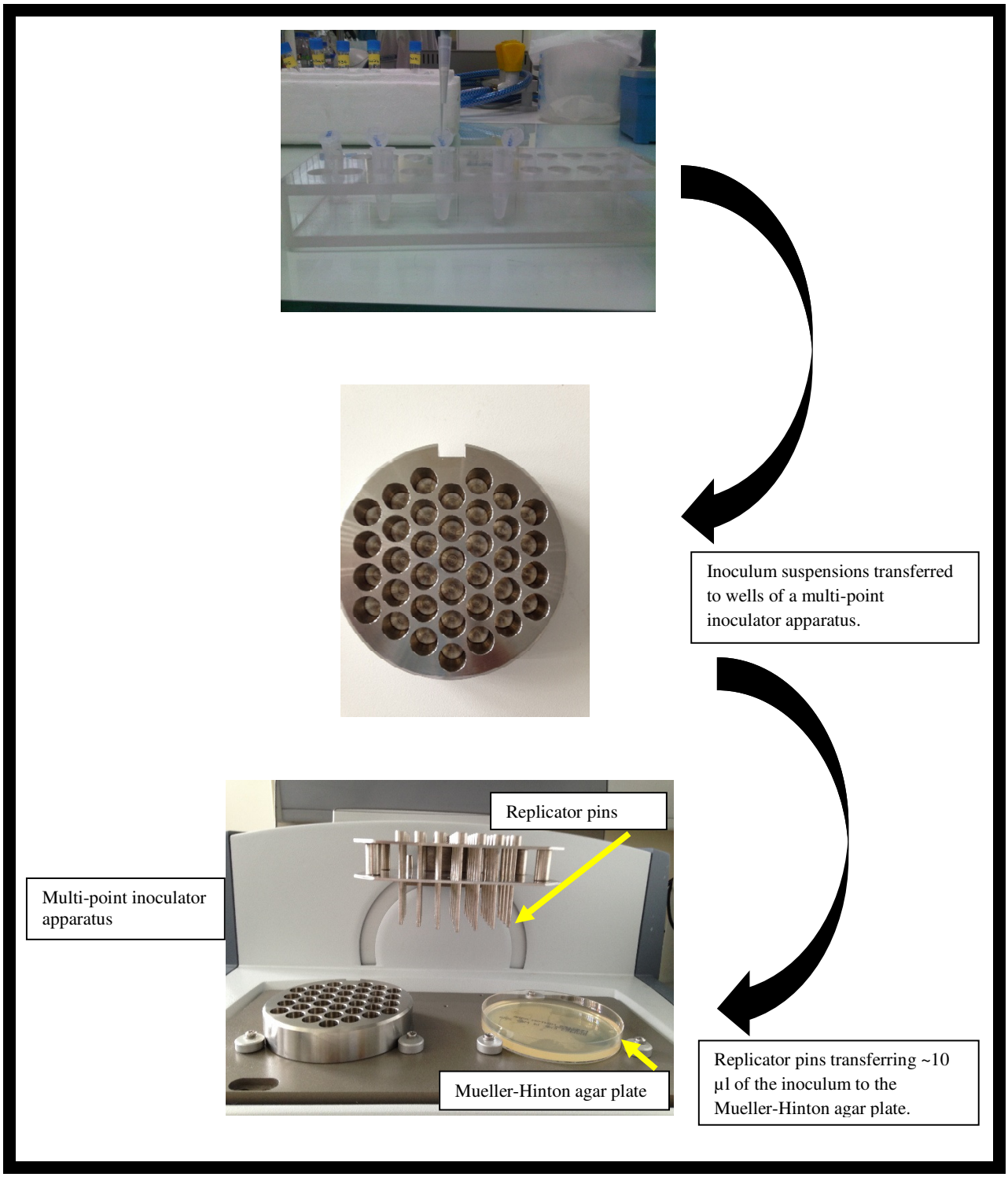

Figure 11 Agar dilution susceptibility testing.

3.2.2 Genotypic characterization

3.2.2.1 Pulsed-field gel electrophorsis (PFGE) analysis

All 31 isolates were characterized by PFGE analysis using the PulseNet standardized protocol for *V. cholerae* (section 2.3).

3.2.2.2 Detection for virulence determinants

Crude DNA extracts were prepared (section 2.4), and these served astemplate DNA in the PCR assays. Conventional PCR was performed on all 31 isolates for the detection of both the *ctxA* and *tcpA* genes respectively (sections 2.5.1 and 2.5.2).

3.2.2.3 Detection for antimicrobial resistance determinants

Ten out of the 31 isolates were selected for further analysis. Due to time and financial constraints, selected isolates were chosen to be representative of different antimicrobial susceptibility profiles and chosen to be representative of different PFGE patterns. Conventional PCR was performed on all ten isolates for the detection of particular antimicrobial resistance determinants (sections 2.5.3 to 2.5.7).

The detection and size of the PCR products were determined using conventional agarose gel electrophoresis (section 2.6).

PCR-positive amplicons (*bla*$_{TEM}$, *gyrA*, *gyrB*, *parC* and *parE*) were purified (section 2.7) and served as template DNA in the cycle sequencing PCR assays (section 2.8). DNASTAR Lasergene® software was used to analyze the nucleotide sequences and sequence identity was determined at the DNA database of the NCBI (section 2.7). Amino acid sequences obtained for GyrA, GyrB, ParC and ParE were compared with a quinolone susceptible isolate of *V. cholerae* O1, N16961 (GenBank accession no. NC_002505).

3.2.2.4 Isolation of plasmid DNA and Southern blot hybridization analysis

Intact plasmid DNA was prepared, as previously described by Kado and Liu (section 2.8). Cell lysates (40 µl) with plasmid DNA were resolved by PFGE analysis (section 2.8). For Southern blot hybridization studies, a DIG-labeled probe *bla*$_{TEM}$ was generated (section 2.9). Southern blot hybridizations on plasmid DNA separated by PFGE analysis was performed to determine the location and size of the plasmid encoding for *bla*$_{TEM}$.

3.3 Results

3.3.1 Bacterial isolates and antimicrobial susceptibility patterns

All 31 isolates were characterized as *V. cholerae* O1 serotype Ogawa. All 31 isolates were shown to have the same antimicrobial susceptibility profile. All were susceptible to ciprofloxacin, erythromycin and imipenem, but resistant to ampicillin, augmentin, trimethoprim, sulfamethoxazole, co-trimoxazole, chloramphenicol, nalidixic acid, tetracycline, and streptomycin. All 31 isolates displayed ESBL activity with further resistance to the third-generation cephalosporin, ceftazidime (MIC 64 µg/ml).

3.3.2 Genes encoding for virulence determinants

PCR analysis revealed that 30 out of 31 (~ 97 %) isolates were positive for the CT and all isolates were positive for the El Tor biotype.

3.3.3 PFGE analysis

All 31 isolates showed a very similar PFGE *Not*I-profile and were determined to be highly clonal at a 95% pattern similarity value on dendrogram analysis.

3.3.4 Genes conferring antimicrobial resistance

PCR analysis of class 1 integrons and class 2 integrons showed that all ten isolates were PCR-negative for the 3'CS, 5'CS and *intI2* respectively. Amplification of the SXT element showed that all ten isolates were PCR-positive for the integrase gene, SXT*int* and associated SXT resistance genes encoding for chloramphenicol (*floR*), sulfamethoxazole (*sul2*), trimethoprim (*dfrA1*) and streptomycin (*strA* and *strB*). All ten isolates were PCR-positive for the *tetA* gene, which confers resistance to tetracycline.

3.3.5 ESBL activity and identified ESBL genes

All ten isolates were PCR-positive for bla_{TEM} but were PCR-negative for bla_{SHV} and $bla_{\text{CTX-M}}$. PCR and nucleotide sequence analysis showed that all ten isolates produced TEM-63 β-lactamase coinciding with a ceftazidime MIC 64 μg/ml. In addition, all ten isolates showed the presence of a single plasmid of ~ 140 kb in size (Figure 12). Southern blotting and DNA probing analysis demonstrated that bla_{TEM} encoding for the TEM-63 β-lactamase, was located on a plasmid (~140 kb) in all ten isolates (Figure 12).

3.3.6 Chromosomal mutations in the QRDR of DNA gyrase and topoisomerase IV

PCR analysis of PMQR genes and the quinolone resistance determinant showed that all ten selected isolates were PCR-negative for *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qepA* and *qnrVC3* respectively. All ten isolates harboured mutations in GyrA and ParC but no changes were observed in GyrB and ParE. In GyrA, an amino acid substitution of serine by isoleucine was detected at codon 83 (S83-I) (Figure 113) and in ParC, an amino acid substitution of serine by leucine was detected at codon 85 (S85-L) (Figure 14).

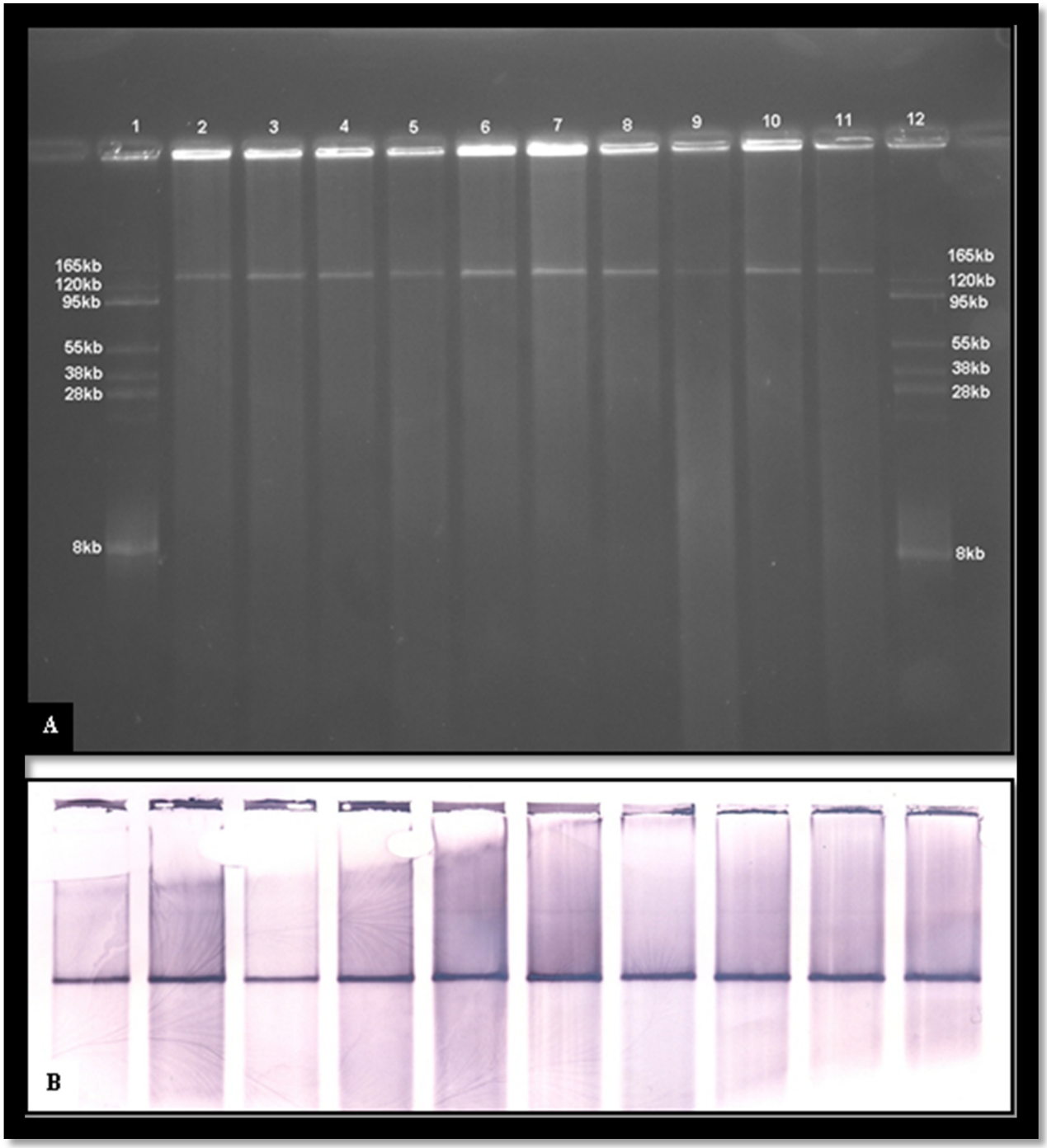

Figure 12 (A) Plasmid DNA isolation from the ten selected *V. cholerae* O1 isolates. (B) Southern blot hybridization of plasmid DNA probed with DIG-bla_{TEM} from ten selected *V. cholerae* O1 isolates.

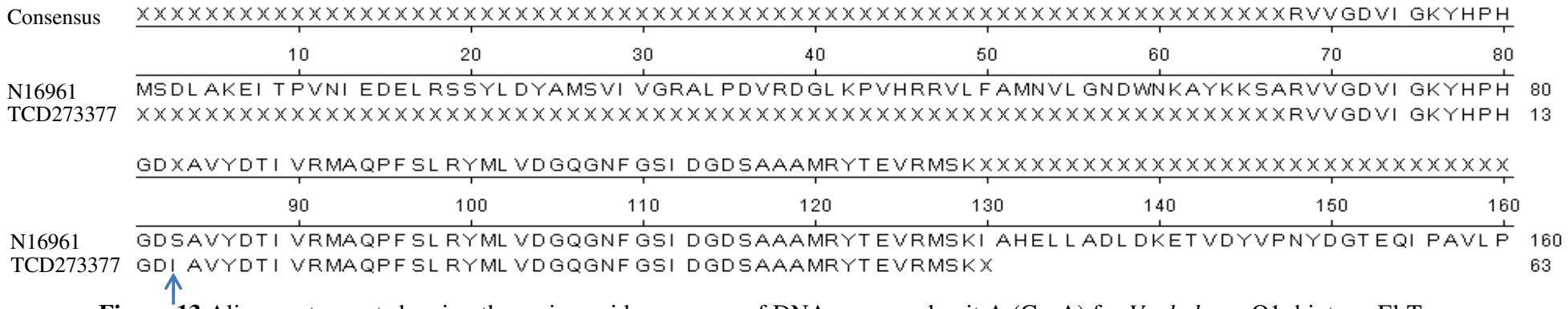

Figure 13 Alignment report showing the amino acid sequences of DNA gyrase subunit A (GyrA) for *V. cholerae* O1, biotype El Tor, strain

Abbreviations: Alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), valine (V), tryptophan (W) tyrosine (Y) and the blue arrow indicates the position of amino acid substitution. N16961 accession number NC_002505 and isolate, TCD273377.

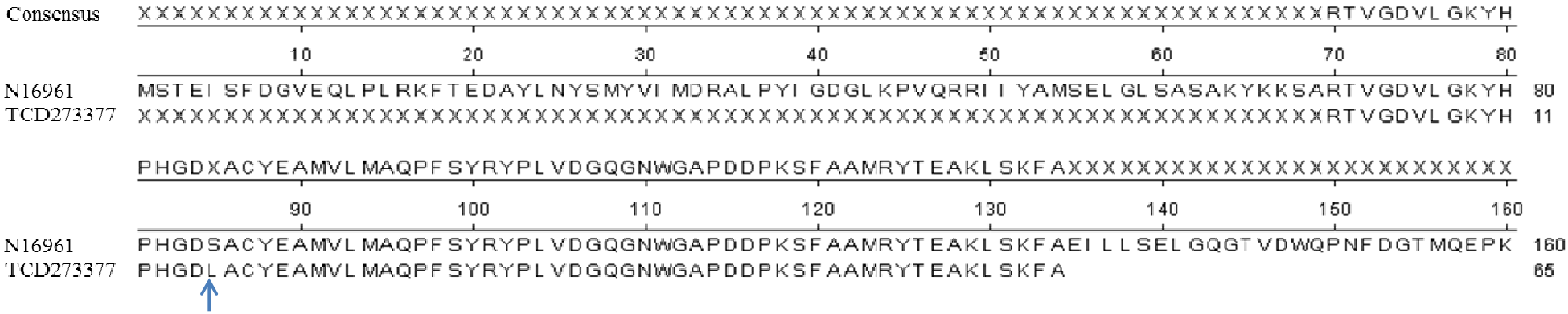

Figure 14 Alignment report showing the amino acid sequences of DNA topoisomerase IV subunit A (ParC) for *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and isolate, TCD273377.

Abbreviations: Alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), valine (V), tryptophan (W) tyrosine (Y) and the blue arrow indicates the position of amino acid substitution.

3.4 Discussion

In this study, all 31 isolates were characterized as *V. cholerae* O1 serotype Ogawa, a serotype, which is generally associated with outbreaks of cholera through Africa [8,9]. Molecular analysis revealed that all 31 isolates were TCP-positive for the El Tor variant and 30 out of the 31 isolates characterized were positive for the CT. This study showed the presence of a TCP-positive, CT-negative *V. cholerae* O1 isolate. Various distributions of virulence determinants (including TCP-positive, CT-negative) have been previously described in *V. cholerae* O1 isolates [10]. Possible explanations for the low prevalence of TCP-positive, CT-negative *V. cholerae* O1 isolates could be (i) decreased enrichment of these isolates in the intestine of the host or (ii) these isolates are converted quickly into TCP-positive, CT-positive *V. cholerae* O1 isolates by the filamentous bacteriophage, CTXΦ either in the intestine of the host or in the environment [10]. It has been suggested that TCP-positive, CT-negative *V. cholerae* O1 isolates do not cause extensive cholera due to the reduced interaction of these isolates in the intestinal environment of the host [10]. In this study, *V. cholerae* O139 Bengal isolates were not identified. Prior to 2010, *V. cholerae* O139 Bengal has particularly not been reported in Africa [11]. A joint study done between Mozambique and South Africa showed the presence of *V. cholerae* O139 Bengal isolated from coastal water in Beira, Mozambique [11].

All 31 isolates displayed the same antimicrobial susceptibility profile and were resistant to multiple classes of antimicrobial agents screened for surveillance purposes. All 31 isolates described in this study were susceptible to ciprofloxacin and imipenem. Widespread resistance to antimicrobial agents namely nalidixic acid, ciprofloxacin, co-trimoxazole, chloramphenicol, tetracycline, cephalexin and ampicillin have been described in *V. cholerae*but have largely remained susceptible to third-generation cephalosporins [12,13]. The use of third-generation cephalosporins is not yet recommended for the treatment of cholera [13] however, a report from Mozambique has suggested the use of the cephalosporins as an option for treatment [8]. Treatment with fluoroquinolones has until recently been the antimicrobial of choice in the management of severe cholera and other diarrhoeal illnesses [9,13,14]. Antimicrobial resistance to ciprofloxacin has been reported in *V. cholerae* O1 in southern Asia [14-16]. In parts of Africa

namely southern and western Africa, reduced susceptibility to ciprofloxacin in *V. cholerae* O1 has also been described [17,18]. To date, reported data or literature to support reduced susceptibility or resistance to imipenem in *V. cholerae* O1 has not been described.

Results obtained in this study have revealed the first incidence of TEM-63 β-lactamase producing, multidrug-resistant toxigenic *V. cholerae* O1 isolates recovered in South Africa. There has been no previous report of TEM-63 β-lactamase producing isolates of *V. cholerae* O1 worldwide. Plasmid-mediated ESBLs have been globally reported and are widespread among members of the family *Enterobacteriaceae* [19,20]. A study done by Petroni *et al*. characterized *V. cholerae* O1 outbreak isolatesrecovered in Argentina where plasmid-mediated CTX-M type and PER-2 type β-lactamases hydrolyzing cefotaxime, ceftazidime and cefepime were detected using isoelectric focusing and PCR-RFLP analysis [19]. More recently, a study done by Mandal *et al*. characterized an AmpC β-lactamase and carbapenemase producing *V. cholerae* O1 pathogen isolated from a paediatric patient in India [21]. Another study done by Walsh *et al.* described New Delhi metallo-β-lactamase (NDM-1) producing *V. cholerae* isolates recovered from seepage water and tap water swab specimens in New Delhi [22]. TEM-63 β-lactamase has previously been described in *Enterobacteriaceae* from South Africa [20,23]. The isolation of TEM-63 β-lactamase producing isolates of *Klebsiella pneumoniae* between 1994 and 1996 was first described from a teaching hospital in Durban, South Africa in 2001 by Essack *et al.*[23]. In this study all ten selected isolates harboured a single ~ 140 kb plasmid. This finding correlates with plasmid profiling analysis done by Essack *et al*, whereby *Klebsiella pneumonia* isolates contained plasmids of a similar size encoding for TEM-63 β-lactamase[23].

PCR analysis for the detection of the class A tetracycline resistance determinant (*tetA*) yielded an amplicon of ~ 950 bp in all ten selected isolates.The presence of the *tetA* gene may possibly confer tetracycline resistance. Tetracycline has been used extensively in Africa, until resistance became common due to the presence of incompatibility group C plasmids[9].Tetracycline resistance determinants are generally found on transposons and as a result are able to move around for instance, by inserting into conjugative plasmids [24]. Tetracycline resistance encoded

by the *tetA* gene in African and Laotian strains of *V. cholerae* O1 was previously reported by Dalsgaard *et al*. in 2001 and Iwanaga *et al*. in 2004 [25,26].

Class 1 and class 2 integrons were not present in all ten selected isolates.This may suggest the involvement of other mechanisms of antimicrobial resistance. Studies have shown that *V. cholerae* O1 isolates harbouring class 1 integrons isolated in Asian countries before 1996 may have spread to Africa [27], as epidemic *V. cholerae* isolates from Africa contain class 1 integrons [9,27]. Since 2000, several studies done in Africa have described class 1 and class 2 integrons responsible for antimicrobial resistance in *V. cholerae* O1 [9,25,28-30]. Gene cassettes conferring antimicrobial resistance to nearly every important class of antimicrobial agent including quinolones have been described [31-33]. The spread of resistance genes is the result of lateral gene transfer (LGT), also known as horizontal gene transfer [31,32].

In this study, antimicrobial resistance to chloramphenicol, sulfamethoxazole, trimethoprim and streptomycin can be explained by the acquisition of the SXT element. PCR analysis for the detection of the SXT integrase gene yielded an amplicon of ~ 592 bp in all ten selected isolates. To determine the contribution of the SXT element to antimicrobial resistance patterns of each isolate, the presence of *floR, sul2, dfrA1, dfr18, strA* and *strB* were investigated respectively. These are typical clustered resistance genes, which are capable of differentiating among SXT variants [30]. All ten selected isolates were PCR-positive for five of the six SXT associated resistance genes with the exception of the *dfr18* gene, as *dfrA1* is characteristic of SXT^{ET} and not SXT^{MO10} [34,35]. Findings from a previous study have shown that R391, an ICE isolated from a clinical strain of *Providencia rettgeri* in South Africa, is functionally and genetically related to SXT^{MO10} and more than 25 related ICEs within the SXT/R391 family [36]. Variant types of the SXT element have previously been described in other *Vibrio* species such as *V. vulnificus*, *V. metschnikovii*, *V. fluvialis* and *V. parahaemolyticus* [34].

The PMQR genes were not detected in all ten selected isolates. To date, five major *qnr* determinants and two additional PMQR genes namely *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS, qepA* and *aac(6')-Ib-cr* have been described [37]. Qnr proteins belong to the pentapeptide repeat family

and are responsible for protecting the DNA gyrase and topoisomerase IV from quinolones [37]. The AAC(6')-Ib-cr protein is responsible for acetylation of ciprofloxacin, while the QepA is a novel efflux pump and is responsible for transporting unwanted compounds [37]. In *V. cholerae*, a *qnr* homolog, *qnrVC1* has been described for isolates recovered in Brazil [33]. This determinant was shown to be located on the superintegron and has not been shown to confer transferable quinolone resistance [33]. A study done by Kim *et al.* showed that *qnrVC3*, which was shown to be 100 % identical to *qnrVC1* in amino acid composition was harboured on the SXT element of clinical *V. cholerae* O1 isolates recovered from patients seen at the Dhaka Hospital in Bangladesh [33,38]. Isolates containing the *qnrVC3* gene were shown to display reduced susceptibility to ciprofloxacin [33]. To date, reported data or literature to support PMQR in *V. cholerae* O1 in Africa have not been described.

This study found that, there was no involvement of an active efflux pump in quinolone resistance, as there was no difference in MIC values obtained following two-fold serial agar dilution testing in the presence and absence of reserpine or PaβN. In addition this study, the proposed mechanism for quinolone resistance to nalidixic acid is the accumulation of two chromosomal mutations detected in GyrA (S83-I) and ParC (S83-L) as PMQR genes were not detected. The findings of these two mutations correlate with previous studies done by Kim *et al.* in 2010 and Baranwal *et al.* in 2002 [16,33]. With regard to quinolone resistance, multiple mutations are usually needed to be clinically significant, as wild-type pathogens are highly susceptible to these antimicrobial agents [37]. It has been reported that the single amino acid mutation in GyrA exhibits reduced susceptibility to ciprofloxacin to a MIC 0.5 µg/ml [6] a finding also shown in this study. In the study done by Baranwal *et al.* in India in 2002, the authors demonstrated that amino acid substitutions within the QRDR (GyrA and ParC) in combination with an active efflux pump may be responsible for elevated MIC levels for quinolones in clinical *V. cholerae* isolates [16].

3.5 Conclusion

The results presented in this study underscore the rapidity at which antimicrobial resistance amongst enteric pathogenic bacteria, *V. cholerae* O1 in particular is developing within South Africa. The isolation of epidemic-prone *V. cholerae* O1 isolates which are resistant to third generation cephalosporins is of public health concern in South Africa and globally. The mechanism of such resistance is plasmid-borne and given the effortless nature in which plasmids can be transferred from one bacterial species to the next, there exist concerns of the transfer of an ESBL-based resistance mechanism to bacteria associated with other community-acquired infections. The available treatment options for the management of such cases will be complicated in an era where new antimicrobial agents are a rare find and advocacy for antimicrobial stewardship is slow to find its feet in developing countries such as South Africa. This is a problem that an already resource constrained health system can do without.

3.6 References

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Chapter Four

Molecular characterization of *Vibrio cholerae* O1 isolates associated with a country wide outbreak of cholera in South Africa, 2008 to 2009

This chapter was published in 2013 in the Journal of Infectious Diseases (volume 208, supplement 1, pages S39-45). The first author was responsible for the molecular characterization of the *V. cholerae* O1 outbreak isolates and preparation of the manuscript for publication. The second author was responsible for the supervision and oversight of methods necessary to complete this chapter. The third author was responsible for molecular characterization of the *V. cholerae* O1 outbreak isolates, which included PFGE analysis and PCR-analysis of the virulence determinants. The fourth author was responsible for the serological characterization and the antimicrobial susceptibility testing of the *V. cholerae* O1 outbreak isolates. The last author was responsible for supervision and guidance on the content of this chapter.

4.1 Introduction

During mid-November 2008 to April 2009, South Africa experienced an outbreak of cholera following the importation of cases (individuals seeking health care in South Africa) associated with an outbreak in Zimbabwe [1-3]. This outbreak was initially identified in Musina of the Limpopo Province, South Africa. All nine Provinces were affected [3]. A total of 12 706 clinical cases meeting the clinical (suspect) case definition were reported by the Department of Health [3]. Of the total number of cases, 1 114 cases (~ 9 %) were laboratory-confirmed and 65 deaths were recorded with a CFR of 0.5 % [3]. The majority of the laboratory-confirmed cases isolated were recovered from both the Mpumalanga Province (~ 33.8 %) and the Limpopo Province (~ 53.5 %) respectively [3]. Local transmission and infection could have been a result of contaminated water supplies, poor sanitation, infrastructure and poor access to potable water sources [3]. Within the same cholera outbreak, a new outbreak was identified in the North West Province [4]. For the period 1 November 2008 to 22 March 2009, the majority of cholera cases (76 out of 90 cholera cases) related to this consecutive outbreak were linked to farms in the Brits

area with no formal sanitation [4]. Source of infection could have been water, which may have been supplied by boreholes [4]. This study proposed to investigate the molecular epidemiology, mechanism of antimicrobial resistance and to characterize seventh pandemic toxigenic *V. cholerae* O1 isolates using various molecular techniques.

4.2 Materials and Methods

4.2.1 Bacterial isolates and phenotypic characterization

For the period, November 2008 to April 2009, a total of 720 isolates, sub-cultured on Dorset's Egg Medium (DMP) were sent for analysis at the CED [5]. *V. cholerae* isolates received were recovered from four environmental (~ 0.6 %), 598 stool (~ 83.0 %) and 118 swab (~ 16.4 %) specimens [5]. Confirmatory identification was done using standard microbiological techniques (section 2.1). MIC testing methods for each isolate were performed as per the CLSI 2008 guidelines (section 2.2).

4.2.2 Genotypic characterization

4.2.2.1 Pulsed-field gel electrophorsis (PFGE) analysis

From the 720 Isolates, 248 (~ 34.4 %) isolates (clinical and environmental) were characterized by PFGE analysis using the PulseNet standardized protocol for *V. cholerae* (section 2.3). Due to time and financial constraints, isolates chosen for PFGE analysis were selected based on antimicrobial susceptibility profiles.

4.2.2.2 Detection for virulence determinants

A random number generator was used to select 90 isolates for further analysis; a criterion for selection was to ensure that all PFGE banding patterns were represented. Isolates chosen were selected based on DNA fingerprint patterns. Crude DNA extracts were prepared (section 2.4),

and these served as template DNA in the PCR assays. Conventional PCR was performed for the detection of both the *ctxA* and *tcpA* genes respectively (sections 2.5.1 and 2.5.2).

4.2.2.3 Detection for antimicrobial resistance determinants

Conventional PCR was performed on all 90 isolates for the detection of particular antimicrobial resistance determinants (sections 2.5.3 to 2.5.7). PCR-positive amplicons (*bla*$_{TEM}$, *gyrA*, *gyrB*, *parC* and *parE*) were purified (section 2.7) and served as template DNA in the cycle sequencing PCR assays (section 2.8). DNASTAR Lasergene® software was used to analyze the nucleotide sequences and sequence identity was determined at the DNA database of the NCBI (section 2.7). Amino acid sequences obtained for GyrA, GyrB, ParC and ParE were compared with a quinolone susceptible isolate of *V. cholerae* O1, N16961 (GenBank accession no. NC_002505).

4.2.2.4 Isolation of plasmid DNA and Southern blot hybridization analysis

Intact plasmid DNA was prepared, as previously described by Kado and Liu (section 2.8). Cell lysates (40 µl) with plasmid DNA were resolved by PFGE analysis (section 2.8). For Southern blot hybridization studies, a DIG-labeled probe *bla*$_{TEM}$ was generated (section 2.9). Southern blot hybridizations on plasmid DNA separated by PFGE analysis was performed to determine the location and size of the plasmid encoding for *bla*$_{TEM}$.

4.2.2.5 Molecular characterization of *Vibrio cholerae* seventh pandemic variants

Four southern African isolates recovered from the two cholera outbreaks in South Africa over the years 2008 to 2009 (Chapter Three [6] and Chapter Four [5]) were further characterized. Extended analysis included PCR amplification and nucleotide sequencing (sections 2.6 and 2.7) of the complete coding regions for both the CT gene, *ctxAB* and TCP gene, *tcpA* as previously described [7] as well as tracking of *V. cholerae* seventh pandemic variants by characterizing highly related genomic sequences as previously described by Spagnoletti *et al*. [8].

4.2.2.5.1 Cholera toxin

A single PCR assay was implemented to detect for the presence of the entire coding region of the CT (Appendix H1). A PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.). A step-down PCR assay consisting of 40 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). The expected size of the *ctxAB* gene is ~ 1350 bp in length. Nucleotide sequences obtained for *ctxAB* were compared with *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and *V. cholerae* O1, biotype classical, strain 569B accession number VCU25679.

4.2.2.5.2 Toxin co-regulated pilus

A single PCR assay was implemented to detect for the presence of the entire coding region of the *tcpA* gene (Appendix H2). A PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.). A standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad).The expected size of the *tcpA* gene is ~ 1 234 bp in length.Nucleotide sequences obtained for *tcpA* were compared with *V. cholerae* O1, biotype El Tor, strain CIRS101accession number ACVW01000010.

4.2.2.5.3 Mobilome

Two multiplex PCR assays aimed at characterizing the mobilome, which consists of genomic islands (GIs), prophages and integrative conjugative elements (ICEs) of *V. cholerae* O1 and O139, were performed (Appendix H3). PCR reactions were prepared in 200 µl thin-walled, flat-capped PCR tubes (Axygen Inc.). Standard-3 PCR assays consisting of 35 amplification cycles were set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad).Expected sizes for the PCR amplicons for both multiplex PCR assays are shown below.

Target genes for multiplex PCR 1	Expected size (~ bp)	Target genes for multiplex PCR 2	Expected size (~ bp)
VSP-II integrase	170	TLC phage	449
Prototypical VSP-II	245	Kappa phage	230
SXT/R391 ICEs integrase	505	GI 12	571
SXT Hotspot IV	357	GI 14	142
ICE*Vch*Ind5 Hotspot IV	423	GI 15	348
ICE*Vch*Moz10 Hotspot IV	712		

4.3 Results

4.3.1 Bacterial isolates

All 720 isolates were confirmed as *V. cholerae* O1. Of the 720 isolates, 708 (~ 98.3%) isolates were characterized as serotype Ogawa, while the remaining 12 isolates (~ 1.7%) were characterized as serotype Inaba. *V. cholerae* O139 isolates were not identified.

4.3.2 Antimicrobial susceptibility patterns

Antimicrobial susceptibility testing was not performed on the four environmental isolates as it was not included in the standard operating procedure implemented in the CED, thus were not included in the study. At the beginning of the cholera outbreak, the antimicrobial susceptibility patterns of the clinical isolates were shown to be identical. However, a few months into the cholera outbreak (January 2009), antimicrobial susceptibility patterns were shown to be altered. All 716 isolates were shown to be susceptible to ciprofloxacin and imipenem. All 716 isolates were shown to be resistant to co-trimoxazole and nalidixic acid. Of the 716 isolates, 298 isolates (~ 41.6 %) were resistant to chloramphenicol and 176 isolates (~ 24.6 %) were resistant to erythromycin. Sixteen isolates (~ 2.2 %) demonstrated resistance to ampicillin and tetracycline.

Six isolates (~ 1.0 %) displayed ESBL activity and showed to be resistant to third-generation cephalosporin, ceftriaxone (MIC ≥ 16 μg/ml).

4.3.3 PFGE analysis

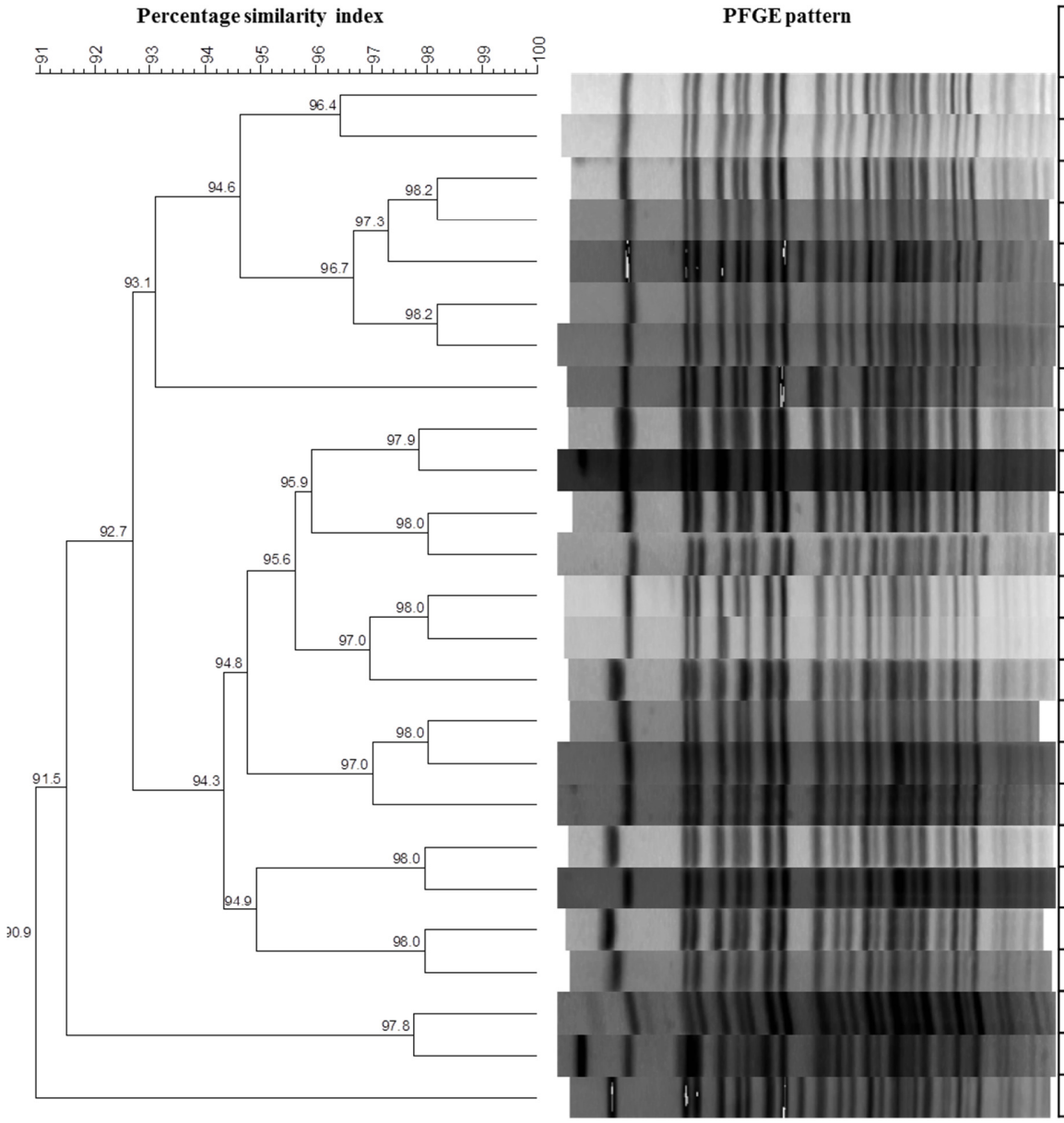
Analysis of the 248 isolates showed 25 DNA fingerprint patterns (Figure 15). Pattern 12 was the most commonly identified pattern in 64 (~ 25.8 %) out of the 248 isolates. The PFGE banding patterns only showed subtle differences with all fingerprint patterns showing similarity at ≥ 90.9 %, which suggested that all isolates were very closely related.

4.3.4 Genes conferring antimicrobial resistance

PCR analysis of class I and class 2 integrons showed that all 90 isolates were PCR-negative for both the 3'-CS and 5'-CS conserved segments as well as the *intI2* gene respectively. Of the 90 isolates analysed, seven isolates (~ 7.8 %) were PCR-positive for the *tetA* gene. All 90 isolates were PCR-positive for the SXT element-integrase and five associated SXT resistance geneswith the exception of the *dfr18* gene.

4.3.5 ESBL activity and identified ESBL genes

Six (~ 6.7 %) out of the 90 isolates that displayed ESBL activity against third generation cephalosporins were further characterized using PCR amplification to detect for the presence of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ respectively. All six isolates were PCR-positive for the bla_{TEM} gene and nucleotide sequencing was shown to encode for the TEM-63β-lactamase. Analysis for plasmid DNA showed that all six isolates harboured a 140 kb plasmid. Southern blot hybridization and DNA probing showed that this bla_{TEM} gene was located on this single plasmid.



Pattern number	Number of isolates showing pattern	Source of isolation
1	2	Human
2	1	Human
3	15	Human
4	7	Human
5	2	Human
6	11	Human
7	1	Human
8	1	Human
9	19	Human and Water
10	1	Human
11	6	Human
12 *	64	Human and Water
13	50	Human
14	1	Human
15	1	Human
16	21	Human
17	8	Human
18	6	Human
19	6	Human
20	2	Human
21	1	Human
22	17	Human
23	1	Human
24	1	Human
25	2	Human

Figure 15 Dendrogram of PFGE fingerprint patterns of *V. cholerae* O1 isolates (*Not*I-digestion) associated with the cholera outbreak in South Africa, November 2008 to April 2009. "*" indicates the most commonly identified pattern.

4.3.6 Chromosomal mutations in the QRDR of DNA gyrase and topoisomerase IV

All 90 isolates were PCR-negative to all five *qnr* genes as well as the quinolone resistance determinant (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qepA* and *qnrVC3*). Nucleotide sequencing results showed that all 90 isolates harboured two amino acid mutations detected in GyrA (S83-I) and ParC (S85-L). Amino acid substitutions were not observed in GyrB and ParE for all 90 isolates.

4.3.7 Molecular characterization of *Vibrio cholerae* seventh pandemic variants

All 90 isolates were virulent as they all possessed the *ctxA* gene encoding for the CT and all were PCR-positive for the *tcpA*-El Tor gene encoding for the TCP.

The complete coding region of the *ctxAB* gene (~ 1 350 bp) was amplified from the four selected *V. cholerae* O1 isolates recovered from the two separate cholera outbreaks in South Africa over the years 2008 to 2009 (section 4.2.2.5). Amino acid sequences of ctxA for all four isolates were shown to be identical to ctxA of *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and *V. cholerae* O1, biotype classical, strain 569B accession number VCU25679. Figure 16 is an alignment report of the ctxB amino acid sequence. As shown, *V. cholerae* O1, biotype El Tor, strain N16961 is differentiated from *V. cholerae* O1, biotype classical, strain 569B by two amino acid substitutions. The first amino acid substitution is located at codon 39. *V. cholerae* O1, biotype El Tor, strain N16961 contains a tyrosine (Y), while *V. cholerae* O1, biotype classical, strain 569B contains a histidine (H). The second amino acid substitution is located at codon 68. *V. cholerae* O1, biotype El Tor, strain N16961 contains an isoleucine (I), while *V. cholerae* O1, biotype classical, strain 569B contains a threonine (T). As shown, all four selected isolates matched the amino acid sequence of ctxB gene of *V. cholerae* O1, biotype classical, strain 569B.

Nucleotide sequences of the *tcpA* gene for the four selected isolates were shown to be identical *V. cholerae* O1, biotype El Tor, strain CIRS101 (*tcpET*^{CIRS}).

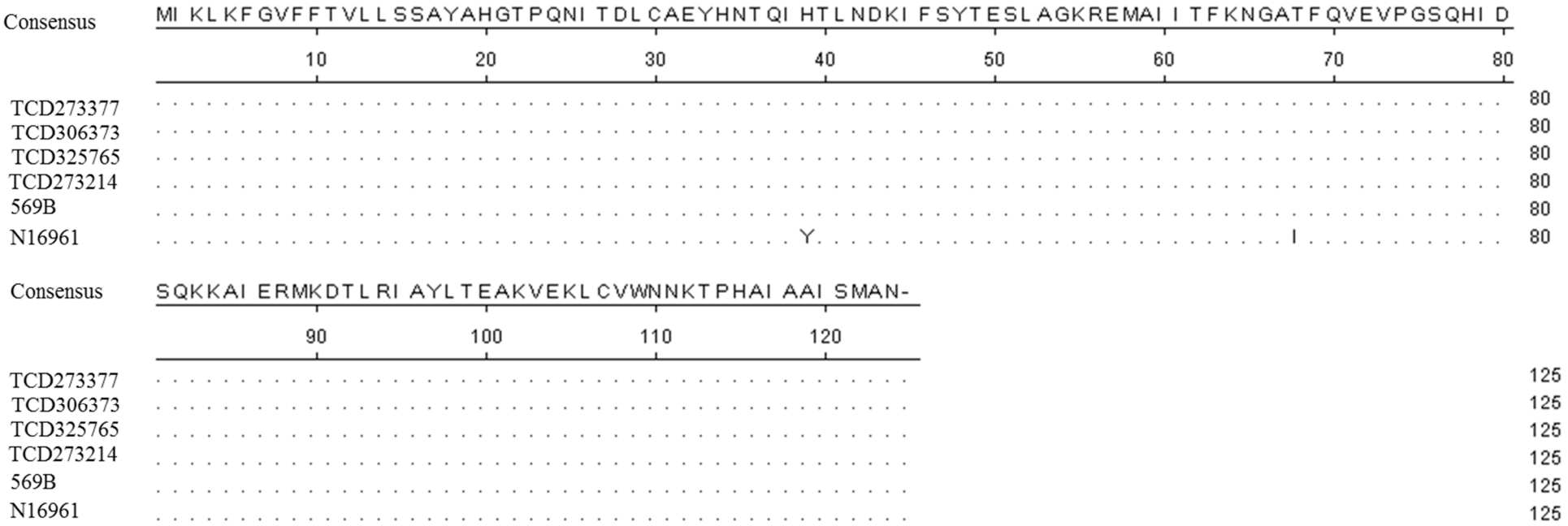


Figure 16 Alignment report showing the amino acid sequence of ctxB of four selected *V. cholerae* O1, serotype Ogawa, biotype El Tor isolates, TCD306373, TCD325765, TCD273214 and TCD273377; *V. cholerae* O1, biotype El Tor, strain N16961 accession number NC_002505 and *V. cholerae* O1, biotype classical, strain 569B accession number VCU25679. Amino acid residues that match the consensus sequence displayed on top are hidden. Amino acid residues that do not match the consensus sequence displayed on top are shown.

Abbreviations: Alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), valine (V), tryptophan (W) and tyrosine (Y).

The mobilome of isolates was characterized by combining the PCR results for the two multiplex PCR reactions. An identical mobilome profile pattern was determined for the selected isolates. Multiplex PCR 1 amplified amplicons for the VSP-II integrase (~ 170 bp), ICE*Vch*Ind5 Hotspot IV (~ 423 bp) and the SXT integrase(~ 505 bp). Multiplex PCR 2 amplified the amplicon for prophage TLC (~ 449 bp). PCR results for both multiplex PCR assays are shown in Figure 17.

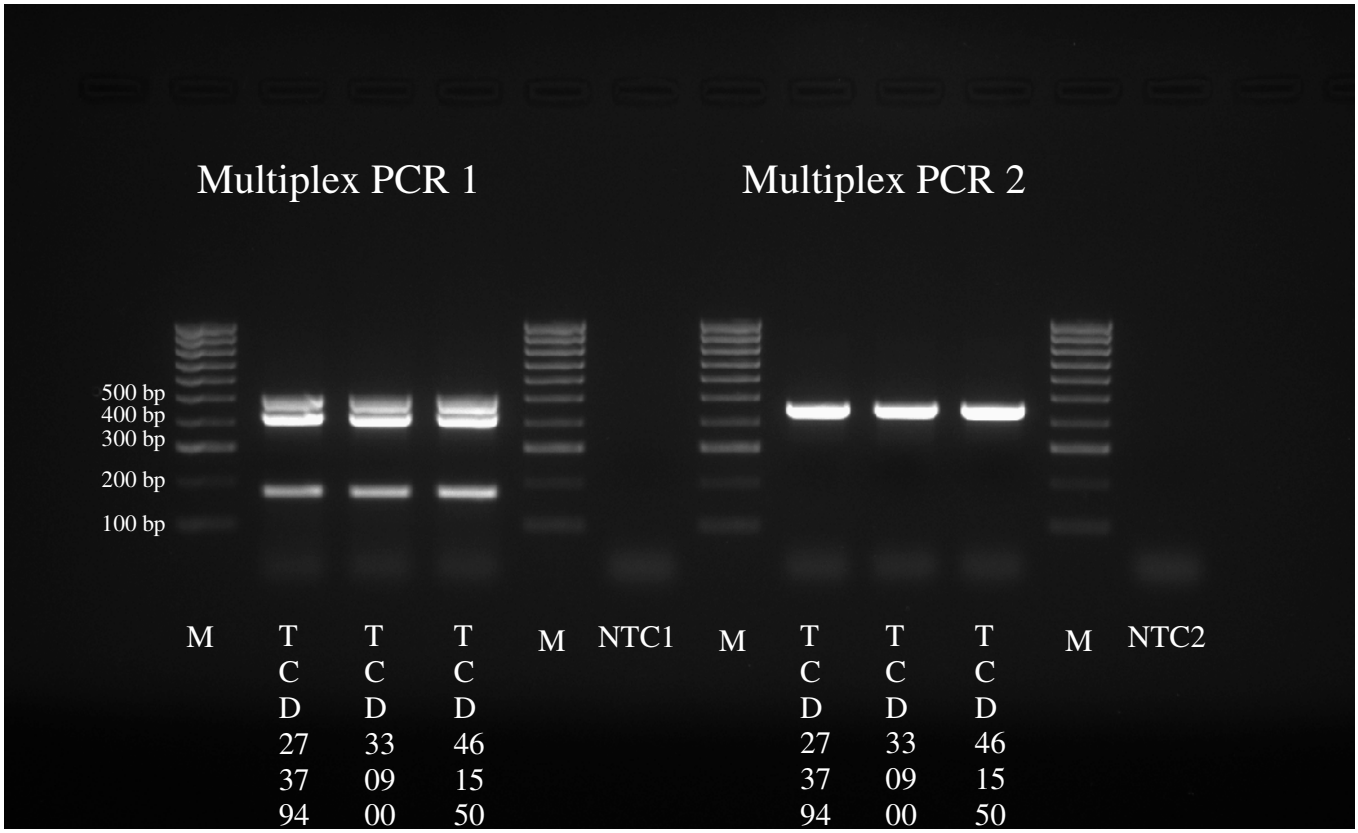


Figure 17 PCR amplification and detection of particular genetic markers in multiplex PCR 1 and multiplex PCR 2 for three *V. cholerae* O1 isolates. PCR-amplified products were separated on a 1.5 % (w/v) agarose gel.

Abbreviations: M HyperLadder™ IV (100 bp molecular weight marker), NTC no template control.

4.4 Discussion

During the cholera epidemic described in South Africa, a small percentage of isolates characterized were serotyped as Inaba. Serotype conversion is due to a mutation in the *rfbT* gene, which is responsible for switching [9,10]. *V. cholerae* O1 classical variants of the TCP were not identified in this study. This finding is supported by previous studies that have showed that *V. cholerae* O1 classical biotype isolates are not recovered in Africa [11].

The initial resistance patterns of isolates analyzed in this study were identical, but a few months into the outbreak (January 2009) it was altered to include reduced susceptibility to chloramphenicol, tetracycline andmacrolides. During antimicrobial treatment all bacteria in the human body are exposed to selective pressure of the antimicrobial agent [12]. The gastrointestinal tract is highly exposed particularly during rehydration therapy [12]. As a result, naturally resistant bacteria harbouring a genetic pool of antimicrobial-resistant determinants have the potential to transfer these genes to other bacteria within the human gastrointestinal tract [12]. In this study, each isolate was resistant to at least three classes on antimicrobial agents namely sulfonamides, quinolones and aminoglycosides. As previously discussed in section 3.4, *V. cholerae* O1 has established different mechanisms to combat the effects of antimicrobial agents.

PFGE analysis was shown to be an effective molecular tool to characterize *V. cholerae* O1 isolates recovered from the outbreak, as it was able to distinguish the PFGE banding patterns evaluated in this study from other patterns in the database of southern African isolates (Results not shown). PFGE analysis identified 25 *Not*I restriction patterns. Isolates displayed minor diversity in their PFGE patterns showing > 90 % similarity value. The predominant PFGE pattern identified, pattern 12 was shown to be related to isolates with the KZGS12.0088/KZGN11.0092 pattern and its close variant, KZGS12.0089/KZGN11.0092 from Afghanistan, Cameroon, India, Nepal, Oman, Pakistan and Haiti [7]. The KZGS12.0088/KZGN11.0092 pattern is recognized as a relatively new PFGE pattern as it was first describedin thePulseNet USAdatabase in 2005 in isolates from travellers returning from India [7].

Although all 90 isolates in this study were PCR-positive for the El Tor variant of the TCP, *ctxB* genotyping of the four selected isolates showed that all four isolates expressed the encoded *ctxB* allele for the CT of the classical biotype. To support this finding, *V. cholerae* O1 isolates from this 2008 to 2009 South African outbreak were further analyzed in a study done by Talkington *et al*. to investigate the similarity to the Haiti *V. cholerae* O1 outbreak isolates using PFGE. They showed that South African *V. cholerae* O1 isolates carried the classical *ctxB-1* allele and the *rstR*^{El} [7]. These characteristics define the isolates as "altered El Tor" [7,13]. Although, *V. cholerae* O1 isolates from South Africa and Haiti share the same PFGE restriction pattern, isolates from Haiti were shown to carry the *ctxB-7* allele, which was first documented in *V. cholerae* O1 isolates recovered from a cholera outbreak in Orissa, India in 2008 [7]. To confirm the similarity in the PFGE fingerprint patterns between the South African and Haiti *V. cholerae* O1 isolates, whole genome sequencing was performed [14]. Comparative analysis showed that the Haiti *V. cholerae* O1 isolates clustered away from the South African isolates [14]. *V. cholerae* O1 biotype El Tor variants expressing a classical CT were first described in 2002 by Nair *et al*. among clinical isolates collected for the years 1991 to 1994 [15]. Several *ctxB* alleles have been have been described among *V. cholerae* isolates of different biotypes and serogroups [13]. These alleles differ by amino acid substitutions within the *ctxB* gene [13]. In Africa, *V. cholerae* O1, biotype El Tor variants expressing a classical CT have been described in Nigeria, Cameroon, Mozambique and Zambia [16-18]. Isolates displayed typical characteristics of the El Tor biotype based on phenotypic assays but carried the CTX$^{class}\Phi$ [16-18].

In order to better understand the genetic organization and track seventh pandemic *V. cholerae* O1, biotype El Tor isolates, southern African isolates, each recovered from the two separate cholera outbreaks in South Africa over the years 2008 to 2009 were further characterized. Findings from Figure 17 formed a small part of the study. It was used as a snapshot to determine if there were differences in the mobilome patterns of the isolates from the two reported outbreaks. An identical mobilome profile pattern was determined for the selected isolates. Combining the PCR results for the isolates in this study showed a profile B. Profile B is one of twelve profiles that have been described, profile A to profile L [8]. Profile B belongs to the

reference strain CIRS101 (*ctxB-1/tcpET*^{CIRS}) as reported by Spagnoletti *et al*. *V. cholerae* O1, biotype El Tor, strain CIRS101 was first isolated in Dhaka, Bangladesh in 2002 [8].

4.5 Conclusion

V. cholerae O1 El Tor variants are well adapted pathogens that have the ability to spread both efficiently and expeditiously. The use of mobilome characterization data enabled us to characterize and uncover the source of the southern African isolates. Global networks such as PulseNet, standardized protocols and innovative epidemiological tools for characterizing pathogenic isolates, are invaluablefor the understanding of how enteric pathogens are transmitted globally. Global travel has decreased the duration under which outbreaks spread and increased the likelihood that a potential pathogen may cross a border. Strengthening global resources such as PulseNet, developing innovative epidemiological tools and adhering to standardized protocols are key as they can all be used a means identifying and managing potential public health concerns such as outbreaks of cholera.

4.6 References

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Chapter Five

Azithromycin susceptibility in recent antimicrobial-resistant *Vibrio cholerae* O1 altered El Tor variant isolates in South Africa.

5.1 Introduction

Protein biosynthesis is an essential process in all living cells [1]. The ribosome, which consists of two subunits is the universal cellular organelle responsible for this process [1]. The bacterial ribosome consists of a small 30S subunit and a large 50S subunit [1,2]. The small 30S subunit consists of a 16S ribosomal ribonucleic acid (rRNA) chain of ~ 1 500 bp together with 20 to 21 ribosomal proteins [1,2]. The large 50S subunit is composed of two RNA chains of ~ 3 000 bp, namely the 23S rRNA and 5S rRNA together with 31 to 35 ribosomal proteins [1,2]. The small 30S subunit provides the decoding centre, which is responsible for the selection of the aminoacyl-transfer RNAs (tRNAs) based on the order of codons in the messenger RNA (mRNA) [1,2]. The amino acids are assembled into a polypeptide chain in the catalytic site known as the peptidyl transferase center (PTC), which is located in the large 50S subunit [1,2]. The newly assembled polypeptide chains exit the ribosome by means of the nascent peptide exit tunnel (NPET), which begins at the PTC and extends through the body of the large 50S subunit [1,2]. The NPET contains a pocket or binding site of high affinity for antimicrobial agents such as kelotides as well as the macrolides, lincosamides and streptogramins (MLS) group [1,3-5]. Although MLS are chemically distinct, they are considered together as most of these antimicrobial agents share overlapping binding sites on the large 50S subunit and many bacteria possess antimicrobial-resistant genes to more than one than antimicrobial agent in this group [4,6].

Macrolides were first introduced into medical practice during the early 1950s and are recognized as an important class of antimicrobial agents [7-9]. Macrolides are viewed as an excellent class of antimicrobial agents with high potency and low toxicity [2,9]. They have an excellent safety and tolerability profile, which includes children and pregnant women and are generally used in

place of penicillin in β-lactam allergic individuals [9]. Macrolide antimicrobial agents are characterized based on their possession of a multi-membered lactone ring consisting of 12 to 16 carbon atoms together with one or more sugars (amino sugars, non-nitrogenous or both) attached [7,10,11]. Erythromycin was the first 14-membered macrolide to be used in medical practice and was shown to be active against Gram-positive and certain Gram-negative pathogenic bacteria such as *Campylobacter* species and *Neisseria gonorrhoeae* [2,7,9,12]. It is still used to treat respiratory tract, skin and soft tissues and genitaltract infections [7]. An important characteristic of macrolides that may contribute to their effectiveness is that they accumulate within the leukocytes and can improve the immune system [10,13]. A study done using mice demonstrated that phagocytes may be a possible mechanism to transport intracellular macrolides and release it to sites of infection [10,13]. Since the target ribosome is intracellular, macrolides must cross either one membrane barrier in Gram-positive bacteria or two membrane barriers in Gram-negative bacteria [14]. Therefore the mechanism of uptake of macrolides by the bacterium is essential [14]. Gram-negative bacilli such as *Escherichia coli* and *Salmonella* species are naturally unaffected by MLS antimicrobial agents due to the impermeability of the outer membrane [10,11,14]. However certain Gram-negative bacteria such as *Haemophilus*, *Bordetella*, *Legionella*, *Campylobacter*, *Chlamydia*, *Treponema*, *Helicobacter*, *Mycoplasma* and *Pasteurella* species are susceptible to them [10,11,14].

Macrolides act by binding to the bacterial ribosome and thereby inhibit protein synthesis [2,9-11,15,16]. This is achieved by binding of the macrolide in the upper chamber of the NPET, between the PTC and the constriction formed by the ribosomal proteins L4 and L22 [1,2,17-19]. The binding site is made up of rRNA nucleotides, which belong to domains II and V of the 23S rRNA [2,17]. The lactone ring of the antimicrobial agent interacts with rRNA residues 2057, 2058 and 2611 (residues are numbered according to *Escherichia coli* 23S rRNA and will used throughout this chapter), which are responsible for the formation of the tunnel wall on the side of the PTC A site [2,17]. Another way for macrolides to inhibit protein synthesis, is to interact with partially assembled 50S precursors causing nucleolytic degradation of unassembled precursors and as a result, stall the assembly process [20].

Azithromycin (CP-62,993; 9a-methyl-9-deoxo-9-dihydro-9a-aza-homoerythromycin A), a semi-synthetic derivative of erythromycin A is a 15-membered macrolide, which was first produced by PLIVA, a pharmaceutical company in 1980 [13]. The interest in this antimicrobial agent was sparked by the investigation on the effects of treatment with azithromycin on Gram-negative bacilli from cystic fibrosis patients [12]. Azithromycin is chemically distinct from erythromycin A by the presence of a methyl-substituted nitrogen in the lactone ring as shown in Figure 18 and was shown to have improved *in vivo* potency over erythromycin A against localized soft tissue infections as well as enhanced activity against a broader range of Gram-negative bacteria [7,9,12,13,21].

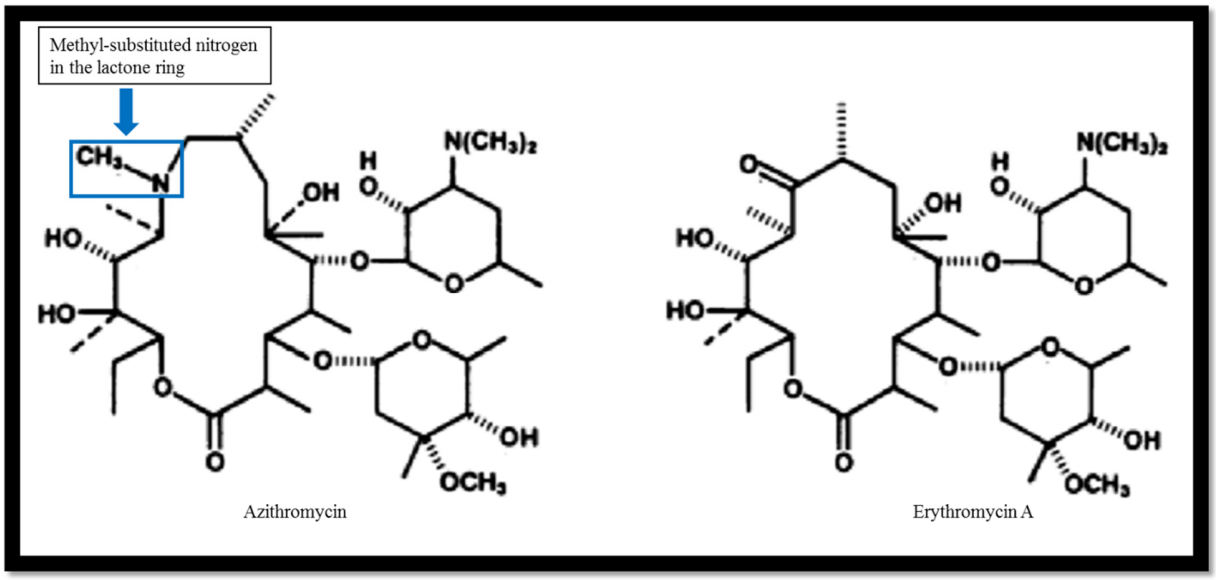

Figure 18 Chemical structures of azithromycin and erythromycin A. Picture adapted from Retsema *et al*, 1987.

Azithromycin is not generally used in the treatment of *Enterobacteriaceae* infections. However, it has been shown to be effective in the treatment of quinolone–resistant typhoid and paratyphoid fever caused by *Salmonella enterica* serotypes Typhi and Paratyphi A, B and C [21,22]. It has been recommended by the American Academy of Paediatrics for the treatment of shigellosis in children and by the WHO as a second-line treatment for adults [22]. Azithromycin has the ability

to penetrate and reach high concentrations within leukocytes, which is why it is effective against intracellular bacteria [9,13]. Though azithromycin is concentrated intracellularly, this creates difficulties in defining clinical MIC breakpoints [9]. Therefore, one cannot assume that bacterial isolates with decreased susceptibility to erythromycin *in vitro* will be resistant to azithromycin *in vivo*[9].

Although, no clinical azithromycin MIC breakpoints are available for *Enterobacteriaceae* by the Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) states that *Salmonella* and *Shigella* isolates with MICs less than or equals to (≤) 16 µg/ml for azithromycin should be considered wild-type organisms that are responsive to treatment [21]. Previous studies have reported MICs for azithromycin in *Salmonella enterica* serotype Typhi isolates ranged from 4 µg/ml to 64 µg/ml[22]. Bacterial resistance to MLSs are due to nucleotide changes in intrinsic genes or by the acquisition of antimicrobial resistance determinants carried on mobile genetic elements [12,23,24]. Mechanisms of macrolide resistance have been well characterized in clinical isolates of Gram-positive bacteria [9,11,25-27]. Macrolide resistance occurs in three ways namely, (i) enhanced efflux activity, (ii) modification of the target site by methylation or amino acid mutations that inhibit binding of the antimicrobial agent to its ribosomal target and (iii) by the inactivation of the antimicrobial agent [11,23-29]. All three resistance mechanisms to macrolides have also been reported in Gram-negative bacteria in particular, the family *Enterobacteriaceae* [12,23-25,29].

The genes involved in the regulation of efflux pump activity produce proteins, which transport the antimicrobial agent out of the bacterial cell and allow for the the ribosomes to function normally [12]. Seventeen different genes have been described, which code for either ATP transporters or Major Facilitator Transporters and the genes necessary to perform this function [4,23]. In Gram-negative bacteria, the two most commonly found efflux genes described, *mef*(A) and *msr*(D) were shown to be responsible for conferring macrolide resistance to erythromycin [12,29].

Ribosomal target modification generally occurs by the acquisition of the erythromycin ribosome methylase (*erm*) gene, which codes for an enzyme that methylates a single adenine at residue 2058 (A2058 in *Escherichia coli*), anessential macrolide target binding site in the PTC of23S rRNA[24]. Erm methylases either add one or two methyl groups to A2058 [6,12,17,24,26-28]. This modification inhibits the binding of MLS to the large 50S subunit [6,12,17,24,26-28]. There are currently 33 different *erm* genes, of which *erm*(A), (B), (C), (F), (G) and (Q) have been identified in Gram-negative bacteria [4,12,23]. In addition, *erm*(B) is the most commonly found gene amongst Gram-positive and Gram-negative bacteria [23,24]. In pathogenic bacteria, *erm* determinants are generally disseminated by conjugal transfer either by plasmids or transposons [23,24]. Ribosomal target modification may also arise from nucleotide changes in the genes encoding for the 23S rRNA domain V and the ribosomal proteins L4 and L22 [12,16,28,30,31]. Mutations in ribosomal proteins L4 and L22 affect the conformation of 23S rRNA and as a result prevent the binding of the antimicrobial agent to the ribosomal target site [12,16,28,30,31]. Nucleotide changes in the 23S rRNA genes have been reported in pathogenic Gram-negative bacteria, for example *Campylobacter* species, *Chlamydia trachomatis*, *Haemophilus influenza*, *Helicobacter pylori* and *Neisseria gonorrhoeae*[12]. Nucleotide changes in the 23S rRNA and the ribosomal proteins L4 and L22 that confer antimicrobial resistance to erythromycin have been reported in laboratory-mutants of *Escherichia coli* [12,24,31].

A total of 19 inactivation enzymes have been described [23]. These include two lyases, 11 transferases, two esterases and four phosphorylases [23]. Antimicrobial inactivation by esterases and phosphorylases confer resistance to 14-membered and 15-membered macrolides but not to lincosamides [12,24]. Erythromycin esterases encoded by the both the *ere*(A) and *ere*(B) genes, break down the lactone ring in the macrolide thus generating an inactive product [12,24,27,29]. These esterases are generally found in Gram-negative bacteria and are spread by plasmids as well as by class 1 and class 2 integrons [12]. A study done in 2002 by Thungapathra *et al*. described a *V. cholerae* isolate carrying the erythromycin esterase encoding *ere*(A2) resistance determinant on a class 1 integron [32]. Macrolide phosphorylases encoded by the *mph*(A), *mph*(B), *mph*(C) and *mph*(D) genes add a phosphate group to the macrolide antimicrobial agent hence inactivating it[12]. Macrolide phosphorylase genes *mph*(A), *mph*(B) and *mph*(D) are

generally found in Gram-negative bacteria[12,29]. The *mph*(C) gene is found in both Gram-positive and Gram-negative bacteria [12,23].

The chapter proposed to investigate the antimicrobial susceptibility to azithromycin amongst South African isolates of *V. cholerae* O1 and to investigate the presence of typical macrolide resistance determinants commonly associated with the family *Enterobacteriaceae*.

5.2 Materials and Methods

5.2.1 Bacterial isolates

One-hundred *V. cholerae* O1 El Tor variant isolates, ten selected isolates further characterized in Chapter three and 90 selected isolates further characterized in Chapter Four were included in this study.

5.2.2 Antimicrobial susceptibility testing

A comparative study using both the Etest and agar dilution methods was conducted. Based on previously published literature for wild type isolates of *Salmonella* and *Shigella* the tentative breakpoint for resistance to azithromycin of > 16 µg/ml was applied to these *V. cholerae* O1 isolates. For quality control purposes, *Staphylococcus aureus* ATCC29213 was used. The target MIC range for the control isolate on Mueller-Hinton agar was between 0.5 µg/ml - 2 µg/ml.

5.2.2.1 Episolimeter test (Etest) method

MICs for azithromycin were determined using the Etest® for all 100 isolates as previously described in section 2.2. The antimicrobial concentration range for azithromycin ETest® strip was between 0.016 µg/ml – 256 µg/ml.

5.2.2.2 Doubling agar dilution method

Susceptibility to azithromycin was investigated using agar dilution MIC testing as described in section 3.2.1.1. A doubling dilution series of increased concentration was prepared for azithromycin (Sigma) (Appendix I1). An antimicrobial-free control plate was included. The antimicrobial concentration for the agar dilution ranged between 0.015 µg/ml and 16 µg/ml.

5.2.3 Genotypic characterisation

5.2.3.1 Detection for macrolide resistance determinants

Single PCR assays were implemented to detect for the presence of seven macrolide resistance determinants, *mefA*, *ereA*, *ereB*, *ermB*, *mphA*, *mphB* and *mphD* respectively (Appendix I2) [29]. A 25 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.). For each PCR assay, a standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad). Positive control *Streptococcus pneumoniae* # 15 (PCR-positive for *mefA* and *ermB*) was kindly provided, courtesy of the Dr. Nicole Wolter from the Centre for Respiratory Diseases and Meningitis. Expected sizes for the PCR amplicons are shown below. For *ereA*, *ereB*, *mphA*, *mphB* and *mphD,* artificial chimeric DNA constructs to serve as positive control DNA as described in section 5.2.3.2 were constructed. The detection and size of the PCR products were determined using conventional agarose gel electrophoresis (section 2.6).

Target gene	**Expected size for wild-type isolates (~ bp)**
mefA	324
ereA	420
ereB	303
ermB	639
mphA	403
mphB	889

mphD 436

5.2.3.2 Internal control DNA constructs

Artificial chimeric DNA constructs were created and these served as internal positive controls for the detectionof *ereA*, *ereB*, *mphA*, *mphB* and *mphD* respectively. The method for creating these constructs was adapted from Hoorfar *et al*. [33]. This method involved two rounds of PCR.

The purpose of the first PCR was to create a chimeric DNA construct, which consisted of a non-relevant template DNA of known size flanked by the target for macrolide specific PCR-primers. Figure 19 illustrates how an internal positive control for *mphA* was created. In this study the *eae* gene, which encodes for an outer membrane protein called intimin for Enteropathogenic *Escherichia coli*, ATCC43887 was used. This gene is ~ 482 bp in size and served as the backbone for designing chimeric primers. Both forward and reverse primers used in the initial PCR contained nucleotide sequences for both the *eae* gene and the macrolide resistance determinant, *mphA*.

A 50 µl PCR reaction was prepared in a 200 µl thin-walled, flat-capped PCR tube (Axygen Inc.). For each PCR assay, a standard-3 PCR assay consisting of 35 amplification cycles was set up using a Bio-Rad (iCycler) thermal cycler (Bio-Rad).The detection and size of the PCR products were determined using conventional agarose gel electrophoresis and the resultant 45 µl PCR-positive product was purified as per the manufacturer's guidelines using the MSB® Spin PCRapace clean-up kit (Invitek, Berlin, Germany) (section 2.7).

Figure 20 shows the PCR amplification and detection of both the *eae* gene (~ 482 bp) and *mphA-eae* chimeric DNA construct (~ 522 bp). The purpose of the second round of PCR was amplify this *mphA-eae* chimeric DNA construct using the initial macrolide specific PCR-primers for *mphA* as described in section 5.3.2.1. Expected sizes for the PCR amplicons are shown below.

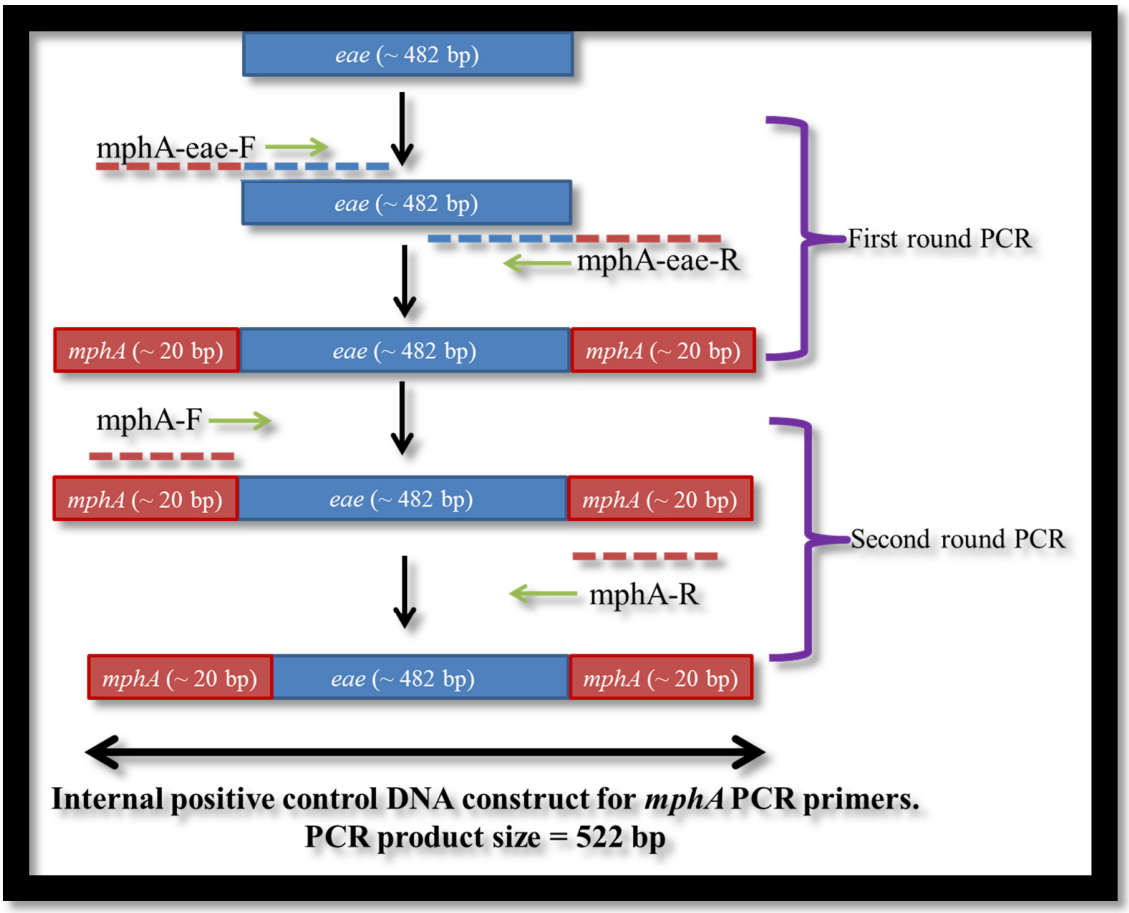

Figure 19 Schematic overview of creating internal positive control DNA for the detection of the macrolide resistance determinant, *mphA*.

Target gene	**Expected size for positive control DNA constructs (~ bp)**
ereA-eae	524
ereB-eae	519
mphA-eae	522
mphB-eae	524
mphD-eae	523

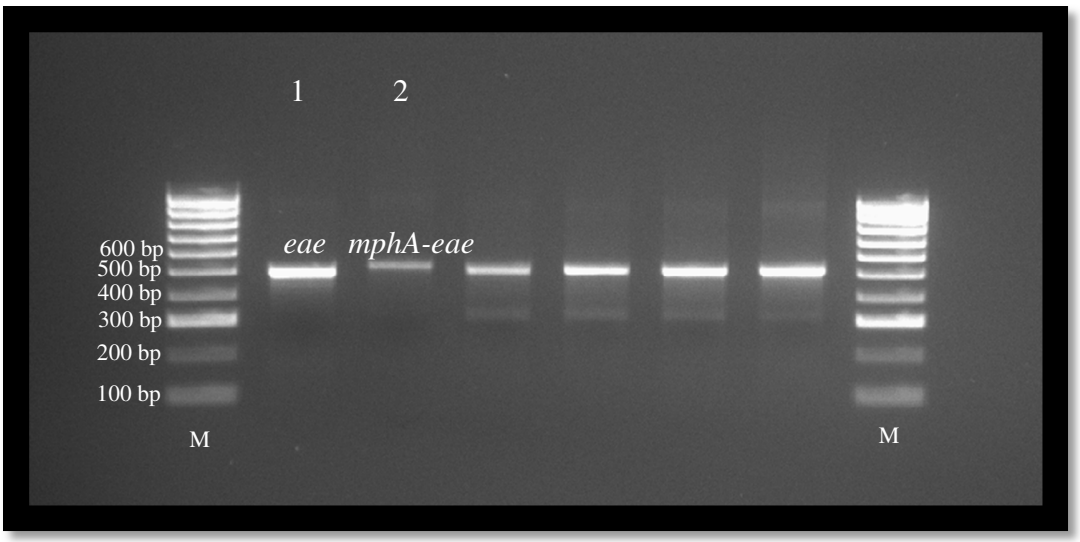

Figure 20 PCR amplification and detection of the *eae* (~ 482 bp) gene in lane 1 and the internal positive control, *mphA-eae* (~ 522 bp) in lane 2. PCR-amplified products were separated on a 1.5 % (w/v) agarose gel. LaneM, HyperLadder™ IV (100 bp molecular weight marker).

5.2.4 Statistical analysis

The modes and ranges of both the Etest and agar dilution method MIC results were calculated from the results of the azithromycin testing. The two methods used in this study were not qualified as "gold standards". A "gold standard" is recognized as a test, which determines absolutely and without error whether an isolate is resistant (sensitivity) or susceptible (specificity) [34]. For consistency of comparison, the upper MIC limit of 16 µg/ml was the greatest value for the MIC range of the agar dilution. To analyze the degree of agreement between the Etest and the agar dilution method results for two independent samples, the Mann Whitney test was performed. Measurement of the association between the tests was provided by performing a Spearman correlation coefficient for the agar dilution method and the Etest from the results of the azithromycin testing.

5.3 Results

5.3.1 Azithromycin susceptibility testing

For all 100 selected isolates, the MIC range for the Etest method was from 0.19 µg/ml to 0.38 µg/ml. No growth was observed at a concentration of 0.5 µg/ml for the agar dilution. For consistency of comparison, the upper MIC limit of 16 µg/ml was the greatest value for the MIC range of the agar dilution. The mode MIC of the agar dilution (0.5 µg/ml) was one MIC dilution factor greater than that of the Etest (0.25 µg/ml).

5.3.2 Detection for macrolide resistance determinants

All 100 selected isolates were PCR-negative for all seven macrolide resistance determinants.

5.3.3 Statistical analysis

Statistical comparison of the results of the Etest and agar dilution showed that the agreement of susceptibility was 100 %. The agreement of MIC values based on the nearest similar dilution factor was 97 % ($P > 0.05$; Mann-Whitney test showed no significant difference) and a positive MIC correlation at 0.99 ($P < 0.01$; Spearman correlation coefficient, highly significant correlation).

5.4 Discussion

The agreement of MIC values between the two azithromycin susceptibility testing methods is evidence of consistency as both methods produced comparable results, as illustrated by the highly significant correlation. All 100 South African *V. cholerae* O1 isolates investigated in this study would be considered susceptible to azithromycin provided that the tentative breakpoint of ≤ 16 µg/ml is applied (the EUCAST value for wild-type isolates of *Salmonella enterica* and *Shigella* species). This may suggest that the South African *V. cholerae* O1 isolates recovered

from patients with cholera have not been subjected to selective pressures (azithromycin exposure). The result of exposure to antimicrobials, including azithromycin, could have led to the selection of antimicrobial-resistant isolates that may have mutated to acquire azithromycin resistance. Although these results are positive from a public health perspective, in terms of treatment strategy, these values need to be correlated against observed clinical responses to azithromycin therapy in patients with cholera in a trial setting as previously described in Bangladesh [35,36].

Without such investigation conjecture regarding the appropriate treatment does and/or duration will remain a contentious issues amongst clinicians. The result of such indecisiveness, due to a lack of convincing evidence, may result in clinicians underdosing and underprescribing azithromycin for the treatment of cholera. The practice of exposing the bacterium to sub-optimal levels of this key antimicrobial will provide the selective pressure for increased susceptibility or full resistance resulting in non-clinical response. From a pubic health perspective, this may lead to the partial or total loss of an antimicrobial to treat an epidemic prone pathogen, polypharmacy and significant increases in costs to treat severe or complicated cases of cholera.Non-clinical response to azithromycin therapy has been described in the United Kingdom from a patient returning from Pakistan [37]. *Salmonella enterica* serotype Paratyphi A was isolated from blood culture specimens with observed MIC values between 64 µg/mland 256 µg/ml [37]. In addition, reduced susceptibility to ciprofloxacin was observed and treatment with azithromycin was changed to intravenous third-generation cephalosporin, ceftriaxone [37]. In the same study done by Rai *et al*., non-clinical response to azithromycin therapy was described in 19 patients infected with nalidixic acid-resistant *Salmonella enterica* serotype Typhi [38]. As a result treatment was changed from azithromycin to oral third-generation cephalosporins or amoxicillin as per the standard guidelines [38]. A recent study done by Kobayashi *et al*. from Japan described non-clinical response to azithromycin therapy in a Japanese patient returning from India [39]. *Salmonella enterica* serotype Paratyphi A was isolated from a blood culture specimen with observed MIC value of 8 µg/ml by Etest [39]. This resulted in a change of treatment from azithromycin to ceftriaxone [39]. A study done by Boumghar-Bourtchai *et al*. in France in 2008 also reported non-clinical response to azithromycin in the treatment of *Shigella sonnei* (MIC ≥

64 µg/ml) isolated from children < 15 years of age during an outbreak [40]. A study done by Howie *et al*. in the United States in 2010, described reduced susceptibility to azithromycin in *Shigella sonnei* isolates from routine surveillance at the National Antimicrobial Resistance Monitoring System, reported outbreaks and historical collections [41]. Antimicrobial susceptibility testing included broth microdilution and doubling agar dilution procedures [41]. Observed MIC values were clustered at 8 µg/ml while three isolates displayed an elevated MIC value of 64 µg/ml [41,42].

Unfortunately there exists a dearth of studies from Africa that describe the azithromycin antimicrobial susceptibility patterns in *V. cholerae* O1 isolates. A study done by Mahmud *et al*. described azithromycin susceptibility patterns of *V. cholerae* O1 isolated from Sierra Leone in 2012 using the Etest method [43]. The MIC values for these isolates ranged from 0.125 µg/ml to 0.5 µg/ml [43]. These MIC values were comparable with results shown in this study. A study done in Zimbabwe described *V. cholerae* O1 El Tor variants collected during the cholera epidemic in 2008 displaying reduced susceptibility to azithromycin but their MIC values were not stated [44]. Studies from Asia show similar results to those in Africa but are also limited in nature.A study done in Vietnam (also using the Etest method) showed that 95 % of the *V. cholerae* O1 isolates displayed MIC values ≤ 2 µg/ml and 1 % of the isolates displayed MIC values ≥ 8 µg/ml (MIC range 0.25 µg/ml to 32 µg/ml) [45]. A study done by Faruque *et al*. reported on the first multidrug-resistant (isolates were resistant to furazolidone, tetracycline, erythromycin and co-trimoxazole) *V. cholerae* O1 isolates observed in Matlab, Bangladesh in October 2004 [46]. Prior to 2004, *V. cholerae* O1 isolates recovered were observed to be sensitive to tetracycline, erythromycin, and ciprofloxacin [46]. Further investigation showed that 17 out of 35 isolates were resistant to both erythromycin and azithromycin based on disc-diffusion and Etest methods [46]. The Etest MIC values of *V. cholerae* O1 isolates shown to be azithromycin-resistant ranged from 0.75 µg/ml to 3 µg/ml [46]. This published study showed how rapidly reduced susceptibility to azithromycin was observed in a period of three years [46]. Azithromycin MIC values obtained in this present study are comparable with previous studies on *V. cholerae* O1 as described above. The results of this present study could be used to inform CLSI and EUCAST in their attempts to determine standardized MIC breakpoints for *V. cholerae*

O1. However, literature suggests that this may not be the pattern for other enteric bacteria of clinical importance.

Reduced susceptibility to azithromycin has been reported in enteric pathogenic organisms belonging to the family *Enterobacteriaceae*. For example, elevated MIC values (Etest MIC ≥ 256 µg/ml, doubling agar dilution MIC range 500 µg/ml to 1000 ug/ml) were in observed in *Campylobacter jejuni* isolates recovered from children less than five years of age in Kolkata, India for the years 2010 to 2012 [47]. A study done in the Netherlands in 2014, described azithromycin MIC values (2 µg/ml to 256 µg/ml) in *Salmonella enterica* serotypes Typhi and Paratyphi A, B and C isolated from ill returning travellers [22]. A study done by Gunell *et al*. investigated the *in vitro* activity of azithromycin in 1 237 non-typhoidal *Salmonella enteric* isolates recovered from Finnish patients for the years 2003 to 2008 [48]. Non-typhoidal *Salmonella enterica* isolates that showed reduced susceptibility to fluoroquinolones displayed azithromycin MIC values ≥ 32 µg/ml based on the doubling agar dilution method [48]. A study from Pakistan investigated the *in vitro* activity of azithromycin in *Salmonella enterica* serotypes Typhi and Paratyphi A, B and C isolates [49]. *Salmonella enterica* serotypes Typhi and Paratyphi A and C displayed azithromycin MICs in the range of 2 µg/ml to 12 µg/ml, while *Salmonella enterica* serotype Paratyphi B displayed azithromycin MICs in the range of 2µg/ml to 48 µg/ml based on the Etest method [49]. A study done by Rai *et al*. cited that azithromycin MIC values obtained for *Salmonella* isolates examined in India (24 µg/ml) were not comparable with MIC values obtained for *Salmonella* isolates from the Western countries (MIC range 4 µg/ml to 8 µg/ml) [38]. Since there are no defined azithromycin MIC breakpoints for *Salmonella enterica*, justifying the treatment with azithromycin becomes a challenge particularly for patients with enteric fever, as well as for patients who have travel history to and from developing countries such as India [38].

In the present study, all 100 South African *V. cholerae* O1 isolates examined were PCR-negative for all seven macrolide resistance determinants (*mefA*, *ereA*, *ereB*, *ermB*, *mphA*, *mphB* and *mphD*), which are known to be commonly found in Gram-negative bacteria respectively. These results augment the azithromycin MIC values obtained in this study for both the Etest (0.19

µg/ml to 0.38 µg/ml) and doubling agar dilution methods (0.5 µg/ml). *Escherichia coli* has been shown to harbour a range of common macrolide resistance determinants associated with increased MICs for erythromycin [25].

One-hundred and ninety *Escherichia coli* isolates recovered from various countries (French Guiana, Senegal, France, Niger and Vietnam) were examined [25]. Twenty isolates examined from Senegal (MIC range 64 µg/ml to 128 µg/ml) were shown to be PCR-negative for the detection of macrolide resistance determinants [25]. However, of the 29 isolates recovered from children in Niger (MIC range 64 µg/ml to 1 024 µg/ml), nine isolates were shown to be PCR-positive for the *mphA* gene [25]. Plasmid-mediated transfer of antimicrobial resistance genes have been described between *Escherichia coli* and *Shigella* species [25,50]. The *Shigella sonnei* isolates described by Boumghar-Bourtchai *et al*. was shown to harbour a 90 kb plasmid containing the the *mphA*gene, which encodes for a macrolide 2'-phosphotransferase [47]. Following the report by Howie *et al*. a publication from the United States reported by Sjölund-Karlsson*et al*., described outbreak *Shigella sonnei* isolates displaying azithromycin MICs > 16 µg/ml as well as harbouring a plasmid containing the the *mphA* gene [49]. Reduced susceptibility to azithromycin in *Campylobacter* is due to nucleotide changes in the genes encoding for the 23S rRNA domain V [42]. Macrolide-resistant *Campylobacter jejuni* isolates examined in the study by Mukherjee *et al*. showed that these isolates contained a point mutation, A2075G and isolates that displayed a MIC of 1000 µg/ml was shown to present with a different point mutation, A2074C [42]. The non-typhoidal *Salmonella enterica* isolates examined by Gunell *et al*. showed that out of the 1 237 isolates, nine isolates presented with point mutations in genes *rlpD* and *rlpV* encoding for the 50S ribosomal proteins, L4 and L22 [43]. Three *Salmonella enterica* Montevideo isolates harboured single point mutations in both genes (*rlpD* C379T and *rlpV*G25A), while the remaining six isolates (three *Salmonella enterica* Blockley, one *Salmonella enterica* Saintpaul and two *Salmonella enterica* Typhimurium) harboured a single point mutation in *rlpD*, G235A [43]. These studies illustrate that further analyses are required if this is to be observed in *V. cholerae*.

5.5 Conclusion

The MIC values and PCR results reported in this study provides a foundation for the surveillance of azithromycin susceptibility and to determine MIC breakpoints for *V. cholerae* O1 isolates circulating in South Africa. The global circulation of *V. cholerae* O1 with increased azithromycin MIC values and different mechanisms of macrolide resistance is of public health importance particularly amongst those at most risk populations such as children and immune suppressed individuals in developing countries. Although the molecular findings following this investigation were negative, from a public health outlook it is a positive report, whereby azithromycin can be used as a treatment option for severe diarrhoeal disease. However, due to a lack of comprehensive linking to patients' clinical history it cannot be determined for certain to what antimicrobial agents or environmental factors these *V. cholerae* O1 isolates were exposed to. These isolates were a subset of the total number of *V. cholerae* O1 isolates submitted to the CED for analysis, therefore additional, resource-intensive examination is required. Investigations of other macrolide resistance determinants such as *mph*(C) or mutations in the 23S rRNA or ribosomal proteins L4 and L22 were not attempted as the study *V. cholerae* O1 isolates displayed low MIC values (≤ 0.38 µg/ml). This is the first report describing azithromycin susceptibility amongst South African *V. cholerae* O1 isolates linking phenotypic observations with molecular characteristics.

5.6 References

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Chapter Six
Limitations

A total of 720 *V. cholerae* O1 isolates were characterized during the country wide outbreak of cholera in South Africa during 2008 to 2009 (section 4.2.1). Therefore only a limited percentage of patients had stool cultures taken for examination and ~ 50 % of the positive stool cultures were received by the CED. It is believed nevertheless that these findings do represent the outbreak and the impact of the disease as a whole given the nature of cholera and the rapidity with which the outbreak spread. Full molecular analysis was not conducted on all *V. cholerae* O1 isolates collected through normal surveillance activities. Surveillance activities carried out by the CED are dependent on peripheral laboratories submitting isolates. Many *V. cholerae* O1 isolates were not submitted (section 4.1).

In this study, PFGE analysis was able to distinguish different *Not*I restriction patterns described in both outbreaks (Chapter Three and Chapter Four). This point can be supported with results obtained from antimicrobial susceptibility testing, suggesting that *V. cholera* O1 isolates collected from both cholera outbreakswere highly clonal originating from an independent point-source. Due to limited resources and time constraints, only one molecular subtyping method was used. It would have been advantageous to compare PFGE analysis with another method such as multiple-locus variable-number of tandem repeat analysis (MLVA) [1,2] or MLST [3] to compare genomic variation within *V. cholerae* O1 isolates collected in South Africa (sections 3.3.3 and 4.3.3).

Antimicrobial susceptibility data for furazolidone were not included in the antimicrobial profile of the isolates described as MIC testing for furazolidone was not determined for all *V. cholerae* O1 isolates. Furazolidone MIC testing was only done for *V. cholerae* O1 isolates collected in 2009 and not for 2008. Data not described in this study showed that ~ 74 % of the isolates from the Limpopo Province and ~ 51 % of the isolates from the Mpumalanga Province displayed reduced susceptibility to furazolidone (MIC $\geq$ 128 µg/ml). Due to financial and time constraints extended analysis was not possible. If funding and time were feasible, it would have been

interesting to further analyze the identified plasmid DNA with the antimicrobial resistance marker (*bla*$_{TEM}$) in order to determine the plasmid incompatibility group as well as the frequency of transfer of theantimicrobial resistance marker [4,5]. Furthermore it would have been interesting to determine whether the *tetA* gene is located on the plasmid or SXT element as previously described by Iwanaga *et al*. [6]. It would have also been valuable to investigate molecular mechanisms of antimicrobial resistance to nitrofurans [7,8]. Antimicrobial resistance genes that are now mobile in clinical isolates probably originated as determinants with a fixed chromosomal origin [9]. Global gene flow occurs in multiple directions, therefore allowing the introduction of new antimicrobial resistance genes into clinical isolates and transporting clinically relevant antimicrobial resistance genes back into the broader bacterial population [9]. *V. cholerae* possess the mechanisms to acquire and share resistance genes from direct contact with intrinsically resistant bacteria through mobile genetic elements and share these genes with other commensal microorganisms or enteric pathogens [10].

In this study, nucleotide sequencing of the *ctxAB* gene (section 4.3.7) of the four selected isolates showed that all four isolates expressed the encoded *ctxB* allele for the CT of the classical biotype and were defined as altered El Tor. It has been shown that typical *V. cholerae* O1, biotype El Tor and classical isolates did not originate from a recent common ancestor but instead, seem to be independent derivatives with distinct phylogenetic histories [11]. Atypical *V. cholerae* O1, biotype El Tor isolates most likely arose through LTG of CTX$^{class}\Phi$ and as a result, genotypes with the classical *ctxB-1* allele (results shown in this study) have spread to Asia and Africa, including South Africa [12]. A report by le Roux *et al*. described whole genome sequencing of the first bacterial genome of a South African *V. cholerae* O1 isolate (G4222) [13]. This clinical isolate was recovered in South Africa during the 2000-2001 cholera epidemic [13]. It would be advantageous to perform whole genome sequencing in order to understand and track emerging epidemic isolates particularly antimicrobial-resistant atypical *V. cholerae* O1, biotype El Tor isolates, which are appearing worldwide [11,13-17].

All 100 selected isolates were PCR-negative for all seven macrolide resistance determinants and were considered susceptible to azithromycin based on the EUCAST criterion; these results have

created a platform whereby further analyses can be done. Doubling agar dilution and Etest methods are not qualified as “gold standards” [18]. Although broth microdilution is recognized as the “gold standard”, it may not have added additional information in the absence of the macrolide resistance genes. This added MIC testing method would lend further strength to future findings related to the azithromycin MIC susceptibility breakpoints. The development of antimicrobial resistance by bacteria in order to survive under environmental pressure may confer a selective advantage [19]. To overcome the loss of fitness and survive in a competitive environment bacteria generate mutations with increased frequency [19-21]. It may be of clinical interest to create and select azithromycin-resistant *V. cholerae* O1 laboratory mutants by exposing azithromycin-susceptible (MICs $\leq$ 16 µg/ml) *V. cholerae* O1 isolates to sub-inhibitory concentrations of azithromycin.

6.1 References

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Chapter Seven

General Discussion and Conclusion

The *V. cholerae* O1 isolates investigated from each outbreak were resistant to multiple antimicrobial agents, including resistance to β-lactam antimicrobial agents and displayed almost similar antimicrobial resistance patterns. Sixteen *V. cholerae* O1 isolates (ten isolates characterized in Chapter Three and six isolates characterized in Chapter Four) were positive for a plasmid-borne ESBL (TEM-63 β-lactamase) resistance mechanism previously associated with hospital acquired infections [1]. A possible explanation for this observation could be the previously unsuccessful management of patients with a sexually transmitted infection (STI) with third-generation cephalosporins [2-4]. A study conducted in the United Kingdom described the anal-oral transmission of antimicrobial-resistant (including resistance to azithromycin and third-generation cephalosporins) *Shigella flexneri* serotype 3a in men-who-have-sex-with-men, suggesting that alternate sexual behaviour provided the catalyst for transmission [4]. Human behaviour may be a more influencial factor than this study was able to ascertain as a possible mechanism contributing to the spread of antimicrobial-resistant TEM-63 producing *V. cholerae* O1 isolates. In South Africa, first-line treatment for a male patient exhibiting a STI is a single dose of cefixime (400 mg) administered orally and doxycycline (100 mg) administered orally, twice a day for seven days (accessed from http://www.nicd.ac.za/assets/files/STI%20Book%202008%20Edited(1).pdf; November 2014). Comment cannot be made on the study isolates ability to share (as donor or receiver) the various resistance genes described, as these characteristics never investigated - usually investigated by bacterial conjugation assays. *V. cholerae* possess the mechanisms to acquire and share resistance genes from direct contact with intrinsically resistant bacteria through mobile genetic elements and share these genes with other commensal microorganisms or enteric pathogens [5].

Traveller's diarrhoea (TD) is one of the most common illnesses affecting individuals who journey across international borders [6,7]. Suspected imported cases of cholera in travellers emphasize the need for rigorous evaluation if the patient with severe watery diarrhoea has recently visited a cholera endemic country [8]. The increase in antimicrobial resistance,

geography and clinical illness influences the selection and use of antimicrobial agents [7,9]. A study using either co-trimoxazole or doxycycline in the treatment of TD showed that they were effective in many parts of the world however; the development of widespread resistance to these antimicrobial agents has emerged [10]. Although, fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin and levocloxacin) have shown to be more effective than rifaxamine in the treatment of inflammatory diarrhoea, antimicrobial resistance has been reported in other enteric pathogens such as *Campylobacter* and *Shigella* species [6,7,10]. Azithromycin has shown to be highly effective in the treatment of TD and likely would be effective as a chemoprophylactic antimicrobial agent [6,7,10]. However antimicrobial resistance to macrolides remains a serious clinical concern [11,12].

The antimicrobial-resistant *V. cholerae* O1 isolates investigated from each outbreak (Chapter Four) were characterized as altered El Tor variants. This altered El Tor variant is currently recognized as the most successful variant in that isolates belonging to this type have almost replaced the prototype El Tor in Asia and many parts of Africa, particularly East Africa and now South Africa [13]. As aforementioned, the CT is accountable for the major clinical signs and symptoms of cholera [14-17] thus, changes in the genetic material encoding for the CT could alter the clinical appearance of cholera [17,18]. A study done by Siddique *et al*. in Bangladesh, showed that a higher proportion of patients presented with severe dehydration following the isolation and identification of *V. cholerae* O1 El Tor variants in 2006 [17,19]. In addition, other studies have also described an increase in severity of disease during cholera outbreaks one of them being the Haitian cholera outbreak [17,20,21]. The differentiation of *V. cholerae* O1 isolates into biotype does not change the clinical management of patients presenting with cholera [17]. However, it is of public health and epidemiological importance in determining the source and spread of infection particularly, in countries where sanitation and access to clean drinking water are limited [17].

South Africa is one of many countries involved in improving health and achieving the eight Millennium Development Goals (MDGs); (1) eradicate extreme poverty and hunger, (2) achieve universal primary education, (3) promote gender equality and promote and empower women, (4)

reduce child mortality, (5) improve maternal health, (6) combat HIV/AIDS, malaria and other diseases, (7) ensure environmental sustainability and (8) global partnership for development by 2015 (accessed from http://www.health-e.org.za/2014/07/01/motsoaledi-sounds-post-mdg-agenda/ and http://www.un.org/millenniumgoals/beyond2015-faqs.shtml; November 2014) [22]. In the MDGs report (accessed from http://mdgs.un.org/unsd/mdg/Resources/Static/Products/Progress2014/English2014.pdf; November 2014) published in July 2014, according to MDG 7C, many people worldwide still rely on unsafe water sources. Between 1990 and 2012, 2.3 billion more people have gained access to cleansources of drinking water. However 748 million peoplestill draw their water from anunimproved source of which, Sub-Saharan Africa was shown to be the highest. In addition, between 1990 and 2012 almost two billion people obtained access to improved sanitation. However in 2012, it was shown that one billion people still make use of open defecation facilities, of which prevalence is greatest in Southern Asia, Oceania and Sub-Saharan Africa. It was also shown that about seven out of ten people without access to improved sanitation facilities and clean drinking waterreside in rural areas. In low-income and middle-income countries, transmission of pathogenic organisms (bacterial, viral or parasitic) responsible for gastrointestinal infections generally occurs through contaminated food or drinking water [23,24]. In addition, the absence of effective public health measures in developing countries significantly impact health burdens following these gastrointestinal infections [24]. In developing countries, the infrastructure required to provide basic sanitation and access to clean drinking water is beyond the countries financial constraints [24].

In South Africa, water quality has severely decreased due to constant disposal of industrial and domestic waste into the Vaal River [25]. The Vaal River supply the water requirements of communities in Gauteng, the Free State, North West and Northern Cape Provinces [25]. Microbiological pollution is one many problems affecting water quality [25]. Water sources such as rivers, boreholes and fountains used by rural communities for domestic and drinking purposes are generally contaminated by faeces and lack treatment [26]. A study done by Keshav *et al*. described the isolation of *V. cholerae* O1 in 17 out of 74 stool samples collected from cows [27]. These samples were collected close to water sources used by villagers in rural areas of the

Limpopo Province in South Africa. Another study done in the Venda region of the Limpopo Province reported on the bacterial contamination of Vhuswa, a local weaning food used by mothers and caretakers to supplement breastfeeding and quality of stored water in rural households [26]. Enteric pathogens namely, *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter* were isolated from the Vhuswa samples [26]. Water samples (spring water and standpipe water) used to prepare Vhuswa and for drinking were shown to be of poor quality as all four enteric pathogens described previously were isolated [26]. A study done by Singh *et al*. investigated the microbiological quality of drinking water from ground-tanks and community tankers and its association to health outcomes (for example diarrhoea and vomiting) in particular water quality, demographic distribution as well as sanitation and hygiene education in two peri-urban areas [28]. The results from this study showed that households with children under five yearsof age using open-topped containers had the poorest water quality overall [28]. Households with ground-tanks had the best water quality at point-of-use, however; did not have the lowest occurrence of health effects [28]. In addition, it was shown that households that practiced open defecation had higher levels of *Escherichia coli* in their drinking water as well as higher rates of adverse health outcomes [28].

This study was conducted in an attempt to describe the molecular epidemiology and mechanism of antimicrobial resistance of *V. cholerae* O1 isolated from outbreaks in South Africa over recent years (2008 to date). Antimicrobials decrease the number of *V. cholerae* bacilli being shed by an infected individual thereby reducing the risk of spread of the disease. In terms of infectious disease epidemic control any intervention at the beginning of the epidemic that can potentially limit the number of cases and effectively reduce overall morbidity and mortality is viewed as essential. Controlling an epidemic of *V. cholerae* with treatment of initial cases with effective antimicrobials in conjunction with rehydration may be the answer. As a result volumes of diarrhoea may be reduced, potentially reducing hospitalization, dependence on intravenous rehydration and reduced risk of downstream complications from dehydration. An added more important advantage is that the initial investment in treating cholera patients with an efficacious antibiotic upfront will save treatment, hospitalization and potential complication costs. The

downstream benefit to the health system is that resources saved can be directed to primary health care or preventative medicine which is of a greater importance.

The priority for investigation of such an intervention, however, is no greater in resource limited settings as more often than not it is these areas that are worst affected by outbreaks of *V. cholerae*. This is due to, either partly or entirely, poor healthcare and sanitation infrastructure, or in vulnerable populations due to natural disaster, e.g. earthquakes, or unnatural disasters such as wars where people are displaced and forced into cramped settlements with poor sanitation. Failure to identify epidemics in their infancy can result in large scale outbreaks within these settings. The priority then quickly shifts to limiting complicated cases, general morbidity and overall mortality and this is where an efficacious antibiotic comes into its own as a tool for public health.

All this is impossible without proper regulations and that means standardized MIC breakpoints to inform treatment strategy. Treatment with the incorrect doses of antibiotics will drive resistance during and after the epidemic resulting in strains that are hard to treat. The results presented in Chapter Five are suggestive that azithromycin could be used as a treatment option for *V. cholerae* infection however the public health usefulness will be lost without aforementioned standardization of MIC breakpoints. However, *V. cholerae* continues to evolve and develop new ways to resist antimicrobial action, therefore highlighting the extent and severity of cholera as well as the rapidity of spread of the disease. Evidence of this was presented in Chapter Three, as resistance to a third generation cephalosporin poses further treatment challenges for severe or complicated cases. The default when faced with a non-clinical response to antimicrobial treatment is to opt for treatment with a third generation cephalosporin, however with mounting evidence that *V. cholerae* isolates harbor ESBL resistance genes will limited treatment options further (Chapter Three). The global public health fear is the introduction of such a highly adaptable pathogen, such as the *V. cholerae* O1 El Tor circulating South Africa, into a vulnerable population, which may give rise to yet another cholera epidemic with increased morbidity and mortality as experienced in the last nationwide outbreak (Chapter Four). Therefore investigating

and understanding the mechanisms of antimicrobial resistance is vital for treatment availability not just for cholera, but other community-acquired infections (Chapter Five).

This study has illustrated that an active laboratory-based surveillance system with clinical support incorporating innovative, cutting-edge molecular and phenotypic assays is an essential element in the process of identification and monitoring of epidemic prone organisms such as *V. cholerae* O1. Evidence of this was presented in Chapters Three and Four, which described the two most prominent outbreaks of cholera South Africa has experienced in recent history. Without the existing surveillance network in South Africa these outbreaks may not have been described in the detail that it has been. The analysis conducted on isolates from these separate outbreaks was key to defining the isolates and sources of the outbreaks as distinct. The public health response as a result was directed accordingly, that is a defined population (illegal miners) versus a nationwide outbreak (Chapters Three and Four). The other key element to an active laboratory-based surveillance system with clinical support is to work in partnership with larger, global networks such as PulseNet International in the monitoring of epidemic-prone organisms isolated from South Africa and globally so that public health interventions can be initiated timeously to limit spread of disease and overall morbidity and mortality (Chapter Four).

As the world reflects on the impending 2015 United Nations deadline for achieving the MDGs, it is with great concern that the countries that will not meet all their targets by 2015 are in Africa. However, in relation to MDG 7, which is to ensure environmental sustainability, South Africa has achieved the Target 7C which is to halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation. Although such reports are promising in terms of service delivery to a vulnerable population and from a public health point-of-view in limiting the spread and of waterborne pathogens, recent reports have suggested that South Africa sits on the cusp of a water crisis due to failing infrastructure, poor planning and inefficient water use. South African water quality can rival any of the developed nations as safe, drinkable water is a mere turn-of-the-tap away however water-borne pathogens such as *V. cholerae* require nothing but a small breakdown in an efficient system to result in a nation-wide outbreak.

7.1 References

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Appendices

Appendix A: Pulsed-field gel electrophoresis (PFGE)

A1: 1.00 M Tris (pH 8.00)

121.1 g Tris (Merck KGaA, Darmstadt, Germany)

Dissolve in 800 ml distilled water

Adjust to pH 8.00 with a laboratory pH meter (WTW GmbH, Weilheim, Germany) using 32 % Hydrochloric acid (HCl) (Merck) or sodium hydroxide (NaOH) pellets (Merck)

Adjust final volume to 1000 ml with distilled water

Sterilize the mixture by autoclaving (120 ºC for 15 minutes at 1 kg/cm^2)

Store at room temperature (+ 25 ºC)

A2: 0.50 M EDTA (pH 8.00)

186.1 g disodium ethylenediaminetetra-acetic acid (EDTA) (Merck)

Dissolve in 800 ml distilled water

Adjust to pH 8.00 with 32 % HCl (Merck) or NaOH pellets (Merck)

Adjust final volume to 1000 ml with distilled water

Sterilize the mixture by autoclaving

Store at room temperature

A3: Cell suspension buffer (100 mM Tris : 100 mM EDTA, pH 8.00)

10 ml of 1 M Tris, pH 8.00

20 ml of 0.5 M EDTA, pH 8.00

Dissolve in 70 ml distilled water

Sterilize the mixture by autoclaving

Store at room temperature

A4: Proteinase-K (20 mg/ml)

20 mg Proteinase-K (Roche Diagnostics, Mannheim, Germany)

Dissolve in 1 ml TE buffer, pH 8.00

Prepare fresh and keep on ice

A5: Proteinase-K (10 mg/ml)

10 mg Proteinase-K (Roche)

Dissolve in 1 ml TE buffer, pH 8.00

Prepare fresh and keep on ice

A6: Sodium dodecyl sulphate (10 %)

10 g sodium dodecyl sulphate (SDS) (Merck)

Dissolve in 100 ml distilled water

Store at room temperature

A7: 1 % SeaKem Gold® agarose (Preparation of agarose plugs for *V. cholerae*)

0.2 g SeaKem Gold® agarose (Lonza, Rockland, USA)

Dissolve in 20 ml of TE buffer

Dissolve agarose by boiling in the microwave

Store at room temperature

A8: 1 % SeaKem Gold® agarose : 1 % SDS (Preparation of agarose plugs for *Salmonella*)

0.25 g SeaKem Gold® agarose (Lonza)

Dissolve in 22.5 ml of TE buffer with 2.5 ml 10 % SDS

Dissolve agarose by boiling in the microwave

Store at room temperature

A9: Cell lysis buffer for *Salmonella* (5 ml/plug)

5 ml of 0.50 M EDTA, pH8.00

1 % (50 mg) N-lauroylsarcosine sodium salt (sarcosyl) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA)

50 µl of 10 mg/ml Proteinase-K (Roche)

Prepare fresh and keep at room temperature

A10: Cell lysis buffer for *V. cholerae* (5 ml/plug) (50 mM Tris : 50 mM EDTA, pH 8.00)

250 µl of 1.00 M Tris, pH8.00

500 µl of 0.50 M EDTA, pH8.00

Dissolve in 4.25 ml distilled water

50 mg sarcosyl (Sigma)

50 µl of 10 mg/ml Proteinase-K (Roche)

Prepare fresh and keep at room temperature

A11: Tris-EDTA (TE) buffer (10 mM Tris : 1 mM EDTA, pH 8.00)

10 ml of 1 M Tris, pH 8.00

2 ml of 0.5 M EDTA, pH 8.00

Dissolve in 988 ml distilled water

Sterilize the mixture by autoclaving

Store at room temperature

A12: Restriction enzyme O-buffer mix (Per agarose slice)

90 µl distilled water

10 µl 10X O-buffer (Fermentas International Inc, Burlington, Canada)

Prepare fresh and keep on ice

A13: Restriction enzyme H-buffer mix (Per agarose slice)

90 µl distilled water

10 µl 10X H-buffer (Roche)

Prepare fresh and keep on ice

A14: Restriction enzyme O-buffer mix with *Not*I restriction enzyme (Per agarose slice)

132 µl distilled water

15 µl 10X O-buffer (Fermentas)

3 µl *Not*I restriction enzyme (Fermentas)

Prepare fresh and keep on ice

A15: Restriction enzyme H-buffer mix with *Xba*I restriction enzyme (Per agarose slice)

132 µl distilled water

15 µl 10X H-buffer (Roche)

3 µl *Xba*I restriction enzyme (Roche)

Prepare fresh and keep on ice

A16: Tris : borate : EDTA (TBE)

10X TBE (Merck)

Store at room temperature

A17: 0.5X TBE

50 ml 10X TBE

Dissolve in 950 ml distilled water

A18: 1 % SeaKem Gold® agarose gel (Preparation for a PFGE run)

1.5 g SeaKem Gold® agarose (Lonza)

Dissolve in 150 ml of 0.5X TBE

Dissolve agarose by boiling in the microwave

A19: Ethidium bromide (EtBr) stock solution (10 mg/ml)

500 mg ethidium bromide (Merck)

Dissolve in 50 ml distilled water

Store in the dark in the fridge at + 2 °C to + 8 °C

A20: EtBr staining solution

25 µl of ethidium bromide (10 mg/ml)

Dissolve in 250 ml of 0.5X TBE

Appendix B: Polymerase Chain Reaction (PCR)

B1: Detection and analysis of the internal amplification control and detection of enzymatic A subunit of the cholera toxin (ctxA)

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
16SrRNA-B	GATTAGATACCCTGGTAGTCC	*16S rRNA*	726	*V. cholerae* O1 ATCC9458
16SrRNA-r	ACGGCTACCTTGTTACGACTT			
ctxA-f	CTCAGACGGGATTTGTTAGGCACG	*ctxA*	301	*V. cholerae* O1 ATCC9458
ctxA-r	TCTATCTCTGTAGCCCCTATTACG			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	14.5
10X PCR Gold Buffer (Applied Biosystems, Foster City, California)	2.5
25 mM Magnesium chloride ($MgCl_2$) (Applied Biosystems)	2
10 mMDeoxynucleoside triphosphate (dNTP) mix (Celtic Molecular Diagnostics, Cape Town, South Africa)	2
Primer mix*	3
AmpliTaq Gold®(Applied Biosystems)	0.3
crude template DNA	1
Total reaction volume	25

Primer mix *****

85 µl autoclaved TE buffer **(Appendix A11)**

2.5 µl of 20 µM 16SrRNA-B

2.5 µl of 20 µM 16SrRNA-r

5.0 µl of 20 µM ctxA-f

5.0 µl of 20 µM ctxA-r

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.15	35
Annealing	55	1.15	
Extension	72	1.15	
Final extension	72	7	1
Hold	4	∞ (Infinite hold)	1

B2: Detection and analysis of the toxin co-regulated pilus (*tcpA*) of El Tor and classical variants

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
tcpA-Classic-F	CACGATAAGAAAACCGGTCAAGAG	*tcpA*-Classical	617	*V. cholerae* O1 ATCC9458
tcpA-Classic-R	ACCAAATGCAACGCCGAATGGAGC			
tcpA-ElTor-F	GAAGAAGTTTGTAAAAGAAGAACAC	*tcpA*-ElTor	471	*V. cholerae* O139 VIBCH05
tcpA-ElTor-R	GAAAGGACCTTCTTTCACGTTG			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	15.0
10X PCR Gold Buffer (Applied Biosystems)	2.5
25 mM Magnesium chloride ($MgCl_2$) (Applied Biosystems)	1.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
Primer mix**	3
AmpliTaq Gold®(Applied Biosystems)	0.3
crude template DNA	1
Total reaction volume	25

Primer mix **

20 µl autoclaved TE buffer **(Appendix A11)**

20 µl of 20 µM tcpA-Classic-F

20 µl of 20 µM tcpA-Classic-R

20 µl of 20 µM tcpA-ElTor-F

20 µl of 20 µM tcpA-ElTor-R

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1	35
Annealing	60	1.15	
Extension	72	1.15	
Final extension	72	7	1
Hold	4	∞	1

B3: Detection and analysis of ESBL genes

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
TEM-F1	ATGAGTATTCAACATTTCCGTG	*bla*TEM	840	*Klebsiella pneumoniae* ATCC51503
TEM-R1	TTACCAATGCTTAATCAGTGAG			
SHV-F1	ATGCGTTATATTCGCCTGTG	*bla*SHV	846	*Klebsiella pneumoniae* ATCC700603
SHV-R1	GTTAGCGTTGCCAGTGCTCG			
CTXM-F1	CGATGTGCAGTACCAGTAA	*bla*CTX-M	550	*Klebsiella pneumoniae* D-17
CTXM-R1	TWRGTSACCAGAAYCAGCGG			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology, Cape Town, South Africa)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	60	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

B4: Detection and analysis of class 1 and class 2 integrons

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	3'-CS	800	*Escherichia coli* 803Rif:p3iANG
sul1-B	GCAAGGCGGAAACCCGCGCC			
inDS-F	CGGAATGGCCGAGCAGATC	5'-CS	870	*Escherichia coli* 803Rif:p3iANG
inDS-B	CAAGGTTCTGGACCAGTTGCG			
in-F	GGCATCCAAGCAGCAAGC	Gene cassette	variable	*Escherichia coli* 803Rif:p3iANG
in-B	AAGCAGACTTGACCTGAT			
INT-2U	ATGTCTAACAGTCCATTTT	*intI2*	450	
INT-2D	AAATCTTTAACCCGCAAAC			
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Gene cassette	variable	
hep51	GAT GCCATCGCAAGTACGAG			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

3’-CS and 5’-CS

PCR Cycle	**Temperature** (°C)	**Time (minutes)**	**# of cycles / reaction**
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	62	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

intI2

PCR Cycle	**Temperature** (°C)	**Time (minutes)**	**# of cycles / reaction**
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	57	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

B5: Detection and analysis of the tetracycline resistance determinant, SXT element-integrase and SXT associated resistance genes

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
TetA-F	GTAATTCTGAGCACTGTCGC	*tetA*	950	TCD273377
TetA-R	CTGCCTGGACAACATTGCTT			
int1-F	GCTGGATAGGTTAAGGGCGG	SXT*int*	592	*Escherichia coli* CAG18439:SXT^{MO10}
Int1-B	CTCTATGGGCACTGTCCACATTG			
FLOR-F	TTATCTCCCTGTCGTTCCAGCG	*floR*	526	*Escherichia coli* CAG18439:SXT^{MO10}
FLOR-2	CCTATGAGCACACGGGGAGC			
SUL2-F	AGGGGGCAGATGTGATCGC	*sul2*	625	*Escherichia coli* CAG18439:SXT^{MO10}
SUL2-B	TGTGCGGATGAAGTCAGCTCC			
dfr1-F	CGAAGAATGGAGTTATCGGG	*dfrA1*	372	TCD273377
dfr1-B	TGCTGGGGATTTCAGGAAAG			
TMP-F	TGGGTAAGACACTCGTCATGGG	*dfr18*	389	*Escherichia coli* CAG18439:SXT^{MO10}
TMP-B	ACTGCCGTTTTCGATAATGTGG			
STRA-F	TTGATGTGGTGTCCCGCAATGC	*strA*	383	*Escherichia coli* CAG18439:SXT^{MO10}
STRA-B	CCAATCGCAGATAGAAGGCAA			
strB-F	GGCACCCATAAGCGTACGCC	*strB*	470	*Escherichia coli* CAG18439:SXT^{MO10}
strB-R	TGCCGAGCACGGCGACTACC			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	60	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

B6: Detection and analysis of PMQR genes and the quinolone resistance determinant

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
QP1	GATAAAGTTTTTCAGCAAGAGG	*qnrA*	657	*Escherichia coli* pMG252
QP2	ATCCAGATCGGCAAAGGTTA			
FQ1	ATGACGCCATTACTGTATAA	*qnrB*	566	*Escherichia coli* pMG298
FQ2	GATCGCAATGTGTGAAGTTT			
qnrS-F	TGGAAACCTACAATCATACATATCG	*qnrS*	585	*Escherichia coli* pMG306
qnrS-R	TTAGTCAGGATAAACAACAATACC			
qnrC-F	GGGTTGTACATTTATTGAATCG	*qnrC*	307	*Proteus mirabils* 06-498
qnrC-R	CACCTACCCATTTATTTTCA			
qepA-F	AACTGCTTGAGCCCGTAGAT	*qepA*	596	*Escherichia coli* pAT851
qepA-R	GTCTACGCCATGGACCTCAC			
qnrVC-F	AATTTTAAGCGCTCAAACCTCCG	*qnrVC3*	521	
qnrVC-R	TCCTGTTGCCACGAGCATATTTT			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

qnrA

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	57	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

qnrB and *qnrS*

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	53	1.3	
Extension	72	1.	
Final extension	72	7	1
Hold	4	∞	1

qnrC, qepA and *qnrVC3*

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	60	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

B7: Detection, nucleotide sequencing and analysis of the QRDR of DNA gyrase and topoisomerase IV

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
gyrA-F1	AATGTGCTGGGCAACGACTGG	*gyrA*	239	TCD273377
gyrA-R1	GTGCGCGATTTTCGACATACG			
gyrB-F1	GGAAATGACTCGCCGTAAAGG	*gyrB*	309	TCD273377
gyrB-R1	GTTGTGATAACGCAGTTTATCTGGG			
parC-F1	GTCTGAGTTGGGTCTCTCGGC	*parC*	248	TCD273377
parC-R1	AGAATCTCGGCAAACTTTGACAG			
parE-F1	ATGCGTGCCAGCAAGAAAGTG	*parE*	268	TCD273377
parE-R1	TTATCGCTGTCAGGGTCAATCC			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

gyrA

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	60	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

gyrB and *parE*

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	0.3	3
Annealing	37	0.3	
Extension	72	1	
Denaturation	95	0.3	30
Annealing	45	0.45	
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	1

parC

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	62	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

Appendix C: Detection of PCR products using conventional agarose gel electrophoresis

C1: 10X Tris : acetate: EDTA (TAE)

48 g Tris (Merck)

7.5 g EDTA (Sigma)

Dissolve in 500 ml distilled water

11 ml of glacial acetic acid (Merck)

Adjust to a final volume of 1000 ml with distilled water

Sterilize the mixture by autoclaving

Store at room temperature

C2: 1X TAE

100 ml 10X TAE

Dissolve in 900 ml distilled water

Store in the fridge at + 2 ºC to + 8 ºC

C3: Bromophenol blue (Loading buffer)

250 mg bromophenol blue (Merck)

40 g sucrose (Merck)

Dissolve in 100 ml distilled water

Store in the fridge at + 2 ºC to + 8 ºC

C4: 1.5% Seakem LE agarose gel

1.5 g Seakem LE agarose (Lonza)

Dissolve in 100 ml of 1X TAE buffer

Dissolve agarose by boiling in the microwave

Add 6 µl of the ethidium bromide stock solution (Appendix A19) to the molten agarose

Appendix D: Nucleotide sequencing using the ABI Prism®BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	7.5
5X Sequencing v1.1, v3.1 Buffer	1.5
ABI Prism®BigDye® Terminator v3.1 Ready Reaction mix	3
5 µM Forward primer or 5 µM Reverse primer	1
Purified template DNA	2
Total reaction volume	15

Cycle sequencing PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	2	1
Denaturation	95	0.5	25
Annealing	50	0.5	
Extension	60	4	
Hold	4	∞	1

Appendix E: Isolation and preparation of plasmid DNA

E1: Luria-Bertani (LB) agar plates

10 g Bacto tryptone (Oxoid, Basingstoke, Hampshire, England)

5 g Yeast extract (Oxoid)

10 g NaCl (Merck)

Dissolve in 500 ml distilled water

Adjust to a final volume of 1000 ml with distilled water

15 g Bacto agar (Becton, Dickinson and Company, Sparks, USA)

Sterilize the mixture by autoclaving

E2: Ceftriaxone (1 mg/ml)

10 mg ceftriaxone (Sigma)

Dissolve in 10 ml distilled water

Solution filtered through a filtered tip syringe and passed through 0.22 μm Millex®-GS filter unit (Millipore S.A., Molsheim, France)

Store in the freezer at minus (-) 10 ºC to - 20 ºC

E3: LB broth

10 g Bacto tryptone (Oxoid)

5 g Yeast extract (Oxoid)

10 g NaCl (Merck)

Dissolve in 500 ml distilled water

Adjust to a final volume of 1000 ml with distilled water

Sterilize the mixture by autoclaving

Store at room temperature

E4: 10X E-buffer (0.5 M Tris-HCl : 10 mM EDTA)

6.05 g Tris (Merck)

372 mg EDTA (Sigma)

Dissolve in 50 ml distilled water

Adjust to a final volume of 100 ml with distilled water

Sterilize by autoclaving

Store at room temperature

E5: 1X E-buffer

10 ml 10X E-buffer

Dissolve in 90 ml distilled water

Sterilize by autoclaving

Store in the fridge at +2 °C to +8 °C

E6: Lysis solution (3 % SDS in 50 mM Tris-HCl, pH 12.56)

605 mg Tris (Merck)

Dissolve in 50 ml distilled water

Adjust to pH 12.56 with 32 % HCl (Merck) or NaOH pellets (Merck)

Adjust to a final volume of 100 ml with distilled water

3 g SDS

Store at room temperature

E7: Phenol : chloroform : isoamyl alcohol

25 ml phenol(Sigma)

24 ml chloroform(Merck)

1 ml isoamyl alcohol (Merck)

Prepare fresh

Appendix F: PCR Digoxigenin (DIG)-labelling and Southern blot hybridization

F1: PCR cocktail mix for DIG-labeled hybridization target gene, *bla*$_{TEM}$ using the PCR DIG Probe Synthesis Kit (Roche)

Reagent	Final concentration	Volume (µl) / reaction
Autoclaved distilled water	-	32.25
PCR buffer with $MgCl_2$ (10X concentration) (Roche)	$1x10^4$	5
PCR DIG mix (10X concentration) (Roche)	200 µM	5
20 µM TEM-F1	1 µM	2.5
20 µM TEM-R1	1 µM	2.5
Enzyme mix, Expand High, Fidelity (Roche)	2.6 units	0.75
Purified template DNA	~ 10 ng	2
Total reaction volume	-	50

F2: PCR cocktail mix for unlabelled control, *bla*$_{TEM}$ using the PCR DIG Probe Synthesis Kit (Roche)

Reagent	Final concentration	Volume (µl) / reaction
Autoclaved distilled water	-	32.25
PCR buffer with $MgCl_2$ (10X concentration) (Roche)	$1x10^4$	5
dNTP stock solution (10X concentration) (Roche)	200 µM	5
20 µM TEM-F1	1 µM	2.5
20 µM TEM-R1	1 µM	2.5
Enzyme mix, Expand High, Fidelity (Roche)	2.60units	0.75
Purified template DNA	~ 10 ng	2
Total reaction volume	-	50

F3: PCR conditions for labeled hybridization target genes and unlabeled controls using the PCR DIG Probe Synthesis Kit (Roche)

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	2	1
Denaturation	95	1	30
Annealing	60	1	
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	1

F4: Denaturation solution (0.5 M NaOH : 1.5 M NaCl)

20 g NaOH (Merck)

87.66 g NaCl (Merck)

Dissolve in 500 ml distilled water

Adjust to a final volume of 1000 ml with distilled water

Sterilize the mixture by autoclaving

Store at room temperature

F5: Neutralization solution (0.5 M Tris-HCl, pH 7.50 : 1.5 M NaCl)

60.55 g Tris (Merck)

Dissolve in 500 ml distilled water

Adjust to pH 7.50 with 32 % HCl (Merck) or NaOH pellets (Merck)

87.66 g NaCl (Merck)

Adjust to a final volume of 1000 ml with distilled water

Sterilize the mixture by autoclaving

Store at room temperature

F6: Sodium chloride, Sodium citrate buffer (SSC)

20X SSC (Roche)

F7: 2X SSC buffer

10 ml 20X SSC (Roche)

Dissolve in 90 ml distilled water

Store at room temperature

F8: 0.5X SSC buffer

2.5 ml 20X SSC (Roche)

Dissolve in 97.5 ml distilled water

Store at room temperature

F9: Low stringency buffer (2X SSC buffer containing 0.1 % SDS)

100 ml 2X SSC buffer

1 ml 10 % SDS

Prepare fresh and keep at room temperature

F10: High stringency buffer (0.5X SSC buffer containing 0.1 % SDS)

100 ml 0.5X SSC buffer

1 ml 10 % SDS

Prepare fresh and keep at + 65 °C

Appendix G: Involvement of efflux pumps

G1: Mueller-Hinton agar plates

1.52 g Mueller-Hinton agar(Oxoid)

Dissolve in 40 ml distilled water

Sterilize the mixture by autoclaving

G2: Reserpine (1 mg/ml stock)

10 mgresperpine (Sigma)

Dissolve in 10 ml glacial acetic acid

Solution filtered through a filtered tip syringe and passed through 0.22 μm Millex®-GS filter unit (Millipore S.A.)

G3: Phenylalanyl arginine-β-naphthylamide (PAβN) (1 mg/ml stock)

10 mgPAβN (Sigma)

Dissolve in 10 ml distilled water

Solution filtered through a filtered tip syringe and passed through 0.22 μm Millex®-GS filter unit (Millipore S.A.)

G4: Nalidixic acid (1 mg/ml stock)

10 mgnalidixic acid (Sigma)

Dissolve in distilled water together with 0.1M NaOH

Solution filtered through a filtered tip syringe and passed through 0.22 μm Millex®-GS filter unit (Millipore S.A.)

Appendix H: Tracking seventh pandemic variants

H1: Detection, nucleotide sequencing and analysis of the cholera toxin, *ctxA*B gene

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
CTXA-comp-F	GGCTGTGGGTAGAAGTGAAACGG	*ctxAB*	1350	*V.. cholerae* O1 ATCC9458 or*V. cholerae* O139 VIBCH05
CTXB-comp-R	CTAAGGATGTGGAATAAAAACATC			
CTX93-F	GGCAGATTCTAGACCTCCT	Internal sequencing primers		*V.. cholerae* O1 ATCC9458or*V. cholerae* O139 VIBCH05
CTX618-R	TCGATGATCTTGGAGCATTC			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	15
10X PCR Gold Buffer (Applied Biosystems)	2.5
25 mM Magnesium chloride ($MgCl_2$) (Applied Biosystems)	1.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
10 µM Forward primer	2
10 µM Reverse primer	2
AmpliTaq Gold®(Applied Biosystems)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	92	0.4	2
Annealing	60	0.4	
Extension	72	1.3	
Denaturation	92	0.4	2
Annealing	58	0.4	
Extension	72	1.3	
Denaturation	95	0.4	2
Annealing	56	0.4	
Extension	72	1.3	
Denaturation	95	0.4	2
Annealing	54	0.4	
Extension	72	1.3	
Denaturation	95	0.4	3
Annealing	52	0.4	
Extension	72	1.3	
Denaturation	95	0.4	30
Annealing	50	0.4	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

H2: Detection, nucleotide sequencing and analysis of thetoxin co-regulated pilus, *tcpA* gene

PCR primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)	PCR-positive control isolate(s)
tcpH1	AGCCGCCTAGATAGTCTGTG	*tcpA*	1 234	*V. cholerae* O1 ATCC9458
tcpA4	TCGCCTCCAATAATCCGAC			

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	18.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)P	2
20 µM Forward primer	0.5
20 µM Reverse primer	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	52	1.3	
Extension	72	2	
Final extension	72	7	1
Hold	4	∞	1

H3: Detectionand analysis of the Mobilome

Primer sets for multiplex PCR 1

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)
VSPIIintF	CCGACAAAGAATACACTCTCTCTGATGG	VSP-II integrase	170
VSPIIintR	ACGTCTTTTCCTTGCCTCGGCAAGAG		
VSPIIcutF	TTATCTACGACCACACCAGACAGC	Prototypical VSP-II	245
VSPIIcutR	ATGGGCATAGCAAAGGCACTTACCCA		
ICEdetF	TCAGTTAGCTGGCTCGATGCCAGG	SXT/R391 ICEs integrase	505
ICEdetR	GCAGTACAGACACTAGGCGCTCTG		
SXTdetF	ACTTGTCGAATACAACCGATCATGAGG	SXT Hotspot IV	357
SXTdetR	CAGCATCGGAAAATTGAGCTTCAAACTCG		
Ind5detF	TGCACATTGAGGCCCTGCAAGCAC	ICE*Vch*Ind5 Hotspot IV	423
Ind5detR	GTGCATTCACCAGCTCTAACGTCG		
Moz10detF	CGGAAGATGACGAAGACCGCCTAAGC	ICE*Vch*Moz10 Hotspot IV	712
Moz10detR	ATTTGCCTTCGAACAAAAGGGGCA		

Primer sets for multiplex PCR 2

Primer pair(s)	Primer sequence (5'to 3')	Target gene	Expected size of PCR product (~bp)
TLCdetF	AATCAACTCACGGGTGCAGACCTC	TLC phage	449
TLCdetR	TCCGCCAAGAAGTGACGTTGTAGC		
KdetF	CGTCCGTAACCTTAAAGATGGCAGC	Kappa phage	230
KdetR	TCGTATGTCCGTGAACTTGCCACC		
GI12detF	CTACGGTTGAGCCGCTCCATTTGTC	GI 12	571
GI12detR	GTGCCTTCTAAATTGACCAAACGCGGCA		
GI14detF	AGACGAGTATCTAGTAAACGCCAAACC	GI 14	142
GI14detR	CTTTGCTTGCACTGGCAACCTCAG		
GI15detF	CAGACCGCGAAGGAAAACGCTCTTTGC	GI 15	348
GI15detR	AGCGTCTCAGATGATGTCCGGCTG		

PCR cocktail mix for multiplex PCR 1 and for multiplex PCR 2

Reagent	Volume (µl) / reaction
Autoclaved distilled water	variable
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
20 µM Forward primer (For each primer)	0.5
20 µM Reverse primer (For each primer)	0.5
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions for multiplex PCR 1

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	59	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

PCR conditions for multiplex PCR 2

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	64.5	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

Appendix I: Azithromycin susceptibility testing

I1:Azithromycin (1 mg/ml stock)

10 mgazithromycin(Sigma)

Dissolve in 10 ml 95 % ethanol

Solution filtered through a filtered tip syringe and passed through 0.22 µm Millex®-GS filter unit (Millipore S.A.)

I2: Detection, nucleotide sequencing and analysis of macrolide resistance determinants

Primer sets

Primer pair(s)	Primer sequence (5'to 3')	Target	Expected size of PCR product(~bp)
mefF	TGTGCATATTTCTATTACG	*mefA*	324
mefR	CCAATTGGCATAGCAAG		
mefI	GCTGTGCAATAATGGGGC	Internal sequencing primer	
ereAF	GCCGGTGCTCATGAACTTGAG	*ereA*	420
ereAR	CGACTCTATTCGATCAGAGGC		
ereAI	TCACTGGCTAGAGCTAGTCTT	Internal sequencing primer	
ereBF	GCCTTGAAGCTATGGCTCC	*ereB*	303
ereBR	GGCCCATTGGTAGGCAAC		
ereBI	TTGGAGATACCCGAGTTGTAG	Internal sequencing primer	
ermBF	GAAAAGGTACTCAACCAAATA	*ermB*	639
ermBR	AGTAACGGTACTTAAATTGTTTAC		
ermBI	AGCCATGCGTCTGACATCTAT	Internal sequencing primer	
mphAF	GTGAGGAGGAGCTTCGCGAG	*mphA*	403
mphAR	TGCCGCAGGACTCGGAGGTC		
mphAI	TGCCGCAGGACTCGGAGGTC	Internal sequencing primer	
mphBF	TTAAACAAGTAATCGAGATAGC	*mphB*	889
mphBR	CCTTGTACTTCCAATGCTTG		
mphBI	GCGTATGGATGCAGTAAGAGC	Internal sequencing primer	
mphDF2	GCGGATCTCCTCCCAGAGTG	*mphD*	436
mphDR2	CTTCGGAAGCATTGGAGGCGC		
mphDI	GCGGATCTCCTCCCAGAGTG	Internal sequencing primer	

Primer sets forinternal positive control DNA constructs

Primer pair(s)	Primer sequence (5'to 3')	Expected size of PCR product (~bp)
mph(A)-eae-F	GTGAGGAGGAGCTTCGCGAGTCAATGCAGTTCCGTTATCAGTT	522
mph(A)-eae-R	TGCCGCAGGACTCGGAGGTCGTAAAGTCCGTTACCCCAACCTG	
mph(B)-eae-F	TTAAACAAGTAATCGAGATAGCTCAATGCAGTTCCGTTATCAGTT	524
mph(B)-eae-R	CCTTGTACTTCCAATGCTTGGTAAAGTCCGTTACCCCAACCTG	
ere(A)-eae-F	GCCGGTGCTCATGAACTTGAGTCAATGCAGTTCCGTTATCAGTT	524
ere(A)-eae-R	CGACTCTATTCGATCAGAGGCGTAAAGTCCGTTACCCCAACCTG	
ere(B)-eae-F	GCCTTGAAGCTATGGCTCCTCAATGCAGTTCCGTTATCAGTT	519
ere(B)-eae-R	GGCCCATTGGTAGGCAACGTAAAGTCCGTTACCCCAACCTG	
mph(D)-eae-F2	GCGGATCTCCTCCCAGAGTGTCAATGCAGTTCCGTTATCAGTT	523
mph(D)-eae-R2	CTTCGGAAGCATTGGAGGCGCGTAAAGTCCGTTACCCCAACCTG	

PCR cocktail mix

Reagent	Volume (µl) / reaction
Autoclaved distilled water	17.2
S-T Gold Buffer with 15 mM $MgCl_2$ (Southern Cross Biotechnology)	2.5
10 mMdNTP mix (Celtic Molecular Diagnostics)	2
10 µM Forward primer	1
10 µM Reverse primer	1
Super-Therm Gold (Southern Cross Biotechnology)	0.3
crude template DNA	1
Total reaction volume	25

PCR conditions

mefA and *ermB*

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	60	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

ereA, *ereB*, *mphA*, *mphB* and *mphD*

PCR Cycle	Temperature (°C)	Time (minutes)	# of cycles / reaction
Initial denaturation	95	10	1
Denaturation	95	1.3	35
Annealing	58	1.3	
Extension	72	1.3	
Final extension	72	7	1
Hold	4	∞	1

Appendix J: Plagiarism declaration with Turnitin report

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PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I **Husna Ismail** (Student number: **0201785A**) am a student registered for the degree of **Doctorate of Philosophy in Medicine** in the academic year **2013**.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

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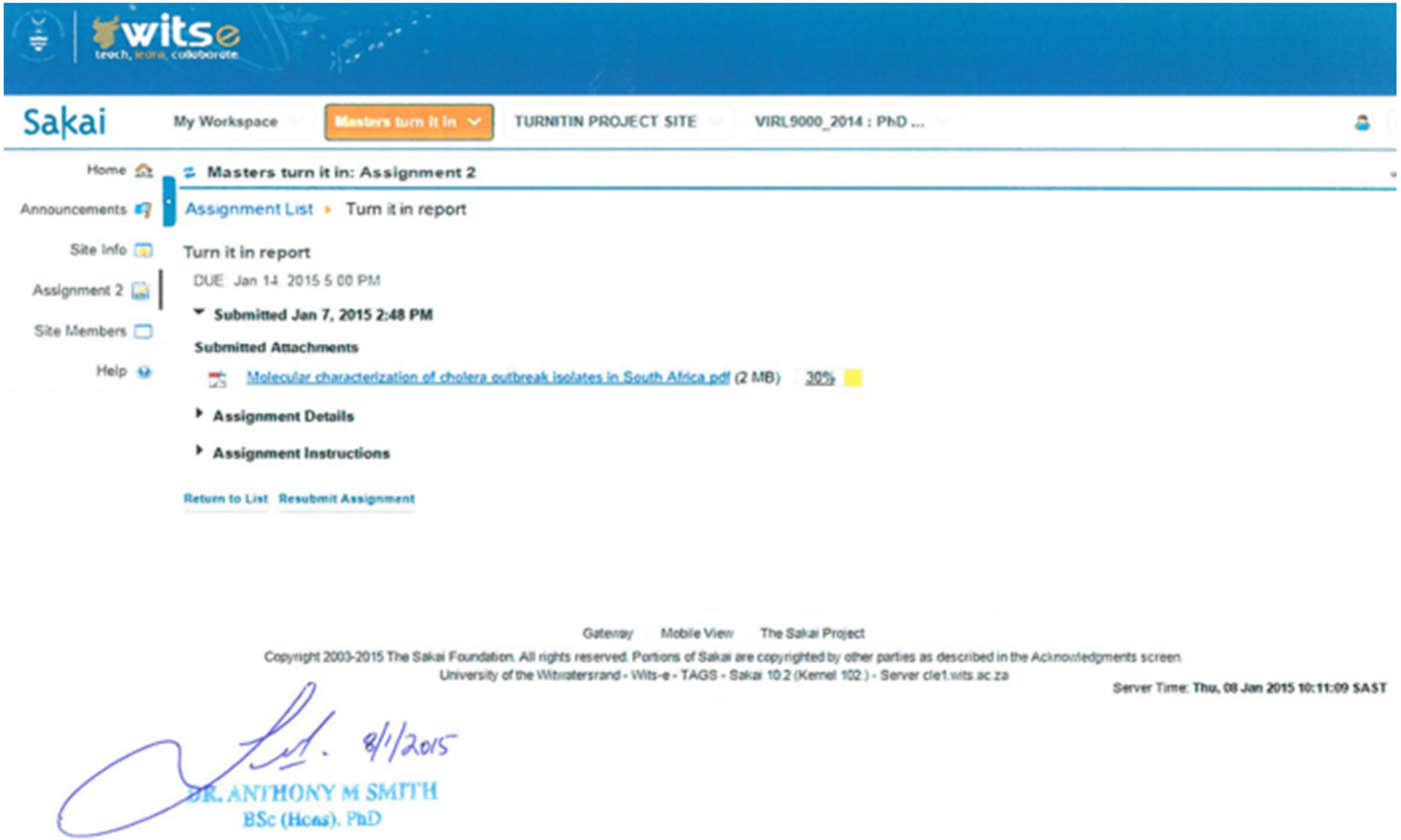

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