Molecular Epidemiology and Mechanism of Resistance of Invasive Quinolone-Resistant South African Isolates of *Salmonella enterica*, 2004-2006

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DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science (Dissertation) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in other University.

(Signature of candidate)

Day 5 of JANUARY 2009

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Abstract

The molecular epidemiology and mechanism of quinolone resistance of South African human isolates of *Salmonella* Typhi for the period 2003-2007, *Salmonella* Enteritidis, *Salmonella* Isangi and *Salmonella* Typhimurium for the period 2004-2006, received by the Enteric Diseases Reference Unit (EDRU) of the National Institute for Communicable Diseases was investigated. Molecular epidemiology was investigated using pulsed-field gel electrophoresis (PFGE) analysis for all four serotypes, as well as multiple-locus variable-number tandem-repeats analysis (MLVA) for *Salmonella* Typhi and *Salmonella* Typhimurium. Three probable mechanisms for quinolone resistance were investigated which included: amino acid mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase (*gyrA/gyrB*) and topoisomerase IV (*parC/parE*), active efflux of antibiotic out the bacterial cell and plasmid-mediated resistance encoded by *qnr* genes. For the period 2003-2007, 498 human isolates of *Salmonella* Typhi were received by the EDRU, of which 27 were resistant to nalidixic acid (MICs, ≥32 μg/ml). Only 19 *Salmonella* Typhi quinolone-resistant isolates were available for analysis. For the period 2004-2006, 329 human isolates of *Salmonella* Enteritidis, 1005 human isolates of *Salmonella* Isangi and 2624 human isolates of *Salmonella* Typhimurium were received by the EDRU. Of these isolates, 119 *Salmonella* Enteritidis, 143 *Salmonella* Isangi and 532 *Salmonella* Typhimurium were invasive, nalidixic acid-resistant. Only 116 *Salmonella* Enteritidis, 137 *Salmonella* Isangi and 516 *Salmonella* Typhimurium invasive, nalidixic acid-resistant isolates were available for analysis. For each respective serotype the isolates were genetically diverse as they could be differentiated into many

PFGE types, suggesting that quinolone-resistant strains have emerged independently of one another for all four serotypes. The use of MLVA for *Salmonella* Typhi and *Salmonella* Typhimurium also illustrated the genetic diversity of the isolates by differentiating the isolates in various MLVA types. The investigation into the contributory mechanisms of resistance showed that an over-active efflux system in combination with mutations in both *gyrA* and *parC* play a major role in facilitating quinolone resistance in *Salmonella* Typhi, *Salmonella* Enteritidis and *Salmonella* Isangi. These very same mechanisms were also found to be responsible for the quinolone resistance in the majority of the *Salmonella* Typhimurium isolates along with the rarely isolated mechanism of resistance, a *qnr* plasmid. This is the first report of any kind identifying the presence of *qnr* genes in South African Enterobacteriaceae isolates. Our study also highlights the need for further work to establish the link amongst the various mechanisms of resistance as their interactions remains unclear.

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Layout of Dissertation

This dissertation consists of a general introduction chapter (Chapter 1), followed by general materials and methods chapter (Chapter 2). The four subsequent chapters (Chapters 3 to 6) are separate results and discussion sections for the four separate study serotypes. These are then followed by a general discussion and conclusion chapter (Chapter 7). A common reference section is presented, followed by six appendices, the last two of which are manuscripts submitted for publication.

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Nomenclature

≈	**Almost equal to**
bp	**Base pair**
β	**Beta**
CDC	**Centres for Disease Control**
CHEF	**Contour-clamped homogenous electric field**
CLSI	**Clinical and Laboratory Standards Institute**
º	**Degree**
ºC	**Degree Celsius**
DMP	**Diagnostic Media Products**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleoside triphosphates**
EPI	**Efflux pump inhibitor**
EC	**Eastern Cape**
EDTA	**Disodium ethylenediaminetetra-acetic acid**
EDRU	**Enteric Diseases Reference Unit**
et al.	**And others**
=	**Equal to**
GA	**Gauteng**
≥	**Greater than or equal to**
H_2S	**Hydrogen sulphide**
HIV	**Human immunodeficiency virus**
kV	**Kilo-Volt**

KZN	**KwaZulu-Natal**
≤	**Less than or equals to**
M	**Mole**
$MgCl_2$	**Magnesium chloride**
MIC	**Minimum inhibitory concentration**
mℓ	**Milliliter**
mg	**Milligram**
µℓ	**Microliter**
µg	**Microgram**
µg/mℓ	**Microgram per milliliter**
mm	**Millimetre**
µM	**Micromolar**
MP	**Mpumalanga**
MLVA	**Multiple-Locus Variable-Number Tandem-Repeats Analysis**
NaCl	**Sodium chloride**
NICD	**National Institute for Communicable Diseases**
NTS	**Non-typhoidal *Salmonella***
NW	**North West**
O	**Outliers**
%	**Percent**
PCR	**Polymerase Chain Reaction**
PFGE	**Pulsed-Field Gel Electrophoresis**
pmol	**Picomol**

pH	**Percentage Hydrogen**
±	**Plus-minus**
PT	**Phage Typing**
QRDR	**Quinolone Resistance Determining Region**
RAPD	**Random amplified polymorphic DNA**
rpm	**Revolutions per minute**
TAE	**Tris-acetate-EDTA**
TBE	**Tris-borate-EDTA**
TE	**Tris-EDTA**
U	**Unit**
UPGMA	**Unweighted pair group method with arithmetic averages**
WP	**Western Cape**
w/v	**Weight to volume**
X	**Not analysed**

Chapter 1: Introduction

1.1 Background

Salmonella is the leading cause of food and water-borne disease worldwide, causing an estimated 16 million annual cases of gastroenteritis and/or enteric fever, resulting in five-hundred thousand deaths. The majority of these infections are among young children and elderly adults in both developing and developed countries, impacting both socially and economically. *Salmonella* has also been identified as an important opportunistic pathogen affecting those individuals with human immunodeficiency virus (HIV) infection (Edgeworth, 2005). The genus *Salmonella*, named after D.E. Salmon an American bacteriologist and veterinarian who studied animal pathogens, can be divided into two species *S. enterica* and *S. bongori*. *S. enterica* can be further divided into seven subspecies assigned by Roman numerals. *S. enterica* subspecies I contains over two-thousand described serotypes, which represent more than 99.5% of clinical isolates, are isolated from warm-blooded animals. The remaining six subspecies, I I, I I Ia, I I Ib, IV, V I and V I I, which represent less than 0.5% of clinical isolates, are isolated from cold-blooded animals. This organism is capable of large community outbreaks of food-borne illness as well as nosocomial outbreaks. *S. enterica* is spread via contaminated food products usually of animal origin resulting in gastroenteritis, while serotypes Typhi and Paratyphi, causing typhoid fever and paratyphoid fever respectively, are spread directly or indirectly from human faeces (Kim *et al.,* 2006).

1.2 Microbiology

S. enterica is a Gram-negative, facultative, rod-shaped bacterium in the same family as *Escherichia coli,* the family *Enterobacteriaceae,* commonly known as enteric bacteria. Most *Salmonella* strains are motile with peritrichous flagella, however, non-motile variants may occur occasionally. Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter. *Salmonella* ferment glucose, use citrate as their sole carbon source, produce H_2S during the fermentation process, are oxidase negative, reduce nitrates to nitrites and do not require sodium chloride (NaCl) to grow. *Salmonella* grow at temperatures between 8 and 45 degrees Celsius (ºC) and in a percentage hydrogen (pH) range of 4-9. *Salmonella* are heat sensitive and are usually killed at temperatures in of 70ºC (Tindall *et al.,* 2005). Although the principal habitat of the *Salmonella* is the intestinal tract of humans and animals, *Salmonella* has been isolated from contaminated environmental sources such as soil. Such sources support the growth of *Salmonella* if the aforementioned conditions are favourable. The Gram-negative *S. enterica* cell wall contains a thin peptidoglycan layer adjacent to the cytoplasmic membrane. In addition to the peptidoglycan layer, the Gram-negative cell wall also contains an additional outer membrane composed by phospholipids and lipopolysaccharides which face the external environment. The highly charged lipopolysaccharides translate a negative charge to the Gram-negative cell wall. The chemical structure of the outer membrane lipopolysaccharides is often unique to specific bacterial strains and is responsible for many of the antigenic properties of these strains (Gootz, 2006).

This antigenicity of *S. enterica* has aided with diagnostic or identifying applications. A serotyping application termed the Kauffman-White uses the three main antigenic factors to determine the isolates serotype phenotypically. Serotypes are identified using specific anti-sera to elicit an agglutination of highly specific somatic or cell wall antigens (O) which are heat stable and alcohol resistant, flagellar antigens (H) which are heat labile proteins and surface or envelope antigens (Vi) which only occur in three serotypes, Typhi, Paratyphi C and Dublin. A given serotype will contain a specific combination of O and H antigens, with possibly the occurrence of the Vi antigen (Kaufmann, 1966).

1.3 Pathogenesis

Salmonella infections occur when a pathogenic strain is ingested through contaminated source. *Salmonella* begin infection by systematically surviving the acidic pH of the stomach, which is dependant on the infectious dose, competing with the normal flora of the small intestine and then translocating across the intestinal mucosa to propagate in the antigen-sampling M cells of the Peyer's patch as well as the draining mesenteric lymph nodes. The pathogenic effect of invading *Salmonella* on M cells is rapid post-infection as significant damage to the intestinal epithelium is observed. This includes the invasion of enterocytes, sloughing of large sections of epithelial cells and the penetration of large numbers of bacteria into underlying tissues. The diarrhoeal symptoms result from the inflammatory reaction that has been elicited in the small intestine due to the presence of invading bacteria. The presence of invading *Salmonella* causes host responses in the form of increasing the number of M cells, increasing the number of CD4+ cells, decreasing the number of CD8+ cells and activating the humoral and cellular divisions of the immune

system. The invading organisms enter the lymphatic system and interact with leukocytes which endeavour to kill the internalised bacteria using oxygen-independent mechanisms. *Salmonella* that survive disseminate via monocytes through the lymphatic system to the thoracid duct into the blood and finally to host sites such as the liver and bone marrow, from where disease symptoms may manifest as an invasive infection (Santos *et al.,* 2003; Zhang *et al.,* 2003; Shakespeare *et al.,* 2005; Srikanth & Cherayil, 2007).

1.4 Clinical Presentation

Infections caused by *Salmonella* may result in gastroenteritis, bacteraemia, local infections, chronic carrier state or enteric fever. The incubation period is approximately 6-72 hours and diagnosis is made by isolating the organism from a food source or from the patient's stool. Gastroenteritis is characterised by abdominal pain and loose, watery stools, low-grade fever and varying severity of nausea and vomiting (Bhan, *et al*., 2005). Bacteraemia is a manifestation of disease characterised by a prolonged fever and a positive blood culture, with symptoms of gastroenteritis occasionally present. *Salmonella* organisms can also infect any anatomical site producing local infections such as abscesses independently of any systemic illness (Shakespeare *et al.,* 2005). Although an individual may recover from a *Salmonella* infection they may still excrete the causative organism in their faeces for up to year. This condition is called the carrier state and is especially prominent in typhoid fever. Enteric fever which is the most severe of all the types of clinical presentations is caused primarily by human specific serotypes, *Salmonella enterica* serotype Typhi, but can also be caused by *Salmonella enterica* serotype Paratyphi A, *Salmonella enterica* serotype Paratyphi B and *Salmonella enterica*

serotype Paratyphi C. Illness is characterized by prolonged fever, rose spots and malaise (Bhan, *et al*., 2005).

1.5 Prevention

Salmonella infections can be prevented by limiting the contact of humans to contaminated food and water. Municipalities should ensure adequate sewage disposal system and clean water supply. Education plays a big role in stemming the spread of infectious disease. The practice of good personal hygiene, good food hygiene and the eradication of the chronic carrier state will decrease the incidence of *Salmonella* infections (Le *et al.,* 2004). Although vaccines for non-typhoidal *Salmonella* infections are still being assessed, enteric fever due to *Salmonella* Typhi can be prevented by immunisation. Vaccines are available in three variants and may confer immunity from infection for varying degrees of time ranging from 2-5 years. These vaccines if administered in areas of where the burden of disease is high (1000 infections per 100 000 of the population) could help decrease the incidence of illness (Connor & Schwartz*,* 2005).

1.6 Treatment

A varying number of antibiotics are used to treat infections due to *Salmonella*. Ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftriaxone and azithromycin are the first choice antibiotics used to treat salmonellosis, but the fluoroquinolone ciprofloxacin has become the popular choice for treatment of invasive infection and enteric fever (Bhan *et al.,* 2005). Treatment of gastroenteritis due to

Salmonella is not essential, as the antimicrobial therapy does not seem to decrease the duration or severity of the gastroenteritis. Rehydration with electrolytes and fluids is the recommended treatment in non-invasive or where enteric fever has not been diagnosed (Bertrand *et al.*, 2006). Although ciprofloxacin is the treatment of choice for invasive disease and enteric fever it has been suggested that treatment with a combination of antibiotics such as ciprofloxacin and cefazolin may be useful in combating infection caused by antibiotic-resistant *Salmonella* strains (Mandal *et al.,* 2004).

1.7 Quinolone Antibiotic Resistance

The prototype for modern day quinolones, nalidixic acid, was discovered in 1962 as a by product of the formation of the anti-malarial drug chloroquine. The 1980s brought about the synthesis of the second-generation quinolones, the fluoroquinolones, including ciprofloxacin and levofloxacin (Zhanel *et al.,* 2004). Quinolones are bactericidal and exhibit concentration-dependent killing. Quinolones enter bacteria through porins or directly through the lipid and cytoplasmic membrane (Nordmann & Poirel, 2005). The targets of quinolone activity are the bacterial deoxyribose nucleic acid (DNA) gyrase and topoisomerase IV, enzymes essential for DNA replication and transcription. Antibiotics are critical in the management of typhoid fever. Various fluoroquinolones such as ciprofloxacin have become the routine treatment for typhoid fever. Internationally there has been a disturbing trend noted of increasing resistance to the quinolones and decreased susceptibility to fluoroquinolones in salmonellosis (Paterson, 2006). This resistance is thought to be driven by the selective pressure of exposure of the organism to antimicrobial agents during therapy. Resistance to quinolones limits drug selection for

treatment of infections as the organisms resistant to quinolones often are resistant to other classes of antimicrobials, either as a result of stepwise evolution of resistance mutations in target genes or due to the selection of an over-active efflux pump in combination with decreased outer membrane permeability (Giraud *et al.,* 2006), which makes the management of infection due *Salmonella*, in particular *Salmonella* Typhi due its clinical implications, all the more difficult (Butt *et al.,* 2003).

Three major mechanisms have been described for the development of quinolone-resistance in *Salmonella* (Jacoby, 2005). The first mechanism involves amino acid mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*) (Eaves *et al*., 2002; 2004): DNA gyrase is the primary target of quinolone antibiotics. Amino acid mutations in the QRDR of *gyrA* results in resistance to the non-fluorinated quinolone, nalidixic acid, while also resulting in reduced susceptibility to fluoroquinolones such as ciprofloxacin. Amino acid mutations at Ser-83 (to Phe, Tyr, or Ala) or at Asp-87 (to Gly, Asn, or Tyr) are the most frequently observed mutations in *gyrA*. Additional amino acid mutations in the QRDRs of *parC*, *gyrB*, and *parE* proteins, results in resistance to fluoroquinolones such as ciprofloxacin (Eaves *et al.,* 2004; Hopkins *et al.,* 2005). Although it is widely believed that quinolone resistance evolves in a stepwise manner as a result of initial mutation in the DNA gyrase genes, followed by mutations in the topoisomerase genes (Nordmann & Poirel, 2005), there are contrary reports that suggest that for *Salmonella* efflux mechanisms are primarily selected (Giraud *et al.,* 2006).

The second mechanism for quinolone resistance in *Salmonella* is the active efflux of antibiotic from the bacterial cell. Efflux pumps are naturally present in bacteria and there function is to eliminate toxic compounds from the bacterial cell. Overproduction of efflux pumps results in removal of quinolones (and other antibiotics) from bacterial cells and contributes to the development of resistance (Saenz *et al.,* 2004). The AcrAB-TolC efflux system is a major player with regards to quinolone resistance in *S. enterica*. AcrAB is a member of the resistance nodulation cell division family of transporters and is encoded by *acrAB*. The pump has three components: a transporter protein in the inner membrane (AcrB), a periplasmic accessory protein (AcrA), and an outer membrane channel (TolC). AcrB captures its substrates within the phospholipid bilayer and transports them into the external medium via TolC. Cooperation between AcrB and TolC is mediated by the periplasmic protein AcrA (Baucheron *et al.,* 2002; Hopkins *et al.,* 2005; Olliver *et al.,* 2005). Alterations in the outer membrane protein and lipopolysaccharide profiles of resistant *Salmonella* have been reported as the possible cause of decreased permeability of the outer membrane and in combination with an active efflux may result in a subsequent decreased accumulation of antibiotic (Giraud *et al.,* 2000). The involvement of the AcrAB-TolC efflux system in the development of quinolone resistance has been proven qualitatively through studies which have taken quinolone-resistant strains, inactivated their efflux systems, and then showed a resultant decrease in resistance (Chu *et al.,* 2005). Inactivation of efflux systems have been performed through inactivation of genes coding for the efflux and through the use of efflux pump inhibitors such as β-*Phe-Arg-naphthylamide* (Saenz *et al.,* 2004). Other researchers have shown the effect of an increased expression AcrA on the efflux system in fluoroquinolone-resistant

isolates using quantitative methods such as Western-blotting (Morgan-Linnell *et al.,* 2009).

The third mechanism described for the development of quinolone resistance in *Salmonella* is that of plasmid mediated quinolone resistance. Three genes have been identified as those responsible for conferring resistance to quinolones. The pentapeptide repeat gene *qnrA* (Jacoby *et al.,* 2003) is responsible for encoding a protein that protects DNA gyrase from inhibition by quinolones. The other pentapeptide repeat genes *qnrB* (Jacoby *et al.,* 2006) and *qnrS* (Kehrenberg *et al.,* 2006; Hopkins *et al.,* 2007) have also been associated with conferring resistance to quinolones. The first *qnr* gene was isolated in 1998 from a *Klebsiella pneumoniae* isolate from Birmingham, Alabama, United States of America, and was called *qnr*A (Martinez-Martinez *et al.,* 1998). *Shewanella algae*, an environmental species from marine and fresh water, was identified as its reservoir (Poirel *et al.,* 2005). *qnr* genes have been found in various bacteria worldwide which include; *Citrobacter freundii*, *Enterobacter* species, *Escherichia coli*, *Klebsiella pneumoniae* and *Providencia stuartii* in the United States (Jacoby *et al*., 2003), *Salmonella* species in the United Kingdom (Hopkins *et al.,* 2007) and *Shigella flexneri* in Japan (Hata *et al.,* 2005).

1.8 Incidence of Quinolone-Resistant *Salmonella*

Quinolone-resistant *Salmonella* has become a global burden with various countries reporting high rates of resistance to quinolones as well as reduced susceptibility to fluoroquinolones. In 1997 Herikstad *et al.* reported on the emergence of quinolone resistant *Salmonella* in the United States of America. Since then a number of reports have

surfaced of quinolone-resistant as well as multi-drug-resistant isolates. A report from Turkey where a random sample of a total number of 73 from various serotypes of *Salmonella* produced a quinolone resistance rate of 12.3% (Albayrak *et al.,* 2004). From a sample of 261 non-typhoidal *Salmonella* (NTS) a Korean study reported a 1.8% increase in quinolone resistance to an overall 21.8% (Choi *et al.,* 2005). A similar Taiwanese study reported a quinolone resistance rate of 69% (Su *et al.,* 2004). National surveillance in Denmark revealed that over a five year period that the incidence of quinolone-resistant *Salmonella enterica* serotype Enteritidis had increased from 0.8% to 8.5% (Mølbak *et al.*, 2002) A retrospective study on 744 typhoid case isolates from Nigeria highlighted a 20% ciprofloxacin resistance rate, which is of concern considering that ciprofloxacin is the drug of choice for treatment of typhoid or enteric fever (Doughari *et al.* 2007). Butt *et al.* (2003) reported a case of ciprofloxacin treatment failure in Pakistan. Renuka *et al*. (2005) reported on high-level ciprofloxacin resistance in *Salmonella enterica* serotype Typhi in India, questioning whether or not fluoroquinolones should be considered as the first line of treatment for enteric fever. A study concentrating on invasive isolates for the years 1997-2001 from Northern India suggested that multi-drug resistant isolates were on the increase (Gautam *et al.*, 2002). These infections may be locally acquired or imported highlighting the global need for reviewed treatment strategies for salmonellosis. In South Africa nalidixic acid resistance incidence rates have consistently remained around 23% for all *Salmonella* serotypes and resistance to the fluoroquinolone ciprofloxacin around 1% (Unpublished).

1.9 Molecular Typing of *Salmonella* Species

Phenotypic typing based on biochemical and serological properties is the traditional method being used to differentiate between strains but current typing methods are based on the characterisation of the genetic traits of the organism by analysis of chromosomal or plasmid DNA. The use of pulsed-field gel electrophoresis (PFGE) analysis as a basis of identification of clones in *Salmonella*, is accepted as the gold-standard for typing *Salmonella* and has proven to be highly discriminatory when applied to most serotypes (Liu *et al.,* 2003). PFGE is helpful for the investigation of clonal relationships between and within serotypes (Ribot *et al.,* 2006).

PFGE differs from standard gel electrophoresis in the following respects. Standard gel electrophoresis employs a uniform electrical field in one direction which allows separation of DNA fragments up to 50000 base pairs (bp) in size. For DNA fragments >50000bp, the sieving action of agarose or polyacrylamide gel is lost because the DNA fragments will then not enter the gel matrix or just run as an resolved smear. PFGE allows one to overcome this problem and separate very large DNA fragments. This is achieved by employing an electrical field that regularly changes direction, called a contour-clamped homogenous electric field, throughout an agarose gel run. When such a pulsing electrical field is applied, DNA molecules move through the pores in a snake-like fashion, facilitating the separation of large DNA fragments. The choice of restriction enzyme for digestion of genomic DNA into several DNA fragments is very important. Rare cutting restriction enzymes are used to produce a limited number of large fragments. Usually a single enzyme is used, as a combination of enzymes can produce too many

small fragments. Usually enzymes with 6 to 8bp recognition sequences are used, as they will cut less frequently compared to enzymes with shorter base-pair recognition sequences. For PFGE analysis, bacterial cells are first immobilized in agarose blocks. This is followed by the digestion of DNA, all occurring within the confines of an agarose block which protects the genomic DNA from any random breaks. This ensures that the resulting DNA fragmentation pattern is the direct result of the occurrence of the digestion of the genomic DNA with the restriction enzyme (Reed *et al.,* 2003).

Other methods such as Phage typing (PT) is a classical method traditionally used for subtype determination of *Salmonella* but has limited discriminatory power and requires specialized phage collections that are available to only a few reference laboratories. Plasmid profiling, single-enzyme ribotyping, and random amplified polymorphic DNA (RAPD) analysis also have limited discriminatory power for *Salmonella* (Kim *et al.,* 2006). Like PFGE, two-enzyme ribotyping has been shown to have a greater discriminatory power but just like PFGE is also labour-intensive (Liu *et al.,* 2003). A relatively newer genotypic typing method called multiple-locus variable-number tandem-repeats (VNTRs) analysis (MLVA) has been established for a number of *Salmonella* serotypes (Lindstedt *et al.,* 2003; Liu *et al.,* 2003; Boxrud *et al.,* 2007). MLVA is a multiplex polymerase chain reaction (PCR) based typing method designed to analyse fragment sizes which contain a variable number of tandem repeat sequences. This method is rapid and highly reproducible with greater discriminatory power (Lindstedt, 2005).

The MLVA technique was made possible through genome sequencing projects which revealed that a high percentage of microbial genomic DNA consists of repeats, where DNA motifs exist in multiple copies (Lindstedt *et al.,* 2003). These repeats are referred to as variable-number tandem-repeats (VNTRs). They are located throughout the genome of bacteria and consist of short nucleotide sequences that are repeated in tandem. Individual strains within a bacterial species often maintain the same sequence element but with different copy numbers. The variation in copy number is caused by slipped-strand mispairing during DNA replication. Since sequence homology exists between strains in the flanking region of the VNTR locus, universal PCR primers can be used to amplify the locus from all strains of a particular species. Variations in copy number of a repeat sequence among individual strains can then be translated into different sized PCR products. To apply MLVA as a genotypic tool, one needs to analyze multiple VNTR loci, of which 3 to 8 loci are typically analyzed. Each VNTR locus is targeted by a specific pair of PCR primers. All the primer pairs are usually combined into a single multiplex-PCR, resulting in the amplification of multiple products. These products are then electrophoretically size separated to produce a DNA fingerprint pattern. Each individual strain of bacteria will produce a unique pattern. The conventional method of MLVA employs agarose gel electrophoresis and ethidium bromide staining to visualize a DNA banding pattern, followed by capturing an image of the patterns (Lindstedt *et al.,* 2003).

Automated MLVA employs capillary electrophoresis of fluorescently labeled PCR products. The labeling of PCR product occurs via PCR primers which are labeled with distinctive fluorescent dyes (fluorophores). Numerous fluorophores exist, so each VNTR

locus can be labeled with a unique fluorophore. This allows detection and classification of multiple PCR products in a single reaction. Capillary electrophoresis of PCR products is performed in the same genetic analyzers which perform automated DNA sequencing. The electrophoresis process moves PCR products past a laser beam which excites the fluorophores, releasing light of distinctive colours (fluorescence) which are detected and translated into detection of specific PCR products. PCR products are also automatically sized via comparison to internal size standards. MLVA results are reported as a MLVA allele profile. For example, where 5 VNTR loci are analyzed, a strain could be defined by the MLVA allele profile '254-330-422-188-550', where the values represent the size (in base pairs) of the 5 loci (PCR products) (Lindstedt, 2005). Although MLVA is an advance in terms of genotyping, a combination of typing methods or the use of one method to supplement the other is seen as the best practise to achieving an accurate result (Liu *et al.,* 2003).

1.10 Aim and Objectives of the Study

The aim of the research presented in this dissertation was to describe the mechanism of resistance and molecular epidemiology of invasive, quinolone-resistant non-typhoidal *Salmonella enterica* serotypes Typhimurium, Enteritidis and Isangi causing infections in South Africa for the years 2004-2006 and the typhoid causing *Salmonella enterica* serotype Typhi isolates causing infections in South Africa for the years 2003-2007. The objectives were as follows:

- To investigate strain relatedness and cluster formation by PFGE and MLVA for each of the above *Salmonella enterica* serotypes;

• To investigate the presence and contribution of amino acid mutations in the QRDR to quinolone resistance in the above *Salmonella* serotypes by PCR and nucleotide sequencing;

• To investigate the involvement of the phenotypic expression of an over-active efflux pump system in quinolone resistance in the above *Salmonella* serotypes, by means of doubling agar dilution minimum inhibitory concentration (MIC) test in the presence of β-*Phe-Arg-naphthylamide,* an efflux pump inhibitor ;

• To investigate the involvement of plasmid mediated quinolone resistance through the identification and characterization of the *qnr* genes by PCR and nucleotide sequencing.

Chapter 2: Materials and Methods

2.1 Bacterial Isolates

Three-thousand and fifteen invasive *Salmonella* isolates were received at the Enteric Diseases Reference Unit (EDRU) of the National Institute for Communicable Diseases (NICD), of the National Health Laboratory Service, Sandringham, South Africa, between 2004 and 2006 as part of ongoing surveillance for salmonellosis. An invasive isolate was deemed one that had been isolated from a normally sterile site (e.g. blood culture, cerebrospinal fluid, pleural fluid or joint fluid) from patients admitted to hospitals throughout South Africa. Of the three-thousand and fifteen invasive isolates, eight hundred and sixty-two isolates were resistant to the quinolone antibiotic, nalidixic acid, (MIC, ≥32 μg/mℓ) as determined by EDRU staff using Etest® strips (AB Biodisk, Solna, Sweden).

Four *Salmonella* serotypes contributed to the majority (818/862; ≈ 94%) of the invasive, nalidixic acid-resistant isolates received by the EDRU from 2004-2006. *Salmonella* Typhi contributed seventeen isolates, to which isolates from years 2003 and 2007 were added for the purpose of this study to result in a total of twenty-seven *Salmonella* Typhi isolates. Only nineteen of the twenty-seven *Salmonella* Typhi quinolone-resistant isolates were available for analysis. The NTS *Salmonella* isolates were dominated by three serotypes, *Salmonella* Enteritidis (329), *Salmonella* Isangi (1005) and *Salmonella* Typhimurium (2624). Most isolates (667/818; ≈ 82%) were received from hospitals in

and around the province of Gauteng with the remaining isolates (≈ 18%) received from hospitals from other provinces in South Africa. For the period 2004-2006, three-hundred and twenty-nine human isolates of *Salmonella* Enteritidis, one-thousand and five human isolates of *Salmonella* Isangi and two-thousand six-hundred and twenty-four human isolates of *Salmonella* Typhimurium were received by the EDRU. Of these isolates, *Salmonella* Enteritidis contributed one-hundred and nineteen, *Salmonella* Isangi one-hundred and forty-three, and *Salmonella* Typhimurium five-hundred and thirty-two isolates, for a total of seven-hundred and ninety-four invasive, nalidixic acid-resistant isolates. Only one-hundred and sixteen *Salmonella* Enteritidis, one-hundred and thirty-seven *Salmonella* Isangi and five-hundred and sixteen *Salmonella* Typhimurium invasive, nalidixic acid-resistant isolates were available for analysis. Due to time and financial constraints all molecular analysis could not be performed on every isolate. Pulsed-field gel electrophoresis was performed on all available isolates but for the subsequent test each isolate was assigned a number and a random number table was used to randomly select isolates for further analysis.

2.2 Pulsed-Field Gel Electrophoresis (PFGE)

Salmonella embedded in agarose blocks were prepared using a previously described procedure with some adaptations (Ribot *et al.,* 2006). Bacterial cultures were grown overnight on 5% Sheep blood agar plates [Diagnostic Media Products, National Health Laboratory Service, Johannesburg, South Africa (DMP)] and incubated at 37ºC. A third of the bacterial culture was resuspended into 800µl of cell suspension buffer (Appendix

A) and adjusted to a cell concentration turbidity of ≈ 0.7 using Microscan Turbidity Meter (Dade Behring, California, USA). Proteinase-K (Appendix A), 20µl, was added to 200µl of the bacterial suspension, gently mixed and incubated at 37ºC for 5 minutes. Two hundred and eighty microliters of 1% SeaKem Gold® (Cambrex Bio Science, Rockland, USA): 1% sodium dodecyl sulphate (SDS) (Appendix A) was mixed with bacterial suspension, and immediately poured into reusable plug moulds (Bio-Rad Laboratories, California, USA) and allowed to solidify (≈10 minutes). To lyse the bacterial cells, the agarose plugs were removed from the moulds and transferred to an appropriately labeled tube containing 5ml cell lysis buffer (Appendix A). The tubes containing the agarose plugs were incubated for 2 hours in a 55°C shaker water bath with constant agitation (70rpm). To wash the plugs, the tubes were removed from the water bath and the lysis buffer decanted. The plugs were washed at 50°C in the water bath, in 15ml sterile deionized water (preheated to 50°C). The water was decanted and followed by four washes with sterile TE (Appendix A) buffer at 50°C.

For bacterial DNA digestion, the plug was removed from the TE buffer and thin (1-2mm) slices of the agarose plugs were cut and added to a new tube containing restriction enzyme buffer called H-buffer (Roche Diagnostics, GmbH, Mannheim, Germany). The plugs were incubated at 37°C for 15 minutes. The H-buffer was removed and 30U/sample *Xba*I (Roche Diagnostics GmbH, Mannheim, Germany) restriction enzyme added and incubated for three hours at 37°C. The restriction enzyme was removed from the tubes and 0.5X TBE buffer (Appendix A) was added to the plugs and incubated for 5 minutes at room temperature. The plugs were removed from the TBE buffer, loaded onto

the bottom of the comb teeth and allowed to air dry for 5-10 minutes. The reference strain CDC-H9812 *Salmonella enterica* Branderup (*Salmonella* Branderup) was included on all gels as a method of quality control and validation (Hunter *et al.,* 2005). Plugs were attached onto a comb, placed in a gel casting tray and allowed to air-dry for 15 minutes before pouring 1% SeaKem Gold® agarose (Appendix A) into the casting tray and allowing to solidify (≈ 25 minutes). The gel was then loaded into the electrophoresis chamber of a contour-clamped homogenous electric field (CHEF-DR) apparatus (Bio-Rad) containing 0.5X TBE (± 3 liters) cooled to a temperature of 12°C and subjected to the following electrophoresis conditions for a run-time of 21 hours:

CHEF-DR II: Initial A time 2.2 seconds, Final A time 63.8 seconds, Start ratio 1.0, Voltage 200 volts. CHEF-DR III: Initial A time 2.2 seconds, Final A time 63.8 seconds, Start ratio 1.0, Voltage 6 volts/centimeter, Angle included 120°.

On completion of electrophoresis the gel was stained using an ethidium bromide solution, (Appendix A) for 30 minutes, gently agitated (12-15rpm) on a Sea-Saw Rocker™ (Stuart Scientic, Stone, Staffordshire, United Kingdom). The staining was followed by a destaining in 500ml deionized water for 20 minutes. The image was captured on the Gel Doc Quantity One documentation system (Bio-Rad) and saved as an uncompressed TIFF image (*.tif) for analysis with the BioNumerics™ (version 5.1) software program (Applied Maths, Sint-Martens-Latem, Belgium). All test patterns were normalized against the pattern of the *Salmonella* Braenderup reference standard. Cluster analysis of the patterns using the unweighted pair group method with arithmetic averages (UPGMA) resulted in the calculation of hierarchical tree-like structures (dendrograms), with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5%

and a position tolerance setting of 1.5%. Seperate dendrograms were created for all the test isolates from each serotype that being, *Salmonella* Enteritidis, *Salmonella* Isangi, *Salmonella* Typhi and *Salmonella* Typhimurium. A PFGE type was defined as a unique PFGE pattern at 90% on the dendrogram. A PFGE cluster was defined as a group of two or more patterns at ≥90% similarity on the dendrogram. A PFGE type may itself be a cluster.

2.3 Preparation of Crude Bacterial DNA

A small loopful of bacteria (2 to 4 colonies) cultured on 5% sheep blood agar (DMP) was resuspended into 400µl of TE and boiled at 95°C for 20 minutes. The suspension was then centrifuged at 12000 rpm for 3 minutes and the resulting supernatant (crude DNA preparation) was used as a template for PCR.

2.4. Multiple-Locus Variable-Number Tandem-Repeats Analysis (MLVA)

2.4.1 MLVA: *Salmonella* Typhi

To supplement the *Salmonella* Typhi PFGE outputs a second genotypic analysis method, MLVA was used to further analyze selected strains. MLVA was based on three VNTR gene loci (TR1, TR2 and TR3) as previously described (Liu, *et al.* 2003). Analysis of the selected strains employed the previously described method with a few adaptations. The previously described method utilized manual agarose gel electrophoresis analysis;

whereas this revised analysis method incorporated automated capillary electrophoresis of fluorescently labelled PCR products. A positive control *Salmonella* Typhi strain NCTC8385 was used a reference. The makeup of PCR primers used to amplify VNTR loci are shown in Appendix B1. The forward primer for each locus was labelled with a distinctive fluorescent dye (Applied Biosystems, Foster City, CA, USA). The reverse primer for each locus was unlabelled (Inqaba Biotechnical Industries, Hatfield, South Africa). Each VNTR locus was amplified in a separate PCR of 25µl final volume containing 1µl crude bacterial DNA, 2mM $MgCl_2$, 0.5µM of each primer, 200µM deoxynucleotide triphosphates (Bioline, London, United Kingdom), 1 U Super-Therm Gold DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa) and 1× Super-Therm Gold DNA polymerase buffer (Southern Cross Biotechnology). Thermal cycling was conducted using an i-Cycler (Bio-Rad) thermal cycler using the following run parameters: hot start at 95°C proceeding to 95°C for 2 minutes , 25 cycles at 95°C for 75 seconds, 55°C for 75 seconds and 72°C for 75 seconds, with a final holding step at 4°C. The three resultant PCR products were pooled as follows: 2µl of TR1 + 2µl of TR2 + 6µl of TR3. This pooled mixture was then diluted 1:40 in deionized water. Two microliters of this diluted mixture was then mixed with 0.7µl of GeneScan 600 LIZ size standard (Applied Biosystems) and 7.5µl of Hi-Di formamide (Applied Biosystems). This 10µl mixture was then incubated at 95°C for 3 minutes and cooled to room temperature before being subjected to capillary electrophoresis using an Applied Biosystems 3130 Genetic Analyzer. Electrophoresis was performed through POP-7 polymer (Applied Biosystems) at a running voltage of 15 kV for 25 minutes at a temperature of 60°C and an injection voltage of 15 kV for 8 seconds. Raw data was

captured and analyzed using GeneMapper (version 4.0) software (Applied Biosystems) which identified each VNTR locus by its distinctive colour (fluorescence) and automatically sized the gene product via comparison to the internal size standard. The resulting fragment sizes were binned into alleles, entered into BioNumerics™ (version 5.1) software program (Applied Maths) and a dendrogram constructed using the Euclidean distance-coefficient and the Ward algorithm. A MLVA type was defined by a unique MLVA allelic profile. For example MLVA type 23 represented the MLVA allele profile 234-443-566 which defined a 234bp PCR product for TR1, a 443bp PCR product for TR2 and a 566bp PCR product for TR3.

2.4.2 MLVA: *Salmonella* Typhimurium

To supplement the *Salmonella* Typhimurium PFGE outputs a second genotypic analysis method, MLVA was used to further analyze selected strains. MLVA was based on five VNTR gene loci (STTR3, STTR5, STTR6, STTR9 and STTR10-pl) as previously described (Lindstedt, *et al.* 2003). Analysis of the selected strains employed the previously described method (Lindstedt, *et al.* 2004) with a few alterations. This analysis method incorporated automated capillary electrophoresis of fluorescently labelled PCR products. The makeup of PCR primers used to amplify VNTR loci are shown in Appendix B2. The forward primer for each locus was labelled with a distinctive fluorescent dye (Applied Biosystems). The reverse primer for each locus was unlabelled (Inqaba Biotechnical Industries). The primers were multiplexed in two solutions using the Qiagen PCR multiplex Kit (Southern Cross Biotechnology) in a total of 50µl as per the

manufacturer's instructions. Multiplex primer mix 1 (M1) consisted of: 10 pico mol (pmol) of the STTR6 and STTR3 primer pairs and Multiplex primer mix 2 (M2) consisted of: 10 pmol of the STTR9, STTR5, STTR10 primer pairs. Thermal cycling was conducted for both multiplex solutions using an i-Cycler (Bio-Rad) thermal cycler, under the same cycling conditions of 95°C for 15 minutes, proceeding to 25 cycles of 94°C for 30 seconds, 63°C for 90 seconds and 72°C for 90 seconds and a final holding step at 72 °C for 10 min. Prior to electrophoresis the two separate PCR products were pooled in the following manner: 10 µl of M1+2.5 µl of M2 were mixed and 87.5µl of deionized water was added to result in a total of 100 µl. Then 1µl of the pooled solution was mixed with 0.7µl of GeneScan 600 LIZ size standard (Applied Biosystems) and 12µl of Hi-Di formamide (Applied Biosystems). This ≈14µl mixture was then incubated at 95°C for 3 minutes and cooled to room temperature before being subjected to capillary electrophoresis using an Applied Biosystems 3130 Genetic Analyzer. Electrophoresis was performed through POP-7 polymer (Applied Biosystems) at a running voltage of 15 kV for 35 minutes at a temperature of 60°C and an injection voltage of 15 kV for 5 seconds. Raw data was captured and analyzed using GeneMapper (version 4.0) software (Applied Biosystems) which identified each VNTR locus by its distinctive colour (fluorescence) and automatically sized the gene product via comparison to the internal size standard. The resulting fragment sizes were binned into alleles according to their repeat sizes, entered into BioNumerics™ (version 5.1) software program (Applied Maths) and a dendrogram constructed using the Euclidean distance-coefficient and the Ward algorithm. A MLVA type was defined by a unique MLVA allelic profile. For example MLVA type E represented the MLVA allele profile 160-248-342-355-390

which defined a 160bp PCR product for STTR9, a 248bp PCR product for STTR5, a 342bp PCR product for STTR6, a 355bp PCR product for STTR10-pl and a 390bp PCR product for STTR3.

2.5 Polymerase Chain Reaction (PCR) and Sequencing

2.5.1 Quinolone Resistance Determining Region (QRDR)

PCR was used to amplify the QRDR of *gyrA, gyrB, parC* and *parE* using previously described methods (Eaves *et al.,* 2004). Primers (Appendix C1) were synthesized by Inqaba Biotechnical Industries. PCR was conducted using an i-Cycler thermal cycler (Bio-Rad) programmed for thermal cycling for 30 cycles, included a denaturation step of 95ºC for 1 minute, a primer annealing step for 1 minute (52ºC for *gyrA,* 55ºC *gyrB,* 58ºC *parC* and 57ºC *parE*) and a primer extension step at 72ºC for 30 seconds. PCR reagents listed in Appendix C1. PCR products were purified using QlAquick® PCR purification Kits (Southern Cross Biotechnology) or MSB® Spin PCRapace purification Kits (Invitek, GmbH, Berlin, Germany). The 16SrRNA gene was targeted as an internal amplification control as all bacterial strains contain this gene in there genome. A no template control was also used.

2.5.2 Plasmid Genes (*qnr*)

PCR was used to screen for three previously described *qnr* genes, *qnrA*, *qnrB* and *qnrS*, using previously described methods (Jacoby, *et al.* 2003; Jacoby, *et al.* 2006; Hopkins, *et al*. 2007). Primers (Appendix C2) were synthesized by Inqaba Biotechnical Industries. PCR was conducted using an i-Cycler thermal cycler (Bio-Rad) programmed for thermal cycling for *qnrA*: 30 cycles, included a denaturation step of 94ºC for 1 minute, a primer annealing step of 57ºC for 1 minute and a primer extension step at 72ºC for 1 minute; and *qnrB* and *qnrS*: 32 cycles, included a denaturation step of 94ºC for 45 seconds, a primer annealing step of 53ºC for 45 seconds and a primer extension step at 72ºC for 1 minute. PCR reagents listed in Appendix C1. PCR products for any resultant positive *qnr* gene were purified using QlAquick® PCR purification Kits (Southern Cross Biotechnology) or MSB® Spin PCRapace purification Kits (Invitek). A positive control was not used as none were available. The 16SrRNA gene was targeted as an internal amplification control as all bacterial strains contain this gene in there genome. A no template control was also used.

2.5.3 Detection of Positive PCR Reactions

Three microliters of PCR reaction product were mixed with 2µl of loading buffer (Appendix C3) and loaded into wells of a 1.2% agarose gel (w/v) to which ethidium bromide had been added (Appendix C3). Three microliters of a 100 base pair DNA size marker, HyperLadder IV [Bioline (Celtic Molecular Diagnostics, Cape Town, South

Africa)] was added to the gel. Electrophoresis was performed at 140 volts for 45 minutes in a running buffer (Appendix C3) and the resulting DNA fingerprint pattern was captured on the Gel Doc Quantity One documentation system (Bio-Rad) and saved as an uncompressed TIFF image (*.tif).

2.5.4. Sequencing

For DNA sequencing, purified PCR product was used as template in a PCR cycle sequencing reaction using the ABI Prism® Big-Dye Terminator version 3.1 cycle sequencing kit as per manufacturer's instruction (Applied Biosystems). PCR reagents listed in Appendix C4. PCR was conducted using an i-Cycler thermal cycler (Bio-Rad) programmed for thermal cycling with and initial denaturation at 95ºC for 2 minutes proceeding to 25 cycles of, a denaturation step of 95ºC for 50 seconds, a primer annealing step of 50ºC for 50 seconds and a primer extension step at 60ºC for 4 minutes. Cycle sequencing products were applied to a DyeEx™ 2.0 Spin Kit as per manufacturer's instruction (Southern Cross Biotechnology) and spun through a filtered column. The filtered sample was then vacuum-dried in Speed Vac Concentrator vacuum centrifuge (Savant, GMI Incorporated, Minnesota, United States of America) for an hour, reconstituted in 15µl of Hi-Di formamide (Applied Biosystems). This mixture was then incubated at 95°C for 3 minutes and cooled to room temperature and subsequently analyzed on an Applied Biosystems 3130 Genetic Analyzer and a sequence was determined. Sequences resulting from the analysis of the QRDR samples were compared to previously PUBMED published sequences

(http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide) to determine mutations using BioEdit version7.5 and the pairwise comparison tool EMBOSS (http://www.ebi.ac.uk/emboss/align/) while sequences resulting from *qnr* positive PCR were confirmed using PUBMED Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide).

2.6 Doubling Agar Dilution Minimum Inhibitory Concentration (MIC) and Efflux Pump Inhibition

Selected strains were subjected to doubling agar dilution MICs for nalidixic acid and ciprofloxacin as previously described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006). The MIC breakpoints used were those recommended by the CLSI. These were defined as strains exhibiting an MIC ≤16µg/ml nalidixic acid were deemed susceptible and those strains exhibiting an MIC ≥32µg/ml were deemed resistant. Ciprofloxacin breakpoints, those strains exhibiting an MIC ≤1µg/ml were deemed susceptible, strains exhibiting an MIC =2µg/ml were deemed intermediately resistant and strains exhibiting and MIC ≥4µg/ml were deemed resistant (CLSI, 2006). These breakpoints were adjusted in both increasing and decreasing fold values as to determine the exact MIC for the test isolates. The extra dilutions ranged from 1µg/ml to 512µg/ml for nalidixic acid and from 0.0625µg/ml to 16µg/ml ciprofloxacin. These agar dilution MICs were repeated within the presence of an efflux pump inhibitor (EPI), namely β-*Phe-Arg-naphthylamide*. Prior to performing efflux pump inhibition tests, a pilot study was performed to determine the appropriate concentration of EPI that could be used which not limit, inhibit or interfere normal growth of the bacteria. A concentration of

40µg/ml β-*Phe-Arg-naphthylamide* was found to be suitable. This concentration fell within a previously tested range (Baucheron, *et al*. 2002). A control strain *Escherichia coli* ATCC® 25922 was included for every test (Kehrenberg *et al.*, 2007). Bacterial cultures were grown overnight on 5% Sheep blood agar plates (DMP) and incubated at 37ºC. Bacterial culture was resuspended into 1000µl of 0.85% sterile saline (Appendix D) and adjusted to a cell concentration turbidity of ≈0.5 McFarland using Microscan Turbidity Meter (Dade Behring). Sterile Mueller-Hinton agar was prepared (Appendix D) and kept molten at 55ºC. The appropriate amount of antibiotic (nalidixic acid or ciprofloxacin) was added to the molten agar, gently swirled, two plates poured (110mm Petri dishes) and the process repeated with the addition of the EPI. Details of the concentrations and makeup of the antibiotics and EPI are provided in Appendix D. Bacterial suspensions (1000µl) were loaded into a 36 well plate spotter (MAST, Meyerside, United Kingdom) with 0.85% sterile saline serving as a negative control. The plates were spotted with equal amounts of all samples and incubated overnight at 37ºC. Results of inhibition of growth were recorded the following day. The MIC value was determined as the minimum concentration of antibiotic that was required to completely inhibit the growth of the bacterium. The involvement of an efflux pump with regard to quinolone resistance was indicated when an isolate showed a decreased MIC in the presence of the EPI. All MIC tests were repeated once per strain.

Chapter 3: *Salmonella* Typhi

3.1. Results

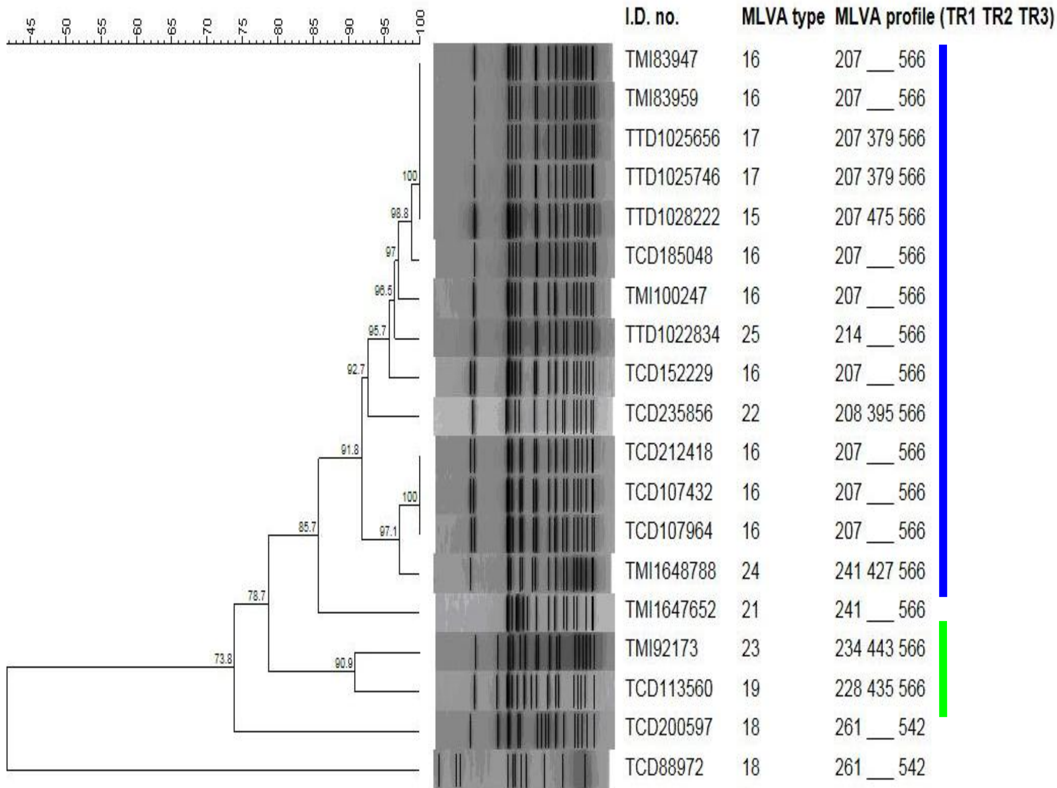


Figure 3.1. PFGE dendrogram of 19 nalidixic acid-resistant *Salmonella* Typhi isolates causing disease in South Africa, 2003 to 2007, showing similarity index, banding pattern, isolate number, MLVA type and MLVA profile. The two main PFGE clusters are highlighted in blue (PFGE type 1) 14 isolates and in green (PFGE type 2) 2 isolates.

Only nineteen of the twenty-seven *Salmonella* Typhi quinolone-resistant isolates were available for analysis. PFGE analysis differentiated the nineteen nalidixic acid-resistant isolates into five PFGE types (Table 3.1). However, MLVA was more discriminatory and differentiated the isolates into 10 MLVA types (Table 3.1). The superior discriminatory power of MLVA was illustrated within the PFGE type 2 cluster (14 isolates), which MLVA was able to divide into 6 MLVA types (Figure 3.1). MLVA type 16 was the most common type accounting for seven out of the fourteen isolates in the PFGE cluster 2. MLVA type 15, 16 and 17 only differ by a single allele (TR2) and may be considered under less strict circumstances as being identical. MLVA allows for a greater reproducibility and accuracy of result.

The investigation into the involvement of an active efflux revealed a 16 or 32-fold decrease in nalidixic acid MIC and a 2 or 8-fold decrease in ciprofloxacin MIC in the presence of EPI. This suggests that an active efflux pump is a major contributory factor in the quinolone resistance of these *Salmonella* Typhi isolates (Table 3.1).

Table 3.1. PFGE types, MLVA types and MIC results for all *Salmonella* Typhi isolates as well as the QRDR results for seven selected isolates.

Isolate	Year	Province[1]	PFGE type[2]	MLVA type[3]	MLVA profile (TR1-TR2-TR3)[4]	*gyrA* mutations	*parC* mutations	Nalidixic acid agar dilution MIC (µg/ml)[5]	Ciprofloxacin agar dilution MIC (µg/ml)[5]	Active Efflux
TMI92173	2005	GA	1	23	234 443 566	X	X	256 (8)	0.5 (0.0625)	Yes
TCD113560	2006	WC	1	19	228 435 566	Asp82-Gly; Ala119-Ser	Tyr57-Ala;Ser80-Phe	512 (32)	0.5 (0.0625)	Yes
TTD1022834	2003	GA	2	25	214 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TTD1028222	2004	GA	2	15	207 475 566	X	X	512 (32)	0.5 (0.0625)	Yes
TTD1025746	2004	KZN	2	17	207 379 566	X	X	512 (32)	0.5 (0.0625)	Yes
TTD1025656	2004	WC	2	17	207 379 566	X	X	512 (32)	0.5 (0.0625)	Yes
TCD83959	2005	EC	2	16	207 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TCD83947	2005	EC	2	18	261 000 542	X	X	256 (8)	0.5 (0.0625)	Yes
TCD100247	2005	KZN	2	16	207 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TMi1648788	2005	KZN	2	24	241 427 566	X	X	128 (8)	0.125 (0.0625)	Yes
TCD152229	2006	GA	2	16	207 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TCD107964	2006	WC	2	16	207 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TCD107432	2006	WC	2	16	207 000 566	Asp82-Gly; Ser83-Ala	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes
TCD212418	2007	KZN	2	16	207 000 566	X	X	512 (32)	0.5 (0.0625)	Yes
TCD185048	2007	WC	2	16	207 000 566	Ser83-Met; Asp87-Cys	Tyr57-Gly; Ser80-Lys	512 (32)	0.5 (0.0625)	Yes
TCD235856	2007	GA	2	22	207 395 566	Ser83-Ala	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes
TCD88972	2005	MP	3	18	261 000 542	Ala119-Gly	Ser80-Ile	>512 (32)	0.5 (0.0625)	Yes
TMI1647652	2005	WC	4	21	241 000 566	Gly81-Ser; Asp82-Gly	Tyr57-Ser	256 (8)	0.125 (0.0625)	Yes
TCD200597	2007	KZN	5	18	261 000 542	Ser83-Phe	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes

1: GA, Gauteng; WC, Western Cape; KZN, KwaZulu-Natal; EC, Eastern Cape; MP, Mpumalanga.
2: PFGE types based on relatedness as based on a dendrogram cluster analysis of the patterns using the unweighted pair group method with arithmetic averages, with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5% and a position tolerance setting of 1.5%. PFGE types defined at ≥90% similarity.

3: MLVA types based on MLVA profiles.

4: 000 represents no amplification of the PCR product at locus TR2.

5: Value in parenthesis represents the MIC with the addition 40 µg/ml *β-Phe-Arg-naphthylamide* as an efflux pump inbihitor.

*: X represents not analysed.
All *qnr* screening results negative for *Salmonella* Typhi.

3.2. Discussion

The PFGE cluster 2 represented the majority of isolates which share a common ancestry. This immediately suggests that clonal spread of nalidixic acid resistance may have occurred. However QRDR sequencing data (discussed later) of selected isolates within this PFGE cluster 2 showed completely different QRDR mutation profiles (Table 3.1). This would suggest that quinolone-resistant isolates have emerged independently of one another, i.e. emergence of quinolone resistance in South African isolates of *Salmonella* Typhi is not clonally driven.

An efflux pump as a mechanism of quinolone resistance has previously been observed in various *Salmonella* serotypes including *Salmonella* Typhi (Piddock *et al.*, 2002; Gaind *et al.,* 2006). Baucheron *et al.* (2002) illustrated the importance of the AcrB in fluoroquinolone resistance and TolC in multidrug resistant *Salmonella* Typhimurium (Baucheron *et al*., 2005). Although the AcrAB-TolC efflux system may vary between isolates (Kehrenberg *et al.,* 2007) it has been shown that quinolone resistance is not as a result of one mechanism but due to a combination of mechanisms (Hopkins *et al.,* 2005). A similar observation can be made for the South African isolates. Gyrase mutations alone do not account for quinolone resistance in *Salmonella* as it is believed that AcrAB pump is the primary mechanism for fluoroquinolone resistance, especially if the QRDR mutations do not sufficiently explain the resistant phenotype (Baucheron *et al.,* 2002; Chu *et al.,* 2005). For South African isolates, an active efflux is seen to be the major contributory mechanism to quinolone resistance; however it is not the only mechanism

because even in the presence of EPI, fifteen out of the nineteen isolates still show nalidixic acid resistance with MICs at 32µg/mℓ (Table 3.1). The role of *qnr* plasmids can be excluded as screening for the pentapeptide repeat genes *qnrA, qnrB* and *qnrS* did not identify the presence of these genes. This leaves mutations in the QRDR as the only other likely mechanism.

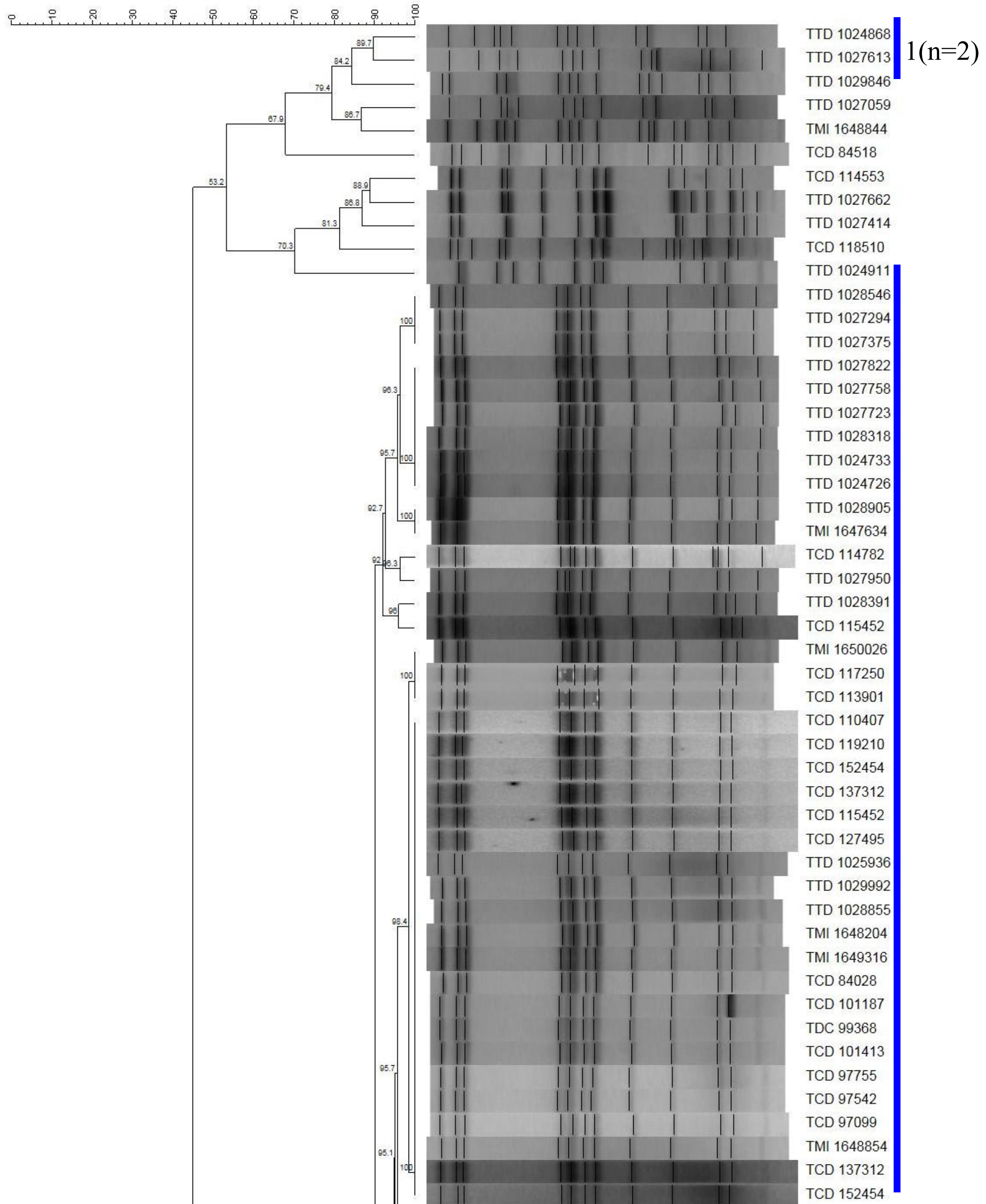
Isolates TCD200597, TCD235856, TCD185048 and TCD107432 show mutations at *gyrA* positions Ser83 and/or Asp87, positions that have been previously described as important for quinolone resistance (Hopkins *et al.,* 2005), therefore these mutations probably play a role in quinolone resistance in these isolates. At these positions, novel mutations (Ser83-Met and Asp87-Cys) were observed in isolate TCD185048. Some of our isolates also show some novel mutations at novel positions in *gyrA*. Isolates 113560 and 88972 showed a novel mutation at position 119 (Ala119-Ser) of *gyrA*. This above mutation was found in combination with a previously described mutation at position 82 (Asp82-Gly) (Ruiz, 2003). Isolate 107432 also showed the Asp82-Gly mutation For isolates 113560, 107432 and 88972, mutations at positions (Asp82 and Ala119) in *gyrA* may be operating as an alternative mechanism of resistance com*parE*d to the commonly reported mutation at positions Ser83 and Asp87. Levy, *et al.* (2004) provided evidence to suggest that selection pressure in terms of the antibiotics the bacteria is exposed to, is a determining factor in the resulting mutations in *gyrA*. Treatment strategies for salmonellosis differ for different regions and since quinolone-resistant *Salmonella* Typhi are not as commonly locally acquired in South Africa as com*parE*d to other *Salmonella* serotypes, the selection pressure may be driven by antimicrobial use in regions with elevated rates of

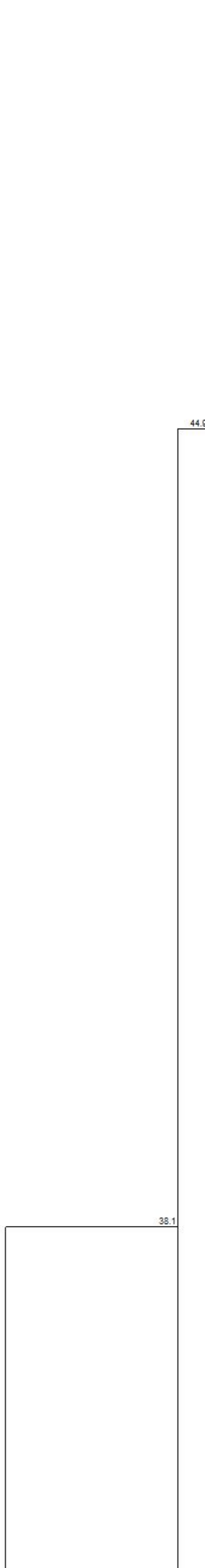
Salmonella Typhi infection. Single point source mutations at codons 83 and 87 of the *gyrA* have over the years received much attention as the cause of quinolone resistance, in particular nalidixic acid and as a result linked to decreased susceptibility of fluoroquinolones (Hirose *et al.,* 2002; Saha *et al.*, 2006). Mutations in *gyrA* are normally associated with nalidixic acid resistance and seen as the precursors to mutations in the topoisomerase IV proteins *parC* and *parE* resulting in fluoroquinolone resistance or decreased susceptibility (Baucheron *et al*., 2005). Conversely it has been strongly suggested that multiple mutations in *gyrA* are more important in conveying fluoroquinolone resistance than any respective combination of mutations in the gyrase and topoisomerase IV proteins. As important as mutations in *gyrA* are, these mutations themselves are not the defining contributory factor for quinolone resistance (Eaves *et al.,* 2002; Hopkins *et al.,* 2005). Sequencing analysis of nalidixic acid-resistant *Salmonella* Typhi isolates showed no amino mutations in either *gyrB* or *parE*. The two regions are homologous with the latter being the secondary target for quinolones (Eaves *et al.,* 2004). Mutations for both *gyrB* and *parE* remain rare for most *Salmonella* isolates as most researchers report mainly on findings of *gyrA* mutations (Hopkins *et al.*, 2007). The role of *gyrB* and *parE* in quinolone resistance is unclear (Piddock *et al.,* 2002), in particular *gyrB*, even in *Salmonella* isolates exhibiting high levels of fluoroquinolone resistance (Hopkins *et al.*, 2007). The present study showed that all nalidixic acid-resistant isolates with amino mutations in *gyrA* also showed amino acid mutations in *parC*. Mutation at position Ser80 of *parC* was commonly present and involved the mutation of Ser80 to either Phe or Arg or Lys or Ile. Some novel *parC* mutations were also identified and these included Tyr57-Gly, Tyr57-Ala, Ser80-Lys and Ser80-Phe. *ParC* mutation at

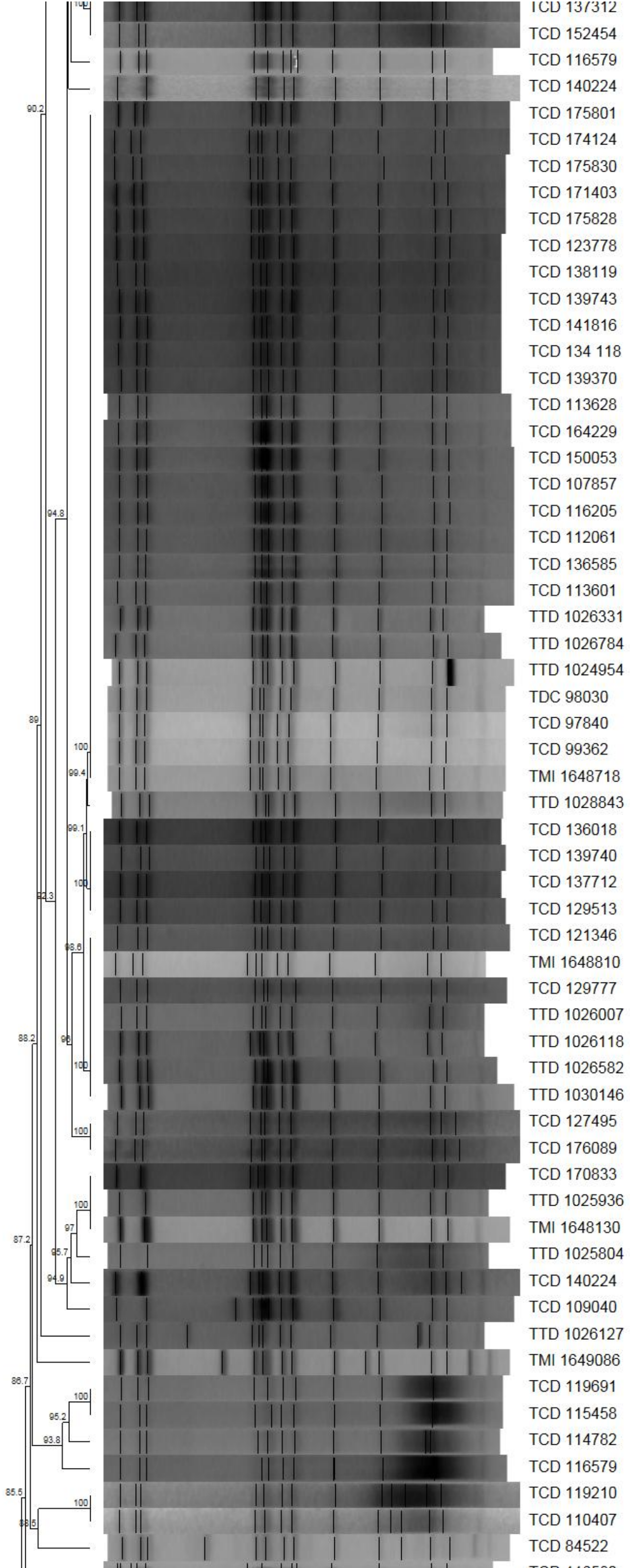
positions Ser80 and Glu84 have previously been shown to be important for quinolone resistance (Hopkins *et al.*, 2005). With regards to the *parC* mutation at position Tyr57, Baucheron *et al.* (2004) suggested that mutation at this position (Tyr57-Ser) is not likely to be involved in quinolone resistance. Results from the present study support this hypothesis. Mutations were found at Tyr57 for three of the isolates. For one of these (isolate 1647652), the mutation at position Ser80 of *parC* was notably absent while a Tyr57-Ser mutation was present. With efflux inhibited, this isolate was nalidixic acid-susceptible (MIC, 8 μg/ml), which would support the view that a mutation at position Tyr57 of *parC* may be inconsequential, but the role of this mutation in combination with concurrent mutations cannot be overlooked. Overall, the role of *parC* mutations in quinolone-resistant *Salmonella* remains unclear. The above results suggest that mutation at position Ser80 of *parC* may be an important role player in quinolone resistance. The question remains concerning the role of mutations in both *gyrA* and *parC*. Sequencing data from our seven isolates showed mutations in both *gyrA* and *parC*. Based upon the results of previous studies (Chu *et al.,* 2005; Hopkins *et al*., 2005) it was therefore expected that our test isolates should be resistant to the fluoroquinolone, ciprofloxacin. However, all seven isolates were susceptible (MICs, ≤0.5 μg/ml) to ciprofloxacin. Our results are therefore in more agreement with the results of Eaves *et al.* (2004) who found that isolates with mutations in both *gyrA* and *parC* were more susceptible to ciprofloxacin than isolates with mutations in *gyrA* alone.

Chapter 4: *Salmonella* Enteritidis

4.1. Results







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3 (n=4)

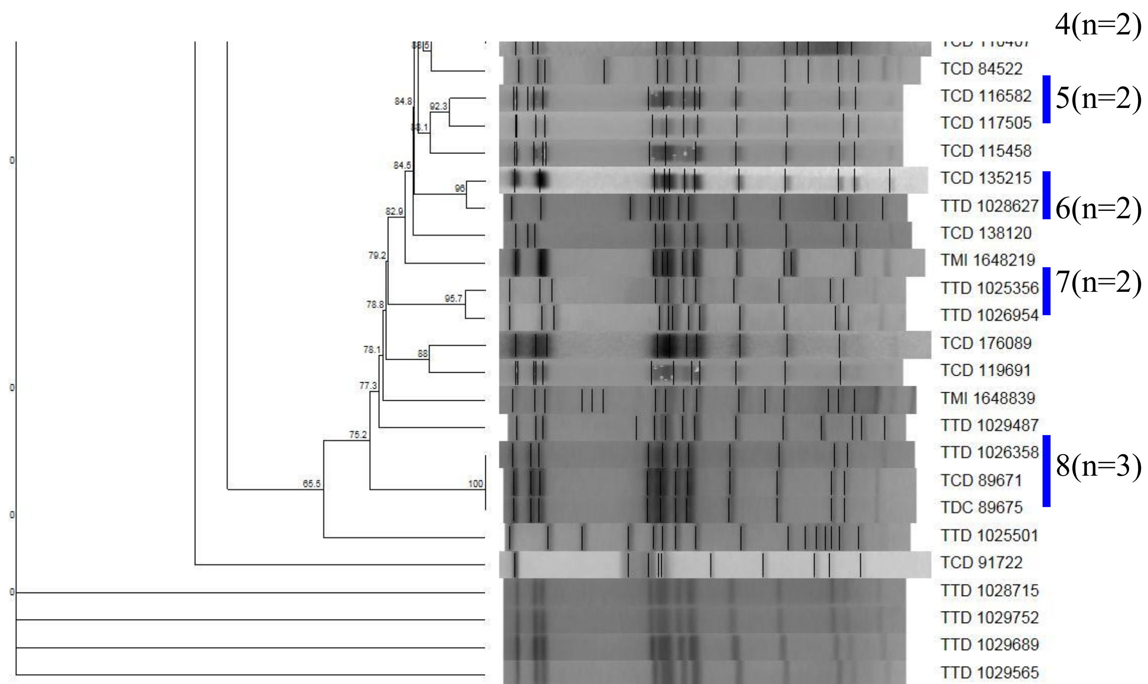


Figure 4.1. PFGE dendrogram of 116 nalidixic acid-resistant *Salmonella* Enteritidis isolates causing invasive disease in South Africa, 2004 to 2006, showing similarity index, banding pattern and isolate number. The dendrogram shows 8 PFGE clusters with PFGE cluster 2 being the largest cluster with 89 isolates.

For the period 2004-2006, three-hundred and twenty-nine human isolates of *Salmonella* Enteritidis, were received by the EDRU, of which one-hundred and nineteen isolates were invasive, nalidixic acid-resistant. Only one-hundred and sixteen isolates were available for analysis. PFGE analysis resolved the invasive, nalidixic acid-resistant *Salmonella* Enteritidis isolates into thirty-six PFGE types and eight PFGE clusters at ≥90% similarity (Figure 4.1). The largest PFGE cluster was the PFGE cluster 2 with eighty-nine isolates. The isolates constituting PFGE cluster 2 are a combination of isolates collected in the period 2004 to 2006 mostly from Gauteng. The distribution of the isolates across the dendrogram to result in one dominant PFGE cluster (89/116), suggests a common ancestry for this group of isolates.

Thirty-seven randomly selected *Salmonella* Enteritidis isolates were screened to determine the contribution of an active efflux system to nalidixic acid resistance. The thirty-seven selected isolates all exhibited an 8 to 32-fold decrease in nalidixic acid MIC and a 2 or 8-fold decrease in ciprofloxacin MIC in the presence of the efflux pump inhibitor (Table 4.1).

Table 4.1. PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Enteritidis isolates.

Isolate	Year	Province[1]	PFGE type[2]	*gyrA* mutations	*parC* mutations	Nalidixic acid agar dilution MIC (µg/ml)[3]	Ciprofloxacin agar dilution MIC (µg/ml)[3]	Active Efflux
TCD117250	2006	GA	2	Ser83-Ala	Tyr57-Arg	>512(32)	1(0.5)	Yes
TCD123778	2006	GA	2	Ser83-Phe	Ser80-Arg	>512(32)	0.5(0.0625)	Yes
TCD175828	2006	GA	2	Ser83-Ala	Tyr57-Arg	>512(32)	0.5(0.0625)	Yes
TCD170833	2006	GA	2	Ser83-Ala	Tyr57-Arg	>512(32)	1(0.5)	Yes
TDC171403	2006	GA	2	Ser83-Phe	Tyr57-Ala	>512(32)	0.5(0.25)	Yes
TCD175830	2006	GA	2	Ser83-Ala	Tyr57-Arg	>512(32)	0.5(0.25)	Yes
TCD116579	2006	GA	2	Ser83-Ala	Tyr57-Gly; Ser80-Lys	>512(32)	0.5(0.25)	Yes
TCD113901	2006	GA	2	Ser83-Phe	Tyr57-Ser	>512(32)	0.5(0.0625)	Yes
TCD136018	2006	GA	2	Ser83-Ala	Tyr57-Phe; Ser80-Pro	>512(32)	0.5(0.25)	Yes
TCD139370	2006	GA	2	Ser83-Ala	Tyr57-Ala	>512(32)	0.5(0.0625)	Yes
TDC129513	2006	GA	2	Ser83-Phe	Tyr57-Arg	>512(32)	0.5(0.25)	Yes
TCD139740	2006	GA	2	Ser83-Ala	Tyr57-Ala	>512(32)	0.5(0.25)	Yes
TCD174124	2006	GA	2	Ser83-Ala	Ser80-Phe	>512(32)	0.5(0.0625)	Yes
TCD139743	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD121346	2006	GA	2	X	X	>512(32)	0.5(0.0625)	Yes
TCD137712	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD138119	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD119210	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD129777	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD141816	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD137312	2006	MP	2	X	X	>512(32)	0.5(0.25)	Yes
TCD115452	2006	MP	2	X	X	>512(32)	0.5(0.25)	Yes
TCD152454	2006	NW	2	X	X	>512(32)	0.5(0.25)	Yes
TCD110407	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD134118	2006	GA	2	X	X	>512(32)	0.5(0.25)	Yes
TCD127495	2006	NW	2	X	X	>512(32)	0.5(0.25)	Yes
TCD140224	2006	WC	2	X	X	>512(32)	0.5(0.25)	Yes

Table 4.1. Continued PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Enteritidis isolates.

Isolate	Year	Province[1]	PFGE type[2]	*gyrA* mutations	*parC* mutations	Nalidixic acid agar dilution MIC (µg/ml)[3]	Ciprofloxacin agar dilution MIC (µg/ml)[3]	Active Efflux
TCD114782	2006	GA	3	X	X	>512(32)	0.5(0.25)	Yes
TCD119691	2006	GA	3	X	X	>512(32)	0.5(0.25)	Yes
TCD115458	2006	GA	3	X	X	>512(32)	0.5(0.25)	Yes
TCD117505	2006	GA	5	X	X	>512(32)	0.5(0.25)	Yes
TCD116582	2006	GA	5	X	X	512(32)	0.5(0.25)	Yes
TCD135215	2006	KZN	6	X	X	128(16)	0.5(0.25)	Yes
TTD1029487	2004	WC	O	Ser83-Ala	Tyr57-Arg	>512(16)	0.5(0.25)	Yes
TTD1029689	2004	GA	O	Ser83-Ala	Ser80-Lys	>512(16)	0.5(0.25)	Yes
TMI1648844	2005	GA	O	Ser83-Phe	Tyr57-Ser	>512(64)	0.5(0.25)	Yes
TCD84518	2005	GA	O	Ser83-Ala	Tyr57-Phe; Ser80-Pro	>512(64)	0.5(0.25)	Yes

1: GA, Gauteng; WC, Western Cape; KZN, KwaZulu-Natal; EC, Eastern Cape; MP, Mpumalanga; NW, North West.
2: PFGE types based on relatedness as based on a dendrogram cluster analysis of the patterns using the unweighted pair group method with arithmetic averages, with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5% and a position tolerance setting of 1.5%. PFGE types defined at ≥90% similarity. PFGE type O, are isolates outliers that did not fit into a PFGE cluster at ≥90% similarity.

3: Value in *paren*thesis represents the MIC with the addition 40 µg/ml *β-Phe-Arg-naphthylamide* as an efflux pump inbihitor.

*: X represents not analysed.
All *qnr* screening results negative for *Salmonella* Enteritidis.

4.2. Discussion

The possible shared common ancestry for the *Salmonella* Enteritidis isolates suggests that a clonal spread of nalidixic acid resistance may have occurred. This thought may be refuted when one considers the number of PFGE types resulting from the dendrogram (Figure 4.1). Thirty-six PFGE types suggest that there is a fair amount of variability amongst the isolates which in turn implies that quinolone resistance with regard to these *Salmonella* Enteritidis isolates was not clonally spread. Analysis of the QRDR mutations provides further clarity (discussed later).

As with the *Salmonella* Typhi isolates, the results of the phenotypic investigation suggests that an active efflux system seems to be the major contributory factor toward the quinolone resistance of these invasive *Salmonella* Enteritidis isolates. This result is in partial agreement with the findings of Braoudaki and Hilton (2004) who have previously shown that the over-expression of an active efflux pump in *Salmonella* Enteritidis isolates is an adaptive mechanism of resistance in response to antimicrobial agents.

Although an active efflux pump is thought to be the major contributory mechanism to quinolone resistance, it cannot be seen as the sole contributory mechanism. In the presence of an efflux pump inhibitor thirty-four out of the thirty-seven screened isolates still showed nalidixic acid resistance with an MIC of 32µg/ml (Table 4.1). All thirty-seven *Salmonella* Enteritidis isolates also showed no positive results for the *qnr* plasmid screening, thus excluding *qnr* plasmids as a contributory mechanism of resistance.

Mutations in the QRDR remain the only explanation for the high level of quinolone resistance observed in these *Salmonella* Enteritidis isolates.

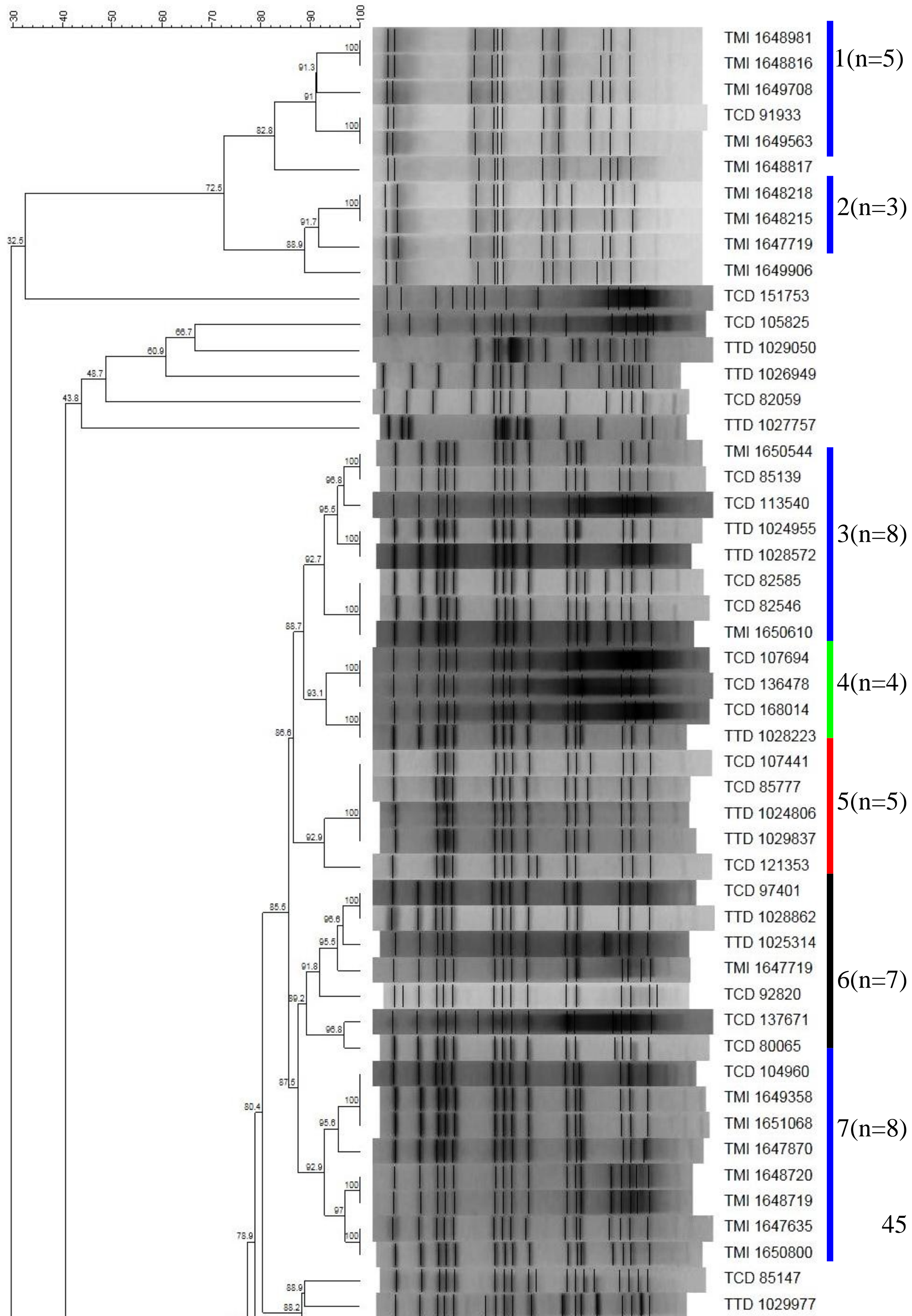
Seventeen isolates selected for sequencing showed mutations in *gyrA* at positions Ser83 (Table 4.1). Mutations in *gyrA* position Ser83 have previously been shown to be an important contributor to quinolone resistance (Bertrand *et al.,* 2006). The commonality of the *gyrA* mutations at position Ser83 for all seventeen isolates [including 13 isolates from PFGE cluster 2 and four outliers (Table 4.1)] provides further evidence to the earlier suggestion, based on PFGE analysis, that these *Salmonella* Enteritidis isolates may have a common ancestry. This finding is in correlation with the suggestion that the distribution of *gyrA* mutations depend on the source of the isolates, but maybe more likely to the exposure of similar selection pressures, that is, antimicrobials (Lindstedt *et al*, 2004). As antimicrobial treatment strategies are similar for most regions quinolone resistance for these *Salmonella* Enteritidis may have occurred independently as suggested by the numerous PFGE types.

Sequencing analysis of the *gyrB* and *parE* regions of the seventeen selected *Salmonella* Enteritidis isolates revealed no amino acid mutations in either *gyrB* or *parE*. In contrast to those findings, sequencing analysis of the *gyrA* homologous region, *parC* of the same isolates showed mutations in *parC* at positions Tyr57 and Ser80. Two isolates, TCD113901 and TMI1648844 showed amino acid changes of Tyr57-Ser which has previously been described (Hopkins *et al*., 2005). Four novel amino mutations at Tyr57 were shown in twelve of the isolates, with the changes being Tyr57-Arg, Tyr57-Ala,

Tyr57-Gly or Tyr57-Phe (Table 4.1). Three novel mutations were also observed at Ser80, with the changes being Ser80-Phe, Ser80-Pro or Ser80-Lys (Table 4.1). One isolate, TCD123778, exhibited a previously described *parC* mutation of Ser80-Arg (Hopkins *et al*., 2005). Three isolates, TCD84518, TCD116579 and TCD136018, showed a combination of mutations at both positions 57 and 80 of *parC*, as well as at position 83 of *gyrA* (Table 4.1). It would be expected that these three isolates along with the other fourteen sequenced isolates would be fluoroquinolone-resistant as a result of the multiple mutations, but the isolates are fully susceptible to the fluoroquinolone ciprofloxacin (Table 4.1). This finding is in accordance with results of Eaves *et al.* (2004) who suggested that isolates with mutations in both *gyrA* and *parC* were more susceptible to ciprofloxacin than isolates with mutations in *gyrA* alone. The contribution of mutations at position Tyr57 of *parC* has come under criticism with Baucheron *et al.* (2004) suggesting that this mutation plays no role in quinolone resistance. For our study the MIC results in the presence of an efflux pump inhibitor showed that for thirteen out the seventeen sequenced isolates, that possessed a mutation at Tyr57 had a nalidixic acid MIC of ≥32μg/ml (Table 4.1). The above finding would suggest that a mutation at position Tyr57 of *parC* may play a role in quinolone resistance, thus contradicting the suggestion of Baucheron *et al.* (2004). However, mutations at Tyr57 of *parC* were found in combination with mutations at position Ser83 of *gyrA* which are associated with quinolone resistance (Cloeckaert & Chaslus-Dancla, 2001). The role of mutations at position Tyr57 of *parC* and the combination of mutations in both *gyrA* and *parC* remain questionable.

Chapter 5: *Salmonella* Isangi

5.1. Results



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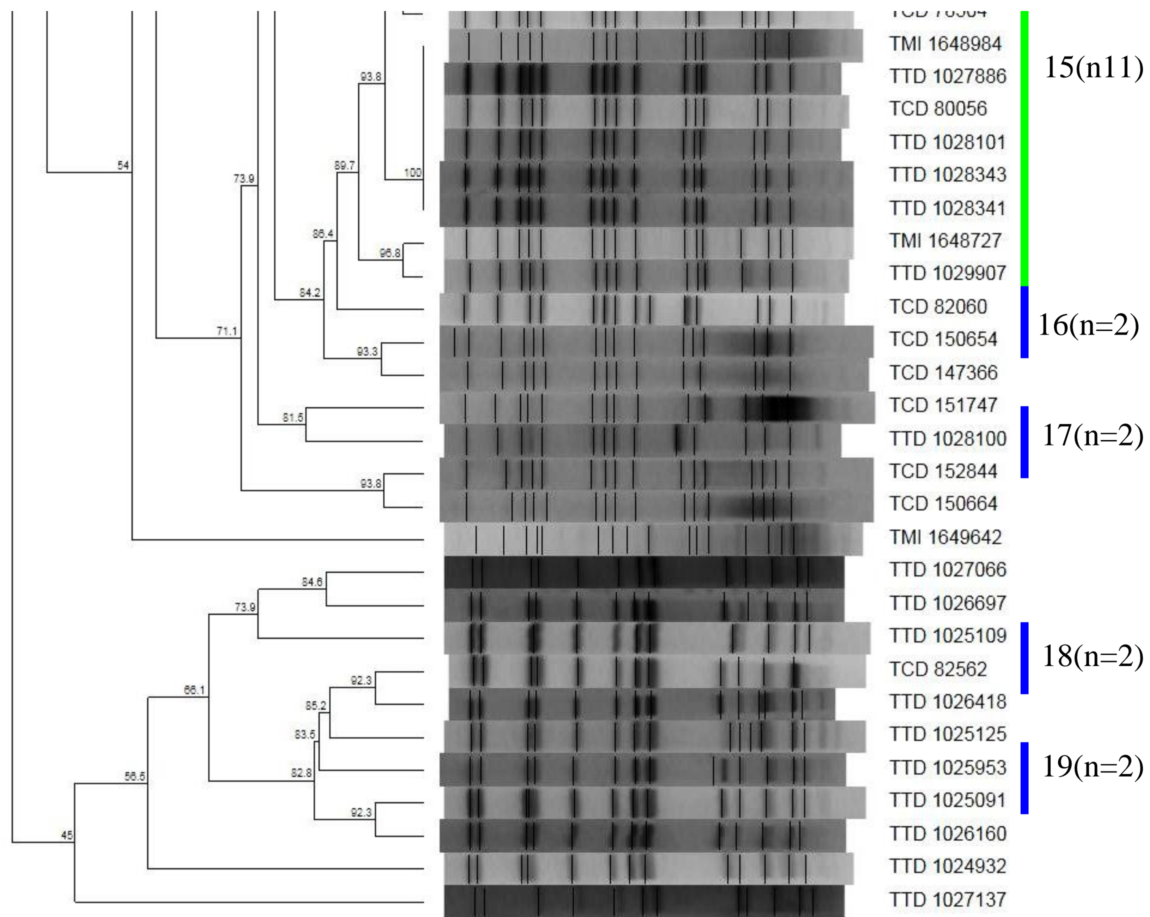


Figure 5.1. PFGE dendrogram of 137 nalidixic acid-resistant *Salmonella* Isangi isolates causing invasive disease in South Africa, 2004 to 2006, showing similarity index, banding pattern and isolate number. The dendrogram shows 19 PFGE clusters with PFGE cluster 8 being the largest cluster with 15 isolates.

For the period 2004-2006, one-thousand and five human isolates of *Salmonella* Isangi, of which, one-hundred and forty-three were invasive, nalidixic acid-resistant. Molecular analysis was conducted on one-hundred and thirty-seven available isolates. Analysis of the PFGE patterns of the nalidixic acid-resistant *Salmonella* Isangi isolates causing invasive disease, for the period 2004-2006, resulted in a dendrogram resolved to fifty-seven PFGE types (As defined in chapter 2) (Figure 5.2). The dendrogram constituted nineteen PFGE type clusters. PFGE cluster 8 was the largest cluster with fifteen isolates and the other eighteen PFGE clusters numbered between two to eleven isolates.

Thirty-two randomly selected isolates were screened for the involvement of an active efflux system as a possible mechanism of quinolone resistance. All thirty-two isolates exhibited the presence of an active efflux system (Table 5.2).

Table 5.1. PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Isangi isolates.

Isolate	Year	Province[1]	PFGE type[2]	*gyrA* mutations	*parC* mutations	Nalidixic acid agar dilution MIC (µg/ml)[3]	Ciprofloxacin agar dilution MIC (µg/ml)[3]	Active Efflux
TTD1024955	2004	GA	3	Gly81-Lys; Asp87-Ile	Tyr57-Ser	>512(16)	0.125(0.0625)	Yes
TCD113540	2006	EC	3	Asp82-His;Ser83-His; Asp87-Cys; Leu98-His; Ala119-Gly	Tyr57-Arg; Thr66-Pro	>512(32)	0.125(0.0625)	Yes
TCD136478	2006	EC	4	Asp82-His;Ser83-His; Asp87-Cys; Leu98-Pro; Ala119-Thr	Tyr57-Arg; Gly78-Cys; Glu84-Ser	>512(32)	0.125(0.0625)	Yes
TCD168014	2006	MP	4	X	X	>512(32)	0.125(0.0625)	Yes
TCD107694	2006	EC	4	Asp82-Pro;Ser83-His; Asp87-Cys; Leu98-His; Ala119-Thr	Tyr57-Ser; Thr66-Pro; Gly78-Phe; Ser80-Pro	>512(32)	0.125(0.0625)	Yes
TCD121353	2006	WC	5	X	X	>512(32)	0.0625(<0.0625)	Yes
TCD97401	2005	EC	6	Gly81-Asp; Ser83-Leu; Asp87-Ala	Tyr57-Arg	>512(8)	0.125(0.0625)	Yes
TCD137671	2006	GA	6	Asp82-His;Ser83-His; Asp87-Cys; Ala119-Phe	Tyr57-Ser; Ser80-Pro	>512(32)	0.5(0.0625)	Yes
TCD104960	2005	EC	7	Gly81-Lys; Asp87-Ile	Tyr57-Ser	>512(32)	0.125(0.0625)	Yes
TCD1028929	2004	EC	8	Gly81-Asp; Ser83-Leu; Asp87-Ala	Tyr57-Arg	>512(32)	0.125(0.0625)	Yes
TCD142070	2006	KZN	8	X	X	>512(32)	0.125(0.0625)	Yes
TCD135180	2006	KZN	8	X	X	>512(32)	0.5(0.0625)	Yes
TCD139881	2006	KZN	10	Ser83-His; Asp87-Cys; Leu98-His; Ala119-Phe	Tyr57-Arg	>512(32)	0.125(0.0625)	Yes
TCD132205	2006	KZN	10	X	X	>512(32)	0.5(0.0625)	Yes
TCD115424	2006	KZN	10	X	X	>512(32)	0.125(0.0625)	Yes
TCD121990	2006	EC	12	Asp82-His;Ser83-His; Asp87-Cys; Leu98-His; Ala119-Ile	Tyr57-Phe; Thr66-Leu	>512(32)	0.125(0.0625)	Yes
TCD122195	2006	GA	12	X	X	>512(32)	0.25(0.0625)	Yes
TCD144912	2006	KZN	13	X	X	>512(32)	0.125(0.0625)	Yes
TCD107441	2006	WC	14	X	X	>512(32)	0.125(0.0625)	Yes
TCD150654	2006	KZN	16	Asp82-Pro;Ser83-His; Asp87-Cys; Leu98-His	Tyr57-Ser; Ser80-Pro	>512(32)	0.125(0.0625)	Yes
TCD147366	2006	KZN	16	X	X	>512(32)	0.125(0.0625)	Yes

Table 5.1. Continued PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Isangi isolates.

Isolate	Year	Province[1]	PFGE type[2]	*gyrA* mutations	*parC* mutations	Nalidixic acid agar dilution MIC (µg/ml)[3]	Ciprofloxacin agar dilution MIC (µg/ml)[3]	Active Efflux
TCD152844	2006	KZN	17	Gly81-Lys; Asp87-Ile; Ala119-Phe	Tyr57-Ser	>512(32)	0.125(0.0625)	Yes
TCD150664	2006	KZN	17	X	X	>512(32)	0.125(0.0625)	Yes
TCD105825	2006	GA	O	Asp82-His;Ser83-His; Asp87-Cys	Tyr57-Phe; Ser80-Pro	>512(16)	0.125(0.0625)	Yes
TCD151747	2006	EC	O	Asp82-His;Ser83-His; Asp87-Cys	Tyr57-Arg; Gly78-Cys; Glu84-Ser	>512(32)	0.125(0.0625)	Yes
TCD168510	2006	KZN	O	Gly81-Lys; Asp87-Ile; Ala119-Phe	Tyr57-Arg	>512(32)	0.125(0.0625)	Yes
TCD151753	2006	EC	O	Gly81-Lys; Asp82-Lys; Leu98-Met; Ala119-Phe	Tyr57-Arg; Gly78-Cys; Glu84-Ser	>512(32)	0.125(0.0625)	Yes
TCD136826	2006	EC	O	Asp82-His;Ser83-His; Asp87-Cys; Leu98-His	Tyr57-Arg; Gly78-Cys; Glu84-Ser	>512(32)	0.125(0.0625)	Yes
TCD155842	2006	KZN	O	X	X	>512(32)	0.125(0.0625)	Yes
TCD152864	2006	KZN	O	X	X	>512(32)	0.125(0.0625)	Yes
TCD150898	2006	WC	O	X	X	>512(64)	0.125(0.0625)	Yes
TCD114240	2006	WC	O	X	X	>512(32)	0.25(0.0625)	Yes

1: GA, Gauteng; WC, Western Cape; KZN, KwaZulu-Natal; EC, Eastern Cape; MP, Mpumalanga.

2: PFGE types based on relatedness as based on a dendrogram cluster analysis of the patterns using the unweighted pair group method with arithmetic averages, with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5% and a position tolerance setting of 1.5%. PFGE types defined at ≥90% similarity. PFGE type O, are isolates outliers that did not fit into a PFGE cluster at ≥90% similarity.

3: Value in parenthesis represents the MIC with the addition 40 µg/ml *β-Phe-Arg-naphthylamide* as an efflux pump inbihitor.

*: X represents not analysed.

All *qnr* screening results negative for *Salmonella* Isangi.

5.2. Discussion

Although PFGE cluster 8 constitutes the largest cluster, the majority of the isolates were distributed across many small clusters. The large number of PFGE types and clusters suggests that these South African *Salmonella* Isangi isolates evolved from different sources. The possible variability of sources of *Salmonella* Isangi implies that nalidixic acid resistance in South African *Salmonella* Isangi evolved independently i.e. is not clonally driven.

This result illustrates that *Salmonella* Isangi also exhibits an active efflux under stress which is in accordance with findings for other different Salmonella serotypes (Piddock *et al.*, 2002; Gaind *et al.* 2006). The effect of the efflux pump can be illustrated by focusing on three isolates, TTD1024955, TCD97401 and TCD105825. These three isolates all exhibit a high level of nalidixic acid resistance (MIC ≥512 µg/ml). The inhibition of the efflux pump in these three isolates decreases their respective nalidixic acid MIC values to within the susceptible range (MIC ≤16µg/ml). This result shows that for these three isolates the efflux pump is the main contributory mechanism of quinolone resistance. Although all three of these isolates exhibit mutations in both *gyrA* and *parC* regions of the QRDR, the fact that their nalidixic acid MIC values were decreased to a value that is within the susceptible range suggests that the mutations in both the *gyrA* and *parC* regions of these three isolates may not play a role in the development of clinical quinolone resistance. These results may be due to the over-expression of the AcrAB-TolC efflux system (Olliver *et al.,* 2005). The remaining twenty-nine isolates although

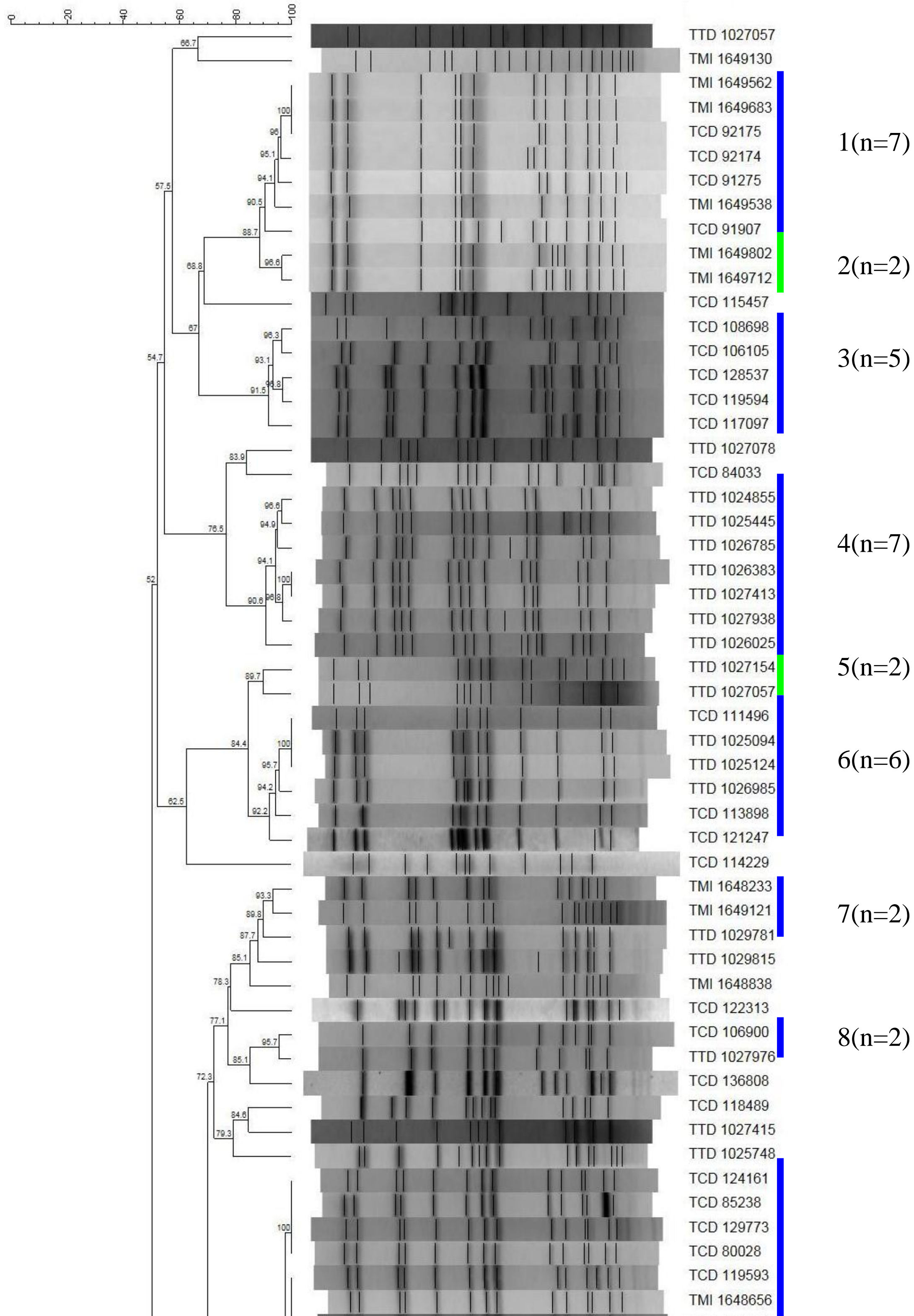
susceptible to ciprofloxacin still exhibit some resistance to nalidixic acid in the presence of an efflux pump inhibitor, suggesting that for these twenty-nine isolates a secondary mechanism is a contributing factor to quinolone resistance.

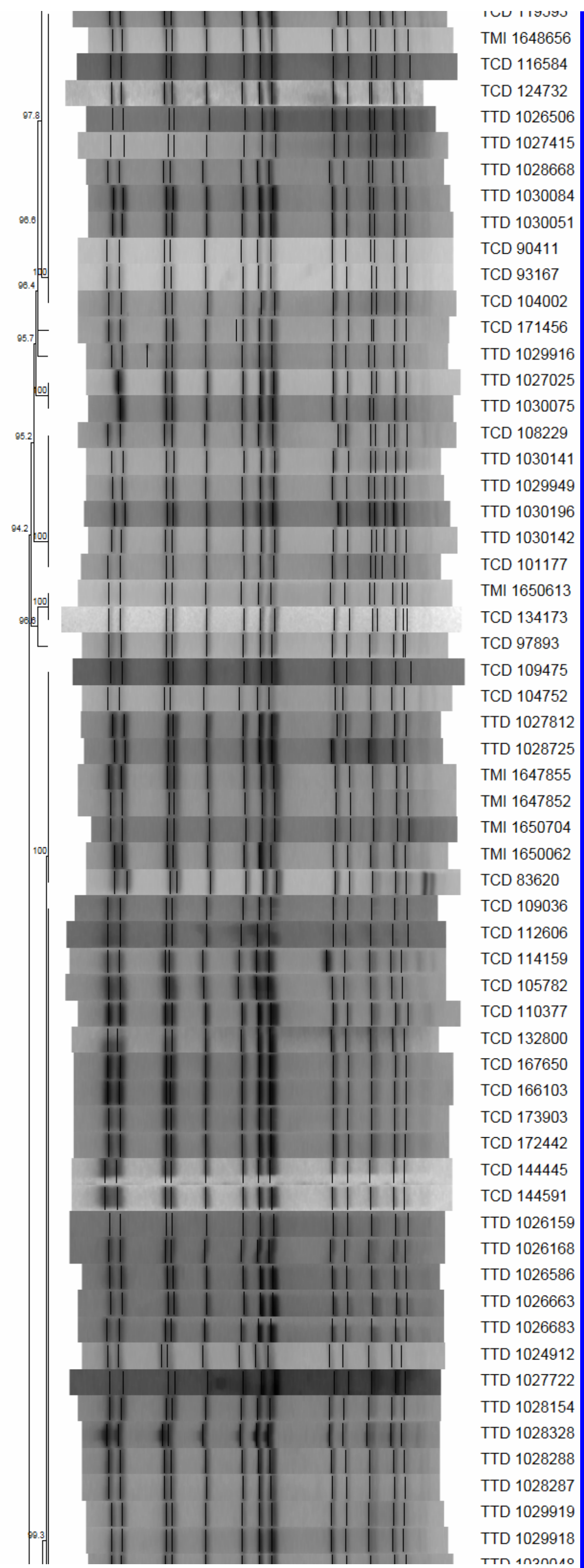
Sequencing of *gyrB* and *parC* revealed no mutations, therefore the role of *gyrB* and *parE* mutations can be excluded as they remain rare as previously suggested (Hopkins *et al.*, 2007). Screening for the pentapeptide repeat genes *qnrA, qnrB* and *qnrS* showed no positive results thus eliminating the *qnr* plasmids as a contributory mechanism of resistance. The seventeen isolates selected for sequencing all exhibited mutations in *gyrA* and *parC*. With the exception of the three aforementioned isolates, it would seem as though the *gyrA* and *parC* mutations in the remaining fourteen isolates contribute to quinolone resistance. All the sequenced isolates exhibited a combination of mutations at *gyrA*. The previously published Gly81-Asp as well as novel mutations Gly81-Lys, Asp82-His/Pro, Ser83-His/Pro, Asp87-Cys/Ile/Ala, Leu98-His/Pro/Met and Ala119-Phe were identified. Seven isolates exhibited a mutation at *gyrA* position Gly81. Another common site for mutation was at *gyrA* position Asp82 (Table 5.2). All seventeen isolates showed mutations in a combination of positions Gly81, Asp82, Ser83, Asp87 and Leu98. These mutations were found in combination involving either all five positions or three of the four positions within the *gyrA* (Table 5.2). The various combinations of mutations in the *gyrA* would imply that all these sequenced isolates should be fluoroquinolone-resistant (Eaves *et al.,* 2004) but they are not (Table 5.2).

This result is in accordance with Eaves *et al.* (2004) who found that isolates exhibiting mutations in both *gyrA* and *parC* are more susceptible to fluoroquinolones. All seventeen sequenced *Salmonella* Isangi isolates exhibited mutations at *parC* position Tyr57, with some isolates showing multiple mutations in the *parC* region (Table 5.2). As previously suggested the role of the Tyr57 mutation in its contribution to quinolone resistance is questionable (Baucheron *et al.*, 2004). Isolate TCD105825 showed a double mutation in *parC* in positions Tyr57 and Ser80 but with the inhibition of the efflux pump the isolates nalidixic acid MIC value was within the susceptible range (Table 5.2). In contradiction isolate TCD150654 with a double mutation at positions Tyr57 and Ser80 of *parC* with the inhibition of the efflux pump was still nalidixic acid resistant. The role of *parC* mutations in quinolone resistance in *Salmonella* is poorly understood (Hopkins *et al.*, 2005), thus it is difficult to conclude that the *parC* mutations found in these South African *Salmonella* Isangi isolates play a role in quinolone resistance. The main contributory mechanisms for quinolone resistance in these South African *Salmonella* Isangi isolates is a combination of an active efflux pump, *gyrA* mutations and possibly *parC* mutations. However there is insufficient evidence from this study to suggest that additional mutations in *gyrA* and/or in *parC* do not extend quinolone resistance to fluoroquinolone resistance. We cannot say that any single unique combination of mutations is the defining factor for quinolone resistance, as there seems to be multiple mutations and multiple combinations of mutations in positions 81 to 119 of *gyrA* and 57 to 84 of *parC* in these *Salmonella* Isangi isolates which can contribute to quinolone resistance.

Chapter 6: *Salmonella* Typhimurium

6.1. Results

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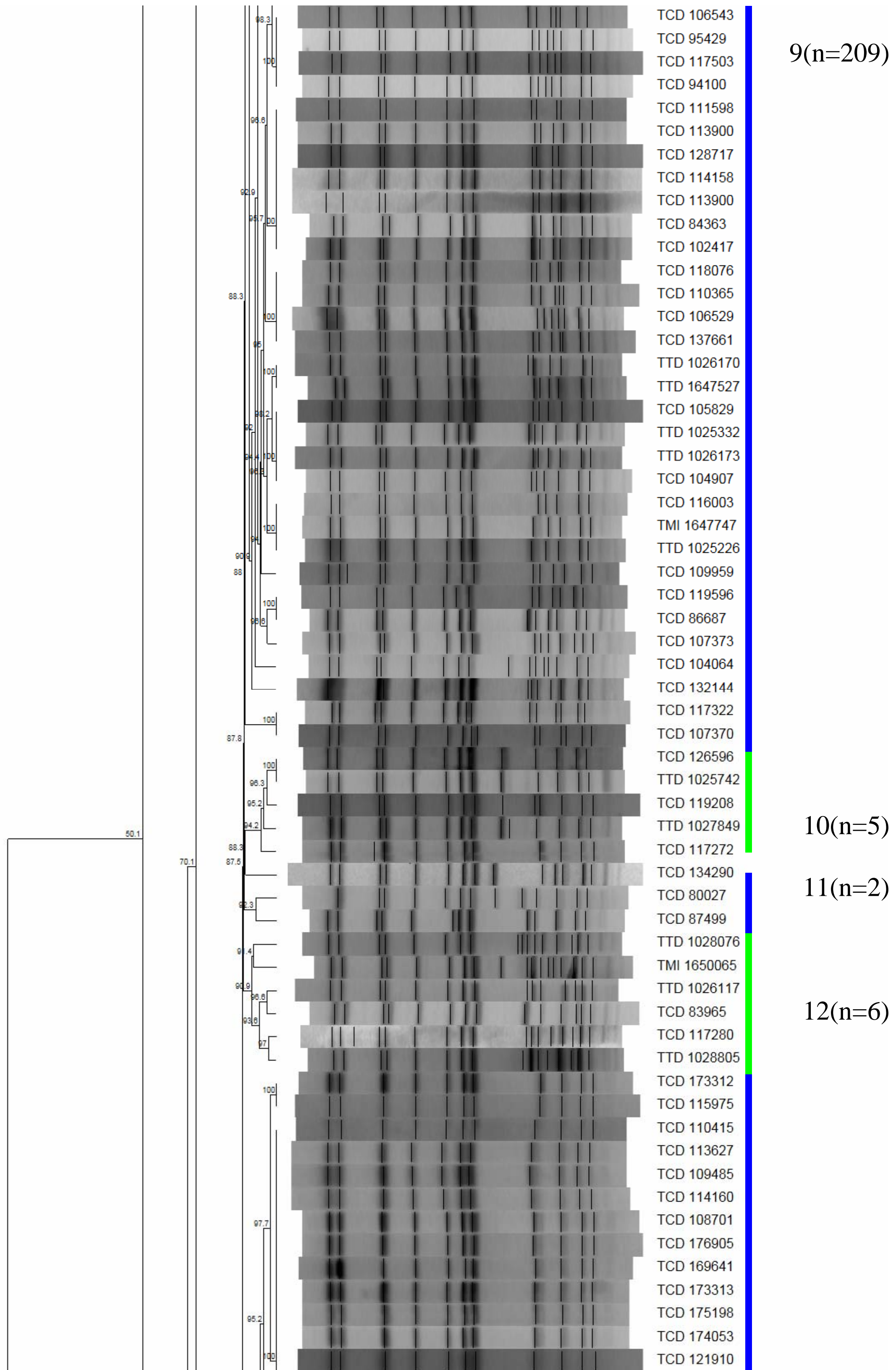

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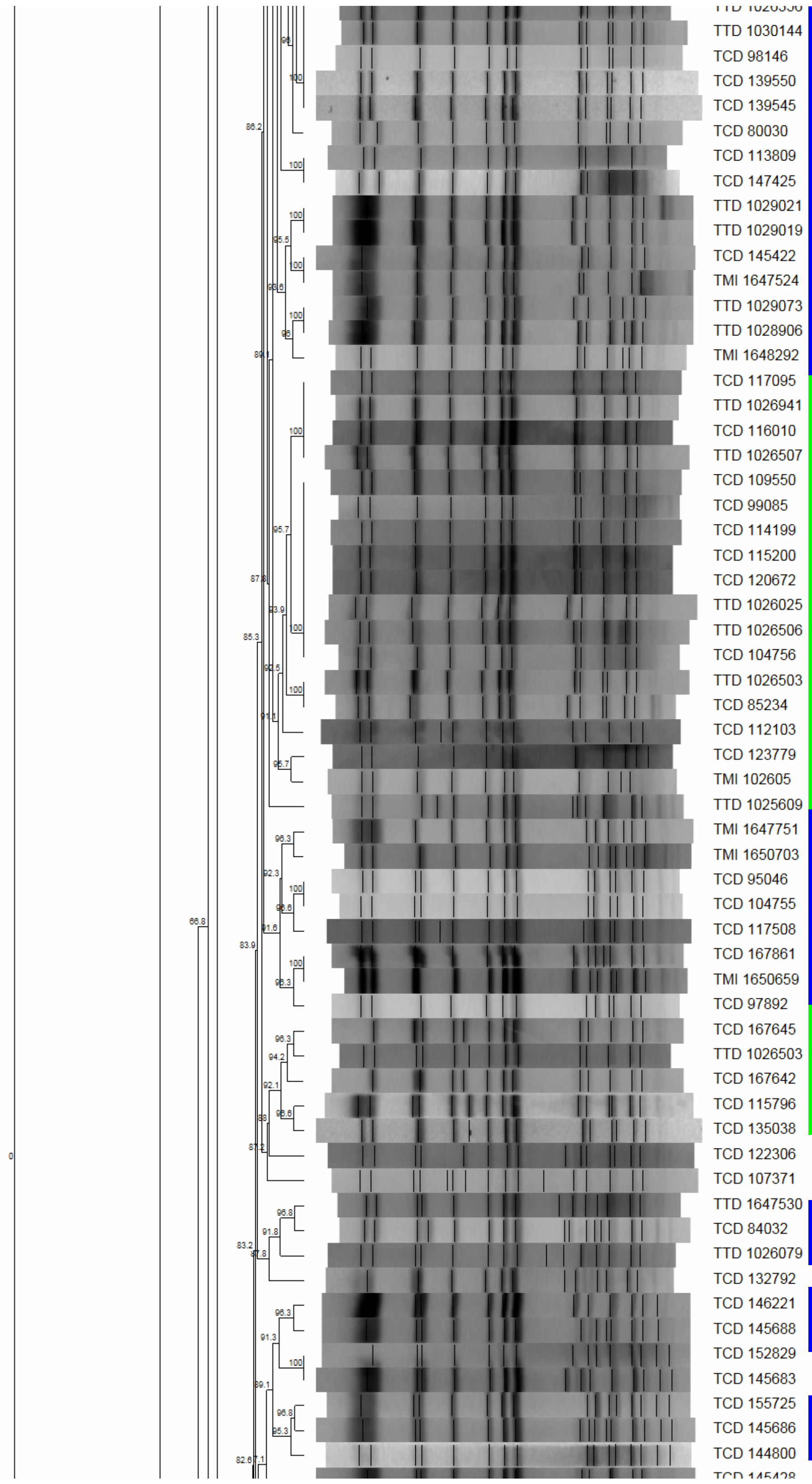
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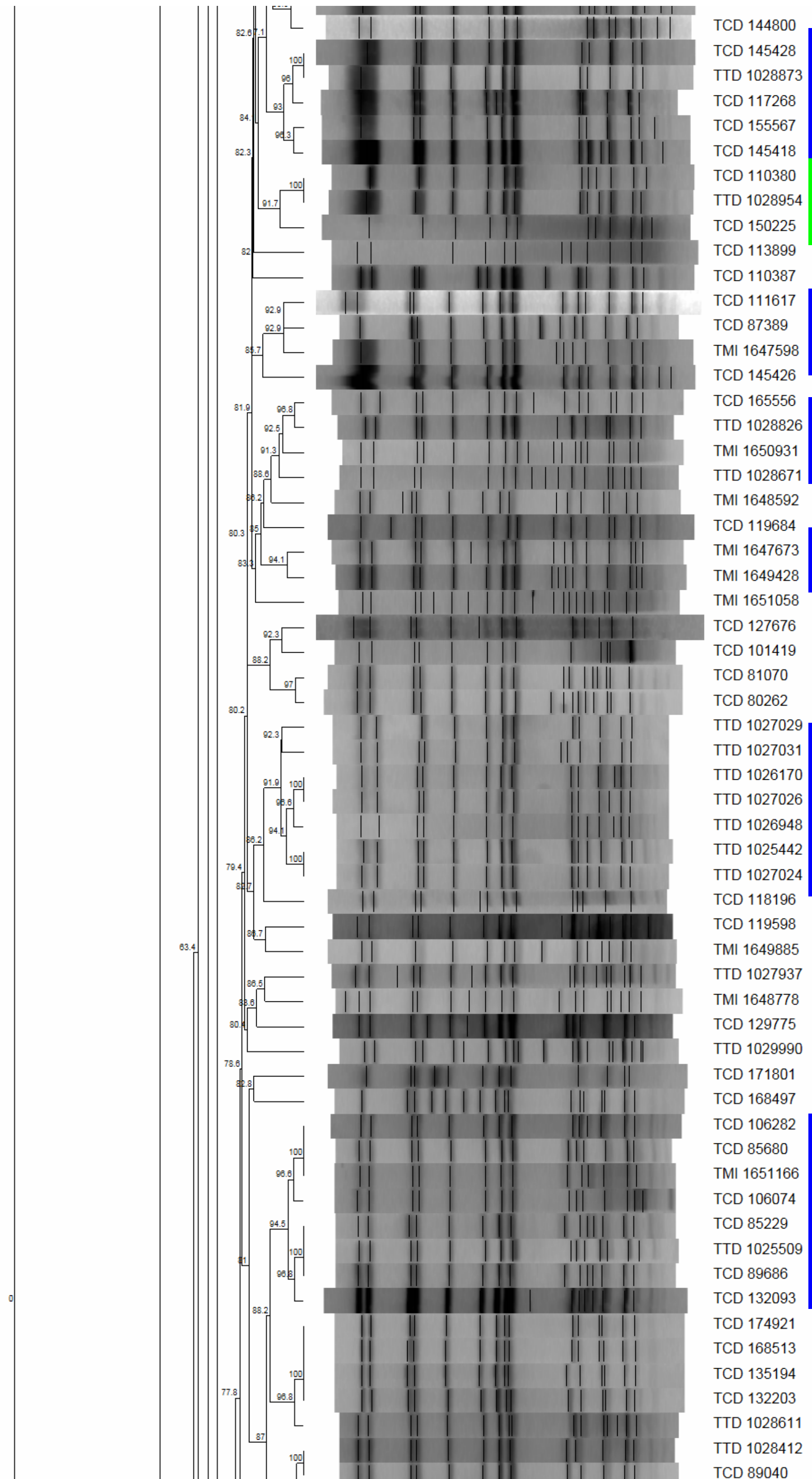

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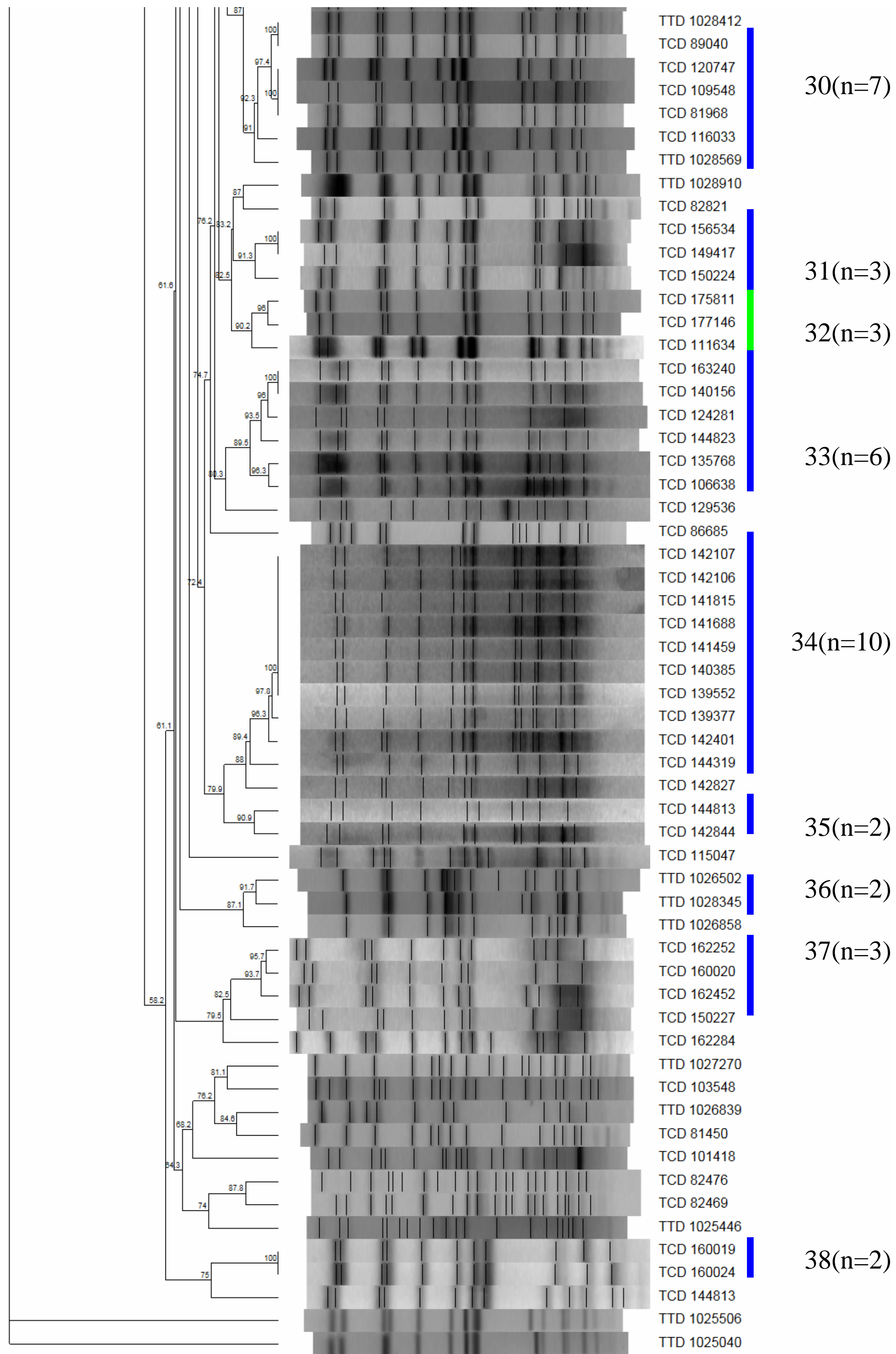
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Figure 6.1. PFGE dendrogram of nalidixic acid-resistant *Salmonella* Typhimurium isolates causing invasive disease in South Africa, 2004 to 2006, showing similarity index, banding pattern and isolate number. The dendrogram shows 38 PFGE clusters with PFGE cluster 9 being the largest cluster with 209 isolates.

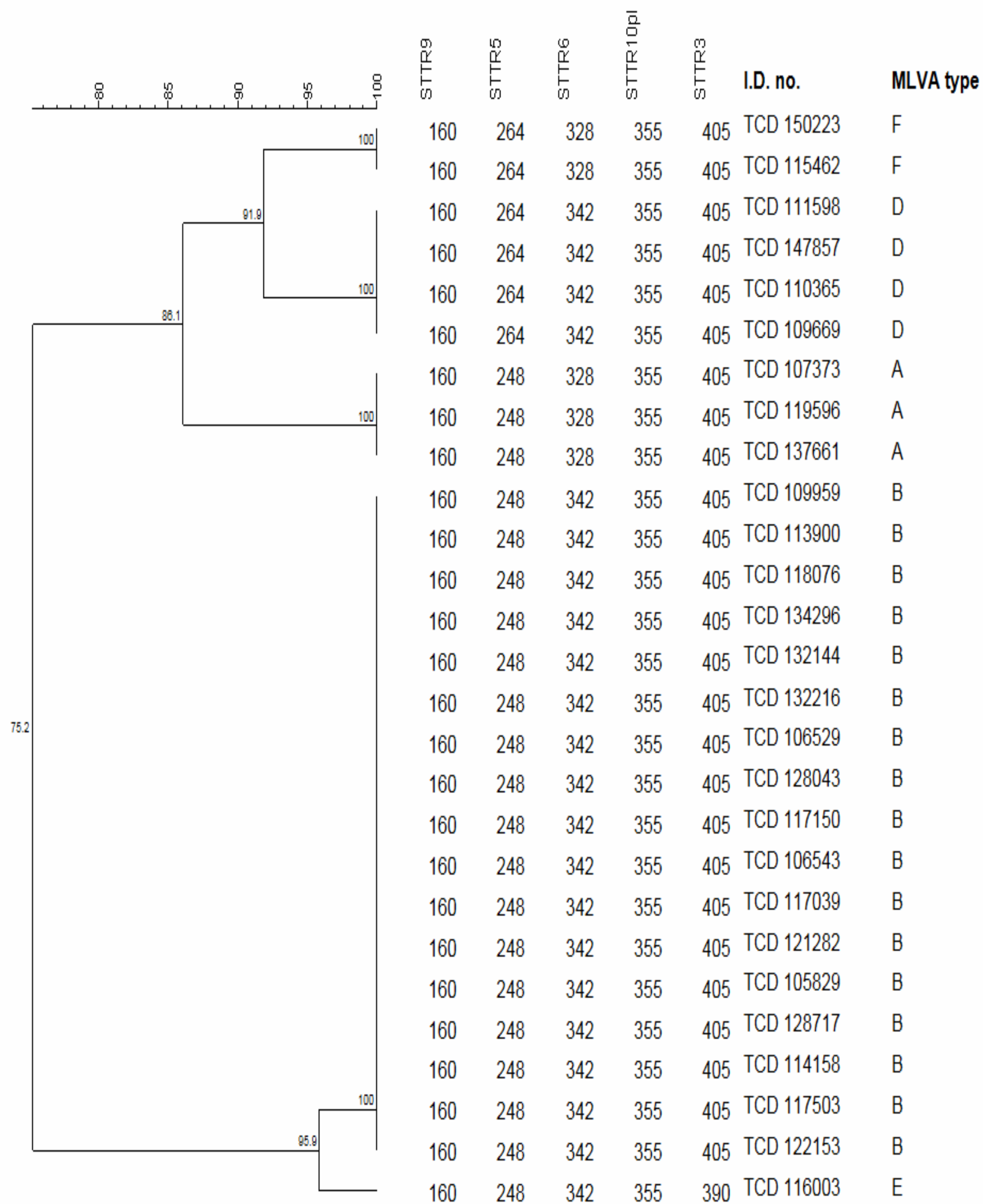

Figure 6.2. MLVA dendrogram of 27 nalidixic acid-resistant *Salmonella* Typhimurium isolates from PFGE cluster 9 causing invasive disease in South Africa, 2004 to 2006, showing similarity index, VNTR loci (STTR9, 5, 6, 10-pl and 3), isolate number and MLVA type.

Two-thousand six-hundred and twenty-four human isolates of *Salmonella* Typhimurium were received by the EDRU for the period 2004-2006, of which, six-hundred and twenty-four were invasive, nalidixic acid-resistant. Molecular analysis was conducted on five-hundred and sixteen available isolates. PFGE analysis resolved the invasive, nalidixic acid-resistant *Salmonella* Typhimurium isolates into ninety-five PFGE types (As defined in chapter 2) (Figure 6.1). The isolates were distributed across thirty-eight PFGE clusters, with two large clusters dominating the dendrogram. PFGE cluster 9 constituted by two-hundred and nine isolates and PFGE cluster 13 constituted by eighty-three isolates (Figure 6.1). Although the dendrogram is dominated by two large cluster, the number immense number of PFGE types and PFGE clusters constituting the dendrogram suggests that nalidixic-acid resistance amongst the South African *Salmonella* Typhimurium isolates is not clonally driven.

Forty-six randomly selected isolates were analysed using MLVA specific for *Salmonella* Typhimurium as a method of evaluating the PFGE results. MLVA was able to group isolates from different PFGE types as either different or similar based on the isolates allelic profile (Table 6.1). Twenty seven isolates from the PFGE cluster 9 were analysed. PFGE grouped all twenty seven isolates as similar but MLVA distinguished the same twenty seven isolates into five MLVA types (Table 6.1). The dendrogram for the twenty-seven PFGE cluster 9 isolates created from the MLVA analysis (Figure 6.2) illustrates the highly discriminatory nature of the analysis as the isolates were selected from different PFGE type clusters. MLVA was able to group PFGE unrelated isolates as either the same MLVA type or as a closely related MLVA type. In contradiction, a number of isolates

belonging to MLVA type 9 were separated into ten different PFGE types (Table 6.1). In this case PFGE can be said to be more discriminatory than MLVA although MLVA can discriminate between isolates with identical PFGE patterns.

The investigation into the possible contributory mechanisms of quinolone resistance for the *Salmonella* Typhimurium isolates showed that out of forty-one randomly selected isolates forty isolates exhibited an over-over-active efflux pump (Table 6.1). Isolates TCD110365, TTD1027026 and TTD1025442 were found to have been harbouring the *qnr*B plasmid.

Table 6.1. PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Typhimurium isolates

Isolate	Year	Province[1]	PFGE type[2]	MLVA type[3]	MLVA profile[4]	*gyrA* mutations	*parC* mutations	*qnr* plasmid	Nalidixic acid agar dilution MIC (µg/ml)[5]	Ciprofloxacin agar dilution MIC (µg/ml)[5]	Active Efflux
TTD1026502	2004	KZN	9	X	X	Ser83-Phe; Asp87-Gly	Ser80-Arg	X	>512(>512)	16(4)	Yes
TMI1649855	2005	GA	9	X	X	Asp87-Gly	Tyr57-Arg	X	>512(32)	0.125(0.0625)	Yes
TCD101416	2005	GA	9	X	X	Asp87-Gly	Tyr57-Arg	X	>512(32)	0.125(0.0625)	Yes
TCD102423	2005	GA	9	X	X	Asp87-Gly	Tyr57-Arg	X	>512(64)	0.125(0.0625)	Yes
TCD117150	2006	GA	9	B	160-248-342-355-405	X	X	X	X	X	X
TCD122153	2006	GA	9	B	160-248-342-355-405	X	X	X	X	X	X
TCD128043	2006	GA	9	B	160-248-342-355-405	X	X	X	X	X	X
TCD119596	2006	GA	9	A	160-248-328-355-405	X	X	X	X	X	X
TCD109669	2006	GA	9	D	160-264-342-355-405	X	X	X	X	X	X
TCD115462	2006	GA	9	F	160-264-328-355-405	X	X	X	X	x	x
TCD134296	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD117039	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD147857	2006	GA	9	D	160-264-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD150223	2006	GA	9	F	160-264-328-355-405	X	x	X	X	X	X
TCD114158	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD128717	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD113900	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD117503	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD107373	2006	GA	9	A	160-248-328-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TCD121282	2006	GA	9	B	160-248-342-355-405	Asp87-Gly	Tyr57-Arg	X	256(32)	0.25(0.125)	Yes
TDC110365	2006	GA	9	D	160-264-342-355-405	Wild Type	Wild Type	*qnr*B	32(32)	0.25(0.25)	No
TCD106543	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.5(0.125)	Yes
TCD105829	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.5(0.125)	Yes
TCD132216	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD118076	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.5(0.125)	Yes
TCD132144	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD109959	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD106529	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.5(0.125)	Yes

Table 6.1. Continued PFGE types, MIC results and QRDR results for randomly selected *Salmonella* Typhimurium isolates

Isolate	Year	Province[1]	PFGE type[2]	MLVA type[3]	MLVA profile [4]	*gyrA* mutations	*parC* mutations	qnr plasmid	Nalidixic acid agar dilution MIC (µg/ml)[5]	Ciprofloxacin agar dilution MIC (µg/ml)[5]	Active Efflux
TCD137661	2006	GA	9	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD111598	2006	GA	9	D	160-264-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD116003	2006	GA	9	E	160-248-342-355-390	X	X	X	X	X	X
TCD147245	2006	GA	13	B	160-248-342-355-405	X	X	X	X	X	X
TCD167861	2006	GA	15	B	160-248-342-355-405	X	X	X	X	X	X
TCD152829	2006	GA	18	B	160-248-342-355-405	X	X	X	X	X	X
TCD145683	2006	GA	18	B	160-248-342-355-405	X	X	X	X	X	X
TCD146221	2006	GA	18	F	160-264-328-355-405	Asp87-Tyr	Tyr57-Gly	X	256(32)	0.25(0.125)	Yes
TCD145688	2006	GA	18	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD145686	2006	GA	19	X	X	Asp87-Tyr	Tyr57-Gly	X	256(32)	0.25(0.125)	Yes
TCD155725	2006	GA	19	B	160-248-342-355-405	Asp87-Tyr	Tyr57-Gly	X	256(32)	0.25(0.0625)	Yes
TCD144800	2006	GA	19	A	160-248-328-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD145418	2006	GA	20	B	160-248-342-355-405	Asp87-Tyr	Tyr57-Gly	X	256(32)	0.25(0.0625)	Yes
TCD111617	2006	GA	22	B	160-248-342-355-405	Asp87-Tyr	Tyr57-Gly	X	256(32)	0.25(0.125)	Yes
TTD1027026	2004	GA	27	X	X	Asp87-Lys	Tyr57-Ser	*qnr*B	>512(32)	4(2)	Yes
TTD1025442	2004	GA	27	X	X	Asp87-Lys	Tyr57-Ser	*qnr*B	>512(32)	4(2)	Yes
TCD106282	2006	GA	28	C	160-216-342-370-450	Asp87-Lys	Tyr57-Ser	X	256(32)	0.25(0.125)	Yes
TCD150224	2006	GA	31	C	160-216-342-370-450	X	X	X	256(32)	0.25(0.125)	Yes
TCD156534	2006	GA	31	A	160-248-328-355-405	Asp87-Lys	Tyr57-Ser	X	256(32)	0.25(0.125)	Yes
TCD111634	2006	GA	32	B	160-248-342-355-405	X	X	X	X	X	X
TCD177146	2006	GA	32	G	160-264-314-355-405	X	X	X	X	X	X
TCD175811	2006	GA	32	G	160-264-314-355-405	Asp87-Lys	Tyr57-Ser	X	256(32)	0.25(0.0625)	Yes
TTD1026858	2004	KZN	O	X	X	Ser83-Phe; Asp87-Gly	Ser80-Arg	X	>512(>512)	16(4)	Yes
TMI1648592	2005	GA	O	X	X	Ser83-Phe	Ser80-Arg	X	>512(32)	0.25(0.125)	Yes
TDC119684	2006	GA	O	A	160-248-328-355-405	X	X	X	256(32)	0.25(0.125)	Yes
TCD113899	2006	GA	O	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.0625)	Yes
TCD118196	2006	GA	O	B	160-248-342-355-405	X	X	X	256(32)	0.25(0.125)	Yes

1: GA, Gauteng; KZN, KwaZulu-Natal

2: PFGE types based on relatedness as based on a dendrogram cluster analysis of the patterns using the unweighted pair group method with arithmetic averages, with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5% and a position tolerance setting of 1.5%. PFGE types defined at ≥90% similarity. PFGE type O, are isolates outliers that did not fit into a PFGE cluster at ≥90% similarity.

3: MLVA types based on MLVA profiles.

4: MLVA profiles in an allelic string of STTR9-STTR5-STTR6-STTR10pl-STTR3

5: Value in *paren*thesis represents the MIC with the addition 40 µg/ml *β-Phe-Arg-naphthylamide* as an efflux pump inbihitor.

*: X represents not analysed.

6.2 Discussion

Lindstedt *et al.* (2003) suggested that PFGE always was not sensitive enough to distinguish amongst strains portraying certain phenotypic characteristics such as antibiotic resistance, as the strains all possessed identical PFGE patterns: thus the development of MLVA. Many further studies have bettered the technique and the upgrade to a more automated MLVA has enhanced the discriminatory power of the technique (Lindstedt *et al*., 2004). The repeatability and consistency of results have made sharing of MLVA data easier for international and outbreak purposes (Lindstedt *et al*., 2007; Torpdahl *et al*., 2007). The use of PFGE as the typing method of *Salmonella* in general is still seen as the "gold standard" and its results still acceptable, especially for routine surveillance (Lindstedt, 2005; Torpdahl *et al*., 2006).

In the presence of EPI, a four-fold decrease in MIC was observed for both nalidixic acid and ciprofloxacin for all forty-one isolates (Table 6.1) indicating that an efflux pump is playing a role in quinolone resistance. However an efflux pump was not the only contributing mechanism of resistance as the MIC for nalidixic acid for all forty-one of the isolates in the presence of an efflux pump inhibitor was as low as 32µg/ml (Table 6.1). Baucheron *et al*. (2004) previously showed that *Salmonella* Typhimurium show signs of an over-over-active efflux pump in response to antibiotics.

In our current study, isolate TCD110365 was the exception to the above. This isolate was PCR positive for the *qnr*B (Table 6.1). The PCR positive *qnr*B result was confirmed by

nucleotide sequence analysis of the gene. QRDR sequences for this isolate showed no mutations in any of the genes (Wild Type; Table 6.1), so mutation in the QRDR was excluded as a mechanism for quinolone resistance. Agar dilution MIC's for nalidixic acid and ciprofloxacin showed no involvement in resistance of an over-active efflux pump, as there was no difference in MIC values following testing in the absence or presence of an efflux pump inhibitor. The agar dilution MIC's did illustrate the isolates low-level of nalidixic acid resistance (MIC, 32µg/ml) and susceptibility to ciprofloxacin (MIC, 0.25µg/ml) (Table 6.1). The low-level nalidixic acid resistance of this *Salmonella* Typhimurium can be attributed to the presence of the *qnr*B. In addition, the ciprofloxacin MIC of 0.25µg/ml is seen by many as an increased MIC and the isolate may be described as having a reduced susceptibility to ciprofloxacin (Hopkins, *et al.*, 2008). Isolates such as these, that confer low-level quinolone resistance due to *qnr* genes, facilitate the selection higher-level resistance mutants. This is the first report of *qnr* plasmid mediated quinolone resistance in South African Enterobacteriaceae.

Two other isolates, TTD1027026 and TTD1025442 were also positive for the *qnr*B. These two isolates were also found to have an over-active efflux pump that was contributory to the development of quinolone resistance and mutations in the *gyrA* (Asp87-Lys) and *parC* (Tyr57-Ser) of the QRDR (Table 6.1). The combination of all these mechanisms contributed to both isolates being not only nalidixic acid-resistant (MIC, ≥512µg/ml), but also ciprofloxacin-resistant (MIC, ≥4µg/ml) (Table 6.1). The inhibition of the efflux pump resulted in the decrease of both nalidixic acid and ciprofloxacin MIC's but the isolates were still nalidixic acid-resistant and exhibited

intermediate resistance to ciprofloxacin (Table 6.1). The combination of the mutations in both the *gyrA* and *parC* regions, as well as the presence of the *qnr*B was sufficient to result in these isolates being nalidixic acid-resistant and intermediately resistant to ciprofloxacin.

The role of the *qnr*B in these two isolates is illustrated when looking at the *gyrA* and *parC* mutations. Both TTD1027026 and TTD1025442 exhibit *gyrA* mutation Asp87-Lys and *parC* mutation Tyr57-Ser. These mutations are also found in ciprofloxacin susceptible isolates TCD106282 and TCD156534 (Table 6.1). These mutations can be excluded as the contributory mechanism for ciprofloxacin resistance in isolates TTD1027026 and TTD1025442. The *qnr*B in TTD1027026 and TTD1025442 plays a different role to the one it did with regard to TCD110365, as in these two isolates it is the defining factor in fluoroquinolone resistance. The combination of the various mechanisms have been previously been touted as the factors required for fluoroquinolone resistance as opposed to the contribution of just a single mechanism (Hopkins, *et al.*, 2007).

For all these sequenced South African quinolone-resistant *Salmonella* Typhimurium isolates, the mutations in the *gyrA* and *parC* regions of the QRDR are fairly uniform (Table 6.1). All sequenced PFGE type 9 isolates with the exception of TTD1026502, displayed mutations at *gyrA* Asp87-Gly and a novel *parC* mutation Tyr57-Arg. All the isolates with the exception of TTD1026502 were susceptible to ciprofloxacin although it

has been previously suggested that isolates with mutations in both *gyrA* and *parC* exhibit a fluoroquinolone resistance (Baucheron *et al*., 2005).

The mutation Asp87-Gly has previously shown to be a contributing factor to quinolone resistance (Hirose *et al*., 2002) but the novel mutation at *parC* Tyr57-Arg may not play a role as Baucheron *et al.* (2004) suggested that mutation at Tyr57 are not important in conferring resistance to fluoroquinolones. This would also provide evidence for the suggestion of Eaves *et al.* (2004), that isolates with mutations in both the *gyrA* and *parC* are more susceptible to fluoroquinolones. This implies that the main contributory mechanism of resistance for the isolates from PFGE cluster 9 is a combination of an over-active efflux pump and mutation(s) in *gyrA*. The same can be said for the other sequenced isolates from different PFGE types or clusters. The commonality amongst the isolates is mutations in *gyrA* Asp87 and *parC* Tyr57 (Table 6.1). Although the amino acid changes differ, the argument remains the same. The role of the *parC* mutation remains unclear as it may not play a role in quinolone-resistant *Salmonella* as these mutations are not found without a *gyrA* mutation (Eaves *et al*., 2004) and may also be dependant on an over-active efflux system. Ling *et al* (2003) showed that the mutation at Tyr57 contributed to an increase in ciprofloxacin in their *Salmonella* but they did not investigate the role of an over-active efflux system.

Isolate TTD1026502 from PFGE type 9 cluster and two outlying isolates TTD1026858 and TMI1648592 possessed different combinations of *gyrA* and *parC* mutations, as com*parE*d to those discussed above. Using agar dilution MIC testing, both TTD1026858

and TTD1026502 were found to be ciprofloxacin resistant (MIC, 16µg/ml). This isolate showed *gyrA* mutations at Ser83-Phe and Asp87-Gly and *parC* mutation Ser80-Arg. The combination of these mutations with the inhibition of the efflux pump, which was only evident with change in ciprofloxacin MIC, still resulted in the isolates being ciprofloxacin resistant (MIC, 4µg/ml) (Table 6.1). All the mutations displayed by TTD1026858 and TTD1026502 have been previously shown to influence quinolone resistance (Hopkins *et al.*, 2005) and with combination of the over-active efflux pump the isolate is highly-resistant to ciprofloxacin (MIC, 16µg/ml). TMI1648592 possessed mutations at *gyrA* Ser83-Phe and *parC* Ser80-Arg but was only resistant to nalidixic acid. The mutation Ser83 in *gyrA* was shown to influence quinolone resistance (Ling et al., 2003) but in combination with the *parC* mutation Ser80-Arg (which was found in TTD1026858 and TTD1026502) one would expect TMI1648592 to be ciprofloxacin resistant but it is not (Table 6.1). The difference between TMI1648592 and, TTD1026858 and TTD1026502 is that, TTD1026858 and TTD1026502 posses a second *gyrA* mutation at Asp87-Gly. Eaves *et al.* suggested that an isolate requires multiple mutations in the *gyrA* to acquire fluoroquinolone resistance rather than a mutation in the topoisomerase region. Thus the second mutation at Asp87-Gly is an important role player in fluoroquinolone resistance.

The probable contributory mechanisms for quinolone resistance in these South African *Salmonella* Typhimurium isolates are a combination of an over-active efflux pump, *gyrA* mutation and possibly *parC* mutation. Low level quinolone resistance may also be conferred via plasmid mediated resistance namely the *qnr*B plasmid. It is apparent that a

combination of mechanisms may be necessary for high-level fluoroquinolone resistance. This could be in the form of (1) *qnr*, efflux pump, *gyrA* mutation and possibly *parC* mutation, or (2) efflux pump, double *gyrA* mutation and possibly *parC* mutation. The evidence presented by this study does not allow for any confident conclusions to be drawn as to what mechanisms play a role in quinolone resistance but only for further hypothesis's to be developed.

Chapter 7: General Discussion and Conclusion

This study was the first attempt to describe the molecular epidemiology and mechanism of resistance of invasive quinolone-resistant South African isolates of any of the four *Salmonella* serotypes subjected to this study. More especially involving the analysis of the large number of isolates collected over a three year period for the NTS serotypes and five year period for the *Salmonella* Typhi. The majority of the isolates analyzed in this study were collected from the Gauteng area resulting in a biased sample, but the EDRU is dependant on isolates received from hospitals participating in routine surveillance. The large number of isolates available for Gauteng may be due to under reporting of salmonellosis in other provinces or poor infrastructure and the unavailability of resources in those provinces for the effective processing of patient samples or that the EDRU is located in Gauteng, an academic center, and for that reason isolates from Gauteng are easily obtained and reported. This phenomenon is noted by the EDRU and they are encouraging increased awareness and surveillance from the other provinces. The sample is Gauteng biased, which is a limitation of the study, and it is believed that the underlying trends shown for all four *Salmonella* serotypes from this study are not a good representation of quinolone-resistant isolates from the entire country of South Africa. Another limitation of this study was the time and financial constraints which resulted in a small number of isolates being exposed to the full scope of molecular analysis in this study.

All four *Salmonella* serotypes from our study showed a similar pattern of independent emergence of quinolone resistance. The PFGE results for all four serotypes revealed dendrograms resolved into a large number of PFGE types in relation to the number of analysed isolates. The large number of PFGE types per serotype suggests that there have been a various number of probable sources for the emergence of quinolone resistance for each respective serotype. This finding suggests that quinolone resistance for these four *Salmonella* serotypes is driven by a selective pressure, in this case the treatment strategies, more than the source of the isolate. The use of MLVA as a secondary technique to PFGE provided evidence that MLVA should be used as a method for evaluating PFGE results for both *Salmonella* Typhi and *Salmonella* Typhimurium. PFGE is subjective as it relies on the researcher to identify and mark resultant bands using a software package. This may lead to discrepancies in results within a lab and between collaborating labs. MLVA allows for a more objective analysis as the analysis is automated and the analysis parameters are adhered to by the machinery and computer system. For *Salmonella*, PFGE remains the primary genotyping technique. Although it has been shown that MLVA exhibits a greater discriminatory power than PFGE (Lindstedt *et al.,* 2003; Liu *et al.,* 2003; Boxrud *et al.,* 2007), the two techniques should be used to complement each other where available as the combination will probably best represent the sample population.

The four study *Salmonella* serotypes also share a common combination of mechanisms of resistance. The major mechanism of resistance for all four serotypes was an over-active efflux system. An over-active efflux system in combination with *gyrA* and *parC*

mutations was identified as contributory mechanisms in all four serotypes. The combination of *gyrA* and *parC* mutations alone was insufficient to explain the quinolone-resistant nature of any of the sequenced *Salmonella* isolates. No individual *gyrA* or *parC* mutation could be isolated as a major role player in quinolone resistance in these *Salmonella* isolates. The isolates from this study that exhibited mutations in both *gyrA* and *parC* were not as resistant as isolates with just a *gyrA* mutation. *parC* mutations are infrequent in *Salmonella* and the mutations at position 57 of *parC* gene occurs outside of the QRDR (Hopkins *et al.*, 2005). It has been suggested that *parC* mutations only occur as a result of exposure to increased levels of quinolones and that *Salmonella* select an over-active efflux system as the primary defense to quinolones and QRDR mutations occur as a secondary mechanism of resistance (Giraud *et al.,* 2006).

It has been established that *gyrA* mutations and an over-active efflux system are contributory mechanisms to quinolone resistance, as well as the fact that *parC* mutations will not occur without an initial *gyrA* mutation (Hopkins *et al.,* 2005). This lends evidence to the thought that isolates resistant to nalidixic acid and that possess *gyrA* mutation(s), with or without an over-active efflux system, are precursors to isolates that may possess *parC* mutations. These resultant isolates may be ciprofloxacin-resistant or may have a reduced susceptibility to ciprofloxacin. It may therefore be important to understand the relationship between *parC* mutations and an over-active efflux system.

The final mechanism of quinolone resistance identified in our study was that of the plasmid gene *qnrB*. Plasmid-mediated quinolone resistance in Enterobacteriaceae has not

previously been described for any South African isolate. All four study *Salmonella* serotypes were screened for the presence of *qnr* plasmid genes with *Salmonella* Typhi, *Salmonella* Enteritidis and *Salmonella* Isangi isolates negative for all screened genes. The presence of the *qnrB* gene was identified in the most commonly isolated South African *Salmonella* serotype, *Salmonella* Typhimurium. Three isolates were positive for the *qnrB* gene. Two of the three isolates were ciprofloxacin-resistant with the *qnrB* found in combination with *gyrA* and *parC* mutations as well as an over-active efflux system in both isolates. The last of the three isolates was the only isolate from all the *qnr* screened studies isolates exhibiting a single mechanism of resistance. The isolate exhibited a low-level quinolone resistance which is indicative of the presence of the *qnrB* plasmid mediated resistance. Isolation of the *qnrB* plasmid in three out of one-hundred and twenty-nine total screened *Salmonella* isolates from our study highlights the fact that *qnr* plasmid mediated resistance remains rare amongst *Salmonella* serotypes (Hopkins *et al.*, 2007).

Irrespective of the combination of the mechanisms of resistance for any of the analysed *Salmonella* isolates attention should not be drawn away from the important fact that these isolates were all nalidixic acid-resistant. Nalidixic acid-resistant isolates pose treatment challenges for healthcare professionals where the routine treatment for salmonellosis is ciprofloxacin. Although these isolates may be susceptible to ciprofloxacin according to the current CLSI breakpoints for ciprofloxacin, treatment of infection due to isolates resistant to nalidixic acid may result in treatment failure (Rupali *et al.,* 2004). This is of particular concern with regard to the treatment of infection due to *Salmonella* Typhi as

the irresponsible use of ciprofloxacin has resulted in an increase in quinolone-resistant isolates. For example administering ciprofloxacin for the treatment of cholera, when it rehydration therapy has been shown to be the best treatment as antibiotics do not seem to decrease the duration or severity of illness (Oliphant & Green, 2002). The indiscriminate use of ciprofloxacin as a routine treatment has created an unfortunate selection pressure that the bacteria have responded to by developing mechanisms of resistance (Chitnis *et al.,* 2006; Gupta and Kaur, 2008). Isolates that are nalidixic acid resistant are believed to exhibit a decreased susceptibility to ciprofloxacin. As a result subsequent treatment with ciprofloxacin could result in patients displaying a delayed response or may even lack a response to treatment (Hakanen *et al*., 2005). The CLSI breakpoints for ciprofloxacin are not accurately reflecting the true nature of the resistance patterns shown by the screened isolates (Crump *et al*., 2003). The CLSI breakpoints require review and nalidixic acid susceptibilities should be used as indicators of possible treatment failure with ciprofloxacin.

The clinical importance of *Salmonella* Typhi and invasive *Salmonella* infection makes the understanding and evolution of antibiotic resistance trends, in respect to treatment of typhoid fever and invasive infection, of paramount importance. Little is known of the role of the AcrAB-TolC tripartite system of *Salmonella* in South Africa, but our work suggests it is important in these quinolone-resistant strains. Since the system may vary from strain to strain it is important to form a basis of understanding of the efflux system of local strains, as the efflux system may be the major contributory mechanism to quinolone resistance. We have shown that an over-active efflux system in combination

with mutations in both *gyrA* and *parC* may play a major role in facilitating quinolone resistance in *Salmonella* Typhi, *Salmonella* Enteritidis and *Salmonella* Isangi. We have also shown that these mechanisms may be responsible for the quinolone resistance in the majority of the *Salmonella* Typhimurium isolates along with the rarely isolated mechanism of resistance, a *qnr* plasmid. Our study also highlights the need for further work to determine regional variability for *gyrA* mutations and to link the various mechanisms of resistance, such as, the relationship between *parC* mutations and an over-active efflux system, as the significance of various mutations and their interactions remains unclear.

References

Albayrak, F., Cokca, F., Erdem, B., and Aysev, A.D. (2004) Predictive value of nalidixic acid resistance for detecting salmonellae with decreased ciprofloxacin susceptibility. International Journal of Antimicrobial Agents. 23, 332–336

Baucheron, S., Imberechts, H., Chaslus-Dancla, E., and Cloeckaert A. (2002) The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium phage type DT204. Microbial Drug Resistance. 8, 281-289

Baucheron, S., Chaslus-Dancla, E., and Cloeckaert, A. (2004) Role of TolC and *parC* mutation in high-level fluoroquinolone resistance in *Salmonella enterica* serotype Typhimurium DT204. Journal of Antimicrobial Chemotherapy. 53, 657-659

Baucheron, S., Chaslus-Dancla, and E., Cloekaert, A. (2005) High-Level Resistance to Fluoroquinolones Linked to Mutations in *gyrA*, *parC*, and *parE* in *Salmonella enterica* Serovar Schwarzengrund Isolates from Humans in Taiwan. Antimicrobial Agents and Chemotherapy. 49, 862-863

Baucheron, S., Mouline, C., Praud, K., Chaslus-Dancla, E. and Cloeckaert, A. (2005) TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype Typhimurium colonization of chicks. Journal of Antimicrobial Chemotherapy. 55, 707–712

Bertrand, S., Weill, F-X., Cloeckaert, A.,Vrints, M., Mairiaux, E., Praud, K., Dierik, K., Wildemauve, C., Godard, C., Butaye, P., Imberechts, H., Grimont, P.A.D., and Collard, J-M. (2006) Clonal Emergence of Extended-Spectrum β-Lactamase (CTX-M-2)-Producing *Salmonella enterica* Serovar Virchow Isolates with Reduced Susceptibilities to Ciprofloxacin among Poultry and Humans in Belgium and France (2000 to 2003). Journal of Clinical Microbiology. 44, 2897–2903

Bhan, M.K., Bahl, R., and Bhatnagar, S., (2005) Typhoid and paratyphoid fever. Lancet. 366, 749-762

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus C., Lyszkowicz, E.,Besser, J., and Bartku, J.M. (2007) Comparison of Multiple-Locus Variable-Number Tandem Repeat Analysis, Pulsed-Field Gel Electrophoresis, and Phage Typing for Subtype Analysis of *Salmonella enterica* Serotype Enteritidis. Journal of Clinical Microbiology. 45, 536-543

Braoudaki, M., and Hilton, A.C. (2004) Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-Resistance to Antimicrobial Agents. Journal of Clinical Microbiology. 42, 73-78

Butt, T., Ahmad, R.N., Mahmood, A., and Zaidi, S. (2003) Ciprofloxacin Treatment Failure in Typhoid Fever Case, Pakistan. Emerging Infectious Diseases. 9, 1621-1622

Chitnis, S., Chitnis, V., Hemvani, N. and Chitnis, D.S. (2006) Ciprofloxacin therapy for typhoid fever needs reconsideration. Journal of Infection and Chemotherapy. 12, 402-404

Choi, S-H., Woo, J.H., Lee, J.H., Park, S.J., Choo, E.J., Kwak, Y.G., Kim, M-N., Choi, M-K., Lee, N.Y., Lee, B.K., Kim, N.J., Jeong, J-Y., Ryu, J., and Kim, Y.S. (2005) Increasing incidence of quinolone resistance in human non-typhoid Salmonella enterica isolates in Korea and mechanisms involved in quinolone resistance. Journal of Antimicrobial Chemotherapy. 56, 1111–1114

Chu, C., Su, L-H., Chu, C-H, Baucheron, S., Cloeckaert, A., and Chiu, C-H. (2005) Resistance to fluoroquinolones linked to *gyrA* and *parC* mutations and overexpression of *acrAB* efflux pump in *Salmonella enterica* serotype Choleraesuis. Microbial Drug Resistance. 11, 248-253

Clinical and Laboratory Standards Institute. (2006) Performance standards for antimicrobial susceptibility testing, document M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA

Clinical and Laboratory Standards Institute. (2006) Performance standards for antimicrobial susceptibility testing, document M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA

Cloeckaert, A. and Chaslus-Dancla, E. (2001) Mechanisms of quinolone resistance in Salmonella. Veterinary Research. 32, 291-300

Connor, B.A., and Schwartz, E. (2005) Typhoid and paratyphoid fever in travelers. Lancet Infectious Diseases. 5, 623-628

Crump, J.A., Barrett, T.J., Nelson, J.T., and Angulo, F.J. (2003) Reevaluating Fluoroquinolone Breakpoints for *Salmonella enterica* Serotype Typhi and for Non-Typhi Salmonellae. Clinical Infectious Diseases. 37, 75-81

Doughari, J.H., Elmahmood, A.M., and Nggada, H.P. (2007) Retrospective study on the antibiotic resistant pattern of *Salmonella typhi* from some clinical samples. African Journal of Microbiology Research. 33-36

Eaves, D.J., Liebana, E., Woodward, M.J., and Piddock, L.J.V. (2002) Detection of *gyrA* mutations in quinolone-resistant *Salmonella enterica* by denaturing high-performance liquid chromatography. Journal of Clinical Microbilogy. 40, 4121-4125

Eaves, D.J., Randall, L., Gray, D.T., Buckley, A., Woodward, M.J., White, A.P. and Piddock, L.J.V. (2004) Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrobial Agents and Chemotherapy. 48, 4012–4015.

Edgeworth, J.D., (2005) Bacterial gastroenteritis. Medicine. 33, 73-77

Gaind, R., Paglietti, B., Murgia, M., Dawar, R., Uzzau, S., Cappuccinelli, P., Deb, M., Aggarwal, P., and Rubino, S (2006) Molecular characterization of ciprofloxacin-resistant *Salmonella enterica* serovar Typhi and Paratyphi A causing enteric fever in India. Journal of Antimicrobial Chemotherapy. 58, 1139-1144

Gautam, V., Gupta, N.K., Chaudhary, U., and Arora, D.R. (2002) Sensitivity Pattern of *Salmonella* serotypes in Northern India. The Brazilian Journal of Infectious Diseases. 6, 281-287

Giraud, E., Baucheron, S., and Cloeckaert, A. (2006) Resistance to fluoroquinolones in *Salmonella*: emerging mechanisms and resistance prevention strategies. Microbes and Infection. 8, 1937-1944

Giraud, E., Cloeckaert, A., Kerboeuf, D., and Chaslus-Dancla, E. (2000) Evidence for Active Efflux as the Primary Mechanism of Resistance to Ciprofloxacin in *Salmonella enterica* Serovar Typhimurium. Antimicrobial Agents and Chemotherapy. 44, 1223–1228

Gootz, T.D. (2006) The forgotten Gram-negative bacilli: What genetic determinants are telling us about the spread of antibiotic resistance. Biochemical Pharmacology. 71, 1073-1084

Gupta, V., and Kaur, J. (2008) A need to revise ciprofloxacin breakpoints for Salmonella in human beings. International Journal of Infectious Diseases. 2, e143-e144

Hakanen, A.J., Lindgren, M., Huovinen, P., Jalava, J., Siitonen, A., and Kotilainen, P. (2005) New Quinolone Resistance Phenomenon in *Salmonella enterica*: Nalidixic Acid-Susceptible Isolates with Reduced Fluoroquinolone Susceptibility. Journal of Clinical Microbiology. 43, 5775-5778

Hata, M., Suzuki, M., Matsumoto, M., Takahashi, M., Sato, K., Ibe, S., and Sakae, K. (2005) Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrobial Agents and Chemotherapy. 49, 801–803

Herikstad, H., Hayes, P., Mokhtar, M., Fracaro, M.L., Threlfall, E.J., and Angulo, F.J. (1997) Emerging Quinolone-Resistant *Salmonella* in the United States. Emerging Infectious Diseases. 3, 371-372

Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H., and Watanabe, H. (2002) DNA Sequence Analysis of DNA Gyrase and DNA Topoisomerase IV Quinolone Resistance-Determining Regions of *Salmonella enterica* Serovar Typhi and Serovar Paratyphi A. Antimicrobial Agents and Chemotherapy. 46, 3249-3252

Hopkins, K.L., Davies, R.H., and Threlfall, E.J., (2005) Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. International Journal of Antimicrobial Agents. 25, 358–373

Hopkins, K.L., Day, M. and Threlfall, E.J. (2008) Plasmid-mediated Quinolone Resistance in *Salmonella enterica*, United Kingdom. Emerging Infectious Diseases. 14, 340-342

Hopkins, K.L., Wootton, L., Day, M.R. and Threlfall, E.J. (2007) Plasmid-mediated quinolone resistance determinant *qnrS*1 found in *Salomonella enterica* strains isolated in the UK. Journal of Antimicrobial Chemotherapy. 59, 1071-1075

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T., and Ribot, E. (2005) Establishment of a universal size standard strain for use with the Pulsenet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. Journal of Clinical Microbiology. 43, 1045-1050

Jacoby, G.A. (2005) Mechanisms of Resistance to Quinolones. Clinical Infectious Diseases. 41, S1120-S126

Jacoby, G.A., Chow, N., and Baites, K.B. (2003) Prevalence of Plasmid-Mediated Quinolone Resistance. Antimicrobial Agents and Chemotherapy. 47, 559-562

Jacoby, G.A., Walsh, K.E., Mills, D.M., Walker, V.J., Oh, H., Robicsek, A., and Hooper, D.C. (2006) *qnrB,* another plasmid mediated gene for quinolone resistance. Antimicrobial Agents and Chemotherapy. 50, 1178-1182

Kauffmann, F. (1966) The bacteriology of *Enterobacteriaceae*. Munksgaard, Copenhagen, Denmark.

Kehrenberg, C., de Jong, A., Friederichs, S., Cloeckaert, A., and Schwarz, S. (2007) Molecular mechanisms of decreased susceptibility to fluoroquinolones in avian *Salmonella* serovars and their mutants selected during the determination of mutant prevention concentrations. Journal of Antimicrobial Chemotherapy. 59, 886-892

Kehrenberg, C., Friederichs, S., de Jong, A., Michael, G.B., and Schwarz, S. (2006) Identification of plasmid-borne quinolone resistance genes *qnrS* in *Salmonella enterica* serovar Infantis. Journal of Antimicrobial Chemotherapy. 58, 18-22

Kim, S., Frye, J.G., Hu, J., Fedorka-Cray, P.J., Gautom, R., and Boyle D.S. (2006) Multiplex PCR-Based Method for Identification of Common Clinical Serotypes of *Salmonella enterica* subsp. *enterica.* Journal of Clinical Microbiology. 44, 3608–3615

Le, T.A.H., Lejay-Collin, M., Grimont, P.A.D., Hoang, T.L., Nguyen, T.V., Francine Grimont, F., and Scavizzi, M.R. (2004) Endemic, Epidemic Clone of *Salmonella enterica* Serovar Typhi Harboring a Single Multidrug-Resistant Plasmid in Vietnam between 1995 and 2002. Journal of Clinical Microbiology .42, 3094–3099

Levy, D.D., Sharma, B., and Cebula, T.A. (2004) Single-Nucleotide Polymorphism Mutation Spectra and Resistance to Quinolones in *Salmonella enterica* Serovar Enteritidis with a Mutator Phenotype. Antimicrobial Agents and Chemotherapy. 48, 2355-2363

Lindstedt, B.A., Aas, L., and Kapperud, G. (2004) Geographically dependent distribution of *gyrA* gene mutations at codons 83 and 87 in *Salmonella* Hadar, and a novel codon 81 Gly to His mutation in *Salmonella* Enteritidis. APMIS. 112, 165–71

Lindstedt, B.A., Heir, H., Gjernes, E., and Kapperud, G., (2003) DNA Fingerprinting of *Salmonella enterica* subsp. *enterica* Serovar Typhimurium with Emphasis on Phage Type DT104 Based on Variable Number of Tandem Repeat Loci. Journal of Clinical Microbiology. 41, 1469-1479

Liu, Y., Lee, M.A., Ooi, E.E., Mavis, Y., Tan, A.L., and Quek, H.H. (2003) Molecular typing of Salmonella enterica serovar typhi isolates from various countries in Asia by a multiplex PCR assay on variable-number tandem repeats. Journal of Clinical Microbiology. 41, 4388-4394.

Ling, J.M., Chan, E.W., Lam, A.W., and Cheng, A.F. (2003) Mutations in Topoisomerase Genes of Fluoroquinolone-Resistant Salmonellae in Hong Kong. 47, 3567–3573

Martinez-Martinez, L., Pascual, A., and Jacoby, G.A. (1998) Quinolone resistance from a transferable plasmid. Lancet. 351, 797–799

Mølbak, K., Gerner-Smidt, P., and Wegener, H.C. (2002) Increasing quinolone resistance in *Salmonella* enterica serotype Enteritidis. Emerging Infectious Diseases. 8, 514-515

Morgan-Linnell, S.K., Boyd, L.B., Steffen, D., and Zechiedrich, L. (2009) Mechanisms Accounting for Fluoroquinolone Resistance in *Escherichia coli* Clinical Isolates. Antimicrobial Agents and Chemotherapy. 53, 235-242

Nordmann, P. and Poirel, L. (2005) Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. Journal of Antimicrobial Chemotherapy. 56, 463-469

Oliphant, C.M., and Green, G.M. (2002) Quinolones: A Comprehensive Review. American Family Physician. 65, 455-464

Olliver, A., Valle, M., Chaslus-Dancla, E., and Cloeckaert, A. (2005) Overexpression of the Multidrug Efflux Operon *acrEF* by Insertional Activation with IS*1* or IS*10* Elements in *Salmonella enterica* Serovar Typhimurium DT204 *acrB* Mutants Selected with Fluoroquinolones. Antimicrobial Agents and Chemotherapy. 59, 289-301

Paterson, D.L. (2006) Resistance in Gram-Negative Bacteria: Enterobacteriaceae. The American Journal of Medicine. 119, S20-S28

Piddock, L.J.V. (2002) Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. FEMS Microbiology Reviews. 26, 3-16

Poirel, L., Rodriguez-Martinez, J-M., Mammeri, H., Liard, A., and Nordmann, P. (2005) Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA. Antimicrobial Agents and Chemotherapy. 49, 3523-3525

Reed, R., Holmes, D., Weyers, J., and Jones, A. (2003) *Practical Skills in Biomolecular Sciences*, 2nd Edition (pp 430-431). United Kingdom. Pearson Education

Renuka, K., Sood, S., Das, B.K., and Kapil, A. (2005) High-level ciprofloxacin resistance in *Salmonella enterica* serotype Typhi in India. Journal of Medical of Microbiology. 54, 999-1000

Ribot, E.M., Fair, M.A., Gautom, R., Cameron. D.N., Hunter, S.B., Swaminathan, B., and Barrett, T.J. (2006) Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella,* and *Shigella* for PulseNet. Foodborne Pathogenic Diseases. 3, 59-67

Ruiz, J. (2003) Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. Journal of Antimicrobial Chemotherapy. 51, 1109-1117

Rupali, P., Abraham, O.C., Jesudason, M.V., John, T.J., Zachariah, A., Sivaram, S., and Mathai, D. (2004) Treatment failure in typhoid fever with ciprofloxacin susceptible *Salmonella enterica* Serotype Typhi. Diagnostic Microbiology and Infectious Disease. 49, 1-3

Saenz, Y., Ruiz, J., Zarazaga, M., Teixido, M., Torres, C., and Vila ,J. (2004) Effect of the efflux pump inhibitor Phe-Arg-β-naphthylamide on the MIC values of the quinolones, tetracycline and chloramphenicol, in *Escherichia coli* isolates of different origin. Journal of Antimicrobial Chemotherapy. 53, 544-545

Saha, S.K., Darmstadt, G.K., Baqui, A.H., Crook, D.W., Islam, M.N., Islam, M., Hossain, M., El Arifeen, S., Santhosham, M., and Black, R.E. (2006) Molecular Basis of Resistance Displayed by Highly Ciprofloxacin-Resistant *Salmonella enterica* Serovar Typhi in Bangladesh. Journal of Clinical Microbiology. 44, 3811-3813

Santos, R.L., Tsolis, R.M., Baumler, A.J. and Adams, L.G. (2003) Pathogenesis of *Salmonella*-induced enteritis. Brazilian Journal of Medical and Biological Research. 36, 3-12

Shakespeare, W. A., Davie, D., Tonnerre, C., Rubin, M. A., Strong, M., and Petti, C. A. (2005) Nalidixic Acid resistant *Salmonella* enterica serotype Typhi presenting as a psoas abscess: case report and review of the literature. Journal of Clinical Microbiology. 43, 996-998

Srikanth, C.V., and Cherayil, B.J. (2007) Intestinal Innate Immunity and the Pathogenesis of *Salmonella* Enteritis. Immunologic Research*.* 37, 61–77

Su, L-H., Chiu, C-H., Chu, C., and Ou, J.T. (2004) Antimicrobial Resistance in Nontyphoid *Salmonella* Serotypes: A Global Challenge. Clinical Infectious Diseases. 39, 546-551

Tindall, B.J., Grimont, P.A.D., Garrity, G.M., and Euzéby, J.P. (2005) Nomenclature and taxonomy of the genus *Salmonella*. International Journal of Systemic Evolution and Microbiology. 55, 521-524

Torpdahl, M., Sorensen, G., Ethelberg, S., Sando, G., Gammelgard, K., and Porsbo, L.J. (2006) A regional outbreak of *S.* Typhimurium in Denmark and identification of the source using MLVA typing. Eurosurveillance. 11, 134-136

Torpdahl, M., Sorensen, G., Lindstedt, B.A., and Nielsen, E.M. (2007) Tandem Repeat Analysis for Surveillance of Human *Salmonella* Typhimurium Infections. Emerging Infectious Diseases. 13, 388-395

Zhanel, G.G., Hobana, D.J., Schureka, K., and Karlowskyd, J.A. (2004) Role of efflux mechanisms on fluoroquinolone resistance in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. International Journal of Antimicrobial Agents. 24, 529–535

Zhang, S., Kingsley, R.A., Santos, R.L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., Nunes, J., Tsolis, R.M., Adams, L.G., and Baumler, A.J. (2003) Molecular Pathogenesis of *Salmonella enterica* Serotype Typhimurium-Induced Diarrhea. Infection and Immunity. 71, 1-12

Appendices

Appendix A: Pulsed-Field Gel Electrophoresis (PFGE)

1M Tris

121.1g Tris [Merck Ltd, Johannesburg, South Africa (Merck)]

800ml deionized water

Adjust to pH 8 with 1M HCl

Adjust volume to 1000ml

Sterilize the mixture by autoclaving

1M HCl

86.2ml HCl (Merck Chemicals, Darmstadt,Germany)

913.8ml deionized water

0.5M EDTA (pH 8)

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

800ml deionized water

Dissolve with the addition of sodium hydroxide pellets (Merck)

Adjust to pH 8

Adjust volume to 1000ml

Sterilize the mixture by autoclaving

Cell suspension buffer (100mM Tris : 100mM EDTA, pH 8)

10ml of 1M Tris, pH 8

20ml of 0.5M EDTA, pH 8

70ml deionized water

Sterilize the mixture by autoclaving

Proteinase-K (10mg/ml)

200mg Proteinase-K [Roche Diagnostics GmbH, Mannheim, Germany (Roche)]

20ml TE buffer

Stored as 1000µl aliquots at ≈ -4ºC.

SDS (10%)

10g sodium dodecyl sulphate (Merck)

100ml deionized water

Cell lysis buffer (5ml/plug)

0.5M EDTA, pH8

1% N-lauroylsarcosine sodium salt (sarcosyl) [Sigma Chemical Co., St.Louis, MO, USA (Sigma)]

0.1 mg/ml Proteinase-K

Tris-EDTA (TE) buffer (10 mM Tris : mM EDTA, pH 8)

10ml of 1M Tris, pH 8

2ml of 0.5M EDTA, pH 8

988ml deionized water

Sterilize the mixture by autoclaving

TBE

10X TBE

108g Tris (Merck)

55g Boric acid (Merck)

7.5g EDTA powder (Merck) or 40ml of 0.5M EDTA, pH 8

Make up to 1000ml with deionized water

Sterilize the mixture by autoclaving

0.5X TBE

50ml 10X TBE

950ml deionized water

1% SeaKem Gold® agarose

1.5g SeaKem Gold® agarose (Cambrex Bio Science, Rockland,USA)

150ml of 0.5X TBE

Dissolve agarose in TBE

Ethidium bromide

Ethidium bromide stock solution

500mg ethidium bromide (Merck)

50ml deionized water

Ethidium bromide staining solution

25µl of ethidium bromide (10mg/ml)

250ml of 0.5X TBE

Store in a dark cupboard

Appendix B: Multiple-Locus Variable-Number Tandem-Repeats Analysis (MLVA)

B1

PCR primers used for *Salmonella* Typhi MLVA.

Name	**Sequence (5' to 3')**
TR1F1	**VIC**-AGAACCAGCAATGCGCCAACGA
TR1R1	CAAGAAGTGCGCATACTACACC
TR2F1	**6FAM**-CCCTGTTTTTCGTGCTGATACG
TR2R2	CAGAGGATATCGCAACAATCGG
TR3F1	**NED**-CGAAGGCGGAAAAAACGTCCTG
TR3R1	TGCGATTGGTGTCGTTTCTACC

B2

PCR primers used for *Salmonella* Typhimurium MLVA.

Name	**Sequence (5' to 3')**
STTR3-F	**6-FAM-**CCCCCTAAGCCCGATAATGG
STTR3-R	TGACGCCGTTGCTGAAGGTAATAA
STTR5-F	**VIC-**ATGGCGAGGCGAGCAGCAGT
STTR5-R	GGTCAGGCCGAATAGCAGGAT
STTR6-F	**NED-**TCGGGCATGCGTTGAAA
STTR6-R	CTGGTGGGGAGAATGACTGG
STTR9-F	**6-FAM-**AGAGGCGCTGCGATTGACGATA
STTR9-R	CATTTTCCACAGCGGCAGTTTTTC
STTR10pl-F	**PET**-CGGGCGCGGCTGGAGTATTTG
STTR10pl-R	GAAGGGGCCGGGCAGAGACAGC

Appendix C: Polymerase Chain Reaction (PCR) and Sequencing

Internal amplification control: 16SrRNA (726bp)

16SrRNA-primerB: GATTAGATACCCTGGTAGTCC

16SrRNA-r: ACGGCTACCTTGTTACGACTT

C1

Primers used for PCR and sequencing of the QRDR.

Name	Sequence (5' to 3')	Product size (bp)
stgyrA1	CGTTGGTGACGTAATCGGTA	251
stgyrA2	CCGTACCGTCATAGTTATCC	
stmgyrB1	GCGCTGTCCGAACTGTACCT	181
stmgyrB2	TGATCAGCGCCACTTCC	
stmparC1	CTATGAGATGTCAGAGCTGG	270
stmparC2	TAACAGCAGCTCGGCGTATT	
stmparE1	TCTCTTCCGATGAAGTGCTG	240
stmparE2	ATACGGTATAGCGGCGGTAG	

PCR reaction mix(25µl reaction)

1. Sterile deionized water 17µl
2. 10X buffer with no $MgCl_2$ 2.5µl
3. $MgCl_2$ (25mM) 1.5µl
4. dNTP mix (25mM) 2µl
5. Forward primer (20µM stock) 0.5µl
6. Reverse primer (20µM stock) 0.5µl

7. Super-Therm DNA polymerase (5U/µl) 0.5µl
8. Crude DNA template 0.5µl

C2

PCR primers used for *qnr* gene screening and sequencing.

Name	**Sequence (5' to 3')**	**Product size (bp)**
QP1	GATAAAGTTTTTCAGCAAGAGG	657
QP2	ATCCAGATCGGCAAAGGTTA	
FQ1	ATGACGCCATTACTGTATAA	566
FQ2	GATCGCAATGTGTGAAGTTT	
qnrS-F	TGGAAACCTACAATCATACATATCG	585
qnrS-R	TTAGTCAGGATAAACAACAATACC	

C3

Bromophenol blue (Loading buffer)

0.25g bromophenol blue (Merck)

40g sucrose (Merck)

Dissolve in 100ml deionized water

1.2% Agarose gel

1.8g agarose (Whitehead Scientific, Johannesburg, South Africa)

150ml of 1X TAE buffer

Dissolve agarose in TAE

Add 12µl ethidium bromide stock solution to molten agarose

TAE

10X TAE

48g Tris (Merck)

7.5g EDTA (Merck)

11ml of glacial acetic acid (Merck)

Adjust to a volume of 1000ml with deionized water

Sterilize the mixture by autoclaving

1X TAE (Running buffer)

100ml 10X TAE

900ml deionized water

Store at 2-8ºC

C4

Cycle sequencing reaction mix (≈15µl)

1.	Sterile deionized water	6µl
2.	ABI Prism® Big-Dye Terminator reaction mix	3µl
3.	5X Buffer	1.5µl
4.	Single primer (forward or reverse-5µM)	1µl
5.	DNA template	4µl

Appendix D: Doubling Agar Dilution Minimum Inhibitory Concentration (MIC) and Efflux Pump Inhibition

0.85% Saline

8.5g Sodium chloride (Merck)

1000ml of deionized water

Sterilize the mixture by autoclaving

1M Sodium hydroxide

20g Sodium hydroxide (Merck)

500ml of deionized water

Sterilize the mixture by autoclaving

Nalidixic acid (10mg/ml)

200mg nalidixic acid powder [Abtek Biological Ltd. Liverpool, England (Abtek)]

10ml of 1M Sodium hydroxide

10ml of sterile deionized water

Solution filtered through a filtered tip syringe and stored as 1000µl aliquots at ≈ -4ºC.

Ciprofloxacin (1mg/ml)

20mg ciprofloxacin powder (Abtek)

20ml of sterile deionized water

Solution filtered through a filtered tip syringe and stored as 1000µl aliquots at ≈ -4ºC.

Efflux pump inhibitor (10mg/ml)

250mg β-*Phe-Arg-naphthylamide* (Sigma)

25ml of sterile deionized water

Solution filtered through a filtered tip syringe and stored as 1000µl aliquots at ≈ -4ºC.

* All filtered solutions were passed through 0.45 micron Millex®-HV filter units (Millipore®, Bedford, Ireland).

Appendix E: Molecular Epidemiology and Mechanism of Resistance of Quinolone-Resistant *Salmonella* Typhi: South Africa 2003-2007

Molecular Epidemiology and Mechanism of Resistance of Quinolone-Resistant *Salmonella* Typhi: South Africa 2003-2007

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Key words: typhoid fever, quinolone resistance, South Africa, pulsed-field gel electrophoresis, PFGE, multiple-locus variable-number tandem-repeats analysis, MLVA, *qnr*, QRDR, efflux pump

Running Title: Quinolone-Resistant Typhi: South Africa

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Abstract

The molecular epidemiology and mechanism of quinolone resistance of South African human isolates of *Salmonella* Typhi received by the Enteric Diseases Reference Unit (EDRU) of the National Institute for Communicable Diseases for the period 2003-2007 was investigated. Molecular epidemiology was investigated using pulsed-field gel electrophoresis (PFGE) analysis and multiple-locus variable-number tandem-repeats analysis (MLVA). Three probable mechanisms for quinolone resistance were investigated which included: amino acid mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase (gyrA/gyrB) and topoisomerase IV (parC/parE), active efflux of antibiotic out the bacterial cell and plasmid-mediated resistance encoded by *qnr* genes. For the period 2003-2007, 498 human isolates of *Salmonella* Typhi were received by the EDRU, of which 27 were resistant to nalidixic acid (MICs, ≥32 µg/ml) and susceptible to ciprofloxacin (MICs, ≤1 µg/ml). Only 19 of these quinolone-resistant isolates were available for analysis. These 19 isolates were genetically diverse as they could be differentiated into 5 PFGE types and 10 MLVA types, suggesting that quinolone-resistant strains have emerged independently of one another. All 19 isolates demonstrated the involvement of active efflux as a mechanism for resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. All 19 isolates were negative for plasmid-mediated *qnr* resistance determinants. Seven isolates were investigated for mutations in the QRDRs of gyrA, gyrB, parC and parE. All seven isolates showed mutations in gyrA and parC. GyrA mutations were located at codons 81, 82, 83, 87 and 119; while parC mutations were located at codons 57 and 80. No mutations were shown in gyrB or parE. Our data show that active efflux of antibiotic out of the bacterial cell in combination with mutations in gyrA and parC, are the mechanisms responsible for quinolone resistance in South African isolates of *Salmonella* Typhi.

61 **Introduction**

62

63 *Salmonella enterica* is the leading cause of food and water-borne disease worldwide and
64 the prevalence of antibiotic-resistant strains remains a serious public health concern (33).
65 *Salmonella enterica* serotype Typhi (*Salmonella* Typhi) is one of over two thousand
66 described serovars of *Salmonella enterica* and the causative agent of typhoid fever, and is
67 transmitted via food or water contaminated with human faeces. The organism is of great
68 clinical importance as humans are the only recognized reservoir of *Salmonella* Typhi
69 (10). *Salmonella* Typhi infects an estimated twenty to thirty million individuals
70 worldwide, resulting in an estimated four hundred thousand deaths annually. Infection
71 occurs in individuals mainly in the age range five to fifteen years old. Infection rates in
72 areas where typhoid fever is endemic may be as high as 1000 per 100 000 of population
73 per year (10). Antibiotics are critical in the management of typhoid fever. Various
74 fluoroquinolones such as ciprofloxacin have become routine treatment for typhoid fever
75 (38). Internationally there has been a disturbing trend noted of increasing resistance to the
76 fluoroquinolones in salmonellosis. Albayrak *et al*, 2004 (2) reported a 12.3% resistance
77 rate to non-fluorinated quinolone nalidixic acid as well as reduced susceptibility to the
78 fluoroquinolone ciprofloxacin, across a range of *Salmonella* serotypes including
79 *Salmonella* Typhi. The value of nalidixic acid to predict a poor clinical response to
80 ciprofloxacin-resistant strains has been recognized (1). Incidence of elevated quinolone
81 resistance rates in *Salmonella* have been reported from various countries around the
82 world: 21.6% in Korea (6), 82.4% in Kenya (24) and 69% in Taiwan (11). Highlighting
83 the global problem best may be the 2007 report from Nigeria where Doughari *et al* (34)
84 found a 20% resistance rate to the fluorinated quinolone ciprofloxacin. Such elevated
85 resistance rates among *Salmonella* species makes the management of infection due to
86 *Salmonella*, in particular *Salmonella* Typhi due its clinical implications, all the more
87 difficult.

88

89 Three major mechanisms have been described for the development of quinolone
90 resistance in *Salmonella* (12; 13; 20). The first mechanism involves amino acid mutations
91 in the quinolone resistance determining regions (QRDRs) of DNA gyrase (gyrA/gyrB)

92 and topoisomerase IV (parC/parE) (13). DNA gyrase is the primary target of quinolone
93 antibiotics. Amino acid mutations in the QRDR of gyrA results in resistance to the non-
94 fluorinated quinolone (12), nalidixic acid, while also resulting in reduced susceptibility to
95 fluoroquinolones such as ciprofloxacin (13). Amino acid mutations at Ser-83 (to Phe,
96 Tyr, or Ala) or at Asp-87 (to Gly, Asn, or Tyr) are the most frequently observed
97 mutations in gyrA (11). Additional amino acid mutations in the QRDRs of parC, gyrB,
98 and parE proteins, results in resistance to fluoroquinolones such as ciprofloxacin (13).The
99 second mechanism for quinolone resistance in *Salmonella* is the active efflux of antibiotic
100 from the bacterial cell. Efflux pumps are naturally present in bacteria and function to
101 eliminate toxic compounds from the bacterial cell. Overproduction of efflux pumps
102 results in removal of quinolones (and other antibiotics) from bacterial cells and
103 contributes to the development of resistance (7).The AcrAB-TolC efflux system is a
104 major player with regards to quinolone resistance in *S. enterica*. AcrAB is a member of
105 the resistance nodulation cell division family of transporters and is encoded by *acrAB* (3;
106 7). The pump has three components: a transporter protein in the inner membrane (AcrB),
107 a periplasmic accessory protein (AcrA), and an outer membrane channel (TolC). AcrB
108 captures its substrates within the phospholipid bilayer and transports them into the
109 external medium via TolC. Cooperation between AcrB and TolC is mediated by the
110 periplasmic protein AcrA (3).The involvement of the AcrAB-TolC efflux system in the
111 development of quinolone resistance has been proven through studies which have taken
112 quinolone-resistant strains, inactivated their efflux systems, and then showed a resultant
113 decrease in resistance. Inactivation of efflux systems have been performed through
114 inactivation of genes coding for the efflux (3) and through the use of efflux pump
115 inhibitors such as *Phe-Arg-naphthylamide* (3; 36).The third mechanism described for the
116 development of quinolone resistance in *Salmonella* is that of plasmid-mediated quinolone
117 resistance (22; 26). Three genes have been identified as those responsible for conferring
118 resistance to quinolones. The pentapeptide repeat gene *qnrA* is responsible for encoding a
119 protein that protects DNA gyrase from inhibition by quinolones. The other pentapeptide
120 repeat genes *qnrB* and *qnrS* have also been associated with conferring resistance to
121 quinolones (20).
122

123 South Africa has an estimated burden of typhoid fever of 100 infections per 100 000 of
124 population per year (10). In addition there exists areas in the eastern parts of the country
125 with a reservoir of typhoid fever (39). In South Africa, molecular epidemiological data
126 for S*almonella* infections is limited. In this country very little data exists on the molecular
127 basis for antibiotic resistance in *Salmonella*, apart from two recent studies describing the
128 molecular basis for extended spectrum β-lactamase activity in South African *S. enterica*
129 strains (16; 28). No study has yet undertaken a large scale genotypic analysis of South
130 African human *Salmonella* strains isolated over a lengthy time period (years).

131

132 The last few years have seen a rapid increase in the prevalence of nalidixic acid resistance
133 amongst human isolates of *S. enterica* (31). In addition, ciprofloxacin-resistant isolates
134 are increasingly being isolated (25). This increasing prevalence of quinolone resistance
135 warrants an investigation into the molecular epidemiology and mechanism of resistance
136 of quinolone-resistant *S. enterica* isolates in South Africa. In this study, we investigated
137 the contribution of various molecular mechanisms to quinolone resistance in *Salmonella*
138 Typhi isolates collected over a five year period.

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154 **Material and Methods**

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156 Bacterial strains:

157 Four hundred and ninety eight *Salmonella* Typhi isolates were received at the Enteric
158 Diseases Reference Unit (EDRU) of the National Institute for Communicable Diseases
159 (NICD), of the National Health Laboratory Service, Sandringham, South Africa, between
160 2003 and 2007 (25). Twenty seven of these 498 isolates were nalidixic acid-resistant
161 [Minimum inhibitory concentration (MIC), ≥32 µg/mℓ], of which 19 nalidixic acid-
162 resistant isolates were available for molecular analysis.

163

164 PFGE analysis:

165 Pulsed-field gel electrophoresis (PFGE) analysis was utilized as our primary genotypic
166 analysis method to investigate the genetic relatedness of all the strains. PFGE analysis
167 was performed using a PulseNet standardized protocol (23) summarized as follows.
168 Bacterial genomic DNA was digested with *Xba*I restriction endonuclease (Roche
169 Diagnostics GmbH, Mannheim, Germany). For control purposes, a strain of *Salmonella*
170 Braenderup, strain H9812 (21), was included as a reference standard and analyzed in
171 parallel with all typhoid strains. Digested DNA was separated on a 1% agarose gel
172 (SeaKem Gold agarose, Lonza, Rockland, ME, USA) using a CHEF-DR III
173 electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed
174 with an electrophoresis gradient of 6 V/cm, an included angle of 120°, an initial switch
175 time of 2.2 seconds, a final switch time of 63.8 seconds and a run time of 21 h. Following
176 electrophoresis, agarose gels were stained with an ethidium bromide and patterns were
177 visualized utilizing UV illumination. Images of the patterns were captured into
178 BioNumerics (version 5.1) Software (Applied Maths, Sint-Martens-Latem, Belgium) for
179 further analysis and comparison. All test patterns were normalized against the pattern of
180 the *Salmonella* Braenderup reference standard. Cluster analysis of the patterns using the
181 unweighted pair group method with arithmetic averages resulted in dendrograms, with
182 analysis of banding patterns incorporating the Dice-coefficient at an optimization setting
183 of 0.5% and a position tolerance setting of 1.5%.

184

185 Preparation of crude bacterial DNA:
186 A small loopfull of bacteria (2 to 4 colonies) cultured on 5% blood agar (Diagnostic
187 Media Products, Sandringham, South Africa) was resuspended into 400 µl of 10 mM Tris
188 - 1 mM EDTA buffer (pH 8) and boiled at 95°C for 20 minutes. The suspension was then
189 centrifuged at 12000 rpm for 3 minutes and the resulting supernatant (crude DNA
190 preparation) was used as a template for PCR.
191
192 MLVA:
193 To supplement our PFGE outputs we used a second genotypic analysis method, multiple-
194 locus variable-number tandem-repeats (VNTRs) analysis (MLVA), to further analyze
195 selected strains. MLVA was based on 3 VNTR gene loci (TR1, TR2 and TR3) as
196 previously described (30). This method employed manual agarose gel electrophoresis
197 analysis whereas we have revised the analysis method to incorporate automated capillary
198 electrophoresis of fluorescently labelled PCR products. The makeup of PCR primers used
199 to amplify VNTR loci are shown in Table 1. The forward primer for each locus was
200 labelled with a distinctive fluorescent dye (Applied Biosystems, Foster City, CA, USA).
201 Each VNTR locus was amplified in a separate PCR of 25 µl final volume containing 1 µl
202 crude bacterial DNA, 2 mM $MgCl_2$, 0.5 µM of each primer, 200 µM deoxynucleotide
203 triphosphates (Bioline, London, UK), 1 U AmpliTaq Gold DNA polymerase (Applied
204 Biosystems) and 1× AmpliTaq Gold DNA polymerase buffer (Applied Biosystems); with
205 thermal cycling (25 times) at 95°C for 75 seconds, 55°C for 75 seconds and 72°C for 75
206 seconds. The 3 resultant PCRs were pooled as follows: 2 µl of TR1 + 2 µl of TR2 + 6 µl
207 of TR3. This pooled mixture was then diluted 1:40 in deionized water. Two µl of this
208 diluted mixture was then mixed with 0.7 µl of GeneScan 600 LIZ size standard (Applied
209 Biosystems) and 7.5 µl of Hi-Di formamide (Applied Biosystems). This 10 µl mixture
210 was then incubated at 95°C for 3 minutes and cooled to room temperature before being
211 subjected to capillary electrophoresis using an Applied Biosystems 3130 Genetic
212 Analyzer. Electrophoresis was performed through POP-7 polymer (Applied Biosystems)
213 at 15 kV for 25 minutes at a temperature of 60°C. Raw data was captured and analyzed
214 using GeneMapper (version 4.0) software (Applied Biosystems) which identified each

215 VNTR locus by its distinctive colour (fluorescence) and automatically sized the gene
216 product via comparison to the internal size standard.
217
218 <u>PCR and Sequencing of the QRDR</u>:
219 Seven isolates were selected for analysis. PCR was used to amplify the QRDR of *gyrA,*
220 *gyrB, parC* and *parE* using previously described methods. Primers (Table 2) were
221 synthesized by Inqaba Biotechnical Industries, Hatfield, South Africa. PCR was
222 conducted using a Bio-Rad Thermal i-Cyler (Bio-Rad Laboratories Inc., Hercules, CA,
223 USA) programmed for thermal cycling (30x) included a denaturation step of 95ºC for 1
224 minute, a primer annealing step for 1 minute (52ºC for *gyrA,* 55ºC *gyrB,* 58ºC *parC* and
225 57ºC *parE*) and a primer extension step at 72ºC for 30 seconds. PCR products were
226 cleaned using Qiaquick PCR purification Kits (Qiagen). For DNA sequencing, PCR
227 product was used as template in a PCR cycle sequencing reaction using the Big-Dye
228 Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Cycle sequencing
229 products were subsequently analyzed on an Applied Biosystems 3130 Genetic Analyzer
230 and a sequence was determined. Sequences resulting from the analysis of the quinolone-
231 resistant strains were compared to a quinolone-susceptible strain to determine mutations
232 using BioEdit v.7.5 and the pairwise comparison tool EMBOSS
233 (http://www.ebi.ac.uk/emboss/align/).
234
235 <u>PCR for *qnr* genes</u>:
236 Screening for *qnrA, qnrB* and *qnrS* was done on all nineteen isolates by PCR using
237 previously described primers (Table 3) and methods (12; 22; 23).
238
239 <u>Antibiotic susceptibility testing and efflux pump screening</u>:
240 The MIC for nalidixic acid and ciprofloxacin of all nineteen isolates was determined by
241 the Etest (AB Biodisk, Solna, Sweden) as instructed by the manufacturer; and by the
242 doubling agar dilution method as previously described by the CLSI. The MIC breakpoints
243 used were those recommended by the CLSI, and adjusted for increased accuracy of
244 results (8; 9). The extra dilutions ranged from 1 µg/ml to 512 µg/ml for nalidixic acid and
245 from 0.0625 µg/ml to 16 µg/ml ciprofloxacin. These agar dilution MICs were repeated

246 within the presence of 40 μg/ml *Phe-Arg-naphthylamide* (EPI), after conducting a pilot
247 study to determine the appropriate concentration of EPI for efflux pump screening. This
248 concentration of 40 μg/ml *Phe-Arg-naphthylamide* falls within a previously tested range
249 (3).
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279 **Results**

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281 PFGE analysis and MLVA:

282 For PFGE analysis, all patterns showing ≥ 90% similarity on dendrogram were regarded
283 as the same PFGE type. PFGE analysis of the 19 strains showed 5 PFGE types (Table 4).
284 PFGE type 1 was represented by 2 strains. PFGE type 2 was the dominant PFGE type and
285 was represented by 14 strains. Lastly, PFGE types 3, 4 and 5 were each represented by 1
286 strain. MLVA of the 19 strains showed 10 MLVA types. MLVA was more
287 discriminatory and was able to discriminate amongst strains showing the same PFGE
288 type. For example, the cluster of strains constituting PFGE type 2 could be differentiated
289 into 6 MLVA types (Table 4).

290

291 Active Efflux:

292 Etest susceptibility testing determined that all 19 strains were resistant to nalidixic acid
293 (MIC ≥32 µg/mℓ) and susceptible to ciprofloxacin (MIC ≤1 µg/mℓ). These results were
294 confirmed using the agar dilution method and results showed that nalidixic acid MICs for
295 all the strains range from 128 µg/ml to ≥512 µg/ml, which confirmed their resistance to
296 nalidixic acid. Agar dilution MICs for ciprofloxacin confirmed susceptibility with MICs
297 of ≤0.5 µg/ml for all the strains. For all isolates, nalidixic acid and ciprofloxacin MICs
298 were decreased when susceptibility testing was conducted in the presence of EPI. For
299 nalidixic acid, the MICs decreased by either 16 or 32-fold; while for ciprofloxacin, the
300 MICs decreased by either 2 or 8-fold. (Table 4).

301

302 Mutations in gyrA and parC:

303 All seven strains selected for QRDR PCR and sequencing exhibited some mutation in
304 gyrA and parC. The gyrA mutations occurred at codons 81, 82, 83, 87 and 119; Gly81-
305 Ser, Asp82-Gly, Ser83-Phe/Ala/Met, Asp87-Cys and Ala119-Ser/Gly. The parC
306 mutations occurred at codons 57 and 80; Tyr57-Ser/Gly/Ala, Ser80-Lys/Phe/Arg/Ile
307 (Table 4).

Mutations in gyrB and parE:

This study revealed no mutations in either gyrB or parE.

Plasmid-mediated quinolone resistance:

Screening for the pentapeptide repeat genes *qnrA*, *qnrB* and *qnrS* showed no positive results.

339 **Discussion**

340

341 PFGE analysis was able to differentiate the 19 nalidixic acid-resistant strains into 5 PFGE
342 types. However, MLVA was more discriminatory and differentiated the strains into 10
343 MLVA types (Table 4). The superior discriminatory power of MLVA is illustrated within
344 the PFGE type 2 cluster (14 strains), which MLVA is able to divide into 6 MLVA types.
345 MLVA type 16 was the most common type accounting for eight out of the fourteen
346 strains in the PFGE type 2 cluster. MLVA type 16, 15 and 17 only differ by a single
347 allele (TR2) and may be considered under less strict circumstances as being identical.
348 MLVA allows for a greater reproducibility and accuracy of result. The PFGE type 2
349 cluster represents the majority of strains of which share a common ancestry. This
350 immediately suggests that clonal spread of nalidixic acid resistance may have occurred.
351 However QRDR sequencing data (discussed later) of selected strains within this PFGE
352 type 2 cluster show completely different QRDR mutation profiles (Table 4). This would
353 suggest that quinolone-resistant strains have emerged independently of one another, i.e.
354 emergence of quinolone resistance in South African strains of *Salmonella* Typhi is not
355 clonally driven.

356

357 The investigation into the involvement of an active efflux revealed a 16 or 32-fold
358 decrease in nalidixic acid MIC and a 2 or 8-fold decrease in ciprofloxacin MIC in the
359 presence of EPI. This suggests that an active efflux pump is a major contributory factor in
360 the quinolone resistance of these invasive *Salmonella* Typhi strains. An efflux pump as a
361 mechanism of quinolone resistance has previously been observed in various *Salmonella*
362 serotypes including *Salmonella* Typhi (14; 32). Baucheron *et al* illustrated the importance
363 of the AcrB in fluoroquinolone resistance (3) and TolC in multidrug resistant *Salmonella*
364 Typhimurium (5). Although the AcrAB-TolC efflux system may vary between strains
365 (27) it has been shown that quinolone resistance is not as a result of one mechanism but
366 due to a combination of mechanisms (18). We believe that a similar observation can be
367 made for the South African strains. Gyrase mutations alone do not account for quinolone
368 resistance in *Salmonella* as it is believed that AcrAB pump is the primary mechanism for
369 fluoroquinolone resistance, especially if the QRDR mutations do not sufficiently explain

370 the resistant phenotype (3; 7). For South African strains, an active efflux is seen to be the
371 major contributory mechanism to quinolone resistance; however it is not the only
372 mechanism because even in the presence of EPI, 15 out of the 19 strains still show
373 nalidixic acid resistance with MICs at 32 µg/mℓ (Table 4). The role of *qnr* plasmids can
374 be excluded as screening for the pentapeptide repeat genes *qnrA, qnrB* and *qnrS* showed
375 no positive results. This leaves mutations in the QRDR as the only other likely
376 mechanism.
377
378 Strains 200597, 235856, 185048 and 107432 show mutations at gyrA positions Ser83
379 and/or Asp87, positions that have been previously described as important for quinolone
380 resistance (18), therefore these mutations probably play a role in quinolone resistance in
381 these strains. At these positions, novel mutations (Ser83-Met and Asp87-Cys) were
382 observed in strain 185048. Some of our strains also show some novel mutations at novel
383 positions in gyrA. Strain 113560 showed novel mutations at position 82 (Asp82-Gly) and
384 position 119 (Ala119-Ser) of gyrA, strain 107432 also showed the novel Asp82-Gly
385 mutation and strain 88972 also showed the novel Ala119-Ser mutation. For strains
386 113560, 107432 and 88972, novel mutations at novel positions (Asp82 and Ala119) in
387 gyrA may be operating as an alternative mechanism of resistance as compared to the
388 commonly reported mutation at positions Ser83 and Asp87. Levy, *et al* (29) provided
389 evidence to suggest that selection pressure in terms of the antibiotics the bacteria is
390 exposed to, is a determining factor in the resulting mutations in gyrA. Treatment
391 strategies for Salmonellosis differ for different regions and since quinolone-resistant
392 *Salmonella* Typhi are rarely locally acquired in South Africa, the selection pressure may
393 be driven by antimicrobial use in regions with elevated rates of *Salmonella* Typhi
394 infection. Single point source mutations at codons 83 and 87 of the gyrA have over the
395 years received much attention as the cause of quinolone resistance, in particular nalidixic
396 acid and as a result linked to decreased susceptibility of fluoroquinolones (17; 37).
397 Mutations in gyrA are normally associated with nalidixic acid resistance and seen as the
398 precursors to mutations in the topoisomerase IV proteins parC and parE resulting in
399 fluoroquinolone resistance or decreased susceptibility (1). Conversely it has been strongly
400 suggested that multiple mutations in gyrA are more important in conveying

401 fluoroquinolone resistance than any respective combination of mutations in the gyrase
402 and topoisomerase IV proteins. As important as mutations in gyrA are, these mutations
403 themselves are not the defining contributory factor for quinolone resistance (12; 18). In
404 the present study, sequencing analysis of nalidixic acid-resistant strains showed no amino
405 mutations in either gyrB or parE. The two regions are homologous with the latter being
406 the secondary target for quinolones (13). Mutations for both gyrB and parE remain rare
407 for most *Salmonella* strains as most researchers report mainly on findings of gyrA
408 mutations (19). The role of gyrB and parE in quinolone resistance is unclear (33), in
409 particular gyrB, even in *Salmonella* strains exhibiting high levels of fluoroquinolone
410 resistance (19). The present study showed that all nalidixic acid-resistant strains with
411 amino mutations in gyrA, also showed amino acid mutations in parC. Mutation at
412 position Ser80 of parC was commonly present and involved the mutation of Ser80 to
413 either Phe or Arg or Lys or Ile. Some novel parC mutations were also identified and these
414 included Tyr57-Gly, Tyr57-Ala, Ser80-Lys and Ser80-Phe. ParC mutation at positions
415 Ser80 and Glu84 have previously been shown to be important for quinolone resistance
416 (18). With regards the parC mutation at position Tyr57, Baucheron *et al* (4) suggested
417 that mutation at this position (Tyr57-Ser) is not likely to be involved in quinolone
418 resistance. Results from our study support this hypothesis. We found mutations at Tyr57
419 for three of our strains. For one of these (strain 1647652), the mutation at position Ser80
420 of parC was notably absent while a Tyr57-Ser mutation was present. With efflux
421 inhibited, this strain was nalidixic acid-susceptible (MIC, 8 μg/ml), which would support
422 the view that a mutation at position Tyr57 of parC is probably inconsequential. Overall,
423 the role of parC mutations in quinolone-resistant *Salmonella* remains unclear. Our study
424 suggests that mutation at position Ser80 of parC may be an important role player in
425 quinolone resistance. The question remains concerning the role of mutations in both gyrA
426 and parC. Our sequencing data from seven isolates showed mutations in both gyrA and
427 parC. Based upon the results of previous studies (7; 18) we therefore expected that our
428 isolates should be resistant to the fluoroquinolone, ciprofloxacin. However, we were
429 surprised to find that our seven isolates were susceptible (MICs, ≤0.5 μg/ml) to
430 ciprofloxacin. Our results are therefore in more agreement with the results of Eaves *et al*

431 (13) who found that isolates with mutations in both gyrA and parC were more susceptible
432 to ciprofloxacin than isolates with mutations in gyrA alone.
433
434 The clinical importance of *Salmonella* Typhi makes the understanding and evolution of
435 antibiotic resistance trends, in respect to treatment of typhoid fever, of paramount
436 importance. Little is known of the role of the AcrAB-TolC tripartite system of
437 *Salmonella* Typhi in South Africa, but our work suggests it is important in these
438 quinolone-resistant strains. Since the system may vary from strain to strain it is important
439 to form a basis of understanding of the efflux system of local strains, as the efflux system
440 may be the major contributory mechanism to quinolone resistance. We have shown that
441 an active efflux system in combination with mutations in both gyrA and parC play a
442 major role in facilitating quinolone resistance. Our study also highlights the need for
443 further work to determine regional variability for gyrA mutations and to link the various
444 mechanisms of resistance, as the significance of various mutations and their interactions
445 remains unclear.
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494 **References**

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496 1. Aarestrup, F.M., Wiuff, C., Molbak, K., and Threlfall, E.J. 2003. Is it time to
497 change fluoroquinolone breakpoints for *Salmonella* spp.?. Antimicrobial Agents
498 and Chemotherapy. **47:** 827-829
499 2. Albayrak, F., Cokca, F., Erdem, B., and Aysev, A.D. 2004. Predictive value of
500 nalidixic acid resistance for detecting salmonellae with decreased ciprofloxacin
501 susceptibility. International Journal of Antimicrobial Agents. **23:** 332–336
502 3. Baucheron, S., Imberechts, H., Chaslus-Dancla, E., and Cloeckaert A. 2002. The
503 AcrB multidrug transporter palys a major role in high-level fluoroquinolone
504 resistance in *Salmonella enterica* serovar Typhimurium phage type DT204.
505 Microbial drug resistance. **8:** 281-289
506 4. Baucheron, S., Chaslus-Dancla, and E., Cloekaert, A. 2005. High-Level
507 Resistance to Fluoroquinolones Linked to Mutations in *gyrA*, *parC*, and *parE* in
508 *Salmonella enterica* Serovar Schwarzengrund Isolates from Humans in Taiwan.
509 Antimicrobial agents and chemotherapy. **49:** 862-863
510 5. Baucheron, S., Mouline, C., Praud, K., Chaslus-Dancla, E. and Cloeckaert, A.
511 2005. TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica*
512 serotype Typhimurium colonization of chicks. Journal of Antimicrobial
513 Chemotherapy. **55:** 707–712
514 6. Choi, S-H., Woo, J.H., Lee, J.H., Park, S.J., Choo, E.J., Kwak, Y.G., Kim, M-N.,
515 Choi, M-S., Lee, N.Y., Kwon Lee, B.K., Kim, N.J., Jeong, J-Y., Ryul, J., and
516 Kim, Y.S. 2005. Increasing incidence of quinolone resistance in human non-
517 typhoid *Salmonella enterica* isolates in Korea and mechanisms involved in
518 quinolone resistance. Journal of Antimicrobial Chemotherapy. **56:** 1111–1114
519 7. Chu, C., Su, L-H., Chu, C-H, Baucheron, S., Cloeckaert, A., and Chiu, C-H. 2005.
520 Resistance to fluoroquinolones linked to *gyrA* and *parC* mutations and
521 overexpression of *acrAB* efflux pump in *Salmonella enterica* serotype
522 Choleraesuis. Microbial drug resistance. **11:** 248-253

523 8. Clinical and Laboratory Standards Institute. 2006. Performance standards for
524 antimicrobial susceptibility testing, document M7-A7. Clinical and Laboratory
525 Standards Institute, Wayne, Pennsylvania, USA
526 9. Clinical and Laboratory Standards Institute. 2006. Performance standards for
527 antimicrobial susceptibility testing, document M100-S16. Clinical and Laboratory
528 Standards Institute, Wayne, Pennsylvania, USA
529 10. Crump, J.A., Luby, S.P., and Mintz, E.D. 2004. The global burden of typhoid
530 fever. Bulletin of the World Health Organisation, May. **82:** 343-356
531 11. Doughari, J.H., Elmahmood, A.M., and Nggada, H.P. 2007. Retrospective study
532 on the antibiotic-resistant pattern of *Salmonella typhi* from some clinical samples.
533 African Journal of Microbiology Research. **1:** 33-36
534 12. Eaves, D.J., Liebana, E., Woodward, M.J., and Piddock, L.J.V. 2002. Detection of
535 *gyrA* mutations in quinolone-resistant *Salmonella enterica* by denaturing high-
536 performance liquid chromatography. Journal of Clinical Microbilogy. **40:** 4121-
537 4125
538 13. Eaves, D.J., Randall, L., Gray, D.T., Buckley, A., Woodward, M.J., White, A.P.
539 and Piddock, L.J.V. 2004. Prevalence of mutations within the quinolone
540 resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with
541 antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrobial
542 agents and chemotherapy. **48:** 4012–4015
543 14. Gaind, R., Paglietti, B., Murgia, M., Dawar, R., Uzzau, S., Cappuccinelli, P., Deb,
544 M., Aggarwal, P., and Rubino, S .2006.Molecular characterization of
545 ciprofloxacin-resistant *Salmonella enterica* serovar Typhi and Paratyphi A
546 causing enteric fever in India. Journal of Antimicrobial Chemotherapy. **58:** 1139-
547 1144
548 15. Giraud, E., Brisabois, A., Martel, J-L., and Chaslus-Dancla, E. 1999. Comparative
549 Studies of Mutations in Animal Isolates and Experimental In Vitro- and In Vivo-
550 Selected Mutants of *Salmonella* spp. Suggest a Counterselection of Highly
551 Fluoroquinolone-Resistant Strains in the Field. Antimicrobial agents and
552 chemotherapy. **43:** 2131-2137

553 16. Govinden, U., Mocktar, C., Moodly, P., Sturm, A.W., and Essack, S.Y. (2006)
554 CTX-M-37 in *Salmonella enterica* serotype Isangi from Durban, South Africa.
555 International journal of antimicrobial agents. **28:** 288–291
556 17. Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H., and
557 Watanabe, H. 2002. DNA Sequence Analysis of DNA Gyrase and DNA
558 Topoisomerase IV Quinolone Resistance-Determining Regions of *Salmonella*
559 *enterica* Serovar Typhi and Serovar Paratyphi A. Antimicrobial agents and
560 chemotherapy. **46:** 3249-3252
561 18. Hopkins, K.L., Davies, R.H., and Threlfall, E.J. 2005. Mechanisms of quinolone
562 resistance in *Escherichia coli* and *Salmonella*: Recent developments. International
563 Journal of Antimicrobial Agents. **25:** 358–373
564 19. Hopkins, K.L., Arnold, C., and Threlfall E.J. 2007. Rapid detection of gyrA and parC
565 mutations in quinolone-resistant *Salmonella enterica* using Pyrosequencing®
566 technology. Journal of Microbiological Methods. **68:** 163–171
567 20. Hopkins, K.L., Wootton, L., Day, M.R. and Threlfall, E.J. 2007. Plasmid-
568 mediated quinolone resistance determinant *qnrS*1 found in *Salomonella enterica*
569 strains isolated in the UK. Journal of Antimicrobial Chemotherapy. **59:** 1071-
570 1075
571 21. Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves,
572 L., Wrigley, D., Barrett, T., and Ribot, E. 2005. Establishment of a universal size
573 standard strain for use with the Pulsenet standardized pulsed-field gel electrophoresis
574 protocols: converting the national databases to the new size standard. Journal of Clinical
575 Microbiology. **43:** 1045-1050
576 22. Jacoby, A., Chow, N., and Baites, K.B. 2003. Prevalence of Plasmid-Mediated
577 Quinolone Resistance. Antimicrobial agents and chemotherapy. **47:** 559-562
578 23. Jacoby, G.A., Walsh, K.E., Mills, D.M., Walker, V.J., Oh, H., Robicsek, A., and
579 Hooper, D.C. 2006. *qnrB,* another plasmid-mediated gene for quinolone
580 resistance. Antimicrobial agents and chemotherapy. **50:** 1178-1182
581 24. Kariuki, S., Revathi, G., Muyodi, J., Mwituria, J., Munyalo, A., Mirza, S., and
582 Hart, A. 2004. Characterization of Multidrug-Resistant Typhoid Outbreaks in
583 Kenya. Journal of Clinical Microbiology. **42:** 1477-1482
584 25. Keddy, K. (2007) Unpublished

585 26. Kehrenberg, C., Friederichs, S., de Jong, A., Michael, G.B., and Schwarz, S.
586 2006. Identification of plasmid-borne quinolone resistance genes *qnrS* in
587 *Salmonella enterica* serovar Infantis. Journal of Antimicrobial Chemotherapy. **58:**
588 18-22

589 27. Kehrenberg, C., de Jong, A., Friederichs, S., Cloeckaert, A., and Schwarz, S.
590 2007. Molecular mechanisms of decreased susceptibility to fluoroquinolones in
591 avian *Salmonella* serovars and their mutants selected during the determination of
592 mutant prevention concentrations. Journal of Antimicrobial Chemotherapy. **59:**
593 886-892

594 28. Kruger, T., Szabo, D., Keddy, K.H., Deely, K., Marsh, J.W., Hujer, A.M.,
595 Bonomo, R.A., and Paterson, D.L. 2004. Infections with nontyphoidal *Salmonella*
596 species producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa.
597 Antimicrobial agents and chemotherapy. **48:** 4263–4270

598 29. Levy, D.D., Sharma, B., and Cebula, T.A. 2004. Single-Nucleotide Polymorphism
599 Mutation Spectra and Resistance to Quinolones in *Salmonella enterica* Serovar
600 Enteritidis with a Mutator Phenotype. Antimicrobial agents and chemotherapy.
601 **48:** 2355-2363

602 30. Liu, Y., Lee, M.A., Ooi, E.E., Mavis, Y., Tan, A.L., and Quek, H.H. 2003.
603 Molecular typing of *Salmonella enterica* serovar Typhi isolates from various
604 countries in Asia by a multiplex PCR assay on variable-number tandem repeats.
605 Journal of Clinical Microbiology. **41:** 4388-4394

606 31. NICD Bulletins March 2007/2008

607 32. Piddock, L.J.V., White, D.G., Gensberg, K., Pumbwe, L., and Griggs, D.J. 2000.
608 Evidence for an Efflux Pump Mediating Multiple Antibiotic Resistance in
609 *Salmonella enterica* Serovar Typhimurium. Antimicrobial agents and
610 chemotherapy. **44:** 3118-3121

611 33. Piddock, L.J.V. 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated
612 from humans and food animals. FEMS Microbiology Reviews. **26:** 3-16

613 34. Preisler, A., Mraheil, M.A., and Heisig P. 2006 Role of novel gyrA mutations in
614 the suppression of the fluoroquinolone resistance genotype of vaccine strain

615 *Salmonella* Typhimurium vacT (gyrA D87G). Journal of Antimicrobial
616 Chemotherapy. **57:** 430-436
617 35. Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan,
618 B., and Barret, T.J. 2006. Standardization of pulsed-field gel electrophoresis
619 protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and
620 *Shigella* for PulseNet. Foodborne Pathogen Diseases. **3:** 59-67
621 36. Saenz, Y., Ruiz, J., Zarazaga, M., Teixido, M., Torres, C., and Vila ,J. 2004 Effect
622 of the efflux pump inhibitor Phe-Arg-β-naphthylamide on the MIC values of the
623 quinolones, tetracycline and chloramphenicol, in *Escherichia coli* isolates of
624 different origin. Journal of Antimicrobial Chemotherapy. **53:** 544-545
625 37. Saha, S.K., Darmstadt, G.K., Baqui, A.H., Crook, D.W., Islam, M.N., Islam, M.,
626 Hossain, M., El Arifeen, S., Santhosham, M., and Black, R.E. 2006. Molecular
627 Basis of Resistance Displayed by Highly Ciprofloxacin-Resistant *Salmonella*
628 *enterica* Serovar Typhi in Bangladesh. Journal of Clinical Microbiology. **44:**
629 3811-3813
630 38. Shakespeare, W. A., Davie, D., Tonnerre, C., Rubin, M. A., Strong, M., and Petti,
631 C. A. 2005. Nalidixic acid-resistant *Salmonella* enterica serotype Typhi
632 presenting as a psoas abscess: case report and review of the literature. Journal of
633 Clinical Microbiology. **43:** 996-998
634 39. Waner, S., Kfir, R., Idema, G.K, Coetzee, D.J., Rasmussen, K., Koornhof H.J.,
635 and Klugman, K.P. 1998. Waterborne outbreak of typhoid fever in Delmas. South
636 African Journal of Epidemiology and Infection.**13:** 53-57
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646 **Table 1.** PCR primers used for MLVA.

Name	**Sequence (5' to 3')**
TR1F1	**VIC**-AGAACCAGCAATGCGCCAACGA
TR1R1	CAAGAAGTGCGCATACTACACC
TR2F1	**6FAM**-CCCTGTTTTTCGTGCTGATACG
TR2R2	CAGAGGATATCGCAACAATCGG
TR3F1	**NED**-CGAAGGCGGAAAAAACGTCCTG
TR3R1	TGCGATTGGTGTCGTTTCTACC

647

648 **Table 2.** Primers used for PCR and sequencing of the QRDR.

Name	**Sequence (5' to 3')**
stgyrA1	CGTTGGTGACGTAATCGGTA
stgyrA2	CCGTACCGTCATAGTTATCC
stmgyrB1	GCGCTGTCCGAACTGTACCT
stmgyrB2	TGATCAGCGCCACTTCC
stmparC1	CTATGAGATGTCAGAGCTGG
stmparC2	TAACAGCAGCTCGGCGTATT
stmparE1	TCTCTTCCGATGAAGTGCTG
stmparE2	ATACGGTATAGCGGCGGTAG

649

650 **Table 3.** PCR primers used for *qnr* gene screening.

Name	**Sequence (5' to 3')**
QP1	GATAAAGTTTTTCAGCAAGAGG
QP2	ATCCAGATCGGCAAAGGTTA
FQ1	ATGACGCCATTACTGTATAA
FQ2	GATCGCAATGTGTGAAGTTT
qnrS-F	TGGAAACCTACAATCATACATATCG
qnrS-R	TTAGTCAGGATAAACAACAATACC

651

Table 4. PFGE types, MLVA types and MIC results for all *Salmonella* Typhi isolates as well as the QRDR results for seven selected isolates.

Isolate	Year	Province[1]	PFGE type[2]	MLVA type[3]	MLVA profile (TR1-TR2-TR3)[4]	gyrA mutations	parC mutations	Nalidixic acid agar dilution MIC (µg/ml)[5]	Ciprofloxacin agar dilution MIC (µg/ml)[5]	Active Efflux
92173	2005	GA	1	23	234 443 566			256 (8)	0.5 (0.0625)	Yes
113560	2006	WC	1	19	228 435 566	Asp82-Gly; Ala119-Ser	Tyr57-Ala;Ser80-Phe	512 (32)	0.5 (0.0625)	Yes
1022834	2003	GA	2	25	214 000 566			512 (32)	0.5 (0.0625)	Yes
1028222	2004	GA	2	15	207 475 566			512 (32)	0.5 (0.0625)	Yes
1025746	2004	KZN	2	17	207 379 566			512 (32)	0.5 (0.0625)	Yes
1025656	2004	WC	2	17	207 379 566			512 (32)	0.5 (0.0625)	Yes
83959	2005	EC	2	16	207 000 566			512 (32)	0.5 (0.0625)	Yes
83947	2005	EC	2	16	207 000 566			256 (8)	0.5 (0.0625)	Yes
100247	2005	KZN	2	16	207 000 566			512 (32)	0.5 (0.0625)	Yes
1648788	2005	KZN	2	24	241 427 566			128 (8)	0.125 (0.0625)	Yes
152229	2006	GA	2	16	207 000 566			512 (32)	0.5 (0.0625)	Yes
107964	2006	WC	2	16	207 000 566			512 (32)	0.5 (0.0625)	Yes
107432	2006	WC	2	16	207 000 566	Asp82-Gly; Ser83-Ala	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes
212418	2007	KZN	2	16	207 000 566			512 (32)	0.5 (0.0625)	Yes
185048	2007	WC	2	16	207 000 566	Ser83-Met; Asp87-Cys	Tyr57-Gly; Ser80-Lys	512 (32)	0.5 (0.0625)	Yes
235856	2007	GA	2	22	207 395 566	Ser83-Ala	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes
88972	2005	MP	3	18	261 000 542	Ala119-Gly	Ser80-Ile	>512 (32)	0.5 (0.0625)	Yes
1647652	2005	WC	4	21	241 000 566	Gly81-Ser; Asp82-Gly	Tyr57-Ser	256 (8)	0.125 (0.0625)	Yes
200597	2007	KZN	5	18	261 000 542	Ser83-Phe	Ser80-Arg	512 (32)	0.5 (0.0625)	Yes

1: GA, Gauteng; WC, Western Cape; KZN, KwaZulu-Natal; EC, Eastern Cape; MP, Mpumalanga.
2: PFGE types based on relatedness as based on a dendrogram cluster analysis of the patterns using the unweighted pair group method with arithmetic averages, with analysis of banding patterns incorporating the Dice-coefficient at an optimization setting of 0.5% and a position tolerance setting of 1.5%. PFGE types defined at ≥90% similarity.

3: MLVA types based on MLVA profiles.

4: 000 represents no amplification of the PCR product at locus TR2.

5: Value in parenthesis represents the MIC with the addition 40 µg/ml *Phe-Arg-naphthylamide* as an efflux pump inbihitor.

Appendix F: First Report of Plasmid-Mediated Quinolone Resistance in Enterobacteriaceae from South Africa

1 # First Report of Plasmid-Mediated Quinolone Resistance in
2 # Enterobacteriaceae from South Africa
3
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7
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19 **To the Editor**: Invasive infections with *Salmonella* species are usually treated with
20 fluoroquinolones or extended spectrum β-lactams. Quinolone resistance results in
21 reduced susceptibility to fluoroquinolones, leaving healthcare professionals with limited
22 resources for treatment of salmonellosis. Three mechanisms contribute to quinolone
23 resistance in *Salmonella,* (I) mutations in the quinolone resistant determining region
24 (QRDR) genes (*gyrA/gyrB*) and topoisomerase IV genes (*parC/parE*) (1), (II) active
25 efflux of antibiotic out of the bacterial cell (1), and (III) plasmid-mediated quinolone
26 resistance encoded by *qnr* genes A, B and S (4). The *qnr* genes encode pentapeptide
27 repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and
28 topoisomerase IV (7). The first *qnr* gene was isolated in 1998 from a *Klebsiella*
29 *pneumoniae* isolate from Birmingham, Alabama, United States of America (8) and was
30 called *qnr*A. *Shewanella algae*, an environmental species from marine and fresh water,
31 was identified as its reservoir (10). *qnr*B is very similar to *qnr*A in that it confers low-
32 level quinolone resistance. *qnr* genes have been found in various bacteria worldwide
33 which include; *Citrobacter freundii*, *Enterobacter* spp., *Escherichia coli*, *K. Pneumoniae*
34 and *Providencia stuartii* in the United States (6), *Salmonella* species in the United
35 Kingdom (4), and *Shigella flexneri* in Japan (3). We report on the first discovery of
36 plasmid-mediated quinolone resistance in Enterobacteriaceae from South Africa, of
37 which this was identified in a clinical isolate of *Salmonella* Typhimurium.
38

39 A subset (48 isolates) of invasive *Salmonella* Typhimurium isolates collected by the
40 Enteric Diseases Reference Unit (EDRU) of the National Institute for Communicable
41 Diseases (NICD) in the year 2006, with minimum inhibitory concentrations (MIC's) to
42 nalidixic acid of ≥32µg/ml (as determined by E-tests), were screened for all known *qnr*
43 genes using the polymerase chain reaction (PCR). Isolates which were PCR positive for a
44 *qnr* gene, had their *qnr* and QRDR sequences determined. Agar dilution MIC's for
45 nalidixic acid and ciprofloxacin were conducted in the presence and absence of the efflux
46 pump inhibitor, *Phe-β-Arg-naphthylamide*.
47
48 For all isolates tested, we identified a single isolate (nalidixic acid MIC, 32µg/ml;
49 ciprofloxacin MIC, 0.38 µg/ml) which was PCR positive for the *qnr*B (Figure 1). This
50 isolate was cultured from a severely immunocompromised HIV seropositive 44 year old
51 female. The patient presented to hospital with chronic diarrhea while being treated for
52 military tuberculosis at an extended care facility. Her clinical condition was compounded
53 by a history of previous alcohol dependency and her CD4 count was 31/µl. Her blood
54 culture was positive for *Salmonella* Typhimurium. Stool culture did not yield an
55 organism. The patient was treated empirically with a combination of ciprofloxacin 500mg
56 bid, which was then continued for 14 days, and metronidazole 400mg tid. She recovered
57 well. Antiretroviral therapy was initiated and she remains well 2 years later.
58
59 The PCR positive *qnr*B result was confirmed by nucleotide sequence analysis of the
60 gene. QRDR sequences for this isolate showed no mutations in any of the genes, so
61 mutation in the QRDR was excluded as a mechanism for quinolone resistance. Agar
62 dilution MIC's for nalidixic acid and ciprofloxacin showed no involvement in resistance
63 of an active efflux pump, as there was no difference in MIC values following testing in
64 the absence or presence of an efflux pump inhibitor. The agar dilution MIC's did
65 illustrate the isolates low-level of nalidixic acid resistance (MIC, 32 µg/ml) and
66 susceptibility to ciprofloxacin (MIC, 0.38 µg/ml). The low-level nalidixic acid resistance
67 of this *Salmonella* Typhimurium can be attributed to the presence of the *qnr*B. In
68 addition, the ciprofloxacin MIC of 0.38 µg/ml is seen by many as an increased MIC and
69 the isolate may be described as having a reduced susceptibility to ciprofloxacin (5).
70
71 Although the patient from whom the isolate was obtained recovered well after receiving
72 ciprofloxacin therapy, isolates such as these, that confer low-level quinolone resistance
73 due to *qnr* genes, facilitate the selection higher-level resistance mutants. The spread of
74 high-level quinolone resistance amongst the serotype, *Salmonella* Typhimurium, as well
75 as other Enterobacteriaceae is highly plausible as *qnr* genes are also found in combination
76 with active efflux systems and QRDR mutated genes. *Salmonella* Typhimurium is the

77 most common serotype isolated in South Africa (9), and South Africa has a high burden
78 of HIV, so the isolation of plasmid-mediated quinolone resistance from an
79 immunocompromised patient illustrates the possible treatment challenges for these
80 patients when drug interactions have to be taken into consideration. Our study shows that
81 the *qnrB* gene is a contributing factor for low-level quinolone resistance in *Salmonella*
82 Typhimurium. This is the first report of any kind identifying the presence of *qnr* genes in
83 South African Enterobacteriaceae isolates.
84
85 This study was funded by the MRC and the NRF, South Africa.
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87 References:
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89 1. Baucheron S, Imberechts H, Chaslus-Dancla E, Cloeckaert A. 2002. The AcrB
90 multidrug transporter palys a major role in high-level fluoroquinolone resistance
91 in *Salmonella enterica* serovar Typhimurium phage type DT204. Microbial drug
92 resistance. **8:** 281-289
93 2. Eaves DJ, Randall L, Gray DT, Buckley A, Woodward MJ, White AP, Piddock
94 LJV. 2004. Prevalence of mutations within the quinolone resistance-determining
95 region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in
96 quinolone-resistant *Salmonella enterica*. Antimicrobial agents and chemotherapy.
97 **48:** 4012–4015
98 3. Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, Ibe S, Sakae K. 2005.
99 Cloning of a novel gene for quinolone resistance from a transferable plasmid in
100 *Shigella flexneri* 2b. *Antimicrobial Agents and Chemotherapy*. **49**: 801–803
101 4. Hopkins KL, Wootton L, Day MR, Threlfall EJ. 2007. Plasmid-mediated
102 quinolone resistance determinant *qnrS*1 found in *Salomonella enterica* strains
103 isolated in the UK. Journal of Antimicrobial Chemotherapy. **59:** 1071-1075
104 5. Hopkins KL, Day M, Threlfall EJ. 2008. Plasmid-mediated Quinolone Resistance
105 in *Salmonella enterica*, United Kingdom. *Emerging Infectious Diseases*. **14**: 340-
106 342
107 6. Jacoby GA, Chow N, Waites KB. 2003**.** Prevalence of plasmid-mediated
108 quinolone resistance. *Antimicrobial Agents and Chemotherapy*. **47:** 559–562
109 7. Kehrenberg C, Friederichs S, de Jong A, Michael GB, Schwarz S. 2006.
110 Identification of plasmid-borne quinolone resistance genes *qnrS* in *Salmonella*
111 *enterica* serovar Infantis. Journal of Antimicrobial Chemotherapy. **58:** 18-22
112 8. Martinez-Martinez L, Pascual A, Jacoby GA. 1998. Quinolone resistance from a
113 transferable plasmid. *Lancet*. **351**: 797–799
114 9. NICD Bulletins March 2007/2008

10. Poirel L, Rodriguez-Martinez J-M, Mammeri H, Liard A, Nordmann P. 2005. Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA. *Antimicrobial Agents and Chemotherapy*. **49**: 3523-3525

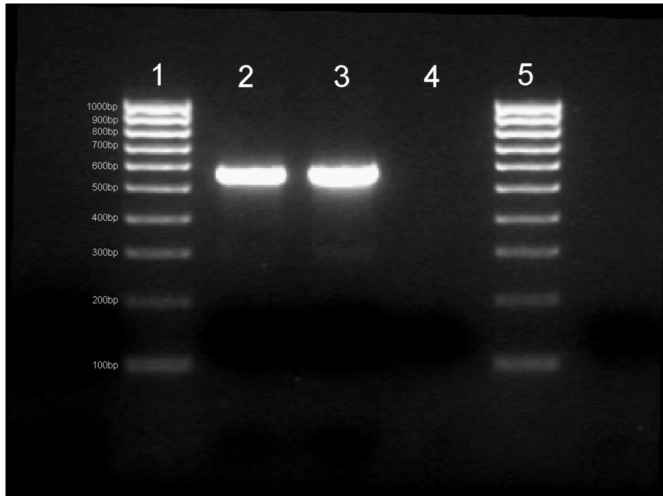

Figure 1: PCR amplification of the *qnr*B. Lane 1. Bioline Hypperladder IV 100bp marker. Lane 2. First positive PCR for *qnr*B, product ≈ 560 bp. Lane 3. Confirmatory positive PCR for *qnr*B, product ≈ 560 bp. Lane 4. Negative control. Lane 5. Bioline Hypperladder IV 100bp marker.