Investigations into Quinolone and Fluoroquinolone Resistance in *Salmonella enterica*

By

Angela Murray BSc

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DECLARATION

This thesis is the original work of the author and the information, ideas and opinions expressed herein are my own unless otherwise stated.

I performed the experimental procedures and the written work of the enclosed papers.

Acknowledgements

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Abstract

Infections with *Salmonella enterica* usually present as a self-limiting diarrhoeal disease. Occasionally, antimicrobial intervention is required for invasive infection or patients with underlying disease or at the extremities of age. In adults the therapeutic agents of choice are the fluoroquinolones, especially ciprofloxacin, which is also used in addition to third generation cephalosporins in children.

Fluoroquinolone resistance is rare and is mostly detected in strains associated with foreign travel. Nevertheless, reports from around the world describing treatment failures with these drugs in nalidixic acid-resistant isolates have been increasing.

To determine the levels of fluoroquinolone resistance in Scotland from 1990-2000, this study examined a collection of 180 isolates of *Salmonella enterica* isolated from human, veterinary and environmental sources. These were characterised using the genotypic methods of Plasmid Profile Analysis and Pulsed Field Gel Electrophoresis and levels of resistance were determined for quinolone and fluoroquinolone antimicrobials by the agar dilution method.

By exposing susceptible isolates to subinhibitory concentrations of fluoroquinolones, resistant mutants were selected. Amplification and sequencing of the topoisomerase genes *gyrA* and *parC* was performed on a number of these mutants in an attempt to characterize the mutations.

The prevalence of the recently described plasmid-borne resistance *qnr* genes was determined for a selection of 53 strains of *Salmonella enterica* from years 1997-2007 susceptible to nalidixic acid (40mg/L) but resistant to ciprofloxacin (0.125mg/L) in

addition to 17 strains of *Salmonella enterica* from years 1997-2007 resistant to ciprofloxacin (0.125mg/L) and cefotaxime (1mg/L).

The determined resistance levels indicated that with the exception of the isolates deemed fully susceptible by breakpoint method, all other isolates examined were resistant to the quinolone nalidixic acid. Resistance to fluoroquinolones was rare during this time as has been previously reported.

With the exception of a single mutation in the *parC* gene, mutations, if present were confined to *gyrA.* Although significant increases in minimum inhibitory concentrations (MICs) were observed between wild-type isolates and selected mutants, only single point mutations were characterised. This, and the absence of mutations in some mutants with raised MICs compared to their wild-type, may indicate additional mechanisms of resistance such as increased efflux or porin changes not investigated in this study.

Thirty-four from a total of 70 strains investigated for the presence of *qnr* genes were shown to harbour *qnrA*, *qnrB*, or *qnrS*. Twelve serotypes were represented, 7 of which have not previously been shown to harbour these genes. Positive strains were from human, environmental and veterinary sources; 58% of the strains of human origin were from patients with a history of foreign travel.

Plasmid-mediated quinolone resistance has recently been identified in isolates of *S. enterica* in a number of countries at low prevalence. This study of Scottish isolates has identified a higher prevalence of *qnr* than expected and a wider dissemination of *qnr* resistance genes among different serotypes.

Abbreviations

BHI Brain Heart Infusion

CLED Cysteine Lactose Electrolyte Deficient agar

CCC DNA Covalently closed circular DNA

DNA Deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

DT Definitive type

kb kilobases

MH Mueller Hinton agar

MIC Minimum inhibitory concentration

PFGE Pulsed Field Gel Electrophoresis

PPA Plasmid Profile Analysis

PT Phage type

SAP Serotype Associated Plasmid

SSRL Scottish Salmonella Reference Laboratory

TBE Tris Borate EDTA buffer

TE Tris EDTA buffer

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Chapter 1: INTRODUCTION AND REVIEW OF THE LITERATURE

1:1 History of the Genus *Salmonella*

In 1885, an American veterinarian named Daniel Elmer Salmon and his colleague, bacteriologist Theobald Smith discovered what they believed to be the causative agent of hog cholera (Salmon & Smith, 1886). The micro-organism they described was ultimately identified as the cause of the secondary infections which were often associated with the disease, which was eventually shown to be of viral origin. The generic designation *Salmonella* was coined in 1900 as a tribute to the veterinarian, and the newly described bacillus was given the name *Salmonella choleraesuis.*

Other bacilli displaying similar phenotypic characteristics were subsequently added to the genus. These included organisms which, prior to the creation of the *Salmonella* genus, had been given names describing the disease they caused or the host from which they were first isolated. As time progressed, these names became frequently inaccurate and so the practice of naming the organism after the geographic location in which it was first isolated was adopted.

1:2 Characteristics of the genus *Salmonella*

The genus *Salmonella* consists of non-sporing, Gram-negative, facultatively anaerobic straight rods that conform to the general definition of the family *Enterobacteriaceae*. With the exception of *S.* Pullorum and *S.* Gallinarum, which are always non-motile, these organisms are usually motile due to peritrichous flagella which exhibit diphasic variation

(Le Minor, 1984). They produce hydrogen sulphide from triple sugar iron agar and can use citrate as their sole carbon source. They produce gas from glucose, mannitol and usually sorbitol, an important exception being *S.* Typhi which never produces gas. They rarely ferment sucrose, lactose or adonitol and are urease- and indole-negative.

These biochemical characteristics are used for the identification of *Salmonella* to subspecies level. Serologic identification of the bacterial surface 'O' and 'H' antigens is then used to distinguish serotypes as described in the Kauffmann-White scheme (Popoff & Le Minor, 1997).

1:3 Nomenclature

Initially, *Salmonella* nomenclature was based on a one serotype-one species concept introduced by P.B. White in 1929 and later modified by Kauffmann in 1966. Each serotype, based on its antigenic structure and biochemical characteristics, was considered a separate species. Following the development of new techniques such as DNA-DNA hybridisation (Crosa, *et* al, 1973), it was demonstrated that all serotypes were closely related at the species level with the exception of subspecies V, which appeared to have evolved sufficiently to be significantly different from all the other subspecies (Reeves, *et al*, 1989).

The nomenclature most frequently used in today's literature is based on the system devised by Le Minor and Popoff of the World Health Organisation Collaborating Centre for Reference and Research on *Salmonella*, at the Institut Pasteur, Paris. In 1987, their group made a proposal as a "Request for an Opinion" to the Judicial Commission of the International Committee on Systematic Bacteriology (Le Minor & Popoff, 1987) that the

genus *Salmonella* consist of a single species and that the name which, up until that time was *S. choleraesuis*, be changed to *S. enterica* since "choleraesuis" was the name of a serotype as well as the name of the species. "Enterica", coined by Kauffmann and Edwards in 1952, had no serotype (Kauffmann & Edwards, 1952). The Commission denied their proposal (Judical Commission of the International Committee on Systematics of Prokaryotes, 2005). In 1999, J.P. Euzéby made an amended request that *S. enterica* be adopted as species name, while conserving the name *Salmonella typhi* as an exception (Euzéby, 1999). The request was successful.

Euzéby proposed that the genus *Salmonella* consist of two species, *S. enterica*, the type species and *S. bongori*, which was formerly subspecies V (Reeves *et al*, 1989). *S. enterica* is divided into six subspecies; (I, *S. enterica* subspecies *enterica*; II, *S. enterica* subspecies *salamae*; IIIa, *S. enterica* subspecies *arizonae*; IIIb, *S. enterica* subspecies *diarizonae*; IV, *S. enterica* subspecies *houtenae*; VI, *S. enterica* subspecies *indica*).

In 2004, a new species was identified through physiological and phylogenetic analyses and was named *Salmonella subterranae* (Shelobolina *et al*, 2004).

The serotypes belonging to *S. enterica* subspecies *enterica* are written in Roman (not italicised) and the first letter is capitalised, for example *S. enterica* subspecies *enterica* serotype Typhimurium. Serotypes belonging to other subspecies are designated by their antigenic formulae.

There are currently 2,463 *Salmonella* serotypes, with the majority of these belonging to *S. enterica* subspecies *enterica* (Popoff *et al*, 2000*).* New serotypes are listed in annual updates of the Kauffmann-White scheme.

This thesis will use the nomenclature *Salmonella enterica* subspecies *enterica* serotype Typhimurium and the shortened version *Salmonella* Typhimurium or *S.* Typhimurium.

1:4 *Salmonella* Pathogenicity

Salmonella is a genus of zoonotic bacteria that is capable of causing disease in a wide range of species. In humans, infection is a result of ingestion of contaminated food or water. There are three types of infection associated with *Salmonella*: gastroenteritis, systemic disease and a carrier state.

Gastroenteritis is usually associated with acute, but mild enteritis with a short incubation period of 6-72 hours after the ingestion of contaminated food or water. For disease to occur in healthy humans an infective dose as low as 10^3 organisms must be ingested (Blaser & Newman, 1982). They must reach the stomach where they are exposed to gastric acid and a pH of 1-2. Viable bacteria must then pass to the small intestine where they must compete with normal microbial gut flora, and adhere to the intestinal epithelia. Factors that reduce gastric acidity such as antacids or underlying disease, immature or compromised humoral and cellular immune responses all aid the passage of the bacteria to the small intestine.

The non-systemic infection of the intestinal tract results in varying degrees of nausea and vomiting followed by abdominal pain and mild to severe diarrhoea. The disease is self-limiting and normally subsides within seven days. Electrolyte and water loss may lead to more severe disease in young children and the elderly.

Severe systemic diseases fall into three main categories: enteric; bacteraemic and focal. The bacteria cross the intestinal epithelium to the reticulo-endothelial system where they are phagocytosed by macrophages. The bacilli multiply within the cells of the liver, spleen,

lymph nodes, gallbladder, bone marrow, lungs and kidneys. After intracellular multiplication, the bacilli re-enter the bloodstream resulting in bacteraemia.

Bacteraemia occurs in approximately 5% of intestinal non-typhoidal gastroenteritis cases. It is a serious and potentially fatal illness that is more likely to occur in immunologically compromised patients (Hohmann, 2001).

Focal lesions may occur, mainly in immunocompromised patients, at any anatomical site after *Salmonella* bacteraemia and include meningitis (Varaiya *et al*, 2001), septic arthritis (Sarguna, 2005), osteomyelitis (Khan, 2006) and pneumonia (Samonis *et al*, 2003). Clinical symptoms depend largely on the site affected, although most patients present with spiking fever in the absence of enteritis.

Faecal shedding of non-typhoidal salmonellae has been demonstrated to be resolved approximately 12 days after a positive culture result (Sirinavin *et al*, 2003), much shorter than previously believed (Buchwald, & Blaser, 1984). In long-term S*almonella* carriage, sufferers are usually asymptomatic and may continue to excrete organisms from the gallbladder, one of the sites of persistent infection.

Enteric fever caused by either *S.* Typhi or *S.* Paratyphi, is a severe and debilitating illness. An infectious dose of approximately 10^2 organisms must be ingested with sufficient numbers surviving long enough to reach the intestinal mucosa. The incubation period is between 7 and 14 days during which time the typhoid bacilli must penetrate the intestinal mucosa and translocate to the lymphoid follicles and the reticuloendothelial cells of the liver and spleen. After multiplication within the mononuclear phagocytic cells, they are released into the bloodstream (Parry *et al*, 2002). The patient is largely asymptomatic during the incubation stage however; once the bacilli enter the bloodstream symptoms

include remittent fever in the range of 38°C-40°C, malaise, myalgia and headache. In untreated cases, once typhoid fever has resolved, convalescent faecal excretion is seen in approximately10% of acute infections for up to 3 months, with 1-4% becoming long-term carriers (Parry *et al*, 2002).

A manifestation of the disease seen frequently in animals that is of great economic importance is the ability of some serotypes to induce abortion. *S*. Dublin, a host-adapted serotype, primarily infects cattle including calves, causing enteritis and/or systemic disease. Fever and anorexia due to severe diarrhoea eventually lead to death. Milder infections result in acute diarrhoea and abortion in pregnant cows; it also induces abortion in sheep, usually accompanied by death of the ewe (Uzzau *et al*, 2000). *S*. Dublin has the ability to cause systemic disease in humans, often resulting in bacteraemia and metastatic sites of infection, especially in immunocompromised individuals (Fang & Fierer, 1991).

S. Choleraesuis is another host-adapted serotype that is mainly associated with disease in pigs. It causes severe systemic disease in weaned pigs between 2 and 4 months of age. Septicaemia, fever and chronic wasting generally lead to death, with abortion induced in pregnant sows (Uzzau *et al*, 2000). *S*. Choleraesuis is associated with bacteraemia and extraintestinal focal infections in immunocompromised humans (Chen *et al*, 2007).

1:5 Epidemiology of *Salmonella*

Salmonellae are essentially intestinal parasites of humans and animals including domestic pets, farm animals, birds, reptiles and rodents. They are found in the environment and have been isolated from rivers, sewage and soil where, under the correct conditions, they can survive for many years. They have been detected in animal feeds as well as food such as

fruit and vegetables. The vast majority of salmonellosis cases are a result of consumption of contaminated food (including milk) or water, or by direct faecal-oral spread.

Members of *Salmonella enterica* are primarily associated with warm-blooded vertebrates, while members of the other five subspecies and *S. bongori* are usually isolated from the environment, cold-blooded animals and exceptionally, humans.

Many serotypes such as Typhimurium and Enteritidis are ubiquitous, inhabiting a wide range of host species, while others known as host-adapted serotypes are restricted to infecting a single host species. For example, *S.* Typhi is almost exclusively associated with systemic disease in humans as is *S.* Gallinarum in fowl (Barrow, 1994). Some host-adapted serotypes are able to cause disease in more than one host species. *S.* Dublin normally associated with systemic disease in cattle has been shown to cause abortion in pregnant ewes (McCaughey *et al*, 1971).

To avoid confusion in the literature, it has been proposed that ubiquitous serotypes be referred to as “un-restricted serotypes”, serotypes almost exclusively associated with a particular host species be referred to as “host-restricted serotypes” and lastly, serotypes usually associated with a particular host species but also able to cause disease in at least one other, be referred to as “host-adapted serotypes” (Uzzau *et al*, 2000).

1.6 Review of Typing methods used in investigating *Salmonella* epidemiology

There are many typing methods to help scientists investigate the origins and relatedness of isolates of *Salmonella*. During outbreak situations, these methods are valuable epidemiological tools that help in locating the source of the infection. Typing methods

should fulfil four main criteria; high discriminatory power (the ability to separate epidemiologically-unrelated organisms), reproducibility (the same conclusion should be reached upon repeat testing of an organism), typeability (all isolates should be assigned a "type" by the method applied) and transportability (the ability to replicate the method and achieve the same results in different laboratories).

Bacterial typing methods fall into two categories; phenotypic and genotypic methods. The former includes traditional methods such as serotyping, bacteriophage typing and antimicrobial sensitivity testing. These methods characterize the products of gene expression and therefore are capable of great variation depending on growth conditions, growth phase and spontaneous mutation. Genotypic methods such as plasmid profile analysis, plasmid restriction analysis and pulsed field gel electrophoresis investigate the genetic structure of the organism and are therefore less subject to natural variation. They can, however, be affected by loss or gain of plasmids, insertions and deletions of DNA into the chromosome or mutations that result in either the loss or the gain of a cutting site by restriction endonucleases.

1:6:1 Phenotypic methods

Serotyping

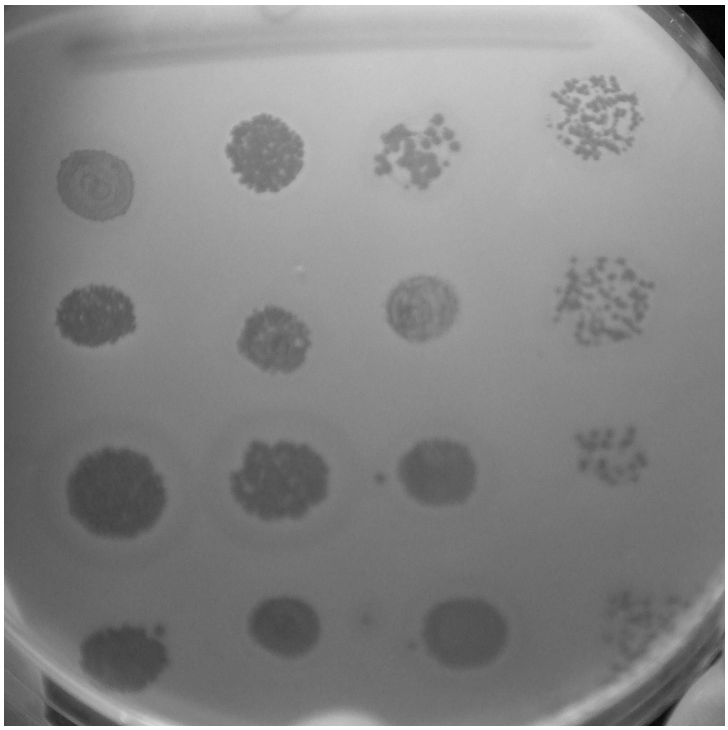
Serotyping of *Salmonella* is used to identify the isolates at subspecies level. A series of antibodies is used to identify the O (somatic) and H (flagellar) antigens on the bacterial cell

surface. In those isolates that possess a capsule or a cell envelope, a third antigen Vi, is also identified. The Kauffman-White scheme is then used to identify the serovar based on the antigens present (Popoff & Le Minor, 1997).

Bacteriophage typing

This method is based on the principle that isolates of *Salmonella* are susceptible to infection by bacteriophages. Bacteriophages are viruses that specifically infect bacteria and either cause lysis of the cell or lysogeny in which they remain inside the cell. This provides a means of further differentiating certain serovars based on their susceptibilities to these bacteriophages. A phage agar plate is flooded with a bacterial culture and allowed to dry at room temperature. Using a multipoint inoculator a series of bacteriophage specific to the serovar is inoculated onto the agar plate. Upon exposure, some (or in some phage types, all) of the bacteriophage lyse the organism creating a lysis pattern that can be interpreted to give the phage type. Phage typing schemes have been developed for, among others; *S.* Enteritidis (Ward *et al* 1987); *S.* Typhimurium (Anderson *et al* 1977); *S.* Typhi (Craigie & Felix, 1947); *S.* Infantis (Kasitya *et al* 1978) and *S.* Virchow (Chambers *et al* 1989).

Figure 1:1 Typical phage reactions of a strain of *S*. Enteritidis PT1b.



Antimicrobial sensitivity testing

Antimicrobial sensitivity testing can provide valuable information for clinicians to use when prescribing antimicrobial therapy and may also be used in surveillance as a means of observing trends in sensitivity patterns among bacterial isolates. Resistance patterns are very changeable mainly due to the methods through which resistance arises such as mutation and plasmid-mediated resistance.

There are various methods used in different laboratories to determine the R-type (resistance pattern) of isolates. For therapeutic advice, the objective is to produce a readily understood value that is both reproducible in the laboratory and relevant to the clinician (Frost, 1994). Minimum Inhibitory Concentration (MIC) determination using a culture grown on a non-selective medium to which an antimicrobial-impregnated strips is a fast and generally accurate method of obtaining such data. However, it can be very expensive if

large numbers of MICs need to be determined. Disks with known concentrations can be used in place of the antimicrobial strips. The zone if inhibition around the disk is measured and compared to published standards set by organisations such as the British Society for Antimicrobial Chemotherapy (BSAC) or the Clinical Laboratory and Standards Institute (CLSI-formally known as NCCLS). Testing by the agar dilution method is useful for determining the MICs of a large number of isolates as multiple isolates can be tested on a single plate using a multipoint inoculator. This is regarded as labour intensive and MICs might be difficult to interpret when they require finer discrimination than the usual two-fold serial dilutions provide.

For epidemiological and surveillance purposes, the MIC of resistant isolates tends not to be so important. It is sufficient to determine whether isolates are sensitive or resistant. For such information, a breakpoint method is very useful. In this method a known concentration of antimicrobial is incorporated into agar such as Isosensitest agar or Diagnostic Sensitivity agar and bacterial cultures are spotted onto the agar plates using a multipoint inoculator. In this way numerous sensitivities/resistances can be determined against multiple antimicrobials with the minimum of effort and time.

1:6:2 Genotypic methods

Plasmid Profile Analysis

In most bacteria plasmids are extrachromosomal covalently-closed supercoiled circular pieces of DNA that are capable of autonomous replication. Many encode products and/or functions that are beneficial to the bacterial host; resistance to antimicrobials or virulence factors are important examples. During cell division, copies of any plasmids present within

the cell are distributed among the daughter cells. In this way, members of the same clonal line should have identical plasmid profiles. However, in the serovars Enteritidis and Typhimurium, the virulence plasmids are present in many different phage types (Brown *et al*, 1993). Plasmids are mobile and can transfer from one bacterial cell to the next usually by means of conjugation, which requires cell-to-cell contact as DNA passes from one bacterial cell to another via a sex pilus. Plasmids that transfer antimicrobial resistance by conjugation are known as 'R-factors' and were first identified by Watanabe in 1963 (Watanabe, 1963). Plasmids can also be "lost"; the bacterial host may eliminate the plasmid from the cell when it is no longer needed, such as in response to the withdrawal of antimicrobial selective pressure (Brown *et al*, 1991).

Pulsed Field Gel Electrophoresis

Standard electrophoresis is unable to resolve fragments of DNA greater than 120kb therefore the technique of pulsed field gel electrophoresis (PFGE) was developed. It first used in 1984 in the examination of yeast chromosomal DNA (Schwartz & Cantor, 1984). PFGE is a highly reproducible genotypic typing method. It has excellent discriminatory power and can be used for almost all bacteria. Depending upon the organism being investigated, methods vary, however they all follow basic principles. Bacterial cells are embedded in agarose and formed into plugs before being lysed, allowing the isolation of the chromosomal DNA. EDTA and detergents are used to inhibit nucleases and Proteinase K is used to digest cellular proteins. The plugs are then washed repeatedly to remove unwanted cell components, leaving only the chromosomal DNA embedded in the agarose. The plugs are cut to the required size and digested by a restriction endonuclease that ideally cleaves the chromosomal DNA infrequently. *Xba*I is the most frequently used

enzyme in the study of S*almonella* as it is inexpensive and has good discriminatory power. However, some very closely related strains, for example the commonly isolated *Salmonella* Enteritidis PT4, may require a second enzyme such as *Spe*I and *Bln*I. These enzymes are much more expensive but often they are able to distinguish between those strains that give identical *Xba*I profiles. Once digested, the plug slices are sealed into the wells of an agarose gel and subjected to pulsed field electrophoresis.

The PFGE apparatus CHEF (contour clamped homogenous electric field) separates the DNA fragments into straight lines. The gel is placed in the centre of the electrophoretic cell between three sets of electrodes that form a hexagon around it. These electric fields alternate; flowing from one direction for a set length of time before switching and flowing from another for the same period. This is known as the switch time which, in addition to other parameters such as voltage, running time and temperature must be optimized. Depending upon the specifications of the CHEF equipment, the angle between the electric fields is fixed at 120ºC. Some models with greater specifications allow the angle to be varied. When the first electric field is applied, the DNA fragments migrate through the gel in the new direction. When the second electric field is applied, the fragments re-orientate in that direction. Larger molecules take longer to re-orientate and therefore migrate through the gel to a lesser extent. Increasing switch times throughout the run allows increasingly larger fragments to migrate, thereby resulting in separation of fragments dependent upon size.

The fragments of DNA can be visualized by staining with a chemical such as ethidium bromide and illuminating on an ultraviolet source. They form a pattern of bands that is known as the pulsed field profile. These pulsed field profiles can be compared with those of other strains for genetic relatedness. Guidelines on the interpretation of the banding

patterns have been published (Tenover *et al*, 1995). The advent of computer software such as Bionumerics (Applied Maths, Kortrijk, Belgium) has greatly enhanced the analysis of these data. Gel images are converted to TIFF files and analysed by the software using statistical formulae. From the results, a Dendrogram may be constructed reflecting the degree of similarity between the patterns obtained.

PulseNet Europe is a collaboration between a number of medical and veterinary laboratories around Europe. Its purpose is the harmonization of protocols and running parameters for the PFGE of *Salmonella.* As each country runs PFGE using identical protocols data may be shared and compared, allowing epidemiological investigation to be run Europe-wide. PulseNet Europe has also established a database to which, the member laboratories submit the pulsed field profiles. A central curator checks the pattern and, if it is novel, assigns a unique PFGE pattern designation, an example of which would be: SENTXB.0001. The letter "S" in the first position designates the genus *Salmonella*. The next three letters, in this case "ENT" representing Enteritidis, designate the serotype using codes preordained by PulseNet Europe. The "XB" represents the enzyme used to generate the pattern, *Xba*I. A full stop separates these characters from the final four numerical characters which are used to distinguish the individual pattern for any particular serotype using any particular enzyme. As a result, laboratories across Europe are able to access this database in order to identify their pulsed field profiles and assign agreed pattern designations.

1:7 History of Quinolones and Fluoroquinolones

Development of the quinolones began with the accidental discovery by George Y. Lesher of a by-product of the synthesis of the anti-malarial drug chloroquine that exhibited some antibacterial activity (Lesher *et al*, 1962). The resulting 1,8-naphthyridine derivative was named nalidixic acid and was used to mainly treat Gram-negative urinary tract infections after it's introduction in the U.S.A. in 1963 and the U.K. in 1964. The next ten years saw a number of related molecules, termed first-generation quinolones, developed. Based on the 4-quinolone nucleus, compounds with minor modifications such as pipemidic acid, oxolinic acid and cinoxacin were all synthesized. These compounds generally displayed increased activity against aerobic Gram-negative bacteria but due to poor serum and tissue kinetics were restricted to treatment of urinary tract infections and sexually transmitted diseases. They still lacked activity against Gram-positive and anaerobic bacteria.

In the early 1980's it was discovered that modifications to the C6 and C7 positions of the 4-quinolone nucleus significantly enhanced the absorption and the activity of these compounds. The addition of a fluorine atom at the C6 position significantly increased the DNA gyrase inhibitory activity and facilitated penetration into the bacterial cell (Domagala *et al*, 1986). This, in addition to a cyclic diamine piperazine at position C7, resulted in the compound norfloxacin-the first fluoroquinolone, so named because of the fluorine atom at C-6 (Hooper & Wolfson, 1985). These modifications resulted in antibacterial activity against aerobic Gram-positives and improved activity against Gram-negatives but made no difference in the activity against anaerobic bacteria.

Since this discovery, chemical modification of the quinolone nucleus has resulted in a broad variety of useful antimicrobial agents. Ciprofloxacin was the result of replacing the N-1 ethyl group of norfloxacin with a cyclopropyl group. This second-generation

quinolone was the first to be useful in treating infections other than those of the urinary tract and sexually transmitted diseases.

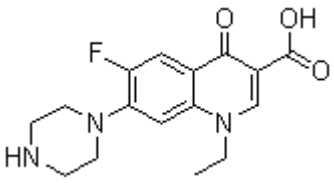
Third- and fourth-generation quinolones have been developed through increasing structural novelty and complexity. These newer compounds have increased activity against Gram-positive cocci and potent activity against anaerobes (Brightly & Gootz, 2000).

Although attempts to further chemically modify this class of drugs continues, the number of ideas for new compounds appears to be relatively small. One of the most promising new ideas is based on the notion of removing the fluorine atom from C6-resulting in "6-desfluoro" compounds (Moellering Jr., 2005). Garenoxacin is a 6-desfluoroquinolone that has broad spectrum activity against both Gram-negative and Gram-positive bacterial infections, including anaerobic organisms (Ameyama *et al*, 2003, Noviello *et al*, 2003, Hoellman *et al*, 2001).

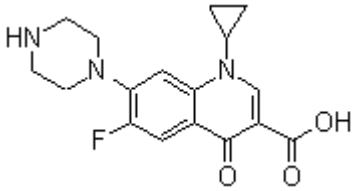
Figure 1:2: Structure of nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin and moxifloxacin.

All diagrams courtesy of http://www.chemblink.com.

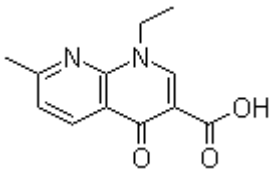
Nalidixic acid



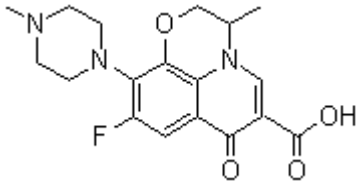
Ciprofloxacin



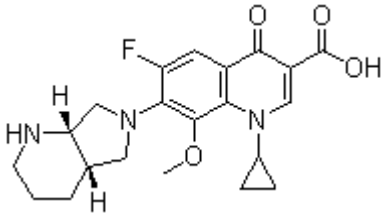
Norfloxacin



Ofloxacin



Moxifloxacin



1:8 The Use of Fluoroquinolones in the Treatment of Human Infections

Fluoroquinolones are broad-spectrum antimicrobials effective in the treatment of a wide variety of clinical infections. They are highly effective in uncomplicated urinary tract infections and gonorrhoea and are the drugs of choice where bacterial resistance compromises routine β-lactam therapy (Nicolle, 2000). The modern fluoroquinolones, including moxifloxacin and gemifloxacin all possess outstanding activity against respiratory pathogens, methicillin-susceptible staphylococci and a variety of important Gram-negative bacilli (Moellering Jr., 2005).

Fluoroquinolones are the drugs of choice for treatment of invasive gastrointestinal infections in adults worldwide. Norfloxacin or ciprofloxacin has been found to be comparable to trimethoprim-sulphamethoxazole in the treatment of diarrhoea caused by *Shigella* species, enterotoxigenic *E. coli* or *Campylobacter jejuni*. Norfloxacin has been found to be superior to both trimethoprim-sulphamethoxazole and doxycycline in the treatment of *Vibrio cholerae* infection (Oliphant & Green, 2002).

The fluoroquinolones have proved to be an invaluable tool in the treatment of moderate to severe enteric infections (Akalin, 1995). They are widely used in the oral management of typhoid fever, reducing complication, relapse and convalescent excretion rates to a greater extent than traditional first-line drugs (Parry *et al*, 2002).

Salmonella gastroenteritis is usually a self-limiting diarrhoeal disease and antimicrobial therapy is usually only indicated when the patient is immunocompromised, has an enteric fever, has an extra-intestinal infection or long-term salmonella carriage. In many such cases, ciprofloxacin is often the drug of choice (Moraitou, 2007, Ridha *et al*, 1996)

1:9 The Use of Fluoroquinolones in Veterinary Medicine

The fluoroquinolones have been used to treat infections in food-producing animals since the early 1990s. The regulation and use of these drugs varies greatly between countries. In Europe, none of the fluoroquinolones used in the treatment of human infections are licensed for use in infections in animals. Nevertheless, there is still concern regarding any usage of this group of antimicrobials in veterinary medicine due to the fear of increasing resistance. Several European countries have reported a significant increase in the resistance of *Salmonella* to quinolones after their use in livestock was approved (Frost *et al*, 1996; Malorny *et al*, 1999; Aarestrup *et al*, 2000). *In vivo* studies have shown that enrofloxacin selects *Salmonella* mutants resistant to nalidixic acid and fluoroquinolones. It has been hypothesized that animals may act as reservoirs for reduced susceptibility mutants that, upon further exposure to fluoroquinolones, may lead to high-level resistance (Giraud *et al*, 1999).

1:10 Quinolone Mechanism of Action

The targets of quinolone action are two bacterial enzymes that are essential for cell growth and division-DNA gyrase and topoisomerase IV. Both are tetramers composed of two pairs of identical subunits, GyrA and GyrB encoded by *gyrA* and *gyrB* in DNA gyrase and ParC and ParE encoded by *parC* and *parE* in topoisomerase IV (Drlica & Zhao, 1997). Gyrase controls DNA supercoiling and relieves topological stress arising from translocation and replication complexes along the DNA; topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication (Drlica & Zhao, 1997). DNA gyrase is the primary target of quinolones in Gram-negative bacteria with

topoisomerase IV acting as a secondary target, the reverse is true for Gram-positive bacteria (Li, 2005).

Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase IV. Shortly after binding, the quinolones induce a conformational change in the enzyme. The enzyme breaks the DNA and the quinolone prevents re-ligation of the broken DNA strands. The enzyme is trapped on the DNA resulting in the formation of a quinolone-enzyme-DNA complex. Quinolone-enzyme-DNA complex formation rapidly inhibits DNA replication and is consistent with gyrase acting ahead of replication forks (Hawkey, 2003). However, inhibition of replication by quinolone-topoisomerase IV-DNA complexes occurs slowly, consistent with the enzyme being located behind the replication forks (Khodursky & Cozzarelli, 1998).

It is thought that cell death arises by more than one mechanism. The release of DNA ends from the quinolone-gyrase-DNA complexes, which are the equivalent of double-strand breaks are thought to induce bacterial apoptosis. It is also believed that quinolone molecules may be able to force gyrase-DNA complexes apart, releasing DNA ends. It is thought that this second mode occurs when cells are treated with high concentrations of fluoroquinolones such as ciprofloxacin (Drlica & Zhao, 1997).

Figure 1:3. DIAGRAMATIC REPRESENTATION OF GYRASE ACTIVITY AND QUINOLONE INHIBITION.

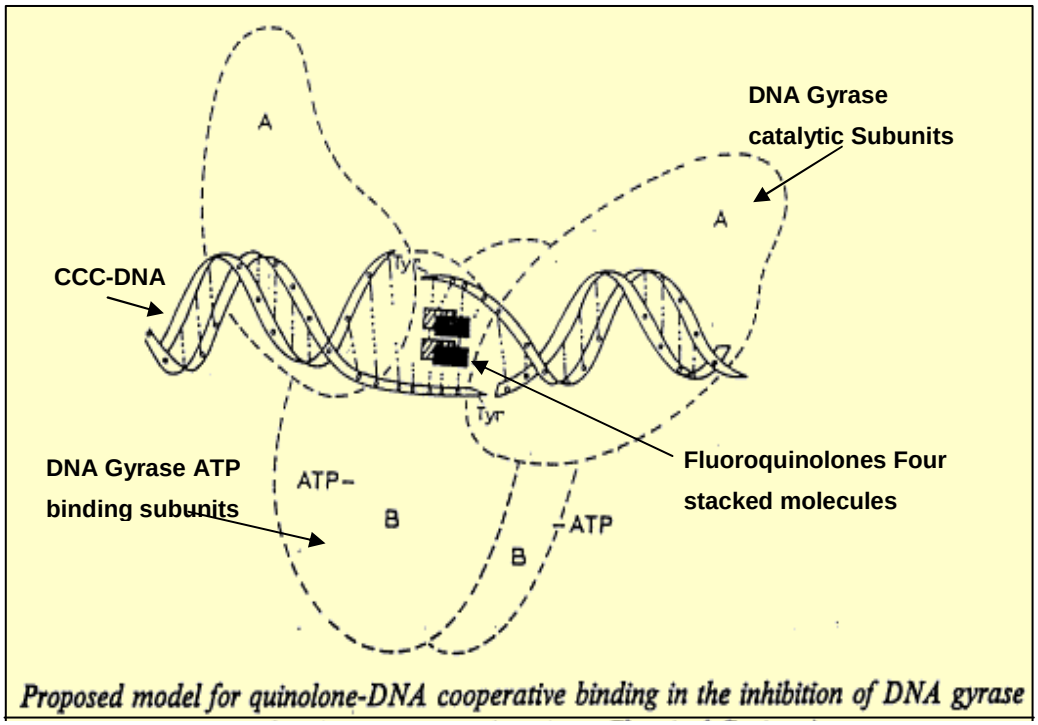


Diagram modified from www.pharmainfo.net

1:11 Resistance to Fluoroquinolones

Before the early 1990s, resistance to fluoroquinolones was rarely witnessed in clinical isolates of Gram-negative bacteria. Successful treatment outcomes resulted in an increase in their use which, in turn, led to an ever increasing rate of resistance (Jacoby, 2005). In the United States an increase in the use of fluoroquinolones of around 40% led to a coincident doubling in the rate of resistance to ciprofloxacin among Gram-negative bacilli

isolated from intensive care units in hospitals (Neuhauser *et al*, 2003). In other parts of the world too, increased rates of resistance are being reported. In Spain, fluoroquinolone resistance had become such a problem that, by the mid 1990s, they were not first choice in the treatment of *E. coli* urinary tract infections (Oteo *et al*, 1999). In Beijing during 1997-1999, approximately 60% of *E.coli* strains isolated from hospital-acquired infections and approximately 50% of community-isolated *E.coli* strains were resistant to ciprofloxacin (Wang *et al*, 2001).

Treatment failures attributed to fluoroquinolone resistance have been reported in cases of typhoid fever. Strains of *S.* Typhi with reduced susceptibility to fluoroquinolones have been reported from amongst others, Vietnam (Parry *et al*, 1998), the Indian subcontinent (Nath *et al*, 2000) and Africa (Kariuki, 2004).

High-level resistance to fluoroquinolones is relatively uncommon among isolates of non-typhoidal *Salmonella*, although a Finnish study associated an increase in ciprofloxacin MIC in *S. enterica* isolates with travellers returning from Southeast Asia (Hakanen *et al*, 2001). Danish scientists also found that quinolone resistance in isolates from patients with a history of foreign travel was more prevalent than those with domestically acquired infections (Molbak *et al*, 2002).

Despite the early promise that came with the licensing of the fluoroquinolones thirty years ago, scientists today are calling for cautious, even restricted, use of these antimicrobials (Bakken, 2004).

1:12 Mechanisms of Quinolone Resistance

There are essentially four types of mechanism by which bacteria evade the action of quinolone antimicrobial agents. They may work independently or in combination, conferring degrees of resistance from reduced susceptibility (not always detected by current antimicrobial susceptibility tests) to clinically relevant resistance.

1:12:1 Mutations in Topoisomerase Genes.

Resistance to quinolones is mainly mediated by chromosomal mutations that alter the targets of these antimicrobials, namely DNA gyrase and topoisomerase IV. In Gram-negative bacteria, gyrase, or more specifically, the *gyrA* subunit, is most frequently the primary target, with topoisomerase IV being the primary target for Gram-positive organisms. The point mutations most often occur within the highly conserved domain of the N-terminus of the *gyrA* gene known as the 'quinolone resistance-determining region' (QRDR) (Piddock, 1999). This region occurs on the DNA-binding surface of the enzyme near the putative active site tyrosine 122 (Piddock, 1999). These mutations give rise to amino acid changes and, in *E.coli*, include amino acids between positions alanine 67 and glycine 106 with mutations at serine 83 and asparagine 87 being the most common. A few mutations have been detected outside the QRDR. An alanine to valine substitutions has been detected in *in-vitro* mutants at position 51 (Friedman *et al*, 1997) and in salmonellae, mutations at Ala131, Glu139 and asp144 have all been detected (Eaves *et al*, 2002).

A few mutations have been mapped to the N-terminus of the *gyrB* gene located between amino acids Asp426-Lys447. This region is known as the QRDR in the *gyrB* gene and all of the mutations give rise to amino acid substitutions. Once a first-step mutation has

decreased susceptibility in a Gram-negative organism, additional mutations in *gyrB* may serve to further increase the resistance.

Point mutations have also been detected in the *parC* and *parE* genes of Gram-negative bacteria but remain much rarer than *gyrA* mutations. Although Ling *et al* detected a *parC* mutation in the absence of a *gyrA* mutation in a strain of *Salmonella* (Ling *et al*, 2003); it is generally believed that *parC* mutations arise later than *gyrA* mutations because in Gram-negative bacteria, gyrase rather than topoisomerase IV is the preferred target of quinolones.

1:12:2 Alterations in Efflux

Increased expression of non-specific, energy-dependant efflux systems allow bacteria to prevent the accumulation of effective concentrations of quinolones inside the cell by actively pumping out the drug. In *E. coli*, the AcrAB-TolC efflux pump plays a major role in quinolone efflux and studies suggest that this may be the primary mechanism of fluoroquinolone resistance in *Salmonella* (Giraud *et al*, 2000). It is thought that these efflux systems cause low-level resistance to quinolones that can become clinically-relevant when combined with mutations in the target enzymes (Hooper, 1999) or alterations in the outer membrane (Giraud *et al*, 2000).

1:12:3 Alterations in the Outer Membrane

Gram-negative bacteria can regulate membrane permeability by altering expression of outer membrane porin (omp) proteins that form channels for passive diffusion. Loss or reduced levels of OmpF has been implicated in antimicrobial resistance (Toro *et al*, 1990),

but in later studies the role of OmpF in reduced levels of quinolone accumulation was less clear with no loss or reduction in levels detected (Piddock *et al*, 1993 ; Ruiz *et al*, 1997).

1:12:4 Plasmid-mediated Resistance

A natural transferable plasmid encoding low-level resistance to quinolones was isolated from a clinical strain of *Klebsiella pneumoniae.* The 56kb plasmid, named pMG252, had a broad host range that included other members of the Enterobacteriaceae and *Pseudomonas aeruginosa* (Martinez-Martinez *et al*, 1998). The *qnrA* gene from the plasmid encodes a protein of 218 amino acids that belongs to the pentapeptide repeat family. *In vitro* studies demonstrated that Qnr protected the *E. coli* DNA gyrase from inhibition by ciprofloxacin but did not protect topoisomerase IV (Tran *et al*, 2005). Further molecular studies demonstrated that *qnr* was collocated with other resistance determinants on *sul*1-type integrons (Rodríguez-Martinez *et al*, 2007). The presence of *qnr* has frequently been found in strains producing extended spectrum β-lactamases (Hata *et al*, 2005).

In 2005, scientists in Japan discovered another *qnr* gene, *qnrS* (now designated *qnrS1*), in a plasmid from a strain of *Shigella flexneri* 2b (Hata *et al*, 2005), and in 2006 a third *qnr* gene, *qnrB1*, was isolated from strains of *K. pneumoniae* from India. These two genes share 41% and 59% amino acid identity with the original gene, now designated *qnrA1* (Nordmann & Poirel, 2005). The sixth variant of *qnrA1*, *qnrA6*, has recently been identified and a variant with 91% homology to *qnrS1* has been identified in the United States and designated *qnrS2* (Gay *et al*, 2006). *qnrB2* and *qnrB5* were identified in non-typhoidal *Salmonella enterica* isolates from the United States (Gay *et al*, 2006), and in Germany *qnrB12* has recently been identified in poultry isolates of *Citrobacter werkmanii* (Kehrenberg *et al*, 2008).

The presence of *qnr* genes themselves do not appear to confer resistance to quinolones; however their importance lies in the augmenting effect of these *qnr* genes and other resistance mutations and facilitating the selection of higher level quinolone resistance from strains harbouring the plasmid than those without it (Martinez-Martinez *et al*, 1998).

1:13 Aims of this study

Fluoroquinolones are being increasingly used in the treatment of invasive gastrointestinal infections. Reports of treatment failures are also increasing. This study aimed to:

1. Determine the levels resistance to quinolone and fluoroquinolone antimicrobials in strains of non-typhoidal *Salmonella* isolated in Scotland.
2. To attempt to select fluoroquinolone-resistant mutants following exposure to subinhibitory concentrations of these drugs.
3. To establish the capacity of susceptible strains of *Salmonella* to develop resistance and to determine whether this capacity is equal in all serotypes examined.
4. To investigate the capacity of certain antimicrobials to select for resistance.
5. To compare the findings to the results of previous investigations into laboratory-selected fluoroquinolone-resistant mutants.

Chapter 2: Characterisation of strains to point of inclusion in this study

2:1 Introduction

The general procedures for the typing of isolates of *Salmonella* have been outlined in Chapter1. In this chapter, the origin of the strains used in this study is given together with the methods used in the Scottish Salmonella Reference Laboratory (SSRL). The characterisation of strains used in this study and described in this chapter comprises identification to reporting stage with the exception of the antimicrobial sensitivity data which are given in Chapter 3.

All isolates of *Salmonella* of human, veterinary and environmental origin isolated in Scotland are submitted to the SSRL for phenotypic and genotypic characterisation. Each isolate is given a unique identifying number. A sweep from this original culture is then inoculated onto a MacConkey agar plate that is incubated overnight at 37°C. A single colony is selected from this plate and inoculated onto a Dorset's Egg slope, which is incubated overnight at 37°C. Inoculated Dorset's Egg slopes are stored at room temperature for future reference. It is from this Dorset's Egg that all subsequent work is performed. Isolates are routinely serotyped, phage typed (where applicable), tested for sensitivity against a panel of fourteen antimicrobials, plasmid profiled and pulse field profiled. Data from these procedures are then stored in the SSRL database and appropriate portions reported to clinicians, Health Protection Scotland (HPS) and the Pulse Field Gel Electrophoresis (PFGE) data are filed with PulseNet Europe. These data were available for each strain prior to selection for this study.

2:2 Materials and Methods

The materials and methods described in this Chapter refer to routine procedures operation in the SSRL and of relevance to the study as a whole. They were used to confirm the properties on file for the isolates of *Salmonella* selected in two laboratory studies.

2:2:1 Isolates of *Salmonella*

The procedure for acquisition, identification, reporting and archiving strains of *Salmonella enterica* has been outlined above in the introduction.

The SSRL received a total of 58,576 isolates in the years 1990-2000. For the purposes of this study quality control strains and duplicate samples were excluded and only a single isolate from epidemiologically-related outbreaks has been included.

These isolates were divided into four subsets based on the following criteria:

- MDR (resistant to multiple antimicrobials) - those isolates resistant by breakpoint method to four or more antimicrobials (two of these being quinolone or fluoroquinolones).
- FQR (Fluoroquinolone resistant) - those isolates only resistant by breakpoint method to nalidixic acid 40mg/L and ciprofloxacin 0.5mg/L.
- NCpL (nalidixic acid and low-level ciprofloxacin resistant)–those isolates only resistant by breakpoint method to nalidixic acid 40mg/L and to low-level ciprofloxacin 0.125mg/L.
- FS (Fully susceptible)–those isolates showing no resistance by breakpoint to any antimicrobials tested.

Inclusion in the final selection was then based on a further set of criteria, namely serotype, phage type, source and year received, to give as varied a collection of strains as possible. As a result 180 isolates of *Salmonella enterica* formed the basis of these studies. The serotype and source distribution of the selected isolates are shown in **Tables 2:1** and **2:2**, respectively and their original breakpoint designations are given in **Appendix I**.

Table 2:1. Serotype distribution of *Salmonella* isolates studied.

Serotype	MDR[a]	FQR[b]	NlCp[c]	FS[d]	Total
Enteritidis	5	45	23	5	78
Typhimurium	12	1	1	10	24
Mbandaka	6	13	2	1	22
Hadar	14	0	1	0	15
Virchow	3	0	4	1	8
Dublin	0	0	2	5	7
Senftenberg	1	0	3	0	4
Thompson	1	0	3	0	4
Liverpool	0	0	3	0	3
Binza	0	0	1	1	2
Blockley	2	0	0	0	2
Brandenburg	1	0	0	1	2
Kottbus	0	0	2	0	2
Antarctica	0	0	1	0	1
Bovismorbificans	1	0	0	0	1
Indiana	1	0	0	0	1
Java	1	0	0	0	1
Monophasic Group C1	1	0	0	0	1
Montevideo	0	0	0	1	1
Oranienberg	1	0	0	0	1
Total	50	59	46	25	180

[a] Resistant by breakpoint testing to 4 or more antimicrobials including nalidixic acid and ciprofloxacin.

[b] Resistant by breakpoint testing to ciprofloxacin 0.5mg/L and nalidixic acid 40mg/L.

[c] Resistant by breakpoint testing to nalidixic acid 40mg/L and ciprofloxacin 0.125mg/L.

[d] Fully sensitive by breakpoint testing.

Table 2:2. Source distribution of *Salmonella* isolates studied.

Source	MDR[a]	FQR[b]	NlCp[c]	FS[d]	Total
Human	31	25	29	13	98
Poultry	11	29	11	3	54
Environmental	4	4	3	0	11
Bovine	1	0	2	4	7
Porcine	0	0	0	1	1
Pheasant	1	0	0	1	2
Canine	0	0	1	0	1
Ovine	0	0	0	1	1
Porpoise	0	0	0	1	1
Canary	0	0	0	1	1
Feline	0	1	0	0	1
Unknown	2	0	0	0	2
Total	50	59	46	25	180

[a] Resistant by breakpoint testing to 4 or more antimicrobials including nalidixic acid and ciprofloxacin.

[b] Resistant by breakpoint testing to ciprofloxacin 0.5mg/L and nalidixic acid 40mg/L.

[c] Resistant by breakpoint testing to nalidixic acid 40mg/L and ciprofloxacin 0.125mg/L.

[d] Fully sensitive by breakpoint testing.

2:2:2 Study 1: Plasmid Profile Analysis of the selected strains

2:2:2:1 Materials

Test organisms	detailed in section **2:2:1**	
Cysteine Lactose Electrolyte Deficient (CLED) agar	Oxoid	CM0301
Brain Heart Infusion Broth (Dehydrated)	Oxoid	CM0225

TE 50:1: Trizma base	Sigma	T6066
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma	E5134
10X TBE Buffer	Invitrogen	15581-044
Phenol Chloroform Isoamyl	Sigma	P2069
Kado & Liu Lysis Buffer: Trizma base	as above	
Sodium Dodecyl Sulfate (SDS)	Sigma	L4390
Sodium Hydroxide	BDH	191533K
Redipac GP agarose	Mast	GP500
Ethidium Bromide	Sigma	E1510
E. coli Control strains, 39R861 & V517	H.P.A. Colindale, England	

Preparation of working solutions can be found in **Appendix I**.

2:2:2:2 Methods

Preparation of Plasmid DNA

Four colonies from an overnight CLED plate were inoculated into 5ml BHI and incubated shaking at 37°C overnight. 0.8ml culture was dispensed into a Treff tube and centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 20µl of TE 50:1 buffer by vortexing. 100µl of Kado and Liu lysis buffer was added and mixed by inversion. The tubes were transferred to a 56°C waterbath for 30 minutes. 100µl

of phenol-chloroform-isoamyl was added to each tube and vortexed. Tubes were transferred to a microcentrifuge and centrifuged at 18,000g for 15 minutes. 40µl of supernatant was carefully transferred to a clean Treff tube to which 6µl of gel loading solution was added. The control strains of *Escherichia coli*, 39R861 (Threlfall *et* al, 1986) and V517 (Macrina, 1978), were also prepared in the same way. These contain plasmids of known sizes and can be used to size the plasmids of the test isolates. The plasmid preparations were stored at 5°C until needed.

Preparation of Gel

The gel was prepared by dissolving 0.7g of general purpose agarose in 100ml of 1X TBE buffer by heating in a microwave oven and then transferring to a 56°C waterbath. The ends of the gel casting tray were sealed using autoclave tape. A twelve well comb was inserted before pouring the cooled agarose into the casting tray. Once the gel was set, the comb and the autoclave tape were removed and the gel was placed in an electrophoresis tank (Bio-Rad) containing 1X TBE buffer to a level 5mm above the gel surface. 25µl of each plasmid preparation was added to wells 2-11, while in wells 1 and 12 the controls, 39R861 and V517, were loaded. The gel was run for 3 hours at 100V and then stained in a 1.25µg/ml solution of Ethidium Bromide for 20 minutes. The gel was then de-stained for a further 20 minutes before being visualized and photographed using a U.V. transilluminator and a Kodak EDAS 120 Digital Camera System (BRL Life Technologies).

Calculation of plasmid sizes

Plasmids migrate at different speeds due to their molecular size and so included on every electrophoresis gel are two standard plasmid preparations with plasmids of known

molecular size. The first, *E. coli* V517 contains 8 plasmids ranging from 2.1kb up to 54kb while the second, *E. coli* 39R861 has 4 plasmids ranging in size from 7kb up to 147kb. Plasmid molecular weights were estimated according to the method of Rochelle *et al*. Briefly, a standard curve of log molecular weight v log relative mobility was generated for the reference plasmids. Relative mobility was calculated for the unknown plasmids and this was used to extrapolate their molecular weights from the standard curve.

2:2:2:3 Results

Plasmid profile results are tabulated in **Appendix II**.

Seventeen different plasmid profiles were identified among the 78 strains of *S.* Enteritidis examined. The most common profile consisted of a single plasmid of approximately 57kb, with 44 strains sharing this profile. Twelve strains harboured a single additional plasmid of 2.1kb and three strains, a single additional plasmid of 100kb or 110kb. Eight strains harboured varying numbers of plasmids that ranged in size from 2.1kb to 70kb in addition to the 57kb plasmid while the remaining 11 strains had profiles that did not include the 57kb plasmid including 4 strains which possessed single plasmids of 120kb, 5.2kb or 2.1kb (2), one strain that harboured 3 plasmids of 45; 6.0 and 4.5kb and 6 strains that harboured no plasmids and were termed plasmid-free.

Of the twenty-four strains of *S.* Typhimurium, twenty possessed a plasmid (or plasmids) of approximately 90kb, eight of which had additional plasmids in the range of 2.0-145kb. One strain of *S.* Typhimurium RDNC (Reacts but Does Not Conform) possessed five plasmids ranging from 2kb up to 145kb. Three strains were plasmid-free.

The plasmid profiles of the *S.* Mbandaka strains varied considerably. The twenty-two strains yielded nineteen different profiles, ranging from one to six plasmids. Plasmid sizes ranged from 2.1kb to 90kb. No strain of *S.* Mbandaka was plasmid-free.

Of the seven *S.* Dublin strains examined, two were plasmid-free and five possessed a single plasmid of approximately 80kb.

The fifteen strains of *S.* Hadar all possessed a small plasmid of 2.1kb. In addition to this, thirteen strains had between one and five larger plasmids ranging from 3.2kb to 45kb. Of the remaining thirty-four isolates examined, nine were plasmid-free. With the exception of three strains of *S.* Liverpool which had identical plasmid profiles, all of the serotypes with greater than one isolate resulted in different plasmid profiles.

2:2:3 Study 2: Pulsed Field Gel Electrophoresis studies of the selected strains

2:2:3:1 Materials

Test organisms		detailed in section **2:2:1**	
Cystine Lactose Electrolyte Deficient (CLED) agar		as in section **2:2:2:1**	
Sodium Chloride		BDH	102414Y
ES Buffer	N-lauryl-sarcosine (Sarcosyl^{R} NL30)	BDH	442753R
	EDTA	as in section **2:2:2:1**	
	Sodium hydroxide solution 40% w/v	BDH	191533K

Proteinase K (stock solution 50mg/ml)		Roche 03 115 852 044
Pulsed Field Certified Agarose		Bio-Rad 162-0137
10X TBE Buffer		as in section **2:2:2:1**
TE (PFGE) Wash Buffer	Trizma Base	as in section **2:2:2:1**
	EDTA	as in section **2:2:2:1**
Ethidium Bromide (10mg/ml solution)		as in section **2:2:2:1**
*Xba*I Restriction Enzyme		Invitrogen 15226-038
React 2 10X Buffer		supplied with *Xba*I.

Preparation of working solutions can be found in **Appendix III**.

2:2:3:2 Method

Four colonies from an overnight CLED plate were inoculated into 5ml BHI and incubated shaking at 37°C overnight. 0.8ml culture was dispensed into a Treff tube and centrifuged in a microcentrifuge at 13,000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 1ml 0.85% saline. After further centrifugation, the supernatant was again removed and the pellet was resuspended in 0.5ml 0.85% saline. The cell suspensions were then transferred to a 40°C heating block and left to equilibrate for 15 minutes.

2% agarose was prepared by dissolving 0.4g Pulsed Field Certified agarose in 20ml 0.85% saline in a microwave oven and allowing it to cool to 56°C in a waterbath. A plug mould was prepared by sealing the underside with autoclave tape.

Agarose plugs were prepared by adding 0.5ml cooled agarose to the cell suspension and mixing well. An aliquot of 120µl was then dispensed into a single well of the plug mould. Once all the wells were full, the mould was then placed at 5°C to allow the plugs to set. Plugs were lysed by transferring each plug to a Treff tube containing 1ml ES buffer to which 20µl of 50mg/ml Proteinase K solution had been added. The tubes were then placed in a 56°C waterbath overnight.

After lysis, the plugs were transferred to sterile disposable 5ml plastic test tubes containing 3ml TE (PFGE) wash buffer. The test tubes were placed in a shaking waterbath at 56°C for 30 minutes. Using fresh wash buffer, this step was repeated 6 times after which, the plugs were stored in 3ml wash buffer at 5°C until digestion.

Using a sterile disposable scalpel, a 1mm slice of plug was taken from each plug and placed into a Treff tube containing 90µl of sterile water and 10µl React 2 10X buffer, and left at room temperature for 1 hour. This was removed and replaced with 85µl sterile water, 10µl React 2 10X buffer and 5µl *Xba*I restriction enzyme. Plugs were incubated for 4 hours at 37°C.

The gel was prepared by suspending 1.3g Pulsed Field Certified agarose in 130ml 0.5X TBE buffer. The agarose was dissolved by heating in a microwave oven and then placed in a waterbath to cool to 56°C. The gel casting mould was assembled and wiped with methylated spirits. A thirty-well comb was inserted before the cooled agarose was poured into the mould. Approximately 1.5ml agarose was returned to the waterbath for later use.

Once the gel was set, the comb was removed and the slices of agarose plugs were removed from the restriction mix and inserted into the wells of the gel using two small spatulas. The remaining agarose was removed from the waterbath and used to seal the plugs inside the wells.

PFGE was performed using a CHEF-DR II system from Bio-Rad. 2.2L of 0.5X TBE buffer was added to the tank. The cooling module and the pump were switched on and set to 14°C and 70 - 80 respectively. The gel and the buffer were left for approximately one hour until both had reached the running temperature of 14°C. The gel was run for 22 hours using the parameters 6V, initial switch 2s and final switch 64s.

The gel was removed and stained in a 1.25µg/ml Ethidium Bromide solution with gentle shaking for approximately 30 minutes and then de-stained in distilled water for a further 30 minutes. The gel was visualized by U.V. transillumination and photographed as previously described. Images of the gel were stored electronically as Tiff files and analysed using Bionumerics software. Where possible, they were given designations applied to identical banding patterns held in the PulseNet Europe database. Any pulsed field profiles (PFP) that failed to match named patterns in this database were given designations unique to this study and are preceded with the letters AM.

2:2:3:3 Results

All 180 isolates of *Salmonella* were analysed by PFGE using the parameters of PulseNet Europe and analysed using Bionumerics software. The pulsed field profiles are listed in **Appendix V.**

Using the Bionumerics software, a Dendrogram was constructed of the 78 strains of *S.* Enteritidis investigated in this study (**Figure 2:1)**. Fifty-five (70%) proved to be identical and were given the designation SENTXB.0001, the most common pulsed field pattern (PFP). Seven strains gave PFPs that differed from SENTXB.0001 by the deletion of the same single band and were assigned the designation SENTXB.0014. Another strain also possessed this deletion but had a smaller single band and was given the designation AMENTX7.The remaining 15 strains of *S.* Enteritidis were divided into a further ten groups some of which, for example, SENTXB.0019, had only one representative. The SENTXB.0001 designation was common among twelve of the thirteen different phage types of Enteritidis.

A discrepancy arose with the Bionumerics analysis of two strains of *S.* Enteritidis assigned the designation SENTXB.0005. On visual comparison the two PFP looked identical, and when analysed they both matched the PulseNet designation SENTXB.0005 and have 98% similarity with each other. However when the *S.* Enteritidis Dendrogram was constructed, the two PFPs were separated into different clusters. Various ways of correcting this very obvious discrepancy were tried but the two PFP remained in separate clusters. The reason for this occurrence is unclear but, as these two strains were run on different gels it may be due to temperature fluctuations during the running time, slight differences in DNA content of the plugs resulting in heavier or lighter bands or perhaps the normalisation step during the Bionumerics analysis where the gels are stretched or compressed to ensure that the

assigned bands of the reference lanes match their corresponding reference positions. Despite this, it is highly likely that these two strains are of clonal origin.

Of the serotypes with multiple examples, *S.* Enteritidis and *S.* Dublin showed least variation by PFGE using *Xba*I, with the seventy-eight strains of the former separated into just thirteen pulsed field patterns and six of the seven strains of *S.* Dublin resulting in identical profiles with the seventh only differing by a single band (**Figure 2:2**).

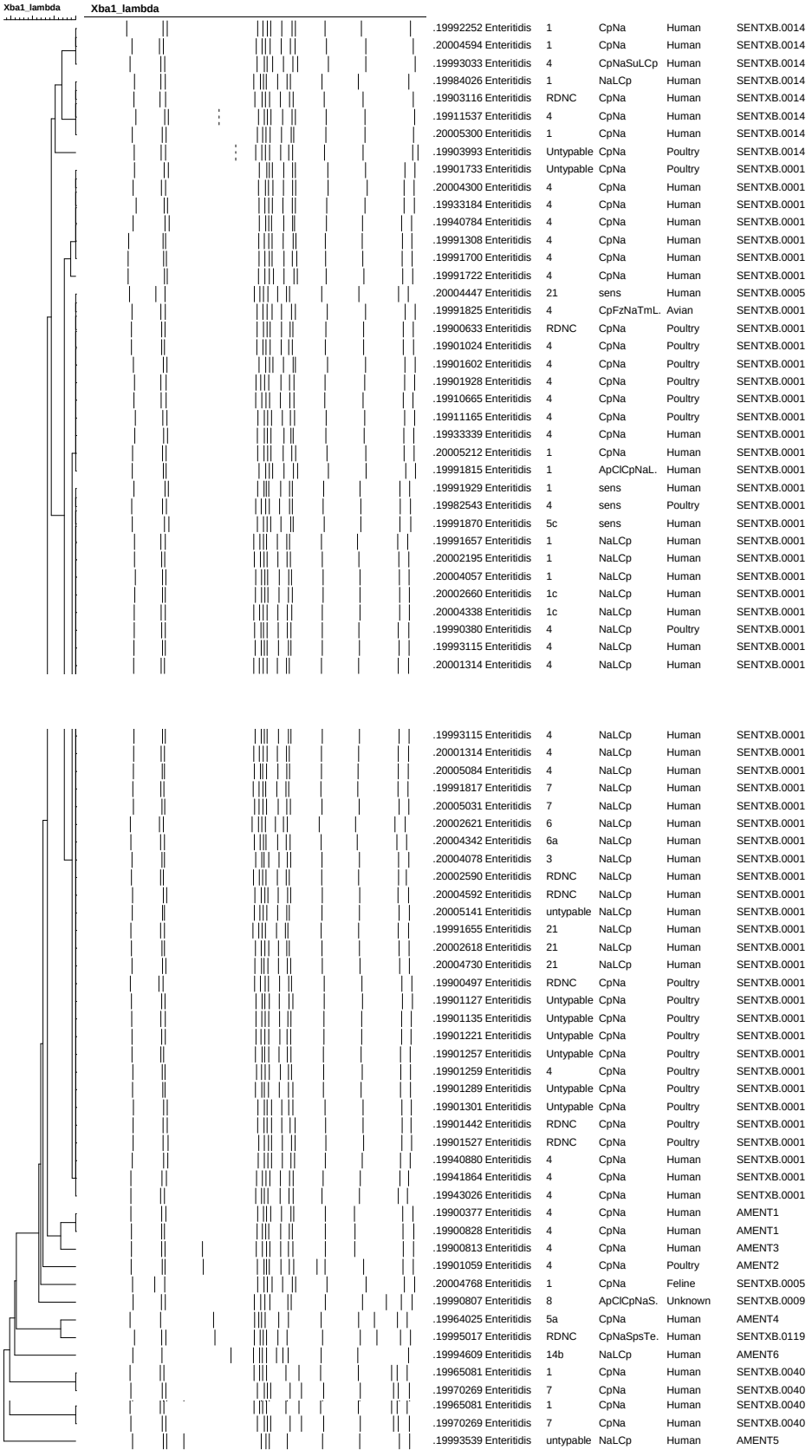
Twenty-four strains of *S.* Typhimurium resulted in fourteen different PFPs (**Figure 2:3)**. However, among these fourteen different PFPs, there are twelve different phage types, the predominant one being DT104, with 9 strains, all of which, when analysed, resulted in the most common Typhimurium PFP-STYMXB.0001.

The Dendrogram for the twenty-two strains of *S.* Mbandaka resulted in thirteen different PFPs the most common of which, was AMMBA3 (a PFP unique to this study) (**Figure 2:4**). Only one strain had an identical pattern in the PulseNet Europe database, while twelve of the fifteen strains of *S.* Hadar were identified as SHADXB.0001 (**Figure 2:5**).

Discrimination among seven strains of *S.* Virchow resulted in 4 different PFP with one, designated AMVIR1, demonstrating only 60% similarity to the other strains (**Figure 2:6**).

In the remaining six serotypes with multiple examples (**Figure 2:7**), only *S.* Liverpool demonstrated 100% identical PFPs and of those serotypes with only a single example, *S.* Java was the only one to have an identical PFP in the PulseNet database.

Figure 2:1 Dendrogram for *S.* Enteritidis



The PFPs of four strains of *S*. Enteritidis (19901754; 19935599; 19940871; 19992150) are not included in this dendrogram as their files were corrupted during analysis in the Bionumerics program.

Figure 2:2 Dendrogram for *S.* Dublin

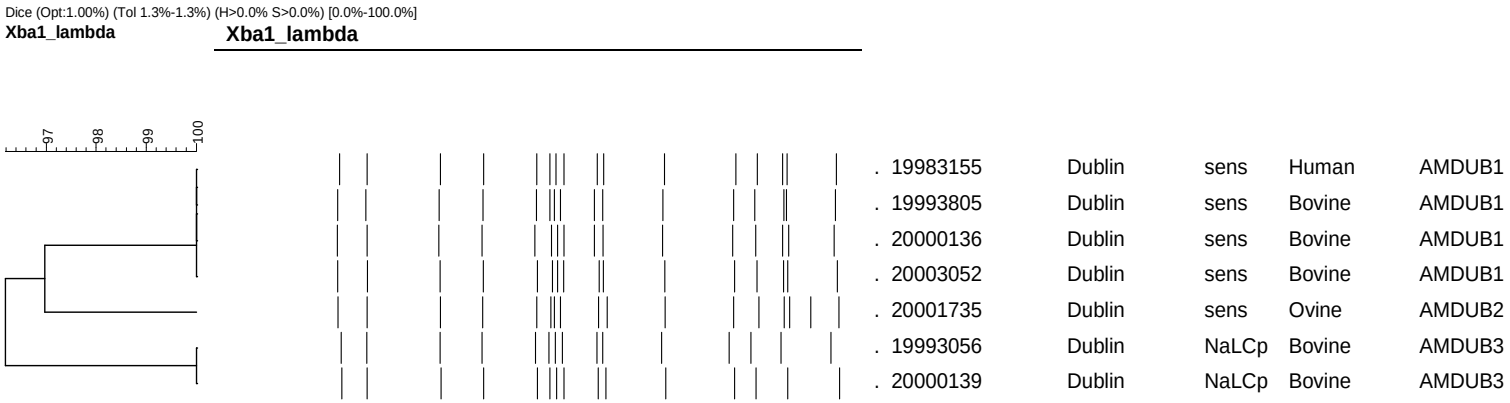


Figure 2:3 Dendrogram for *S.* Typhimurium

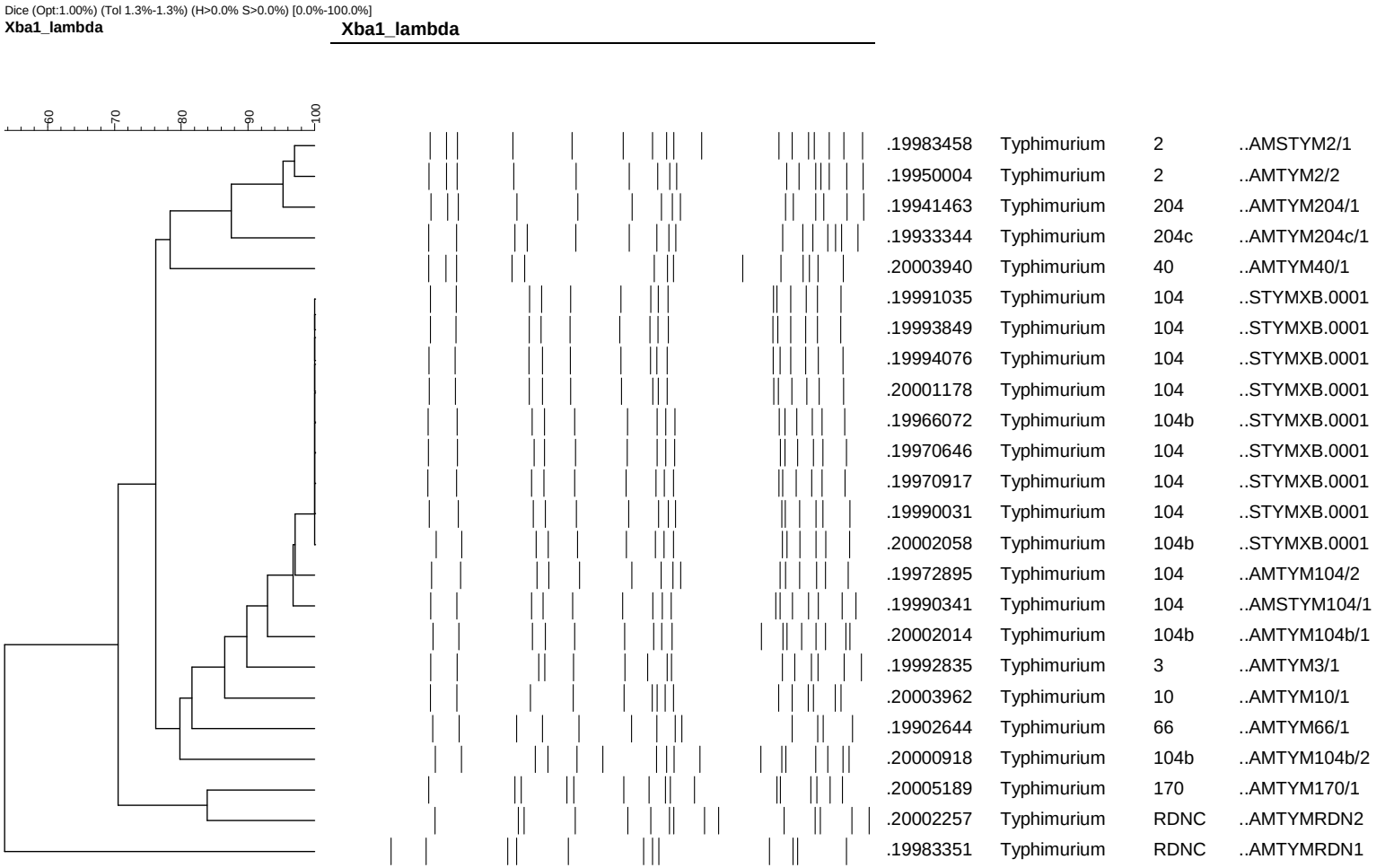


Figure 2:4 Dendrogram for *S.* Mbandaka

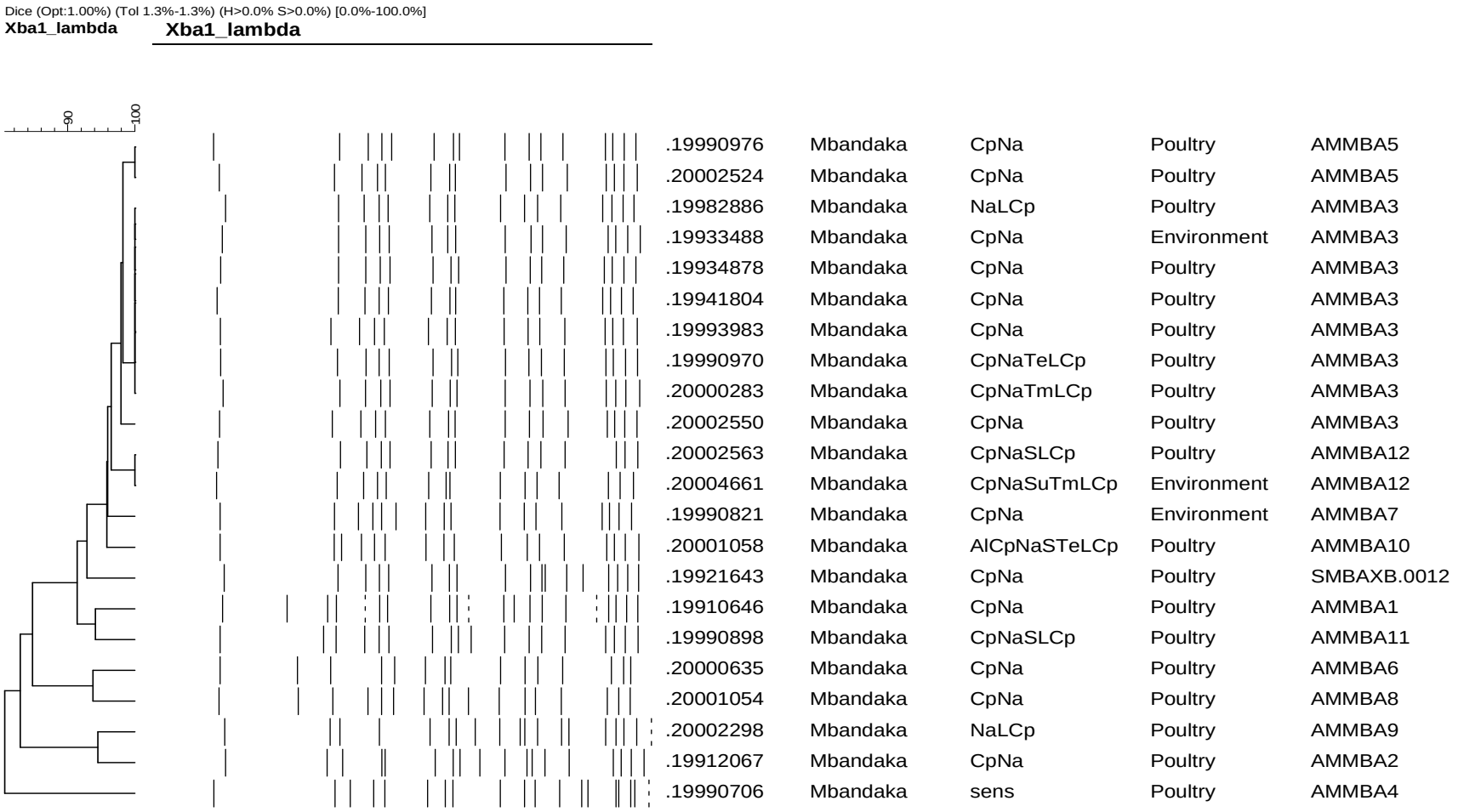


Figure 2:5 Dendrogram for *S*. Hadar

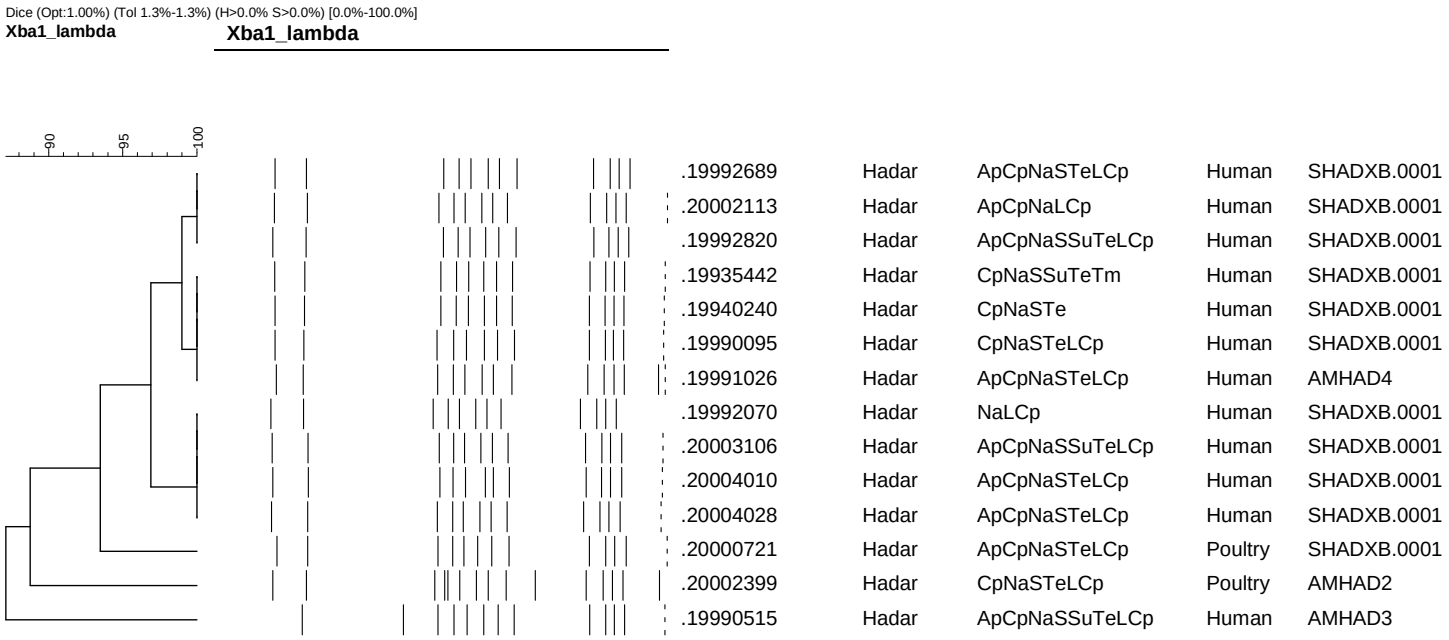


Figure 2:6 Dendrogram for *S.* Virchow

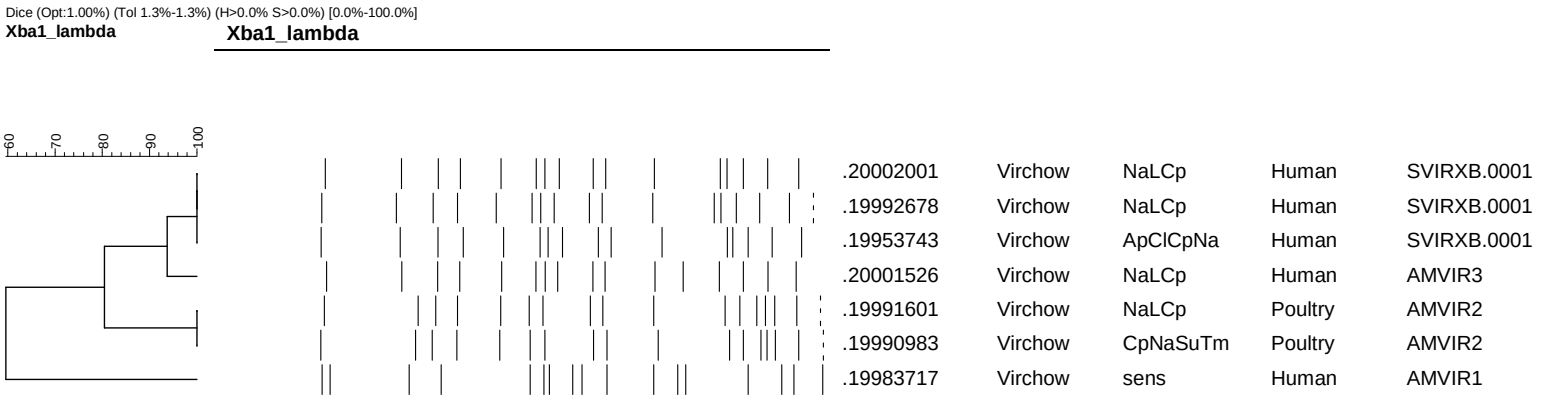
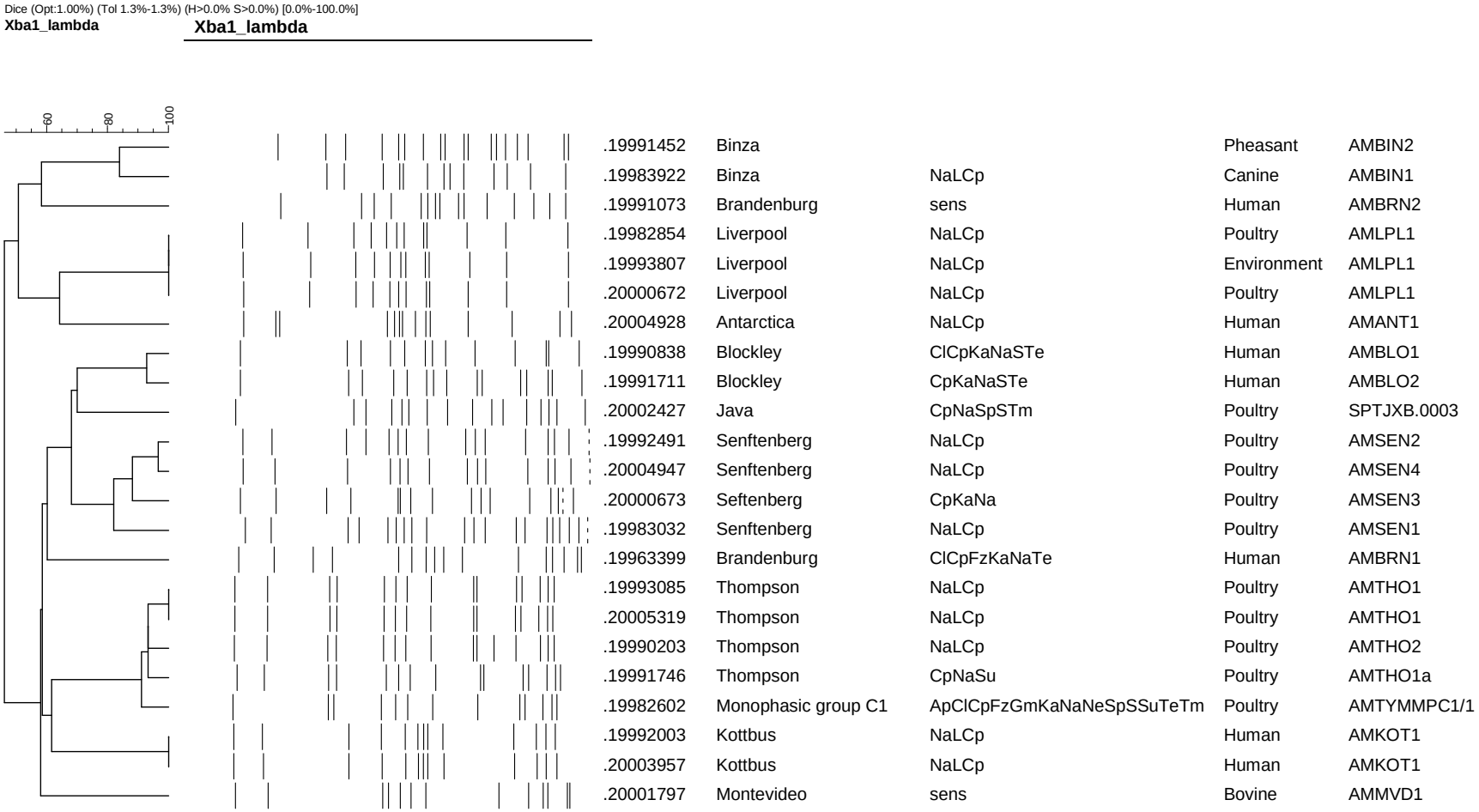


Figure 2:7 Dendrogram for multiple serotypes



2:3 Discussion

The 180 strains of *Salmonella* comprised 20 serotypes selected primarily for their phenotypic resistance types (R-types). The analyses performed in studies 2:2:2 (Plasmid profile analysis) and 2:2:3 (PFGE) provided more information.

2:3:1 Study 1 Plasmid profile analysis

In this study 320 plasmids were isolated from 160 strains of *Salmonella* (20 were plasmid-free). The plasmids varied widely in terms of number, ranging from strains harbouring single sized plasmids to strains harbouring 6 different sized plasmids, and size, from 2.0kb up to 160kb however, very few strains possessed a large plasmid and of the thirteen that did, eight were resistant to four or more antimicrobials.

High molecular-mass plasmids (that is, greater than 100kb) represent a considerable biosynthetic burden to the organism as they may constitute up to 5% of the total genome (Rychlik *et al*, 2006). These plasmids are potentially unstable in maintenance and replication functions. When present, it is most likely that they bestow a selective advantage through encoding beneficial phenotypes for the host, such as resistance to antimicrobials (R-plasmids) and heavy metals (Martinez *et al*, 2007; Ghosh *et al*, 2000).

A total of 78 of the strains studied harboured at least one low molecular-mass plasmid, there appeared to be little correlation between the individual strains. For example, a 2.1kb plasmid appeared to be the most frequently isolated small plasmid, being present in 38 of the eight-one strains (being the sole plasmid in six instances). Although low molecular-mass plasmids are more prolific and diverse than high molecular-mass plasmids, little is known about their biological function. One such plasmid has been shown to influence resistance to phage infection in isolates of *S*. Enteritidis whilst another, designated as pRF-

1, was found to confer resistance to sulphathiazole in isolates of *S.* Choleraesuis (Rychlik *et al*, 2001; Haneda *et al*, 2004). As this 2.1kb plasmid was present in seven different serotypes (and different phage types where applicable) with various R-types, there is no obvious phenotypic marker associated with it.

Whilst the plasmids of *Salmonella enterica* have the ability to transfer between organisms, the mere presence or absence of these extrachromosomal pieces of DNA can be of epidemiological importance. Although the results of plasmid profile analysis can yield plasmids of varying size and number, for those strains that are suspected of being part of an outbreak, a distinctive plasmid profile can provide an essential link. In one case, the absence of a plasmid linked an outbreak of *S.* Senftenberg in infants to the consumption of one brand of baby cereal (Rushdy, 1998).

The most intensively investigated group of plasmids are the serotype-associated plasmids (SAPs-also known as the virulence plasmids) of *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Choleraesuis, *S.* Gallinarum, *S.* Pullorum and *S.* Abortusovis the first three of which have been examined in this study.

The 90kb, 57kb and 80kb plasmids of *S.* Typhimurium, *S.* Enteritidis and *S.* Dublin respectively are very stable. A study examining a strain of *S.* Enteritidis demonstrated that there was no loss or genomic rearrangement of the 57kb plasmid during a 2.5 year period under different storage conditions (Olsen *et al*, 1994). Similarly, studies on a collection of enterobacteria from the pre-antimicrobial era, known as the “Murray collection”, demonstrated no differences to isolates from recent years (Jones & Stanley, 1992); findings reflected in this study with 86% of *S.* Enteritidis strains harbouring the 57kb SAP; 83% of *S.* Typhimuium harbouring the 90kb SAP and 71% of *S.*Dublin harbouring the 80kb SAP.

These plasmids, usually between 50-100kb, are important as they encode genes that contribute towards the virulence of these serotypes. For example, when a 22kb region of the 90kb virulence plasmid of *S.* Typhimurium was cloned into a mobilization vector, the resulting recombinant plasmid restored full virulence to a plasmidless strain in a mouse model (Norel *et al*, 1989). A 7.8kb region, *spv*, which is required for bacterial multiplication in the reticulo-endothelial system, is common to all the virulence plasmids (Rotger, 1999).The virulence plasmids of *S.* Enteritidis, *S.* Typhimurium and *S.* Dublin also contribute to the invasiveness of these strains. It has been demonstrated that only the SAP containing strains of these serotypes were capable of invading the livers of orally-infected mice. The virulence properties of other serotypes with no associated plasmid could not be correlated with their heterogeneous plasmid populations (Helmuth, 1985).

The ability of some isolates of *Salmonella enterica* to cause disease, survive in the presence of antimicrobials and heavy metals and resist infection by bacteriophages can all be attributed to the presence of certain plasmids. It is obvious that the acquisition of plasmids allow their host to survive in environments that would otherwise prove fatal and that the plasmids must continually evolve to meet this challenge, as demonstrated by the 140kb self-transferable plasmid of *S.* Typhimurium encoding both the characteristic *Salmonella* virulence plasmid genes and the genes coding for chloramphenicol and tetracycline resistance (Guerra *et al*, 2002).

2:3:2 Study 2 Pulsed Field Gel Electrophoresis

Nine strains of *S.* Enteritidis were unable to be subtyped by phage typing. PFGE in this instance, using *Xba*I, was only slightly better at differentiating between the strains, separating just 2 strains (SENTXB.0014 & AMENT7) from the others (SENTXB.0001). The difference between SENTXB.0001 and SENTXB.0014 was the loss of a single band of approximately 57kb, in the latter. This is, in fact, the virulence plasmid of *S.* Enteritidis which is remarkably stable (Olsen, 1994). This then implies that the single band difference between SENTXB.0001 and SENTXB.0014 is significant and had this been an outbreak situation, this difference may have been interpreted as indicating different strains. AMENT7 is also lacking a single band of approximately 57kb; however this strain has an additional smaller band compared to SENTXB.0014, resulting in a new PFP designation.

Discrimination between the remaining 69 strains of *S.* Enteritidis using only *Xba*I was not much better with only a further 10 PFP being differentiated. This was applicable to some of the other serotypes also. Twenty-four strains of *S.* Typhimurium resulted in 14 different PFPs however 9 strains had the commonest PFP STYMXB.0001. Of the fifteen strains of *S.* Hadar, twelve had an identical profile. This was also the situation in *S.* Dublin where, from a total of seven strains, six strains were indistinguishable by PFGE.

The results for *S.* Enteritidis and *S.* Typhimurium DT104 were somewhat expected due to the clonal nature of these strains (Davis *et al*, 2002), although, still an improvement on the other typing methods such as plasmid analysis and ribotyping (Ridley *et al*, 1998). The results for *S.* Hadar and *S.* Dublin were disappointing. Using a second and perhaps even a third restriction endonuclease in combination would increase the discriminatory power of this technique. This would especially be an appropriate measure for the previously

mentioned common pulsed field types of *S.* Enteritidis or *S.* Typhimurium such as SENTXB.0001 and STYMXB.0001 as it is imperative to be absolutely certain that the high incidence of these strains does not mask a genuine outbreak and for *S.* Dublin which has important economic implications in the farming industry.

Twenty-two strains of *S.* Mbandaka were divided into thirteen distinct profiles using *Xba*I. Fourteen strains were divided into twelve separate profiles. This is a good example of the discriminatory power of PFGE. As these Mbandaka strains originated mainly from poultry sources, the PFGE results provide reassurance that the incidence of Mbandaka is not due to clonal spread. Eight strains of *S.* Mbandaka had a unique PFP, AMMBA3 (**Figure 2:4**). These strains were isolated over a period of 7 years and all were of poultry origin with the exception of one environmental isolate which was taken from a poultry farm. All have similar R-types which, when added to the unique PFP suggests that these strains were of clonal origin.

The purpose of PFGE in this study was merely to fully characterize the strains and not to identify epidemiological relationships. It was therefore considered sufficient to employ only a single restriction endonuclease.

Chapter 3: Phenotypic determination of resistance

3:1 Introduction

Each isolate the Scottish Salmonella Reference Laboratory receives is routinely tested against a panel of 14 antimicrobials, including nalidixic acid and ciprofloxacin, by an in-house breakpoint method. This generates epidemiological and surveillance data which are reported to Health Protection Scotland. An agar dilution method was used to confirm these breakpoint designations and determine the Minimum Inhibitory Concentrations (MIC) for nalidixic acid, ciprofloxacin and 3 other fluoroquinolones, using first Diagnostic Sensitivity agar and then Mueller Hinton agar.

3:1:1: Study 1 Materials

Test organisms		detailed in section **2:2:1**	
CLED agar		as in section **2:2:2:1**	
Diagnostic sensitivity (DST) agar		Oxoid	CM0301
Mueller Hinton (MH) agar		Oxoid	CM0337
BHI broth		as in section **2:2:2:1**	
Sterile distilled water		In-house	
Antimicrobial powders	Nalidixic acid	Sigma	N-4382
	Ofloxacin	Sigma	O-8757
	Norfloxacin	Sigma	N-9890
	Ciprofloxacin	provided courtesy of Bayer	
	Moxifloxacin	provided courtesy of Bayer	

3:1:2 Method

MIC determination was performed using the technique described by Frost, (1994). Stock solutions of 1024mg/L were made for each of the five antimicrobials. Nalidixic acid, ciprofloxacin, norfloxacin and moxifloxacin were diluted with sterile distilled water to achieve the desired concentration. Ofloxacin required the addition of sodium bicarbonate before dilution with sterile distilled water. A series of two-fold dilutions was carried out on each of the stock solutions until a series of agar plates with final concentrations ranging from 512mg/L to 0.03mg/L were prepared for ciprofloxacin, norfloxacin, ofloxacin and moxifloxacin by adding an aliquot of each antimicrobial to sterilized Diagnostic Sensitivity Agar (DST) that had been cooled to 50ºC. For nalidixic acid the concentration of the agar plates ranged from 512mg/L to 8mg/L. Excess stock solution was stored at -85ºC.The plates were allowed to set at room temperature before being stored at 4ºC. All agar plates were used within one week to ensure stability of the antimicrobial.

A single colony from an overnight CLED plate was inoculated into 3mls of BHI broth and incubated on a shaker at 37ºC for two hours. 0.5ml of sterile saline was aseptically dispensed into a well of a Perspex block. 10µl of culture was then added to the well. The agar plates containing known concentrations of antimicrobial were placed in an incubator at 37ºC for 1 hour before use. They were then inoculated with the cultures from the Perspex block using the Lidwell multipoint inoculator and allowed to dry at room temperature. The plates were then stacked and incubated at 37ºC overnight. The next day the plates were removed from the incubator and the results recorded. The lowest concentration at which there was no visible growth on the plate was recorded as the MIC for that antimicrobial.

3:1:3 Results

MIC results are tabulated in **Table 3:1**. All but one of the 25 strains selected as "fully susceptible" (FS) by breakpoint method were fully susceptible to nalidixic acid. Twenty-four strains had an MIC of 8mg/L, the exception had an intermediate MIC of 16mg/L. Seventeen had MICs of 0.03mg/L-0.06mg/L of ciprofloxacin; the remaining 8 strains had reduced susceptibility at a concentration of 0.125mg/L. The strains showed varying degrees of susceptibility to the three remaining fluoroquinolones. The majority of MICs of ofloxacin ranged from 0.03-0.5mg/L, with the exception of a single strain of *S.* Dublin which was inhibited at a concentration of 2mg/L. The majority of strains demonstrated reduced susceptibility to norfloxacin with 19 strains having an MIC of 0.125mg/L and a further 4 with a MIC of 0.25mg/L. The aforementioned strain of *S.* Dublin was also resistant to norfloxacin, again at a concentration of 2mg/L. The MICs of moxifloxacin ranged from<0.06-0.25mg/L, with a single strain representing each extreme. The majority of strains had MICs of 0.03mg/L.

Of the 46 strains selected as resistant to nalidixic acid at 40mg/L and low-level ciprofloxacin at 0.125mg/L (NCpL), 44 were resistant to nalidixic acid at MICs of 256mg/L or above, with two strains showing reduced susceptibility at a concentration of 16mg/L. The same two strains were fully susceptible to ciprofloxacin with MICs of 0.06mg/L while the remaining 44 strains had MICs ranging from 0.25-2mg/L. The MICs of ofloxacin and norfloxacin ranged from 0.5-4mg/L and 0.25-4mg/L respectively, however in both cases, the predominant MIC was 2mg/L. The majority of the strains were resistant to moxifloxacin, with 40 being resistant at a concentration of 1mg/L. Two were resistant at 2mg/L, with only a single strain susceptible at 0.25mg/L.

Fifty-nine strains were selected as both resistant to nalidixic acid and to ciprofloxacin at a concentration of 0.5mg/L (FQR). All of the strains examined were resistant to nalidixic acid at a concentration of ≥512mg/L. They were also resistant to ciprofloxacin, with MICs ranging from 1-8mg/L. The strains were also cross-resistant to the three other fluoroquinolones, with MICs ranging from 2-32mg/L for both ofloxacin and norfloxacin, and between 1 and 8mg/L for moxifloxacin.

For the 50 strains selected as resistant to multiple antimicrobials (MDR), the most prevalent MIC of nalidixic acid was >512mg/L, with 41 strains being inhibited at this concentration. Six strains demonstrated an MIC of only 8mg/L. MICs of ciprofloxacin ranged from 0.06-32mg/L however, only 2 strains proved to be susceptible (<0.5mg/L) with the majority demonstrating MICs between 1-2mg/L. MICs of ofloxacin and norfloxacin ranged from 0.125-256mg/L, while the MICs of moxifloxacin were slightly lower at 0.125-32mg/L. The majority of these MDR strains were however, resistant to these three fluoroquinolones at concentrations of 8mg/L or 4mg/L respectively.

Table: 3.1: MIC results for strains of *S. enterica* to nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin and moxifloxacin using DST agar.

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19900377	Enteritidis	4	Human	FQR	>512	1	8	8	1
19900497	Enteritidis	RDNC	Poultry	FQR	>512	4	8	4	2
19900633	Enteritidis	RDNC	Poultry	FQR	>512	2	8	8	2
19900813	Enteritidis	4	Human	FQR	>512	1	4	4	1
19900828	Enteritidis	4	Human	FQR	>512	2	4	8	2
19901024	Enteritidis	4	Poultry	FQR	>512	1	8	8	1
19901059	Enteritidis	4	Poultry	FQR	>512	2	4	2	2
19901127	Enteritidis	Untypable	Poultry	FQR	>512	2	4	4	2

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19901135	Enteritidis	Untypable	Poultry	FQR	>512	2	4	4	2
19901221	Enteritidis	Untypable	Poultry	FQR	>512	2	4	4	2
19901257	Enteritidis	Untypable	Poultry	FQR	>512	2	8	4	2
19901259	Enteritidis	4	Poultry	FQR	>512	2	4	4	1
19901289	Enteritidis	Untypable	Poultry	FQR	>512	2	8	4	2
19901301	Enteritidis	Untypable	Poultry	FQR	>512	2	4	4	2
19901442	Enteritidis	RDNC	Poultry	FQR	>512	1	8	8	2
19901527	Enteritidis	RDNC	Poultry	FQR	>512	2	8	4	2
19901602	Enteritidis	4	Poultry	FQR	>512	1	8	8	1
19901733	Enteritidis	Untypable	Poultry	FQR	>512	2	4	4	2

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19901754	Enteritidis	Untypable	Poultry	FQR	>512	1	2	4	1
19901928	Enteritidis	4	Poultry	FQR	>512	1	2	4	2
19902644	Typhimurium	66	Human	FQR	>512	2	8	8	2
19903116	Enteritidis	RDNC	Human	FQR	>512	2	4	4	2
19903865	Bovismorbificans		Human	MDR	>512	4	16	4	8
19903993	Enteritidis	Untypable	Poultry	FQR	>512	1	8	8	1
19910646	Mbandaka		Poultry	FQR	>512	4	8	8	4
19910665	Enteritidis	4	Poultry	FQR	>512	2	4	8	4
19910748	Virchow		Not stated	MDR	>512	2	8	4	8
19911165	Enteritidis	4	Poultry	FQR	>512	2	4	4	2

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19911537	Enteritidis	4	Human	FQR	>512	1	8	8	1
19912067	Mbandaka		Poultry	FQR	>512	1	8	8	2
19921643	Mbandaka		Poultry	FQR	>512	2	8	8	4
19932001	Oranienburg	Human	Poultry	MDR	>512	8	4	2	4
19932300	Hadar		Poultry	MDR	8	32	16	16	4
19933184	Enteritidis	4	Human	FQR	>512	1	4	4	2
19933339	Enteritidis	4	Human	FQR	>512	1	8	4	1
19933344	Typhimurium	204c	Human	MDR	8	32	256	256	16
19933488	Mbandaka		Environment	FQR	>512	2	8	8	4
19934878	Mbandaka		Poultry environment	FQR	>512	2	8	16	2

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19935442	Hadar		Human	MDR	256	32	32	32	16
19935599	Enteritidis	4	Human	FQR	>512	2	4	4	2
19940240	Hadar		Human	MDR	256	8	32	32	32
19940784	Enteritidis	4	Human	FQR	>512	1	8	8	2
19940871	Enteritidis	4	Human	FQR	>512	2	8	8	2
19940880	Enteritidis	4	Human	FQR	>512	1	4	4	2
19941463	Typhimurium	204	Environment	MDR	>512	1	4	2	1
19941804	Mbandaka		Poultry	FQR	>512	2	4	4	2
19941864	Enteritidis	4	Human	FQR	512	2	32	16	2
19943026	Enteritidis	4	Human	FQR	512	1	8	4	2

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19950004	Typhimurium	2	Human	MDR	>512	2	4	4	4
19953743	Virchow		Human	MDR	>512	1	4	8	2
19963399	Brandenburg		Human	MDR	>512	2	8	8	4
19964025	Enteritidis	5c	Human	FQR	>512	4	8	8	8
19965081	Enteritidis	1	Human	FQR	>512	4	32	16	4
19966072	Typhimurium	104b	Bovine	MDR	>512	2	4	16	2
19970269	Enteritidis	7	Human	FQR	>512	8	32	32	8
19970646	Typhimurium	104	Human	MDR	>512	2	8	8	4
19970917	Typhimurium	104	Human	MDR	>512	2	8	8	4
19972895	Typhimurium	104	Environment	MDR	>512	4	8	8	4

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19982543	Enteritidis	4	Human	FS	8	0.125	0.25	0.25	0.25
19982602	Monophasic group C1		Poultry	MDR	8	0.06	4	0.25	0.125
19982854	Liverpool		Poultry	NaCpL	>512	1	4	2	1
19982886	Mbandaka		Poultry environment	NaCpL	>512	2	4	4	2
19983032	Senftenberg		Poultry	NaCpL	>512	0.5	4	4	1
19983155	Dublin		Human	FS	8	0.06	0.25	0.125	0.03
19983351	Typhimurium	RDNC	Canary	FS	8	0.06	0.25	0.125	0.03
19983458	Typhimurium	2	Human	FS	8	0.125	0.25	0.125	0.03
19983717	Virchow		Human	FS	8	0.03	0.125	0.125	0.125

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19983922	Binza		Canine	NaCpL	>512	2	2	2	1
19984026	Enteritidis	1	Human	NaCpL	>512	0.5	2	2	1
19990031	Typhimurium	104	Human	MDR	>512	4	4	0.125	4
19990095	Hadar		Human	MDR	>512	4	8	8	4
19990203	Thompson		Poultry	NaCpL	>512	1	4	2	1
19990341	Typhimurium	104	Human	FS	8	0.125	0.25	0.125	0.06
19990380	Enteritidis	4	Poultry	NaCpL	512	0.5	2	2	1
19990515	Hadar		Human	MDR	>512	2	4	4	8
19990706	Mbandaka		Poultry environment	FS	8	0.03	0.06	0.06	0.06
19990807	Enteritidis	8	Not stated	MDR	>512	2	4	8	4

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19990838	Blockley		Human	MDR	>512	2	4	2	2
19990898	Mbandaka		Poultry	MDR	>512	2	8	8	4
19990970	Mbandaka		Poultry	MDR	>512	2	8	8	2
19990976	Mbandaka		Poultry	FQR	>512	1	16	16	2
19990983	Virchow		Poultry	MDR	>512	0.5	2	2	1
19991026	Hadar		Human	MDR	>512	1	4	4	4
19991035	Typhimurium	104	Porpoise	FS	8	0.06	0.25	0.125	0.03
19991073	Brandenburg		Human	FS	8	0.06	0.125	0.125	0.125
19991308	Enteritidis	4	Human	FQR	>512	1	4	4	1
19991452	Binza		Pheasant	FS	8	0.03	0.125	0.125	0.125

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19991601	Virchow		Poultry	NaCpL	>512	0.5	2	4	1
19991655	Enteritidis	21	Human	NaCpL	>512	0.5	0.5	1	1
19991657	Enteritidis	1	Human	NaCpL	>512	0.5	2	2	1
19991700	Enteritidis	4	Human	FQR	>512	1	4	2	1
19991711	Blockley		Human	MDR	>512	1	4	4	1
19991722	Enteritidis	4	Human	FQR	>512	1	4	4	1
19991746	Thompson		Poultry	MDR	>512	1	8	2	1
19991815	Enteritidis	1	Human	MDR	>512	2	8	16	4
19991817	Enteritidis	7	Human	NaCpL	>512	0.5	2	2	1
19991825	Enteritidis	4	Pheasant	MDR	8	0.25	8	4	0.125

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19991870	Enteritidis	5c	Human	FS	8	0.125	0.25	0.125	0.03
19991929	Enteritidis	1	Human	FS	8	0.125	0.25	0.125	0.03
19992003	Kottbus		Human	NaCpL	256	0.25	2	2	1
19992070	Hadar		Human	NaCpL	>512	1	4	2	2
19992150	Enteritidis	6a	Human	FS	8	0.125	0.5	0.25	0.03
19992252	Enteritidis	1	Human	FQR	>512	2	4	4	2
19992491	Senftenberg		Poultry environment	NaCpL	256	0.5	4	2	1
19992678	Virchow		Human	NaCpL	>512	1	4	2	1
19992689	Hadar		Human	MDR	>512	2	8	16	8
19992820	Hadar		Human	MDR	>512	2	8	4	4

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19992835	Typhimurium	3	Poultry	NaCpL	16	0.06	2	2	0.25
19993033	Enteritidis	4	Human	MDR	>512	1	4	2	2
19993048	Indiana		Human	MDR	>512	32	64	128	16
19993056	Dublin		Bovine	NaCpL	>512	0.5	2	2	1
19993085	Thompson		Poultry environment	NaCpL	>512	1	4	2	1
19993115	Enteritidis	4	Human	NaCpL	>512	0.5	2	2	1
19993539	Enteritidis	untypable	Human	NaCpL	>512	0.5	1	2	1
19993805	Dublin		Bovine	FS	8	0.06	2	2	0.125
19993807	Liverpool		Environment	NaCpL	>512	0.5	2	2	1
19993849	Typhimurium	104	Human	FS	8	0.06	0.25	0.125	0.03

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19993983	Mbandaka		Poultry environment	FQR	>512	2	8	4	2
19994076	Typhimurium	104	Human	FS	8	0.06	0.25	0.125	0.03
19994609	Enteritidis	14b	Human	NaCpL	>512	0.5	2	2	1
19995017	Enteritidis	RDNC	Human	MDR	>512	4	16	8	8
20000136	Dublin		Bovine	FS	8	0.06	0.125	0.125	0.125
20000139	Dublin		Bovine	NaCpL	512	0.25	2	2	1
20000283	Mbandaka		Poultry environment	MDR	>512	2	4	2	2
20000635	Mbandaka		Poultry environment	FQR	>512	2	8	8	2
20000672	Liverpool		Poultry environment	NaCpL	512	0.25	2	2	1

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20000673	Senftenberg		Poultry environment	MDR	8	0.125	0.5	0.125	1
20000721	Hadar		Poultry	MDR	>512	2	8	8	4
20000821	Mbandaka		Environment	FQR	>512	2	8	8	2
20000918	Typhimurium	104b	Human	MDR	>512	2	8	8	4
20001054	Mbandaka		Poultry environment	FQR	>512	2	2	8	2
20001058	Mbandaka		Poultry environment	MDR	>512	1	8	4	4
20001178	Typhimurium	104	Poultry	FS	16	0.125	0.25	0.25	0.125
20001314	Enteritidis	4	Human	NaCpL	>512	0.5	2	2	1
20001526	Virchow		Human	NaCpL	>512	0.5	0.5	2	1
20001735	Dublin		Ovine	FS	8	0.06	0.25	0.125	0.125

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20001797	Montevideo		Bovine	FS	8	0.03	0.125	0.125	0.125
20002001	Virchow		Human	NaCpL	>512	1	4	4	1
20002014	Typhimurium	104b	Human	MDR	>512	2	8	8	4
20002058	Typhimurium	104b	Human	MDR	8	0.06	0.25	4	1
20002113	Hadar		Human	MDR	>512	1	8	4	4
20002195	Enteritidis	1	Human	NaCpL	>512	1	2	2	1
20002257	Typhimurium	RDNC	Human	MDR	>512	1	2	2	1
20002298	Mbandaka		Poultry environment	NaCpL	512	1	1	2	2
20002399	Hadar		Poultry	MDR	>512	1	4	8	2
20002427	Java		Poultry	MDR	>512	1	4	4	1

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20002524	Mbandaka		Poultry environment	FQR	>512	2	8	4	2
20002550	Mbandaka		Poultry environment	FQR	>512	2	4	4	2
20002563	Mbandaka		Environment	MDR	>512	2	8	8	2
20002590	Enteritidis	RDNC	Human	NaCpL	>512	0.5	1	2	1
20002618	Enteritidis	21	Human	NaCpL	>512	0.5	2	4	1
20002621	Enteritidis	6	Human	NaCpL	16	0.06	0.5	0.25	0.25
20002660	Enteritidis	1c	Human	NaCpL	>512	0.5	2	2	1
20003052	Dublin		Bovine	FS	8	0.06	0.125	0.125	0.06
20003106	Hadar		Human	MDR	>512	2	8	8	8
20003940	Typhimurium	40	Human	FS	8	0.06	0.25	0.25	0.03

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20003957	Kottbus		Human	NaCpL	256	0.25	2	1	1
20003962	Typhimurium	10	Human	FS	8	0.06	0.5	0.125	0.06
20004010	Hadar		Human	MDR	>512	1	8	8	4
20004028	Hadar		Human	MDR	>512	2	8	8	4
20004057	Enteritidis	1	Human	NaCpL	>512	0.5	2	2	1
20004078	Enteritidis	3	Human	NaCpL	>512	0.5	1	2	1
20004300	Enteritidis	4	Human	FQR	>512	2	8	8	4
20004338	Enteritidis	1c	Human	NaCpL	>512	0.5	2	2	1
20004342	Enteritidis	6a	Human	NaCpL	>512	1	2	4	1
20004447	Enteritidis	21	Human	FS	8	0.125	0.25	0.125	<0.03

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20004592	Enteritidis	RDNC	Human	NaCpL	>512	0.5	1	2	1
20004594	Enteritidis	1	Human	FQR	>512	1	4	4	2
20004661	Mbandaka		Poultry environment	MDR	>512	1	8	4	2
20004730	Enteritidis	21	Human	NaCpL	>512	0.5	2	2	1
20004768	Enteritidis	1	Feline	FQR	>512	2	8	4	4
20004928	Antarctica		Human	NaCpL	>512	0.5	2	2	1
20004947	Senftenberg		Poultry	NaCpL	>512	0.5	1	2	1
20005031	Enteritidis	7	Human	NaCpL	>512	0.5	2	2	1
20005084	Enteritidis	4	Human	NaCpL	>512	0.5	2	2	1
20005141	Enteritidis	untypable	Human	NaCpL	>512	0.5	1	2	1

SSRL Reference	Serotype	Phage type	Source	Breakpoint designation	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
20005189	Typhimurium	170	Porcine	FS	8	0.06	0.5	0.125	0.03
20005212	Enteritidis	1	Human	FQR	nd	2	4	4	2
20005300	Enteritidis	1	Human	FQR	nd	2	2	4	2
20005319	Thompson		Poultry environment	NaCpL	>512	1	4	4	1

3:2:1: Study 2 Materials & Method

This experiment was performed using the materials and method previously described. Mueller Hinton agar (MH) was used instead of DST as the latter was found to give inaccurate MIC results (discussed in section **3:3:1**).

3:2:2 Results

The MIC results are listed in **Table 3:2**. All FS strains tested were fully susceptible to nalidixic acid with MICs of 8mg/L. MICs of ciprofloxacin and moxifloxacin were 0.03 or 0.06mg/L with those of ofloxacin and norfloxacin measuring slightly higher at 0.125 or 0.25mg/L for most strains.

For the NCpL strains, the majority of the strains examined were highly resistant to nalidixic acid at a concentration of 128mg/L or above. A single strain of *S*. Typhimurium DT 3 had an MIC of 16mg/L. Most of these strains demonstrated reduced susceptibility to ciprofloxacin at concentrations of 0.125-0.25mg/L; 6 strains were susceptible with MICs of 0.06mg/L. For the remaining three fluoroquinolones, the MICs ranged from 0.125-0.5mg/L. An MIC of 1mg/L was recorded for a single strain for ofloxacin and moxifloxacin however 8 strains had an MIC of 1mg/L for norfloxacin.

All FQR strains selected for this experiment were highly resistant to nalidixic acid with MICs of 256mg/L or above. The MICs of ciprofloxacin ranged from 0.125-0.5mg/L, the majority of strains having MICs of 0.25mg/L. For ofloxacin, the MICs were either 0.5 or 1mg/L, with MICs of norfloxacin measuring higher at 1 or 2mg/L. MICs of moxifloxacin were similar to those of ciprofloxacin with the exception of 5 strains whose MICs were 1-2mg/L.

The MDR strains were all resistant to nalidixic acid with MICs of 512mg/L and demonstrated reduced susceptibility to ciprofloxacin with MICs of 0.125 or 0.25mg/L. A single strain had an MIC of 0.5mg/L. MICs of ofloxacin and norfloxacin ranged from 0.125-2mg/L. The MICs of moxifloxacin were considerably higher, ranging from 0.5-4mg/L.

Table 3.2: A Comparison of the MICs of nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin and moxifloxacin for 55 strains of *S. enterica* using Diagnostic Sensitivity agar and Mueller Hinton agar.

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
19900497	Enteritidis	RDNC	FQR	>512	>512	4	0.25	8	1	8	2	2	0.5
19900633	Enteritidis	RDNC	FQR	>512	>512	2	0.25	8	1	8	1	2	0.5
19900828	Enteritidis	4	FQR	>512	512	2	0.25	4	0.5	8	1	2	0.5
19901024	Enteritidis	4	FQR	>512	512	1	0.25	8	0.5	8	1	1	0.5
19901059	Enteritidis	4	FQR	>512	256	2	0.125	4	0.5	2	1	2	0.5
19901127	Enteritidis	Untypable	FQR	>512	512	2	0.25	4	1	4	1	2	0.5
19901135	Enteritidis	Untypable	FQR	>512	512	2	0.25	4	0.5	4	1	2	0.5
19901259	Enteritidis	4	FQR	>512	512	2	0.125	4	0.5	4	1	1	0.5
19901289	Enteritidis	Untypable	FQR	>512	512	2	0.25	8	1	4	1	2	0.5

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
19901442	Enteritidis	RDNC	FQR	>512	512	1	0.25	8	1	8	1	2	0.5
19901527	Enteritidis	RDNC	FQR	>512	512	2	0.25	8	1	4	1	2	0.5
19901602	Enteritidis	4	FQR	>512	512	1	0.25	8	1	8	1	1	0.5
19901928	Enteritidis	4	FQR	>512	512	1	0.25	2	0.5	4	1	2	0.5
19902644	Typhimurium	66	FQR	>512	512	2	0.5	8	1	8	2	4	2
19903116	Enteritidis	RDNC	FQR	>512	512	2	0.125	4	0.5	4	2	2	0.5
19903993	Enteritidis	Untypable	FQR	>512	512	1	0.125	8	0.5	8	1	1	0.5
19933339	Enteritidis	4	FQR	>512	512	1	0.25	8	0.5	4	1	1	0.5
19933488	Mbandaka		FQR	>512	512	2	0.5	8	1	8	2	4	1
19940784	Enteritidis	4	FQR	>512	512	1	0.25	8	0.5	8	1	2	0.5
19941463	Typhimurium	204	MDR	>512	512	1	0.25	4	0.5	2	1	1	0.5

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
19940871	Enteritidis	4	FQR	>512	512	2	0.25	8	1	8	1	2	0.5
19941804	Mbandaka		FQR	>512	512	2	0.25	4	1	4	1	2	1
19941864	Enteritidis	4	FQR	512	512	2	0.25	32	1	16	1	2	0.5
19943026	Enteritidis	4	FQR	512	512	1	0.25	8	0.5	4	1	1	0.5
19964025	Enteritidis	5c	FQR	>512	512	4	0.125	8	0.5	8	1	8	0.5
19982543	Enteritidis	4	FS	8	8	0.125	0.03	0.25	0.25	0.25	0.25	0.25	0.06
19982886	Mbandaka		NaCpL	>512	512	2	0.25	4	1	4	1	2	0.5
19983032	Senftenberg		NaCpL	>512	128	0.5	0.125	4	0.5	4	1	1	0.25
19984026	Enteritidis	1	NaCpL	>512	512	0.5	0.125	2	0.25	2	1	1	0.25
19990341	Typhimurium	104	FS	8	8	0.125	0.03	0.25	0.125	0.25	0.125	0.06	0.06
19990515	Hadar		MDR	>512	512	2	0.5	4	2	4	2	8	4

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
19990807	Enteritidis	8	MDR	>512	512	2	0.5	4	2	8	2	4	4
19990976	Mbandaka		FQR	>512	512	1	0.25	16	1	16	2	2	1
19991655	Enteritidis	21	NaCpL	>512	512	0.5	0.125	0.5	0.25	1	0.125	1	0.25
19992003	Kottbus		NaCpL	256	128	0.25	0.125	2	0.25	2	1	1	0.25
19992070	Hadar		NaCpL	>512	512	1	0.25	4	0.5	4	1	2	1
19992835	Typhimurium	3	NaCpL	16	16	0.5	0.06	2	0.125	2	0.25	0.25	0.125
19993033	Enteritidis	4	MDR	>512	512	1	0.5	4	1	4	2	2	1
19993056	Dublin		NaCpL	>512	128	0.5	0.125	2	0.25	2	1	1	0.25
19993085	Thompson		NaCpL	>512	512	1	0.25	4	0.5	2	1	1	0.5
19993115	Enteritidis	4	NaCpL	>512	256	0.5	0.125	2	0.25	2	1	1	0.25
19993983	Mbandaka		FQR	>512	512	2	0.25	8	1	4	1	2	1

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
19994609	Enteritidis	14b	NaCpL	>512	512	0.5	0.125	2	0.5	2	0.5	1	0.5
20000283	Mbandaka		MDR	>512	512	2	0.125	4	0.125	2	0.25	2	0.5
20001178	Typhimurium	104	FS	8	8	0.25	0.03	0.5	0.06	0.5	0.125	0.25	0.06
20001314	Enteritidis	4	NaCpL	>512	256	0.5	0.125	2	0.5	2	0.5	1	0.25
20001526	Virchow		NaCpL	>512	256	0.5	0.125	0.5	0.25	2	0.5	1	0.25
20002195	Enteritidis	1	NaCpL	>512	512	1	0.125	2	0.25	2	0.5	1	0.25
20002660	Enteritidis	1c	NaCpL	>512	>512	0.5	0.125	2	0.25	2	0.5	1	0.25
20003940	Typhimurium	40	FS	8	8	0.06	0.03	0.25	0.125	0.25	0.25	0.06	0.03
20004338	Enteritidis	1c	NaCpL	>512	512	0.5	0.125	2	0.5	2	0.5	1	0.25
20004592	Enteritidis	RDNC	NaCpL	>512	512	0.5	0.125	1	0.5	2	0.5	1	0.25
20004928	Antarctica		NaCpL	>512	512	0.5	0.125	2	0.25	2	0.5	1	0.25

SSRL Reference	Serotype	Phage type	Breakpoint Designation	DST Nal	MH Nal	DST Cp	MH Cp	DST Oflox	MH Oflox	DST Norflox	MH Norflox	DST Moxi	MH Moxi
20005084	Enteritidis	4	NaCpL	>512	512	0.5	0.125	1	0.5	2	0.5	1	0.25
20005141	Enteritidis	Untypable	NaCpL	>512	512	1	0.125	1	0.25	2	0.5	1	0.25

Table abbreviations: DST, Diagnostic sensitivity agar; MH, Mueller Hinton agar; Nal, nalidixic acid; Cp, ciprofloxacin; Oflox, ofloxacin; Norflox, norfloxacin; Moxi, moxifloxacin

3:3 Discussion

3:3:1 Agar dilution method using DST

The SSRL employs a breakpoint method for sensitivity testing as it is a very efficient method of screening large numbers of isolates with minimal time and effort. The data generated are used for epidemiological surveillance purposes and therefore it is perfectly sufficient to know whether an isolate is sensitive, resistant or intermediate. Breakpoints are determined by organisations such as the British Society for Antimicrobial Chemotherapy (BSAC) or CLSI (Clinical Laboratory Standards Institute). Recently national susceptibility testing committees from Britain, France, The Netherlands, Norway, Sweden and Germany have collaborated on a project to harmonise antimicrobial breakpoints including previously established MIC values that differed between countries. The result is EUCAST (European Committee on Antimicrobial Susceptibility Testing) which has produced a list of MIC breakpoints for antimicrobials for which there has been international agreement. It is the breakpoints produced by EUCAST that have been used for the interpretation of the results of this experiment; resistant at ≥1mg/L for ciprofloxacin, ofloxacin, norfloxacin and moxifloxacin.

For the strains selected from the SSRL database as “fully-susceptible”, the agar dilution experiment confirmed the breakpoint results for nalidixic acid, as all of the strains were fully susceptible at 40mg/L. The current recommended breakpoint for nalidixic acid is 32mg/L; some strains in this study date back to 1990 when the SSRL employed a breakpoint of 40mg/L, therefore this value was chosen for the sake of consistency throughout the experiment. The MIC results for ciprofloxacin confirmed the susceptibility of the majority of the strains however 8 strains that were susceptible by breakpoint had reduced sensitivity to ciprofloxacin at a concentration of 0.125mg/L. With the exception of

a single strain of *S*. Dublin which was resistant to ofloxacin and norfloxacin at a concentration of 2mg/L, all the strains were susceptible to ofloxacin, norfloxacin and moxifloxacin at the breakpoint of 0.5mg/L. With the exception of the 8 strains with reduced susceptibility to ciprofloxacin, the breakpoint data for the 25 “fully susceptible” strains were confirmed by this experiment.

Two strains selected as resistant to nalidixic acid and low-level ciprofloxacin by breakpoint had reduced susceptibility to nalidixic acid with MICs of 16mg/L. One was also susceptible to all four fluoroquinolones, while the other was additionally susceptible to ciprofloxacin and moxifloxacin only. The remaining forty-four strains were as expected, resistant to nalidixic acid at a concentration, for the majority of these strains, of >512mg/L. The MICs of ciprofloxacin were slightly higher than anticipated. As they had been selected on the basis of reduced susceptibility, MICs of 0.125mg/L were expected. Instead, 38 of the 46 strains had MICs of ≥0.5mg/L and 37 of these strains were resistant to ofloxacin at ≥2mg/L while 43 were resistant to norfloxacin at ≥2mg/L.

The MICs for the strains selected as resistant to both nalidixic acid and ciprofloxacin (0.5mg/L) confirmed that all 59 strains were resistant to both antimicrobials and that they also exhibited cross-resistance to ofloxacin, norfloxacin and moxifloxacin.

For the last group of strains, selected on the basis of their multi-drug resistance, 44 of the 50 strains were resistant to nalidixic acid at concentrations of 256- >512mg/L; 6 were found to be fully susceptible with MICs of 8mg/L. Details of these 6 can be found in **Table 3:3**.

The strain of *S*. Typhimurium DT204c was resistant to an additional 8 antimicrobials and had very high MICs for three of the four fluoroquinolones, while a strain of monophasic

group C1 *Salmonella*, resistant to 13 of the 14 antimicrobials tested was only additionally resistant to ofloxacin with an MIC of 4mg/L. Four of these 6 strains are fully susceptible to ciprofloxacin. On the basis of their MIC for nalidixic acid it could be assumed that these six strains do not have mutations in their *gyrA* genes as usually even a single point mutation is sufficient to confer reduced susceptibility to quinolones (Levy *et al*, 2004). This does not explain the high MIC to ciprofloxacin demonstrated in two of the strains. MICs of this level are generally thought to require the presence of gyrase mutations and not be solely the result of increased efflux pump activity (Chen *et al*, 2007). The MICs for ofloxacin and norfloxacin were extensive and ranged from 0.25-256mg/L and 0.125-256mg/L respectively. The MICs for moxifloxacin were slightly lower ranging from 0.125-32mg/L. The "higher-than-expected" ciprofloxacin MIC results for the NCpL strains coupled with the very high MICs for ofloxacin and norfloxacin in the MDR strains highlighted some discrepancies in this experiment. Why were these MIC results so different from the breakpoint results? In case of any experimental error, the experiment was repeated but the same results were obtained. After consulting a member of the BSAC committee (Dr. Jennifer Andrews), it was concluded that Diagnostic sensitivity agar may not be suitable for use with fluoroquinolones as the high concentrations of magnesium and calcium ions may have an inhibitory effect on the fluoroquinolones causing raised MICs. In light of this information, a random selection of 55 strains from the collection was investigated for MIC on Mueller Hinton agar.

3:3:2 Study2 Agar dilution method using MH

Of the 55 strains studied, 26 were from the group resistant to nalidixic acid and ciprofloxacin at a concentration of 0.5mg/L, FQR; 20 were from the nalidixic acid and low-level ciprofloxacin (0.125mg/L) group NCpL; 5 were from the multi-resistant group MDR and 4 were from the “fully susceptible” group FS.

Once again the FS strains investigated were shown to be sensitive to all five antimicrobials. The strain of *S.* Typhimurium DT 104, with the intermediate MIC of 16mg/L for nalidixic acid using DST agar, proved to have the same MIC of 8mg/L as the other FS strains using Mueller Hinton agar.

Nineteen of the 20 NCpL strains were confirmed as resistant to nalidixic acid and had at least reduced susceptibility to the four fluoroquinolones. A single strain of *S.* Typhimurium DT 3 was susceptible to nalidixic acid and ciprofloxacin but had reduced susceptibility to ofloxacin, norfloxacin and moxifloxacin. This suggests that the results of the breakpoint testing were wrong and that this strain, since it is susceptible to all the antimicrobials at EUCAST breakpoints, should be classed as “fully susceptible”.

All 26 (FQR) strains exhibited resistance to nalidixic acid, ofloxacin, norfloxacin and moxifloxacin. Only one strain was cross-resistant to ciprofloxacin with an MIC of 0.5mg/L; the other 25 classed as having reduced susceptibility with MICs of 0.125-0.25mg/L. Although the majority of these strains are classed as resistant by EUCAST standards, the highest MIC recorded was 2mg/L in only five strains; not nearly as high as those recorded when using DST agar. This is a good indication of the inhibitory effect of the DST agar when fluoroquinolone antimicrobials are incorporated for the purposes of MIC determination.

On the basis of these findings, the Scottish Salmonella Reference Laboratory conducted a study into the agar used in the breakpoint method of susceptibility testing. The results indicated that Mueller Hinton and Isosensitest agar gave indistinguishable results therefore the agar used in the routine susceptibility testing by breakpoint method was changed from Diagnostic Sensitivity agar to Isosensitest agar as recommended by the BSAC organisation.

The MDR strains investigated were all resistant to nalidixic acid with MICs of 512mg/L and, although the majority of the strains were categorised as resistant to the fluoroquinolones (with the exception of ciprofloxacin which exhibited reduced susceptibility), they were not highly resistant. These results mirror those of a previous study which found that a single point mutation in *gyrA* resulted in resistance to nalidixic acid (MIC ≥128mg/L) and reduced susceptibility in ciprofloxacin (MIC between 0.125 and 0.25mg/L). It was also discovered that the parental clone of the strains of *S.* Virchow under investigation overproduced the AcrAB efflux pump which, in this study would account for the multiple resistances to the non-fluoroquinolone antimicrobials (Solnik-Isaac *et al*, 2007).

Table 3:3: Additional resistances of the six nalidixic acid-susceptible multi-drug resistant strains of *Salmonella*.

SSRL Reference	Serotype	Phage type	Additional resistances[a]	Nalidixic acid	Ciprofloxacin	Ofloxacin	Norfloxacin	Moxifloxacin
19932300	Hadar		St;Te	8	32	16	16	4
19933344	Typhimurium	204c	Ap;Cl;Ka;Sp;St;Sx;Te;Tm	8	32	256	256	16
19982602	Monophasic group C1		Ap;Cl;Cp;Fz;Gm;Ka;Ne;Sp;St;Sx;Te;Tm	8	0.06	4	0.25	0.125
19991825	Enteritidis	4	Fz,Tm	8	0.25	8	4	0.125
20000673	Senftenberg		Ka;Te	8	0.125	0.5	0.125	1
20002058	Typhimurium	104b	Ap;St;Sx;Tm	8	0.06	0.25	4	1

[a] Antimicrobials: Ap, ampicillin; Cl, chloramphenicol; Cp, ciprofloxacin; Fz, furazolidone; Gm, gentamicin; Na, nalidixic acid; Ne, netilmycin; Sp, spectinomycin; St, streptomycin; Sx, sulphamethoxazole; Te, tetracycline; Tm, trimethoprim.

Chapter 4: Selection and study of Spontaneous Mutants I

4:1 Introduction

With the incidence of treatment failures with fluoroquinolones increasing, the occurrence of *Salmonella* with reduced susceptibility to these drugs has become a real concern (Hakanen *et al*, 2001; Mehta *et al*, 2001). The Luria-Delbruck dogma states that resistant mutants occur spontaneously before the exposure of bacteria to the antimicrobial. If this is true, then *Salmonella* deemed susceptible to antimicrobials may potentially harbour a subpopulation of mutants with reduced susceptibility.

In *Staphylococcus aureus*, the presence in some strains, of heterogeneous resistance to vancomycin has been noted. Hetero-Van-resistant-*Staphylococcus aureus* (hVRSA) strains are defined as those strains that contain non-susceptible subpopulations at a frequency of 10^{-6} or higher **(**Jung, 2002**)**. It is possible that these hVRSA strains are precursors of vancomycin-intermediate-susceptible *Staphylococcus* aureus strains, and therefore the early detection of these strains is imperative. Similarly, the early detection of non-susceptible subpopulations of bacteria in otherwise susceptible strains of *Salmonella* is imperative if an even higher incidence of treatment failures is to be avoided.

The purpose of these experiments was to investigate the possibility that resistant subpopulations of bacteria exist in ostensibly susceptible strains of *Salmonella*. If so, then to calculate the frequency with which they appear and to characterize the mutations, if any, that are present.

4:2 Study 1: Population analysis experiment

4:2:1 Materials

Test organisms	Strains of *Salmonella* susceptible to fluoroquinolones as detailed previously in section **2:2:1**
MH agar	as in section **3:1:1**
CLED agar	as in section **2:2:2:1**
BHI broth	as in section **2:2:2:1**
Glycerol	Sigma Aldrich G-6279
Sterile distilled water	as in section **3:1:1**
Etests (ciprofloxacin)	Biostat-51000686

Antimicrobials:

Ciprofloxacin powder	as in section **3:1:1**
Ofloxacin	as in section **3:1:1**
Norfloxacin	as in section **3:1:1**
Moxifloxacin	as in section **3:1:1**

4:2:2:1 Method

MH agar plates were prepared by adding a 0.25ml aliquot of 1000mg/L antimicrobial stock solution to 500ml of sterilized molten agar that had been cooled to 50ºC. From this 20 plates with a final antimicrobial concentration of 0.5mg/L were poured and allowed to set at room temperature. In addition, plates with final concentrations of 0.125mg/L ciprofloxacin and 1mg/L norfloxacin were similarly prepared, as were non-selective plates with no added antimicrobial.

Several colonies were taken from an overnight CLED plate, inoculated into 5 ml Brain Heart Infusion (BHI) broth and incubated with shaking at 37°C overnight. The broths were centrifuged at 18,000g for 5 minutes and the pellet was resuspended in 1ml BHI broth. The culture was adjusted to an optical density of 0.15 at 540nm, which is approximately 10^8colony forming units (cfu) per ml.

Viable cell counts were determined by performing a series of 1:10 dilutions. 0.1ml aliquots of the dilutions were inoculated onto two non-selective MH agar plates for each dilution and incubated overnight. The number of colonies from the non-selective plates where the inoculum had yielded growth that was easily quantifiable was recorded and an average cfu/ml was determined from the two plates.

Aliquots of 0.1ml were inoculated onto the antimicrobial-containing MH agar plates in duplicate and incubated at 37ºC. The plates were examined after 24 hours. If no growth was detected then the plates were replaced in the incubator for a further 24 hours and re-examined. If colonies were detected, they were considered to be mutants and were subcultured onto MH agar containing the same concentration of relevant antimicrobial to confirm resistance. The plates were incubated at 37°C and examined for growth 24 hours

later. If the mutants had grown on the confirmatory plates then the MIC of the selecting antimicrobial was determined before being inoculated into 5mls BHI and incubated with shaking at 37ºC overnight before being stored at -80°C in 15% glycerol. These were later recovered and subjected to PFGE to ensure they were unchanged from the wild-type strain before being sequenced.

The MICs of ciprofloxacin were determined by Etest for a number of mutants selected on ofloxacin, norfloxacin and moxifloxacin.

4:2:2:2 Determination of MIC by Etest

Colonies taken from a fresh overnight CLED plate were resuspended in 3mL of sterile 0.85% saline to a turbidity of 0.5 McFarland units and inoculated onto Mueller-Hinton agar plates using a cotton wool swab and a RP 454 rotary plater (Denley, England). Ciprofloxacin Etest strips were placed onto the plates, which were then incubated at 37°C for 18 hours. MIC values were determined according to the manufacturer's instructions.

4:2:2:3 Determining the frequency of spontaneous fluoroquinolone-selected mutants.

The mutants were counted and the rate of spontaneous mutation was determined with regards to the total cell population inoculated onto the antimicrobial-containing agar plates determined by the viable cell count.

4:2:3 Results

A total of 46 spontaneous mutants with raised fluoroquinolone MICs were recovered from 6 strains of *Salmonella enterica*. An isolate of *S*. Typhimurium DT104 yielded the highest

number of mutants (n=33) recovered using norfloxacin (n=16), ofloxacin (n=10) and moxifloxacin (n=7). A strain of *S.* Dublin was the only other organism to have mutants recovered using all three of these fluoroquinolones; the numbers were smaller yielding just 2, 1 and 1 respectively. Five ofloxacin-selected mutants were obtained from a strain of *S.* Typhimurium DT40 with a further single mutant obtained from each of the strains of *S.* Typhimurium DT10 and *S.* Virchow. Two *S.* Typhimurium DT170 mutants were selected using moxifloxacin (n=1) and ofloxacin (n=1). No mutants were obtained when using ciprofloxacin as the selecting fluoroquinolone.

Table 4.1 shows the frequencies of spontaneous mutants with raised MICs to norfloxacin, ofloxacin and moxifloxacin. In the strain of *S.* Dublin, the frequency of mutants resistant to norfloxacin was $4x10^{-9}$ which was the lowest frequency detected. Conversely, the highest frequency of $1x10^{-6}$was detected in the mutants spontaneously resistant to ofloxacin in the strain of *S.* Typhimurium DT104.

All of the mutants selected on ofloxacin, norfloxacin and moxifloxacin exhibited a decrease in susceptibility to ciprofloxacin. Results are tabulated in **Table 4:2**.

Table 4:1. Frequencies of spontaneous mutants resistant to ofloxacin, norfloxacin and moxifloxacin.

SSRL No.	Organism	Wild-type MIC (mg/L)	No. Cells per ml inoculum	Selecting antimicrobial[a]	Total No. Mutants obtained	Mutant MIC (mg/L)	Frequency of spontaneous FQ mutation[b]
19993849	*S*. Typhimurium DT104	0.06	$1x10^{8}$	ofloxacin	10	1	$1x10^{-6}$
		0.094	$1x10^{8}$	moxifloxacin	7	1-2	$7x10^{-7}$
		0.25	$1x10^{8}$	norfloxacin	16	>2	$1.6x10^{-6}$
20003940	*S*. Typhimurium DT 40	0.06	$5x10^{9}$	ofloxacin	5	1-2	$1x10^{-8}$
20005189	*S*. Typhimurium DT170	0.03	$8x10^{7}$	moxifloxacin	1	1	$1.25x10^{-7}$
		0.06	$8x10^{7}$	ofloxacin	1	1	$1.25x10^{-7}$
20003962	*S*. Typhimurium DT 10	0.06	$6.35x10^{9}$	ofloxacin	1	1	$1.6x10^{-9}$
19993805	*S*. Dublin	0.25	$5x10^{9}$	norfloxacin	2	1-2	$4x10^{-9}$
		0.125	$5x10^{9}$	ofloxacin	1	1	$2x10^{-9}$
		0.06	$5x10^{9}$	moxifloxacin	1	2	$2x10^{-9}$
19983717	*S*. Virchow	0.06	$5x10^{9}$	ofloxacin	1	2	$2x10^{-9}$

[a] All concentrations of selecting antimicrobial are 0.5mg/L except norfloxacin at 1mg/L

[b] Mutants resistant to fluoroquinolones at BSAC/EUCAST breakpoints.

Table 4:2. Ciprofloxacin MICs of spontaneous fluoroquinolone-resistant mutants selected on ofloxacin, norfloxacin and moxifloxacin.

SSRL Reference	Mutant	Organism	Selecting antimicrobial	WT Cp MIC (mg/L)	Mutant Cp MIC (mg/L)
19993849	P13	*S.* Typhimurium 104	Moxifloxacin	0.012	0.38
20003940	P35	*S.* Typhimurium 40	Ofloxacin	0.023	0.19
	P36	-	-	-	0.38
2005189	P41	*S.* Typhimurium 170	Ofloxacin	0.016	0.19
	P42	-	-	-	0.25
20003962	P43	*S.* Typhimurium 10	Ofloxacin	0.016	0.25
19993805	P47	*S.* Dublin	Ofloxacin	0.016	0.38
	P48	-	Norfloxacin	-	0.19
	P50	-	Moxifloxacin	-	0.5
19983717	P53	*S.* Virchow	Ofloxacin	0.016	0.5

Cp-ciprofloxacin

Dashes indicate identity to parameter above

4:3 Study 2: Amplification and Sequencing of *gyrA* and *parC* genes in selected mutants exhibiting decreased susceptibility to fluoroquinolones.

4:3:1 Materials

Test organisms

5 wild-type strains of *Salmonella*	as detailed in section **2:2:1**

9 mutants exhibiting decreased resistance to ciprofloxacin isolated in study **4.2**:

(19993849, *S*. Typhimurium 104 (n=2); 20003940, *S*. Typhimurium 40; 20005189, *S*. Typhimurium 170; 20003962, *S*. Typhimurium 10; 19993805, *S*. Dublin (n=3); 19983717, *S*. Virchow)

Preparation of Template DNA

CLED agar	as detailed in section **2:2:2:1**
PCR grade water	Sigma Aldrich-W3500

Polymerase Chain Reaction

Deoxynucleotide triphosphates (dNTPs)	ABgene	AB-0196
Taq polymerase	GE Healthcare	27-0799-04
10X Reaction Buffer	supplied with *Taq*	
PCR grade water	as detailed above	
Redipac GP Agarose	as detailed in section **2:2:2:1**	
10X TBE buffer	as detailed in section **2:2:2:1**	
Ethidium Bromide	as detailed in section **2:2:2:1**	

Superladder-Low 100bp	ABgene	SLL-100
6X Loading buffer	supplied with ladder	

Primers

The primers used in this experiment for the detection of the *gyr*A and *par*C genes were made by MWG Biotech (Milton Keynes, U.K.) and were as follows:

gyrA Primer 1	Forward-[5'-TGTCCGAGATGGCCTGAAGC-3']
gyrA Primer 2	Reverse-[5'-CGTTGATGACTTCCGTCAG-3']
parC Primer 1	Forward-[5'-ATGAGCGATATGGCAGAGCG-3']
parC Primer 2	Reverse-[5'-TGACCGAGTTCGCTTAACAG-3']

These primers were described previously by Giraud *et al* 1999.

DNA Purification

QIAquick PCR Purification Kit.	Qiagen 28104

4:3:2 Methods

4:3:2:1 Preparation of Template DNA

The organisms under investigation were inoculated onto CLED agar plates and incubated at 37°C overnight. A single colony was then suspended in a 1.5ml microcentrifuge tube containing 1ml of sterile distilled water and placed on a heating block at 100°C for 12

minutes. The tubes were then centrifuged at 18,000g for 10 minutes. 80µl of supernatant was transferred to a clean tube and stored at -20ºC until needed.

4:3:2:2 Polymerase Chain Reaction

Reagents were prepared as a master mix in a "clean laboratory", i.e. one that was free from possibly contaminating DNA. This master mix comprised: 25pmoles of both forward and reverse primer; 200µM dNTPs; 0.5U *Taq* polymerase; 10X Reaction buffer (that included 1.5mM $MgCl_2$) and sterile water.

In the "dirty" laboratory, 20µl of the PCR master mix was aliquoted to 0.5ml PCR tubes. After adding 5µl of template DNA the tubes were immediately stored on ice. To the negative control, 5µl of sterile water was added to the tube in place of DNA. This would confirm that any amplification was the target DNA and not contaminating DNA. The tubes were immediately transferred to the GeneAmp PCR System 9700, which had been pre-programmed with the relevant parameters.

PCR Parameters

Following an initial denaturation step of 3 minutes at 94°C, amplification was performed over 30 cycles, each one consisting of 1 minute at 94°C, 1 minute at hybridisation temperature specific to the target gene (55°C for *gyrA*, and 52°C for *parC*) and 1 minute at 72°C, with a final extension step of 10 minutes at 72°C.

Gel electrophoresis was performed on the resulting amplicons using a 1.5% agarose gel in 1X TBE buffer at 80V for approximately 45 minutes. 100bp molecular weight marker was

added to wells at regular intervals and used to size the 470-bp and 412-bp products of the QRDR regions of *gyrA*, and *parC* respectively. The amplicons were visualised using ethidium bromide.

4:3:2:3 DNA Purification

The PCR product was purified using the QIAquick Purification Kit according to the manufacturer's instructions.

4:3:2:4 DNA Sequencing

Sequencing of the PCR product was performed in both directions by GATC-Biotech, Lake Constanz, Germany on an ABI 3730XL capillary sequencer.

4:3:2:5 Analysis of the sequence data

GATC-Biotech provided chromatogram files containing the sequence data in addition to FAS files. The chromatogram files were checked using the software provided by GATC-Biotech to ensure the correct oligonuclotide was assigned.

Both the forward and reverse complement sequences were compared to the published *gyrA* or *parC* sequences of an Enteritidis isolate (Accession No. AM933172.1) and *Salmonella* Typhimurium LT2 (Accession No.AE006468.1) respectively, using BLAST software (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A point mutation was accepted as genuine if it was present in both the forward and reverse complement sequences of the isolate.

4:3:3 Results

The results of the sequence analysis are listed in **Table 4.3**. The 5 wild-type sequences were identical to published *gyrA* and *parC* sequences. All 9 mutants examined in this experiment had amino acid substitutions as a result of point mutations within the QRDR of *gyrA*. Four within Ser83 encoded phenylalanine residues, 4 within Asp87 resulted in substitutions to asparagine (2) and tyrosine (2) residues and one at Asp82 encoded an asparagine residue. These substitutions have been described previously. No mutants selected contained mutations within the QRDR of *parC*.

Table 4.3: MICs for wild-types and mutants and nucleotide changes in the QRDR region of *gyrA* with deduced amino acid substitutions.

SSRL Reference	Mutants	Serotype	Phage type	Selecting antimicrobial	WT MIC (mg/L)	Mutant MIC (mg/L)	*gyrA* sequencing results								
	Codons						80	81	82	83	84	85	86	87	88
							CAC (His)	GGC (Gly)	GAT (Asp)	TCC (Ser)	GCA (Ala)	GTG (Val)	TAT (Tyr)	GAC (Asp)	ACC (Thr)
19983717	P53	Virchow	n/a	Oflox	0.06	2	-	-	-	-	-	-	-	**T**AC (Tyr)	-
19993849	P13	Typhimurium	104	Moxi	0.125	2	-	-	-	T**T**C (Phe)	-	-	-	-	-
-	P20	-	-	Norflox	0.25	>2	-	-	-	T**T**C (Phe)	-	-	-	-	-
20003940	P36	Typhimurium	40	Oflox	0.06	2	-	-	**A**AT(Asn)	-	-	-	-	-	-
20005189	P41	Typhimurium	170	Ofllox	0.06	1	-	-	-	-	-	-	-	**A**AC (Asn)	-
20003962	P43	Typhimurium	10	Oflox	0.06	1	-	-	-	-	-	-	-	**A**AC (Asn)	-
19993805	P47	Dublin	n/a	Oflox	0.125	1	-	-	-	T**T**C (Phe)	-	-	-	-	-

SSRL Reference	Mutants	Serotype	Phage type	Selecting antimicrobial	WT MIC (mg/L)	Mutant MIC (mg/L)	*gyrA* sequencing results								
-	P48	-	-	Norflox	0.25	1	-	-	-	T**T**C (Phe)	-	-	-	-	-
-	P50	-	-	Moxi	0.06	2	-	-	-	-	-	-	-	**T**AC (Tyr)	-

Antimicrobial abbreviations: Cp, ciprofloxacin; Oflox, ofloxacin; Norflox, norfloxacin; Moxi, moxifloxacin

Dashes indicate identity to parameter above

4:4 Discussion

4:4:1 Study 1 Population analysis

Spontaneous quinolone-resistant mutants were recovered from only 6 out of 25 strains of *Salmonella enterica*. This may be explained by a previous study which demonstrated that *in vivo*, resistance mechanisms resulting in high-level fluoroquinolone-resistance may be deleterious, as *in vitro* high-level fluoroquinolone resistant mutants exhibited drastically altered growth on solid media relative to the wild-type organism (Giraud *et al*, 1999). The 46 mutants that were selected were obtained from strains that were ostensibly susceptible by the breakpoint and agar dilution methods of susceptibility testing, to norfloxacin, ofloxacin and moxifloxacin, and were selected on concentrations of antimicrobial at least twice their MIC. A selection of these mutants was sequenced to determine the mechanisms responsible for the resistance and will be discussed in the next section. Whatever the mechanism, their existence clearly has serious implications for the use of these antimicrobials in clinical therapy. Suboptimal dosing can enrich these resistant subpopulations resulting in overall resistance which in turn, has been shown to reduce the efficacy of other members of the same class of antimicrobial compound (Drlica , 2003).

All three fluoroquinolones elicited low frequencies of spontaneous mutation. Among the six strains from which mutants were obtained, selection rates were between $1x10^{-6}$ and $4x10^{-9}$. Previous studies have reported that spontaneous bacterial mutants usually arise at a low frequency between 10^{-6} and 10^{-8} (Zhao & Drlica, 2001). Mutation rates can be variable, and the lower frequencies of spontaneous mutation obtained in this experiment may have been a result of differences in experimental process, as previous studies have demonstrated. Experimental parameters such as the antimicrobial, the concentration of the

antimicrobial, physiological conditions, bacterial stress, the existence of mutations that produce mutator phenotypes and the capability of some antimicrobials to increase mutability are all inconsistent factors that can have a significant effect on the rate of selection making it difficult to achieve an accurate evaluation (Martinez & Baquero, 2000).

The increase in cross-resistance to ciprofloxacin exhibited by the spontaneous ofloxacin-, norfloxacin- and moxifloxacin-resistant mutants confirms earlier reports that resistance in one fluoroquinolone confers resistance to all other fluoroquinolones (Hopkins *et al*, 2005).

4:4:2 Study 2 Amplification and Sequencing of *gyrA* and *parC* genes

The mutants characterised were selected from an ostensibly susceptible population as previously discussed. All were selected on agar plates containing at least 3 doubling dilutions of antimicrobial above their measured MIC. The mutations have all been observed in previous studies examining clinical isolates (Escribano *et al*, 2004; Eaves *et al*, 2004), however one of these strains was of porcine origin and another of bovine origin.

Ser83→Phe, found in the strains *S.* Typhimurium and *S.* Dublin, is the most common mutation described and is frequently associated with fluoroquinolone resistance (Ruiz *et al*, 1995). In this instance, it was responsible for resistance to ofloxacin, norfloxacin and moxifloxacin. Asp87→Asn and Asp87→Tyr, the mutations described in the strains of *S.* Typhimurium DT 10, *S.* Typhimurium DT170 and *S.* Virchow were responsible for resistance to ofloxacin, as was the less frequently described mutation Asp82→Asn responsible for resistance in a strain of *S.* Typhimurium DT40. It may be noted that no mutants were selected using ciprofloxacin which has been the drug of choice in the treatment of severe or systemic *Salmonella* infections. This may be due to the fact that resistance to fluoroquinolones usually requires the acquisition of a number of mutations in

gyrA (Casin *et al*, 2003). Perhaps the single mutations described in this study were just first step mutations that required additional mutations, possibly in another of the topoisomerase genes, before clinical resistance to ciprofloxacin could be achieved.

No mutants were selected from any strain of *S*. Enteritidis examined. It has been noted in a previous study that this serotype has the least capacity to generate mutants (Cebrían *et al*, 2003).

Chapter 5: Selection and study of Spontaneous Mutants II

5:1 Study 1: Selection of ciprofloxacin-resistant mutants using an antimicrobial concentration gradient.

5:1:1 Introduction

Due to the increase of multi-resistant isolates of *Salmonella*, fluoroquinolones are considered to be the drugs of choice in the treatment of infections where antimicrobial intervention is warranted. Unfortunately in recent years, resistance to this class of drugs has developed and treatment failures are being reported with increasing regularity (Boswell *et al*, 1997; Chandel & Chaudhry, 2001).

In *Salmonella*, one mechanism of resistance is a result of a sequential accumulation of mutations in the topoisomerase genes. Most to date have been found to occur in a specific region of the *gyrA* gene of topoisomerase II which encodes the A subunit of gyrase, the primary target of fluoroquinolones; *parC* and *parE* genes of topoisomerase IV are considered to be secondary targets for fluoroquinolone action in *Salmonella* and a number of mutations have now been documented in these genes.

The following two experiments were designed to illustrate the progressive accumulation of mutations and the concomitant decrease in susceptibility to fluoroquinolones by repeated exposure of a susceptible isolate of *Salmonella*, to subinhibitory concentrations of antimicrobial. The third experiment characterized the mutations of the selected mutants.

5:1:2 Materials

Test Organisms	21 fully sensitive strains of *Salmonella enterica* as detailed in section **2:2:1**
DST agar	as in section **3:1:1**
Ciprofloxacin powder	as in section **3:1:1**
Sterile distilled water	as in section **3:1:1**
CLED agar	as in section **2:2:2:1**
BHI broth	as in section **2:2:2:1**
Glycerol	as in section **4:2:1**
Etests (ciprofloxacin)	as in section **4:2:1**
Mueller Hinton agar	as in section **3:1:1**

5:1:3 Method

Antimicrobial gradient plates were prepared using a modified version of the technique of Szybalski and Bryson (Szybalski & Bryson, 1952). A 0.1ml aliquot of a 100mg/L stock solution of ciprofloxacin was added to a glass universal containing sterilized molten DST agar that had been cooled to 50°C, to give a final concentration of 0.5mg/L. This was then poured into a square Petri dish, 15cm x 15cm, that had been raised to an angle of 5° at one side and allowed to solidify at room temperature in the angled position. The Petri dish was then levelled and a second 20ml amount of DST agar containing no antimicrobial was poured over the bottom layer. This was also allowed to solidify at room temperature. Plates were left overnight to allow diffusion of antimicrobial concentration gradients. Gradient

plates with final concentrations of 0.25mg/L, 0.75mg/L and 1.0mg/L were also prepared in this way.

Several colonies were taken from an overnight CLED plate and inoculated into 5 ml BHI broth and incubated on a shaker at 37°C overnight. Using a sterile cotton swab, the bacterial culture was plated across the gradient plate in the direction of low to high antimicrobial concentration. The plates were allowed to dry and then incubated at 37°C overnight. If strains failed to grow on the 0.5mg/L gradient plates, they were re-inoculated into BHI and again shaken at 37°C overnight for plating out onto a 0.25mg/L gradient plate the next day. From those strains in which growth occurred, colonies growing nearest the high concentration end of the plate were subcultured into 5ml BHI and incubated overnight on a shaker at 37°C. If no individual colonies were present, a small sweep was taken using a plastic loop at the highest concentration end of the growth streak. This procedure was repeated until there was confluent growth along the entire concentration gradient. At this point a plastic loop was used to sample the growth at the high concentration end of the plate and subcultured into 5ml BHI, which was incubated on a shaker at 37°C overnight. This was then plated onto a gradient plate with a final concentration of 0.75mg/L ciprofloxacin. Plates were incubated at 37°C overnight. The experiment was repeated as many times as required to obtain confluent growth along the concentration gradient. Once this was achieved, the experiment was repeated using gradient plates with a final concentration of 1.0mg/L ciprofloxacin.

Once growth had been established at least 25% along the concentration gradient of the agar plate MICs were determined using Etests for a number of mutants selected on 0.25mg/L (after 1 exposure), 0.5mg/L (after 1 and 2 exposures), 0.75mg/L (after 2 exposures) and 1.0mg/L (after 2 exposures) gradient plates.

Throughout the experiment, colonies that were subcultured for plating out onto the gradient plates were also stored at -85°C in 15% glycerol. These were later recovered and characterised using PFGE to ensure the strains remained unchanged.

Determination of ciprofloxacin MIC by Etest

As detailed in section **4:2:2:2**

5:1:4 Results

Results can be found in **Table 5.1**. On the first exposure on the 0.5mg/L gradient plate, 4 of the 21 strains examined grew near to the lowest ciprofloxacin concentration end. From these, 5 individual mutant colonies and light confluent growth from the strain of *S.* Typhimurium DT40 were selected for further exposure on 0.5mg/L plates. The 17 strains that failed to grow were inoculated onto a 0.25mg/L gradient plate. As a result individual colonies were obtained from a further 5 strains, while a sweep of confluent growth in addition to single colonies was taken from the other 12 strains.

Upon exposure on the 0.75mg/L gradient plates, no individual colonies were obtained and the growth was slight near the low concentration end of the plate. On the second exposure, good confluent growth was obtained along at least 1/3 of the gradient plate for all the strains.

The same pattern of growth was observed following exposure on the 1.0mg/L gradient plates. Initially the strains failed to grow well with only slight growth, but upon the second exposure, confluent growth was obtained along at least ¼ of the gradient plate for most of

the strains with only a single strain of *S.* Dublin still only growing slightly at the low concentration end.

All mutants subjected to PFGE had pulsed field profiles indistinguishable from their parent strains.

The ciprofloxacin MICs were determined for the mutants selected from each of the 21 strains. These results can be found in **Table 5.2.** In every case, the MIC of the mutant was greater than that of the wild-type strain. For 3 strains the difference is very slight with wild-type MICs of 0.012-0.023 increasing to 0.094mg/L in the mutants. Considerable differences were seen in strains of *S.* Enteritidis PT21, *S.* Typhimurium DT RDNC and *S.* Dublin, with mutant MICs of 0.75-1mg/L 10-fold greater than the original wild-type MICs of 0.016, 0.008 and 0.023mg/L respectively. The majority of mutants had MICs ranging between 0.125-0.5mg/L with their wild-type MICs ranging between 0.008-0.047mg/L.

Table 5.1: Mutants recovered from gradient plates using ciprofloxacin as the selecting antimicrobial

			Growth from 0.5mg/L plates			Growth from 0.25mg/L plates		Growth from 0.75mg/L plates		Growth from 1mg/L plates	
SSRL Reference	Serotype	Phage type	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
19991929	Enteritidis	1		ng		1/g	1/g	g	G	g	G
19982543	Enteritidis	4		ng		1/g	1/g	g	G	g	G
19992150	Enteritidis	6a		ng		2/g	2g	g	G	g	G
20004447	Enteritidis	21		ng		1/g	1/g	g	G	g	G
19990341	Typhimurium	104		ng		1/g	1/g	g	G	g	G
19993849	Typhimurium	104		ng		1/g	1/g	g	G	g	G
20001178	Typhimurium	104		ng		1	G	g	G	g	G
19983458	Typhimurium	2		ng		3	2/g	g	G	g	G
19983155	Dublin			ng		1	G	g	G	g	G
19993805	Dublin			ng		1/g	1/g	g	G	g	G

			Growth from 0.5mg/L plates			Growth from 0.25mg/L plates		Growth from 0.75mg/L plates		Growth from 1mg/L plates	
SSRL Reference	Serotype	Phage type	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
20000136	Dublin			ng		1/g	1/g	g	G	g	ng
20001735	Dublin			ng		1/g	1/g	g	g	g	G
20003052	Dublin			ng		1	g	g	G	g	G
19983717	Virchow			ng		1/g	1/g	g	G	g	G
19990706	Mbandaka			ng		1	G	g	G	g	G
19991073	Brandenburg			ng		1/g	1/g	g	G	g	ng
19991452	Binza			ng		1/g	1/g	g	G	g	g
19994076	Typhimurium	104	1	1	3		-	g	G	g	G
19983351	Typhimurium	RDNC	1	1	3		-	g	G	g	G
20003962	Typhimurium	10	3	3	3		-	g	G	g	G
20003940	Typhimurium 40	40	g	1	1		-	g	G	g	G

Numerical value indicates number of single colonies recovered from the gradient plate.

Exp, exposure; ng, no growth; g, light confluent growth; G, heavy confluent growth.

Table 5.2: MICs of mutants selected across ciprofloxacin concentration gradient determined by Etest (mg/L).

SSRL Reference	Serotype	Phage type	Wild type MIC	0.25mg/L	0.5mg/L	0.75mg/L	1.0mg/L
20003940	Typhimurium	40	0.023	n/d	0.047	0.094	0.094
19994076	Typhimurium	104	0.016	n/d	0.047	0.25	0.5
20003962	Typhimurium	10	0.016	n/d	0.047	0.064	0.094
19983351	Typhimurium	RDNC	0.023	n/d	0.125	1	0.5
19993849	Typhimurium	104	0.012	0.064	-	-	0.38
19983155	Dublin		0.008	0.012	-	-	0.75
1999 0706	Mbandaka		0.008	0.064	-	-	0.19
19992543	Enteritidis	4	0.016	0.064	-	-	0.25
19983717	Virchow		0.016	0.047	-	-	0.38
19991452	Binza		0.012	0.047	-	-	0.125
19991929	Enteritidis	1	0.012	0.064	-	-	0.094
19983458	Typhimurium	2	0.016	0.125	-	-	0.5
19992150	Enteritidis	6a	0.047	0.064	-	-	0.125
20004447	Enteritidis	21	0.016	0.125	-	-	0.75
19990341	Typhimurium	104	0.023	0.06	-	-	0.125
20001178	Typhimurium	104	0.023	0.125	-	-	0.19
19993805	Dublin		0.016	0.06	-	-	0.19
20001735	Dublin		0.016	0.06	-	-	0.125
20003052	Dublin		0.016	0.06	-	-	0.5
19991073	Brandenburg		0.032	0.125	-	-	ng
20000136	Dublin		0.023	0.06	-	-	ng

ng: no growth

-: mutants not stored

5:2 Study 2: Selection of mutants by exposure to subinhibitory concentrations of fluoroquinolones.

5:2:1 Materials

Test organisms: 20000041, strain of *S.* Typhimurium DT104 R-type ACSSxT [Tym^R]

3 "FS" strains previously detailed in section **2:2:1**

(19991929, *S.* Enteritidis PT1; 19994076, *S.* Typhimurium DT104 [Tym^S]; 19990706, *S.* Mbandaka)

DST agar	as detailed in section **3:1:1**
CLED agar	as detailed in section **2:2:2:1**
PCR grade water	as detailed in section **4:3:1**
BHI broth	as detailed in section **2:2:2:1**
Glycerol	as detailed in section **4:2:2**

Antimicrobials:

Ciprofloxacin	as detailed in section **3:1:1**
Ofloxacin	as detailed in section **3:1:1**
Norfloxacin	as detailed in section **3:1:1**
Moxifloxacin	as detailed in section **3:1:1**

5:2:2 Method

For each individual antimicrobial, an aliquot of stock solution was added to a glass universal containing sterilized molten DST agar that had been cooled to 50ºC to give final concentrations of 0.125mg/L and 0.5mg/L and ⅛ the measured MIC for each antimicrobial

determined for each one of the *Salmonella* isolates. Plates with no antimicrobial added were also prepared as control plates. Experiments were performed in duplicate.

Several colonies were taken from an overnight CLED plate and inoculated into 5ml BHI broth and incubated on a shaker at 37°C overnight. The culture was adjusted to an optical density of 0.15 at 540nm, containing approximately 10^8 colony forming units (cfu). 0.1ml aliquots of culture were plated out using a plastic spreader over the surface of a non-selective DST plate and plates containing 0.5mg/L, 0.125mg/L and the subinhibitory concentration of antimicrobial. These were allowed to dry at room temperature and incubated overnight at 37°C. The entire growth was harvested from the subinhibitory plate using a sterile cotton swab and resuspended in 3ml sterile saline. After vortexing to ensure thorough resuspension, the culture was again adjusted to 0.15 at 540nm. 0.1ml aliquots were again plated out onto new plates and incubated overnight at 37°C after drying. The 0.125mg/L and 0.5mg/L plated were examined for growth. When growth was observed on either plate, the complete population was subcultured into 5mls BHI and incubated on a shaker overnight at 37ºC. The MIC was measured at various points and if determined to be at least twice the original MIC value, was subjected to three exposures in the absence of antimicrobial to ensure stability of the mutation. This mutant was then characterized using PFGE and stored at –85ºC in 15% glycerol to await further investigation. This experiment was repeated 18 times or if less, as many times as required to obtain growth on the 0.125mg/L and 0.5mg/L concentration plates, indicating a reduction in susceptibility to the antimicrobial.

5:2:3 Results

Antimicrobial susceptibility was found to be reduced following repeated exposure to subinhibitory concentrations of fluoroquinolones. Results for the selection of mutants are

recorded for growth on both 0.125mg/L and 0.5mg/L ciprofloxacin. The MIC results for a selection of these mutants are shown in **Table 5:3** and **Figure 5:1**.

Confluent growth was obtained on each of the agar plates containing the subinhibitory concentrations of antimicrobial. Repeated exposure on these plates resulted in a decrease in fluoroquinolone susceptibility in each of the 4 strains examined. Two strains of *S.* Typhimurium were included in this experiment. Both were indistinguishable by PFGE and plasmid profile analysis. One was fully susceptible to antimicrobials (Tym^S) whilst the other had the pentaresistant R-type (Tym^R) common to *S.* Typhimurium DT104. Both strains showed a decrease in susceptibility to each of the 4 fluoroquinolones (**Figures 5:2 & 5:3**). For ciprofloxacin, Tym^R required the shortest time before growing on the 0.125mg/L plates, taking just three exposures to display a reduction in susceptibility from an initial MIC of 0.012 to 0.25mg/L. After eighteen exposures, 0.5mg/L was the highest MIC recorded for the mutants tested. Although the initially susceptible Tym^S had a slightly higher initial MIC of 0.016mg/L, it took five subinhibitory exposures before an increase in MIC to 0.19mg/L was observed, however MICs of 1.5mg/L, 0.5mg/l, and 1.0mg/L were recorded in mutants isolated from further subinhibitory exposures. For moxifloxacin and ofloxacin, the MICs of both Tym^S and Tym^R increased from 0.064mg/L and 0.047mg/L respectively, to 0.38mg/L after 16 exposures in Tym^S and only 9 exposures in Tym^R (0.25mg/L for moxifloxacin). The MIC of Tym^S rose from 0.047mg/L to 0.25mg/L, while that of Tym^R rose from 0.125mg/L to 0.38mg/L, each after 11 exposures to subinhibitory concentrations of norfloxacin. The strain of *S.* Enteritidis PT1 increased from an initial MIC of 0.012mg/L for ciprofloxacin to 0.38mg/L in 4 subinhibitory exposures, rising to 1mg/L after just 5 exposures. The MIC of the *S.* Kottbus, already resistant to low-level ciprofloxacin (0.125mg/L) upon its introduction to this experiment, increased to 0.25mg/L

after only 4 subinhibitory exposures. Despite this, the MIC failed to increase beyond 0.25mg/L, with no colonies recovered from the 0.5mg/L plates.

All of the mutants were characterised by PFGE and were confirmed as having profiles indistinguishable from those of the parent strains except for those of *S*. Mbandaka, which had several band differences that appeared to be a result of contamination and were therefore disregarded for the remainder of the experiment.

Table 5.3. Selection of mutants using subinhibitory concentrations of fluoroquinolones and the MICs achieved.

SSRL Reference	Organism	Selecting Antimicrobial	Selecting Concentration (mg/L)	Number of subinhibitory subcultures	Number of colonies isolated	Parent strain MIC(mg/L)	Mutant MIC (mg/L)
19991929	*S.* Enteritidis PT1	Ciprofloxacin	0.125	4	6	0.012	0.38
-	-	-	-	5	8	-	0.38
-	-	-	0.5	5	4	-	0.75-1.0
19994076	*S.* Typhimurium DT 104	Ciprofloxacin	0.125	4	2	0.016	0.19
-	-	-	-	5	2	-	0.19
-	-	-	0.5	10	2	-	1.5
-	-	-	-	12	2	-	1.0
-	-	-	-	16	1	-	0.5
-	-	-	-	18	1	-	1.0
-	-	Moxifloxacin	0.125	2	1	0.064	0.064
-	-	-	-	3	1	-	0.19
19994076	*S.* Typhimurium DT104	Moxifloxacin	0.5	16	1	0.064	0.38
-	-	Ofloxacin	0.125	2	1	0.047	0.047
-	-	-	0.5	16	1	-	0.38
19994076	*S.* Typhimurium DT104	Norfloxacin	0.125	1	1	0.047	0.047

SSRL Reference	Organism	Selecting Antimicrobial	Selecting Concentration (mg/L)	Number of subinhibitory subcultures	Number of colonies isolated	Parent strain MIC(mg/L)	Mutant MIC (mg/L)
-	-	-	0.5	11	1	-	0.25
20030041	*S.* Typhimurium DT 104[a]	Ciprofloxacin	0.125	3	1	0.012	0.25
20030041	*S.* Typhimurium DT 104[a]	Ciprofloxacin	0.125	5	2	-	0.25
-	-	-	-	6	2	-	0.25
-	-	-	-	18	1	-	0.5
-	-	Moxifloxacin	0.125	4	2	0.064	0.064
-	-	-	-	9	1	-	0.25
-	-	Ofloxacin	0.125	4	1	0.047	0.047
-	-	-	-	9	1	-	0.38
-	-	-	0.5	9	1	-	0.25
-	-	Norfloxacin	0.125	1	1	0.125	0.125
-	-	-	0.5	11	1	-	0.38
19992003	*S.* Kottbus	Ciprofloxacin	0.125	4	16	0.125	0.25
-	-	-	-	8	29	-	0.25

[a] Resistant to ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, tetracycline.; Dashes indicate identity to the parameter above

Figure 5:1

Diagramatic representation of the decrease in suceptibility of 4 strains of *S. enterica* following exposure to subinhibitory concentrations of ciprofloxacin.

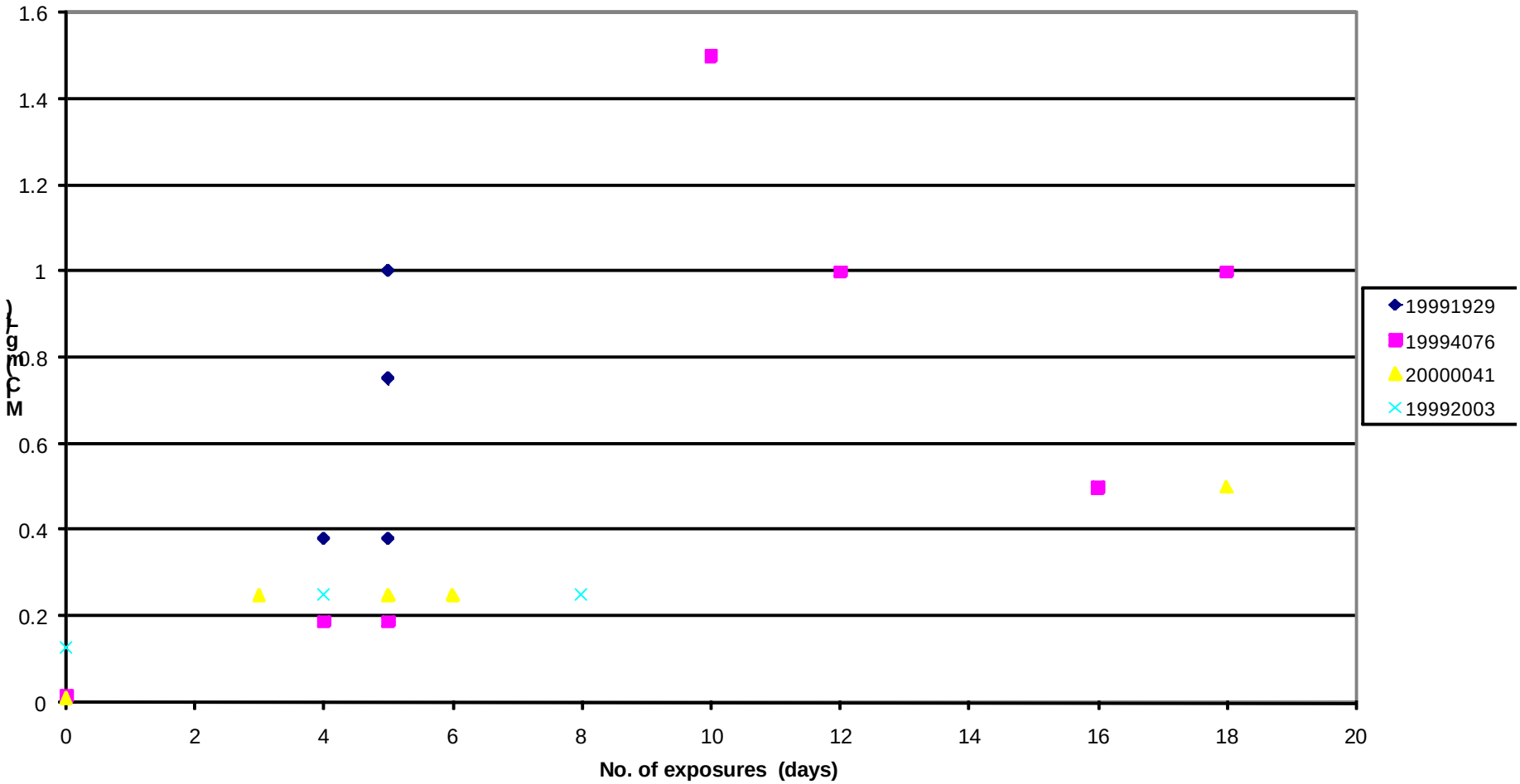


Figure 5:2

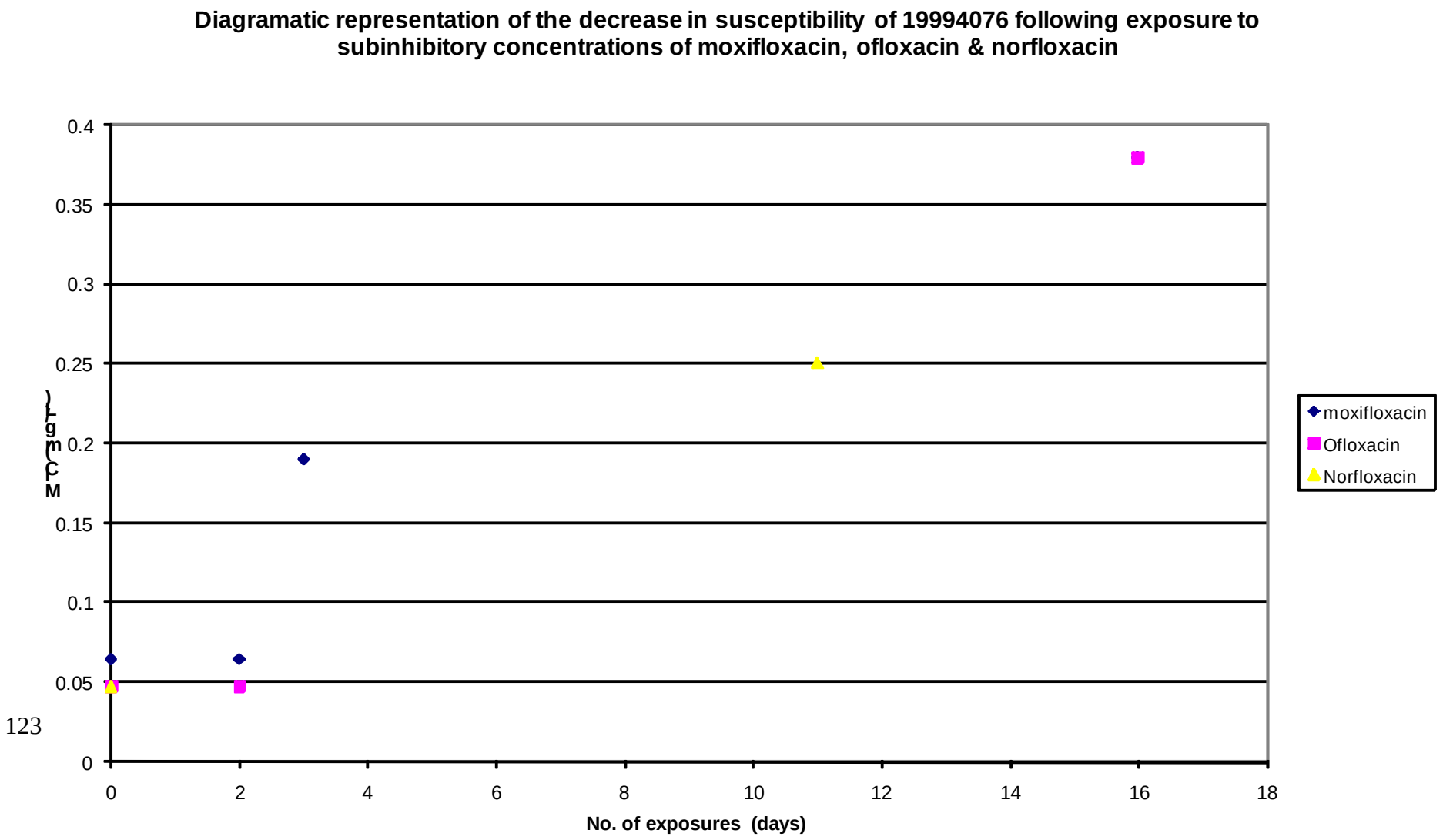
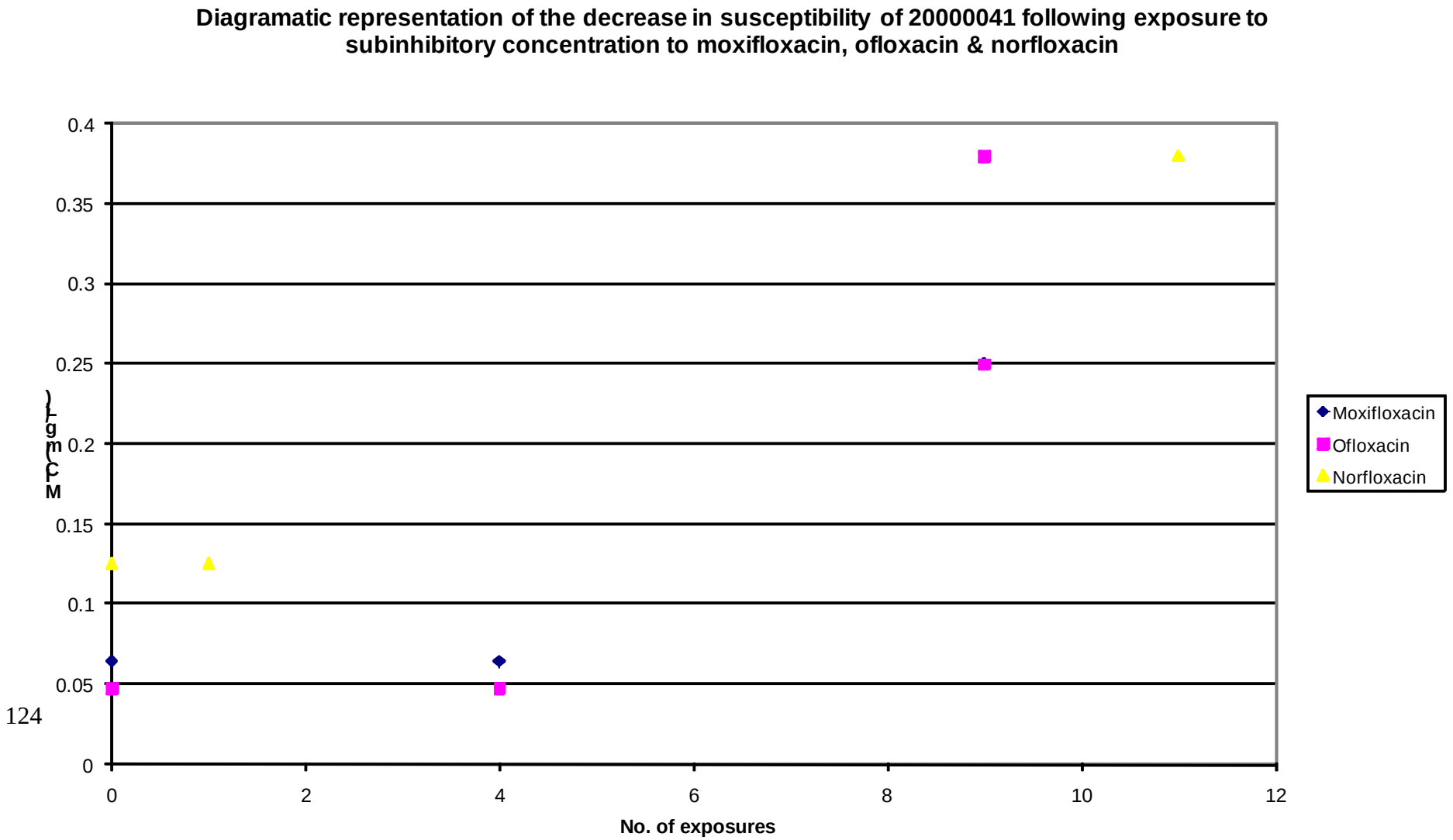

Diagramatic representation of the decrease in susceptibility of 19994076 following exposure to subinhibitory concentrations of moxifloxacin, ofloxacin & norfloxacin
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
16
18
No. of exposures (days)
moxifloxacin
Ofloxacin
Norfloxacin

Figure 5:3


Diagramatic representation of the decrease in susceptibility of 20000041 following exposure to subinhibitory concentration to moxifloxacin, ofloxacin & norfloxacin
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
No. of exposures
Moxifloxacin
Ofloxacin
Norfloxacin

5:3 Study 3: Amplification and Sequencing of *gyrA* and *parC* genes in selected mutants exhibiting decreased susceptibility to fluoroquinolones.

5:3:1 Materials

Test organisms

6 wild-type strains of *Salmonella* as detailed in section **2:2:1**

12 mutants exhibiting decreased susceptibility to ciprofloxacin isolated in experiment **5:1**:

(19994076, *S*. Typhimurium DT104 (n=3); 19983351, *S*. Typhimurium RDNC (n=3); 20004447, *S*. Enteritidis PT21 (n=2); 19982543, *S*. Enteritidis PT4 (n=2); 19983155, *S*. Dublin; 19983717, *S*.Virchow).

All other materials as described in section **4:3:1**

5:3:2 Methods

All methods previously described in section **4:3:2**

5:3:3 Results

The sequencing results can be found in **Table 5.4**. Mutations resulting in amino acid changes in *gyrA* were found in 5 of the 12 mutants examined; the remaining 7 had sequences that were identical to the *gyrA* of the wild-type *S*. Enteritidis. Two mutations within the Asp87 codon of a strain of *S*. Typhimurium DT104 encoded glycine residues and 2 mutations in a strain of *S*. Typhimurium DT RDNC encoded tyrosine residues. A fifth mutation at Ser83 in a strain of *S*. Dublin encoded a phenylalanine residue. All of these substitutions have been described previously.

Only a single mutant of a strain of *S*. Virchow contained a mutation that encoded an amino acid substitution at Thr57→Ser within the QRDR of *parC*

Table 5.4: MICs for wild-types and mutants and nucleotide changes in the QRDR region of *gyrA* with deduced amino acid substitutions.

SSRL Reference	Mutant	Organism	Selecting antibiotic	WT MIC (mg/L)	Mutant MIC (mg/L)	*gyrA* sequencing results								
					Codons	80	81	82	83	84	85	86	87	88
						CAC (His)	GGC (Gly)	GAT (Asp)	TCC (Ser)	GCA (Ala)	GTG (Val)	TAT (Tyr)	GAC (Asp)	ACC (Thr)
19994076	M2	Typhimurium 104	Cp	0.016	0.047	-	-	-	-	-	-	-	-	-
	M10				0.25	-	-	-	-	-	-	-	G**G**C (Gly)	-
	M52				0.5	-	-	-	-	-	-	-	G**G**C (Gly)	-
19983351	M3	Typhimurium RDNC		0.023	0.064	-	-	-	-	-	-	-	-	-
	M14				1	-	-	-	-	-	-	-	**T**AC (Tyr)	-
	M54				1	-	-	-	-	-	-	-	**T**AC (Tyr)	-
20004447	M27	Enteritidis 21		0.016	0.125	-	-	-	-	-	-	-	-	-
	M66				0.75	-	-	-	-	-	-	-	-	-
19982543	M22	Enteritidis 4		0.016	0.064	-	-	-	-	-	-	-	-	-

SSRL Reference	Mutant	Organism	Selecting antibiotic	WT MIC (mg/L)	Mutant MIC (mg/L)	*gyrA* sequencing results								
19982543	M64	Enteritidis 4	Cp	0.016	0.25	-	-	-	-	-	-	-	-	-
19983155	M72	Dublin		0.008	0.75	-	-	-	T**T**C(Phe)	-	-	-	-	-
19983717*	M77	Virchow		0.016	0.38	-	-	-	-	-	-	-	-	

Antimicrobial abbreviation: Cp, ciprofloxacin;

Dashes indicate identity to wild-type codons above;

*Mutation Thr57→Ser detected in QRDR of *parC*

5:4 Discussion

5:4:1: Selection of ciprofloxacin-resistant mutants using an antimicrobial concentration gradient.

The EUCAST MIC breakpoint for resistance to ciprofloxacin in strains of *Salmonella enterica* is 1mg/L, with a reduced susceptibility breakpoint of ≥0.5mg/L. Previous susceptibility testing by breakpoint method and Etest had categorised the strains as susceptible to ciprofloxacin. Heterogeneous populations of bacteria usually demonstrate slight variations in susceptibility to antimicrobial compounds. This study has demonstrated that by exposing these susceptible strains to a concentration gradient of ciprofloxacin, mutants could be selected that demonstrated sufficiently increased MICs to be categorised as intermediately-resistant and in one case resistant to ciprofloxacin. This phenomenon may account for the increasing number of reports of treatment failures with ciprofloxacin in purportedly susceptible strains of *S.* Typhi and other strains of *Salmonella* (Rupali *et al*, 2004; Chang *et al*, 2006). In these reports however, the strains that failed to be resolved with ciprofloxacin therapy were resistant to nalidixic acid at a concentration of 32mg/L. This is a well-documented occurrence in that these strains usually have reduced susceptibility to ciprofloxacin that is overlooked by current routine laboratory testing methods (Oteo *et al*, 2000; Albayrak *et al*, 2004). Nevertheless, the strains in this study were susceptible to nalidixic acid, which suggests that a minority of mutants with reduced susceptibility to ciprofloxacin exist within the susceptible population. This has clinical implications in that it would be imperative that the correct dosage of ciprofloxacin be given in order to eradicate the less susceptible mutants. This would also be an argument in favour of using the "mutant prevention concentration" (MPC) when administering ciprofloxacin for the treatment of *Salmonella* infections. The MPC is defined as "no colony recovery

when 10^{10} cells are applied to an agar plate" (Zhao & Drlica, 2001). A recent study has proposed an MPC value of 0.25mg/L ciprofloxacin for fully susceptible strains of *S.* Typhimurium and *S.* Enteritidis (Randall *et al*, 2004).

5:4:2: Selection of mutants by exposure to subinhibitory concentrations of fluoroquinolones

Resistance to fluoroquinolones mainly occurs as a result of an accumulation of mutations in the topoisomerase genes of *Salmonella*. Mutants were selected following the repeated exposure of susceptible strains of *Salmonella* to subinhibitory concentrations of a fluoroquinolone. The strain of *S.* Enteritidis required the same number of exposures to show a reduction in susceptibility to ciprofloxacin as the three other strains examined. Additionally, the MIC increased to 1mg/L in just five exposures, making this serotype the fastest to achieve resistance. This is contrary to a previous study, which found that the strain of *S.* Enteritidis required more exposures than the other serotypes studied (Cebrian *et al*, 2003).

Of the two strains of *S.* Typhimurium DT104 examined, the one with the pentaresistant R-type (Tm^R) was the first to show a decrease in susceptibility to ciprofloxacin but over eighteen subinhibitory exposures the MIC did not increase beyond 0.5mg/L, whereas the fully susceptible *S.* Typhimurium (Tm^S) took slightly longer to display a decrease in susceptibility but the MIC increased to 1.5mg/L in just eleven exposures. It may be that the initial decrease in ciprofloxacin susceptibility in the pentaresistant strain of *S.* Typhimurium was due to increased efflux as a previous study has demonstrated that increased efflux resulting in multidrug resistance in a strain of *S.* Typhimurium also

resulted in an increase in unrelated antimicrobials such as quinolone/fluoroquinolones (Abouzeed *et al*, 2008).

The MIC of the *S.* Kottbus, although resistant to low-level ciprofloxacin at the beginning of the experiment, did not rise beyond 0.25mg/L despite being subjected to eighteen exposures. This is reflected in the clinical strains of *S.* Kottbus received by the SSRL. From a total of eighteen isolates received between the years of 1990 and 2000, only one additional isolate was classed as resistant to low-level ciprofloxacin.

Subinhibitory exposure of the two strains of *S.* Typhimurium to the three other fluoroquinolones all resulted in a decrease in susceptibility. With the exception of an increase in moxifloxacin MIC for Tm^S in four exposures, each the strains required more exposures to the subinhibitory concentrations of each of the fluoroquinolones than for ciprofloxacin, before displaying an increase in MIC, which did not reach resistance levels.

This experiment was an attempt to mimic the conditions bacteria are sometimes subjected to *in vivo* when dosing regimens are inadequate. In an attempt to determine the order in which bacteria acquire the mutations that result in resistance, the QRDR regions of the *gyrA* and *parC* genes of the mutants recovered were sequenced.

5:4:3: Sequencing of *gyrA* and *parC* genes in selected fluoroquinolone-resistant mutants.

Exposure of these susceptible strains of *Salmonella* to either a concentration gradient or subinhibitory concentrations of fluoroquinolones resulted in mutants being recovered with reduced susceptibility to these antimicrobials. Sequencing of the QRDR of the *gyrA* gene revealed five strains to each have one of the most commonly isolated mutations associated with reduced susceptibility to fluoroquinolones; either Asp87→Tyr or Ser83→Phe (Griggs

et al, 1996) or Asp87→Gly (Ling *et al*, 2003). Compared to the wild-type MICs, the elevated MICs for the mutants were cause for concern in that they were determined to be ≥0.5mg/L; further, the *S*. Typhimurium RDNC mutants with MICs of 1.0mg/L were considered resistant by EUCAST breakpoints. Perhaps in this instance, the overproduction of an active efflux pump worked synergistically with *gyrA* mutations to result in ciprofloxacin resistance (Giraud *et al*, 2000). Alternatively it may be that additional mutations were present in one or more of the other topoisomerase genes not examined.

The mutant of *S*. Virchow which did not exhibit a mutation in the *gyrA* gene was the only mutant to have a *parC* mutation. The Thr57→Ser mutation is considered somewhat unusual in that single point mutations in topoisomerase IV genes are more common in Gram-positive species and in Gram-negative species, second-step mutations after those of gyrase leading to high-level fluoroquinolone resistance (Heisig, 1996). It may be that there are mutations within the *gyrB* and *parE* genes which were not examined, however this single mutation has been described previously in a strain of *S*. Virchow and no mutations were detected in any other topoisomerase gene. The Thr57→Ser mutation of *parC* has been thought not to be responsible for the decrease in susceptibility to quinolones as it has been detected in nalidixic acid-susceptible isolates (Weill *et al*, 2006). This would suggest that perhaps the increase in resistance to ciprofloxacin may be a result of a different mechanism of resistance such as the previously mentioned increased efflux or altered membrane permeability.

Overall, these studies have demonstrated that strains of *Salmonella enterica* susceptible to fluoroquinolones by both breakpoint and agar dilution method of sensitivity testing do harbour mutants with decreased susceptibility to these antimicrobials. If exposed to subinhibitory concentrations of these same antimicrobials, the bacteria will not be inhibited

but will survive due to an enhanced fitness as a result of previous acquisition of first-step mutations and may, with prolonged exposure, continue to acquire mutations ultimately resulting in resistance to fluoroquinolone antimicrobials.

Chapter 6: Amplification and Sequencing of plasmid-mediated resistance genes *qnrA, qnrB* and *qnrS*.

6:1 Introduction

In *Salmonella,* quinolone resistance is mostly attributed to point mutations within the quinolone resistance-determining region (QRDR) of the *gyrA* gene and to a lesser extent the *gyrB* and topoisomerase IV genes. Other resistance mechanisms include alterations in efflux systems and outer membrane porins.

Plasmid-mediated resistance was first reported in 1998 in a clinical strain of *Klebsiella pneumoniae* which carried a plasmid termed pMG252 (Martinez-Martinez, *et al*, 1998). This plasmid was found to contain the gene, now termed *qnrA*1 that is responsible for quinolone resistance. Since then a number of genes with varying degrees of similarity to *qnrA*1 have been discovered: *qnrA*1-6; *qnrB*1-20; *qnrC*; *qnrD* and *qnrS*1-4. Many *qnr* genes have been associated with strains of *Salmonella* that produce extended-spectrum β-lactamases (ESBLs) and have been identified collocated with other resistance determinants on sul1-type integrons (Lavigne *et al*, 2006; Garnier *et al*, 2006). This is cause for concern as these mobile genetic elements could greatly increase the dissemination of *qnr* genes whose clinical importance lies in their ability to facilitate the selection of quinolone resistance mutations in the presence of bactericidal levels of antimicrobial.

This purpose of this study was to determine the prevalence of *qnr* genes among *Salmonella* isolates submitted to the Scottish Salmonella Reference Laboratory from human, veterinary and environmental sources in the years 1997-2007.

6:2 Materials

Test organisms: Detailed in **Table 6:1**. 53 strains of *Salmonella enterica* susceptible to nalidixic acid (40mg/L) but with reduced susceptibility to ciprofloxacin (0.125mg/L) by breakpoint method;

Detailed in **Table 6:2**. 17 strains of *Salmonella enterica* resistant to ciprofloxacin (0.125mg/L) and cefotaxime (1mg/L) by breakpoint method.

Determination of MIC by Etest

Etests (ciprofloxacin)	as detailed in section **4:2:2:2**
Mueller Hinton agar	as detailed in section **3:1:1**

Preparation of template DNA

CLED agar	as detailed in section **2:2:2:1**
PCR grade water	as detailed in section **4:3:1**

Polymerase Chain Reaction

Illustra PureTaq Ready-To-Go Beads	GE Healthcare 27-9558-01
PCR grade water	as detailed in section **4:3:1**

Gel Electrophoresis

Redipac GP Agarose	as detailed in section **2:2:2:1**
10X TBE buffer	as detailed in section **2:2:2:1**
Ethidium Bromide	as detailed in section **2:2:2:1**
Superladder-Low 100bp ladder	as detailed in section **4:3:1**
6X Loading buffer	supplied with ladder

Primers

The primers used for the detection of the *qnr* genes were manufactured by VH Bio (Gateshead, U.K.) and have been described previously (Gay *et al*, 2006) and were as follows:

qnrA	Forward 5'-ATTTCTCACGCCAGGATTTG-3'
	Reverse 5'-GATCGGCAAAGGTTAGGTCA-3'
qnrB	Forward 5'-GATCGTGAAAGCCAGAAAGG-3'
	Reverse 5'-ACGATGCCTGGTAGTTGTCC-3'
qnrS	Forward 5'-ACGACATTCGTCAACTGCAA-3'
	Reverse 5'-TAAATTGGCACCCTGTAGGC-3'

DNA Purification

QIAquick PCR Purification Kit.	as detailed in section **4:3:1**

Table 6:1 Strains of *Salmonella enterica* with reduced susceptibility to ciprofloxacin (0.125mg/L) but susceptible to nalidixic acid (40mg/L)

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
19973002	Human	Typhimurium	104	R		R	S	S	S	S	S	S	R	R	R	R	S	R
19974670	Human	Hadar		R		S	S	S	S	S	S	S	S	R	S	R	S	R
19990154	Porcine	Typhimurium	RDNC	R	S	S	S	S	S	S	S	S	S	R	S	R	S	R
19991811	Human	Enteritidis	1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20003353	Human	Braenderup		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20003619	Human	Braenderup		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20011005	Human	Mucoid : eh :1,5		S	S	S	R	S	S	S	S	S	S	S	S	S	R	R
20013147	Human	Virchow		R	S	S	I	R	R	R	S	R	R	R	R	R	R	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20014543	Human	Braenderup		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20023201	Poultry	Non-motile groupC4		R	R	R	S	S	S	S	S	S	S	S	S	S	R	R
20023466	Human	Braenderup		S	S	S	I	S	S	S	S	S	S	S	S	S	S	R
20023518	Human	Enteritidis	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20030081	Environment	Typhimurium	104b	R	S	R	R	S	S	S	S	S	R	R	R	R	R	R
20032653	Environment	Non-motile groupC4		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20040636	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20041345	Environment	Rough :k :-		S	S	S	S	S	S	S	S	S	S	S	S	S	R	R
20042428	Human	Enteritidis	13a	S	S	S	S	S	S	R	S	S	S	S	S	S	R	R
20050040	Human	Typhimurium	RDNC	S	S	S	R	S	S	S	S	S	S	R	R	R	S	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20050075	Ovine	Typhimurium	Untypable	R	S	R	I	S	S	S	S	S	S	R	R	R	R	R
20050123	Human	Typhimurium	Untypable	R	S	R	R	S	S	S	S	S	S	R	R	R	R	R
20050192	Ovine	Typhimurium	Untypable	R	S	R	R	S	S	S	S	S	S	R	R	R	R	R
20050193	Ovine	Typhimurium	Untypable	R	S	R	R	S	S	S	S	S	S	R	R	R	R	R
20050275	Bovine	Typhimurium	Untypable	R	S	R	R	S	S	S	S	S	S	R	R	R	R	R
20050378	Human	Corvallis		S	S	S	I	S	S	S	S	S	S	R	R	R	S	R
20050446	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20050669	Human	Stanley		S	S	S	S	S	S	S	S	S	R	R	R	R	R	R
20050682	Human	Typhimurium	RDNC	S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20050875	Human	Corvallis		S	S	S	S	S	S	S	S	S	S	R	R	R	S	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20051150	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20051267	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20052136	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	S	R	S	S	R
20052260	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20052592	Human	Stanley		S	S	R	R	S	R	R	S	S	R	R	R	R	S	R
20060113	Human	Newport		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20060787	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20060807	Human	Corvallis		S	S	S	S	S	S	S	S	S	S	R	R	R	S	R
20060845	Human	Typhimurium	120	R	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20062459	Human	Braenderup		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20062576	Human	Corvallis		S	S	S	I	S	S	S	S	S	S	R	R	R	S	R
20062603	Human	Braenderup		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20063292	Human	Enteritidis	RDNC	R	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20070289	Human	Typhimurium	120	S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20070308	Human	Reading		S	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20070317	Human	Corvallis		S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20070339	Human	Bareilly		S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20070467	Environment	Gaminara		S	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20070484	Human	Blockley		R	S	S	R	S	S	S	S	S	S	S	S	S	S	R
20070485	Human	Corvallis		S	S	S	I	S	S	S	S	S	S	R	R	R	S	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20070494	Bovine	Typhimurium	104b	S	R	R	S	S	S	S	S	S	S	S	R	R	S	R
200700598	Human	Typhimurium	Untypable	S	S	S	R	S	S	S	S	S	S	R	R	R	S	R
20071604	Human	Mbandaka		R	S	R	S	S	S	S	S	S	S	S	R	R	R	R
20071727	Human	Java		R	S	S	S	S	S	S	S	S	S	S	S	S	S	R
20071810	Human	Mbandaka		R	S	R	S	S	R	S	S	S	R	R	R	S	R	R

[1]Cefotaxime sensitivity testing began in SSRL in 1999

Table 6:2 Strains of *Salmonella enterica* with reduced susceptibility to ciprofloxacin (0.125mg/L), resistant to cefotaxime (1mg/L).

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
19992535	Human	Virchow		R	R	S	S	S	S	R	R	S	R	R	R	R	R	R
20021751	Human	Virchow		R	R	S	S	S	S	S	R	S	S	R	R	S	R	R
20022460	Human	Virchow		R	R	S	S	S	S	S	R	S	S	R	R	S	R	R
20022461	Human	Virchow		R	R	S	S	S	S	S	R	S	S	R	R	S	R	R
20023201	Poultry	Non-motile group C4		R	R	R	S	S	S	S	S	S	S	S	S	S	R	R
20023730	Environment	Non-motile group C4		R	R	R	S	S	S	S	S	S	S	S	S	S	R	R
20031051	Poultry	Non-motile group C4		R	R	R	S	R	S	S	S	S	S	S	S	S	R	R
20032581	Human	Haifa		R	R	R	R	S	R	R	R	S	R	R	R	R	R	R
20032864	Human	Enteritidis	1b	R	R	R	R	S	S	S	R	S	S	S	S	S	R	R
20050127	Human	Haifa		R	R	R	R	S	R	R	R	S	R	R	R	R	R	R

SSRL No.	Isolate Source	Organism Name	PT	Ap	Cx	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp
20061399	Poultry	Java		R	R	S	R	S	S	S	R	S	R	R	S	S	R	R
20061425	Human	Agona		R	R	S	R	S	R	R	R	S	R	R	R	R	R	R
20062244	Human	Virchow		R	R	S	S	R	S	R	R	S	R	R	R	R	R	R
20070145	Human	Typhimurium	RDNC	R	R	R	R	S	R	S	R	S	R	R	R	R	S	R
20070591	Human	Rissen		R	R	R	I	S	R	I	S	S	S	R	R	R	S	R
20071213	Human	Typhimurium	193	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R
20072161	Human	Colindale		R	R	S	S	S	S	S	R	S	S	S	S	S	S	R

6:3 Methods

Determination of MIC by Etest

Method previously described in section **4:2:2:2**

Preparation of Template DNA

Method previously described in section **4:3:2:1**

Polymerase Chain Reaction

All six primers, each at a concentration of 25pmol, were added to Illustra PureTaq Ready-To-Go beads. After adding 1µl of template DNA and sterile PCR grade water to give a final volume of 25µl, the tubes were immediately stored on ice. To the negative control, an additional 1µl of sterile PCR grade water was added to the tube in place of DNA; this would confirm that any amplification was the target DNA and not contaminating DNA. A touchdown PCR was carried out on a GeneAmp PCR System 9700, with denaturation at 94°C for 45 seconds followed by 32 cycles of 94°C for 45seconds, a ramped annealing step of 45seconds at 67.4°C to 53°C for 12 cycles, 53°C for 20 cycles and elongation at 72°C for 60seconds. A final extension step of 72°C for 7 minutes was carried out.

Gel electrophoresis was performed on the resulting amplicons using a 1.5% agarose gel in 1X TBE buffer at 80V for approximately 45 minutes. 100bp molecular weight marker was added to wells at regular intervals and used to size the 516bp *qnr*A, 469bp *qnr*B and 417bp *qnr*S products. The amplicons were visualised using ethidium bromide.

Purification

As detailed in section **4:3:2:3**.

DNA Sequencing

As detailed in section **4:3:2:4**

Analysis of the sequence data

As detailed in section **4:3:2:5**

6:4 Results

Thirty-four of the 70 strains were found to harbour *qnr* genes. The MICs of ciprofloxacin for these strains ranged from 0.125-1mg/L; thus all were categorized as having either reduced susceptibility or resistance to ciprofloxacin, despite being susceptible to nalidixic acid. These results can be found in **Table 6.3**.

The majority of these strains were of human origin; one strain of *S.* Gaminara was of environmental origin and three strains of *S.* Typhimurium, untypable by phage typing, were from ovine (2) and bovine sources. Of the strains from human sources, 58% of patients had a history of foreign travel.

qnr genes were identified in twelve different serotypes. *S.* Corvallis was the most common with thirteen strains accounting for 38%, followed by nine *S.* Typhimurium (26%), and two *S.* Stanley and *S.* Enteritidis (6%). The eight remaining serotypes, *S.* Virchow, *S.* Haifa, *S.* Agona, *S.* Gaminara, *S.* Blockley, *S.* Rissen, *S.* Colindale and a strain of *S.* Java were represented by a single strain (3%).

qnrA1 was identified in strains of *S.* Virchow and *S.* Stanley. *qnrB1* was identified in *S.* Colindale, with *qnrB2* being identified in *S.* Agona and S. Haifa. *qnrB5* was identified in *S.* Gaminara . With the exception of these six strains, all other qnr genes were identified as *qnrS1*. Four of the seventeen cefotaxime-resistant strains harboured *qnr* genes.

Table 6:3. Details of *qnr*-positive strains of *Salmonella enterica*

Salmonella serotype	Phage type	Number of isolates	Source	Foreign travel	Year of isolation	Range of Cp MICs (mg/L)	Additional resistances	*qnr* gene isolated
Virchow	-	1	human	Oman	2001	0.125	ApFzGmKaNeSpStSxTeTm	A1
Haifa	-	1	human	-	2003	1	ApCxClGmKaNaSpStSxTeTm	B2
Corvallis		9	human	Malaysia(4), Cambodia (1), Thailand (2), Brazil/Chile(1)	2004-2007	0.125-0.5	StSxTe	S1
Corvallis	-	3	human	Bali/Singapore(1)	2005	0.25	-	S1
Corvallis	-	1	human	Thailand	2005	0.25	Sx	S1
Enteritidis	13a	1	human	-	2004	0.25	ApGmSpStSxTm**Ka**	S1
Enteritidis	RDNC	1	human	-	2006	0.5	Ap	S1
Typhimurium	RDNC	2	human	Malaysia(1)	2005	0.25-0.38	StSxTe	S1
Typhimurium	Untypable	4	human,ovine(2) bovine	-	2005	0.25-0.5	ApClSpStSxTeTm	S1
Typhimurium	Untypable	1	human	Singapore/Thailand	2007	0.38	StSxTe	S1

Salmonella serotype	Phage type	Number of isolates	Source	Foreign travel	Year of isolation	Range of Cp MICs (mg/L)	Additional resistances	*qnr* gene isolated
Typhimurium	120	1	human	Singapore	2006	0.75	ApStSxTe	S1
Typhimurium	120	1	human	-	2007	0.38	StSxTe	S1
Stanley	-	1	human	-	2005	0.125	SpStSxTeTm	A1
Stanley	-	1	human	Thailand	2005	0.19	ClGmKaSpStSxTe	S1
Agona	-	1	human	Egypt	2006	1	ApCxGmKaNaSpStSxTeTm	B2
Gaminara	-	1	environment	-	2007	0.25	-	B5
Blockley	-	1	human	Nigeria/Angola	2007	0.5	Ap	S1
Rissen	-	1	human	Thailand	2007	0.125	ApCxClGmStSxTe**Ka**	S1
Java	-	1	human	-	2007	0.19	Ap	S1
Colindale	-	1	human	-	2007	0.19	ApCx	B1

Antimicrobials in **bold** indicate intermediate susceptibility

Additional resistances: Ap, ampicillin; Cx, cefotaxime; Cl, chloramphenicol; Cp, ciprofloxacin; Fz, furazolidone; Gm, gentamicin; Ka, kanamycin; Na, nalidixic acid; Ne, netilmycin; Sp, spectinomycin; St, streptomycin; Sx, sulphamethoxazole; Te, tetracycline; Tm, trimethoprim.

6:5 Discussion

The majority of strains harboured *qnrS1* genes, with *qnrA1*, *qnrB1*, *qnrB2* and *qnrB5* identified in only six strains. The thirty-four *qnr*-positive strains belonged to twelve serotypes, including *S*. Gaminara, *S*. Rissen, *S*. Agona *S*. Haifa, *S*. Blockley, *S*. Colindale and a strain of *S*. Java which, at this time, have not previously been found to harbour *qnr* genes.

Of the eighteen strains isolated from patients with a history of foreign travel, sixteen were identified as *qnrS1*, one was *qnrA1* and another was *qnrB2*. With the exception of one from Brazil, all *qnrS1*-positive strains originated from Thailand or Malaysia

Plasmid-mediated quinolone resistance has serious implications for the use of this group of antimicrobials in the future. More importantly, the fact that these genes have been found on integrons together with ESBLs supports the requirement for ongoing surveillance of resistance. Surveillance will be problematic and it was thought that this problem could be addressed by the recommendation that nalidixic acid disk screening was indicative of fluoroquinolone susceptibility (Hakenen, 1999). However, as this experiment has shown, isolates that are nalidixic acid-susceptible can possess *qnr* genes and have a MIC as high as 0.75mg/L for ciprofloxacin.

Chapter 7: General Discussion

7:1 General Discussion

This study was the first investigation into the incidence of resistance to quinolone and fluoroquinolone antimicrobials in strains of *Salmonella enterica* isolated from human, veterinary and environmental sources in Scotland; to determine the capacity of the fluoroquinolones to select resistant mutants in these isolates and to characterize some of the genetic mechanisms through which any such resistance arises. In this chapter the experimental results of this study and others will be compared and discussed.

All fluoroquinolones, whether used in clinical or veterinary medicine, have an identical mechanism of action in that they inhibit the action of bacterial topoisomerase genes, topoisomerase II (DNA gyrase) in Gram-negative species and in Gram-positive species, topoisomerase IV, leading to the inhibition of DNA replication and ultimately resulting in cell death.

The emergence of resistance in *Salmonella* to this class of antimicrobials was first reported in 1990 in strains of *S.* Typhimurium (Piddock *et al*, 1990). Since then reports of resistance to quinolones and fluoroquinolones have continued to increase from around the world (Frost *et al*, 1996; Hakenen *et al*, 2001; Herikstad, 1997; Molbak *et al*, 2002). Reasons behind this increase in resistance are thought to include previous treatment with ciprofloxacin (Muder *et al*, 1991) and the licensing of enrofloxacin (a ciprofloxacin analogue specifically for use in animal medicine) for use in animal husbandry in 1993 in the U.K. (Threlfall *et al*, 1999).

At the start of this study in 2001, relatively little was known about the mechanisms of quinolone and fluoroquinolone resistance in the genus *Salmonella*. Point mutations in the quinolone resistance-determining region (QRDR) of the GyrA subunit of the topoisomerase II enzyme DNA gyrase (Yoshida *et al*, 1990) were well documented and were shown to result in high-level resistance to nalidixic acid in addition to cross-resistance to the fluoroquinolones (Crumplin, 1990; Reyna *et al*, 1995), with the GyrB subunit being implicated to a lesser degree (Yamagishi *et al* 1981). Despite several studies (Ruiz, 1997; Piddock *et al*, 1998; Giraud *et al*, 1999) no mutations had been detected in the subunits of topoisomerase IV, *parC* and *parE*.

In the following years, additional mutations both within and outwith the QRDR of the topoisomerase genes were described. Mutations outside the QRDR of *gyrA* at codons, Ala131, Glu139 and Asp144 have been described (Eaves *et al*, 2002) in addition to mutations within the QRDR regions of the *parC* and *parE* genes (Casin *et al*, 2003; Ling *et al*, 2003). These mutations, and those of *gyrB* are frequently isolated second to either one or two *gyrA* mutations and are therefore considered second-step mutations thought to be required before high-level fluoroquinolone resistance can be achieved (Hansen & Heisig, 2003; Baucheron *et al*, 2004). Some of the mutations to date are listed in **Table 7:1**.

Table7:1. Some of the mutations previously detected in the topoisomerase genes of *Salmonella* isolates

Gene	Amino acid substitution	Reference[a]
gyrA	Ala67→Pro	Reyna, 1995
	Asp72→Gly	Eaves, 2002
	Val73→Ile	Eaves, 2004
	Gly81→Ser	Reyna, 1995
	Gly81→Cys	Yoshida, 1990
	Gly81→Asp	Piddock, 1998
	Gly81→His	Lindstedt, 2004
	Asp82→Gly	Allen, 2002
	Asp82→Asn	Eaves, 2002
	Ser83→Phe	Casin, 2003
	Ser83→Tyr	Eaves, 2002
	Ser83→Ala	Levy, 2004
	Asp87→Asn	Casin, 2003
	Asp87→Tyr	Eaves, 2002
	Asp87→Gly	Eaves, 2002
	Asp87→Lys	Miró, 2004
	Leu98→Val	Eaves, 2004
	Ala119→Gly	Eaves, 2002
	Ala119→Ala	Eaves, 2002
	Ala131→Gly	Eaves, 2002
	Glu139→Ala	Eaves, 2002
gyrB	Tyr420→Cys	Eaves, 2004
	Arg437→Leu	Eaves, 2004
	Ser464→Tyr	Gensberg, 1995
	Ser464→Phe	Casin, 2003

Gene	Amino acid substitution	Reference[a]
parC	Tyr57→Ser	Ling, 2003
parC	Thr66→Ile	Eaves, 2004
	Gly78→Asp	Hansen, 2003
	Ser80→Arg	Casin, 2003
	Ser80→Ile	Baucheron, 2002
	Glu84→Lys	Casin, 2003
	Glu84→Gly	Miró, 2004
parE	Glu453→Gly	Eaves, 2004
	Ser458→Pro	Ling, 2003
	His461→Tyr	Eaves, 2004
	Ala498→Thr	Eaves, 2004
	Val512→Gly	Eaves, 2004

[a] Reference list is not exhaustive

Other mechanisms of resistance to have been identified include decreased accumulation mediated by the active efflux pump AcrAB which uses the outer membrane protein TolC as an extrusion channel with one study showing that the quinolone resistance and decreased fluoroquinolone susceptibility of the strains studied were highly dependent on the AcrAB-TolC efflux system and that the single *gyrA* mutations had little effect in determining the resistance levels (Baucheron *et al*, 2004); and plasmid-mediated quinolone resistance (PMQR). New variants of the gene *qnr* have been described and a website at the Lahey Clinic in the U.S.A has been set up to keep track as new *qnr* sequences are published (http://www.lahey.org/qnrStudies).

One hundred and eighty strains of *Salmonella enterica* were characterised using plasmid profile analysis and PFGE. These techniques performed in isolation give limited information about the strains being examined. It is useful to know that strains harbour multiple plasmids as this may prompt further investigations such as antimicrobial resistance determination but, if the strains only possess the serotype-associated plasmid (SAPs-as discussed in Chapter 2) then this knowledge may be of less value. This also holds true for PFGE. Standardisation of protocol and the enhanced analysis software now available helps generate banding patterns that are both replicable and easily analyzed; and while a unique pulsed field profile reflects genomic diversity; certain serotypes such as *S.* Enteritidis are clonal and very often result in indistinguishable pulsed field profiles.

Seventy-eight strains of *S.* Enteritidis were examined; forty-four possessed a single plasmid of approximately 57kb. Since this is the approximate size of the serotype associated plasmid (SAP), the 57kb plasmid of this study will be referred to as the SAP. Thirty-four of these strains had the most common *S.* Enteritidis pulsed field profile (PFP) of SENTXB.0001, with a further twelve strains with the same PFP harbouring an

additional plasmid of approximately 2.1kb. In this case the additional plasmid has had no obvious effect on the PFP. In the instance of the SAP being absent from the strain, as is the case for nine of the *S.* Enteritidis examined, the PFP changes to SENTXB.0014 were due to a single band deletion (this being the approximate size of 57kb). The PFP can also be different independent of possession of the 57kb plasmid. Nine strains possessed the SAP yet had PFPs other than SENTXB.0001. This is an example of the benefits of pairing these two genotypic methods together to fully characterize the strains so that in subsequent experiments, these profiles can be used as reference standards. While these data provided details of genetic relationship, there was no information about the phenotypic resistance levels to quinolone or fluoroquinolone antimicrobials, therefore investigations into the MICs were performed using an agar dilution method.

In 1990, 2% of all human, veterinary and environmental isolates of *Salmonella enterica* received by the Scottish Salmonella Reference Laboratory (SSRL) were found to be resistant to nalidixic acid at a concentration of 40mg/L. During 1997, the laboratory introduced the testing of strains for cross-resistance to low-level ciprofloxacin at a concentration of 0.125mg/L, by which time the levels of nalidixic acid resistance had risen to 4.2%. Of these, 51.5% were cross-resistant to low-level ciprofloxacin. By 2000-01, nalidixic acid resistance was demonstrated in 392 isolates, with 88% of these resistant to low-level ciprofloxacin. Since 2003, any isolate found to be resistant to nalidixic acid at a concentration of 40mg/L and/or low-level ciprofloxacin at a concentration of 0.125mg/L, has been subjected to Etests to determine the ciprofloxacin MIC.

Diagnostic Sensitivity agar (DST) or Mueller Hinton agar is the preferred basal medium for the determination of MIC in some laboratories particularly in the United States (Frost, 1994); therefore investigations to determine MICs were performed using DST agar also in

accordance with SSRL standard operating procedures. The fluoroquinolone MIC results, listed in Table 3:1, obtained for the NCpL and MDR strains in particular, were inconsistent with the breakpoint designations assigned to them and therefore further investigation was warranted. No literature could be found advising against the use of DST with fluoroquinolones. On the contrary, the International Centre for Science and High Technology (ICS) recommended using DST in agar diffusion susceptibility tests of norfloxacin (Grimm, 1983) but information from BSAC suggested that the high concentration of magnesium and calcium ions present in DST may have an inhibitory effect on the fluoroquinolones resulting in raised MICs (Dr. Jennifer Andrews, personal communication). Previous studies used Mueller Hinton agar when determining the MIC values for quinolone and fluoroquinolones (Giraud *et al*, 2003; Kumar, 2002). Therefore, the MIC by agar dilution method was determined on Mueller Hinton agar for a random selection of fifty-five of the strains previously examined.

These results confirmed the breakpoint designations for the FS strains as being fully susceptible to nalidixic acid and the four fluoroquinolones tested. With the exception of a single strain of *S*. Typhimurium DT3 (MIC of 16mg/L), all of the other strains were confirmed as resistant to nalidixic acid with MICs of ≥ 128mg/L. The NCpL strains demonstrated reduced susceptibility to ciprofloxacin and the three other fluoroquinolones.

The majority of FQR strains did not confirm the breakpoint designation in that they exhibited only reduced susceptibility to ciprofloxacin and moxifloxacin; however resistance to ofloxacin and norfloxacin was confirmed. The breakpoint designations of the MDR strains were also not corroborated for ciprofloxacin. As with the FQR strains, reduced susceptibility was demonstrated for ciprofloxacin, but the majority were resistant to the other fluoroquinolones tested.

Cross-resistance to fluoroquinolones is a well documented phenomenon in multi-drug resistant isolates. One study demonstrated that mutants selected with tetracycline or chloramphenicol were cross-resistant to norfloxacin and ciprofloxacin. (Cohen *et al*, 1989). In that study norfloxacin MICs were higher than those of ciprofloxacin and overall resistance of the mutants was attributed to decreased drug accumulation due to outer membrane changes and loss of OmpF. Since the MICs of norfloxacin were also higher than those of ciprofloxacin, it may be that the mechanism of resistance of the MDR strains is also decreased drug accumulation. Later studies have confirmed that active efflux pumps such as the AcrAB-TolC system are responsible for decreased susceptibility to fluoroquinolones and that their increased expression may serve as an initial step in allowing the isolate to survive environmental exposures to fluoroquinolone selection pressures (Chen *et al*, 2007).

None of the 31 strain that were resistant to high-level ciprofloxacin by breakpoint method exhibited resistance to ciprofloxacin at EUCAST values. These results reflect the findings of a recent study which suggested that high-level fluoroquinolone resistance is rare in clinical strains of *Salmonella enterica* isolated in Scotland (Murray *et al*, 2005), however all 31 were resistant to at least one of the other fluoroquinolones tested, which reinforces the need for surveillance of these antimicrobials in the treatment of *Salmonella* infections.

Nalidixic acid-resistance has been extensively used as an indicator of decreased fluoroquinolone susceptibility in clinical isolates of *Salmonella* (Hakanen *et al*, 1999) and the MIC data from this study appear to confirm this. While there was a consistent relationship between the nalidixic acid MICs and those of the fluoroquinolones studied in that those strains resistant to nalidixic acid exhibited at least reduced susceptibility to the fluoroquinolones, all of the MIC values for high-level ciprofloxacin indicated that the

assigned breakpoint designations were incorrect. This would corroborate the data that were demonstrated in the original agar dilution experiment, where the MICs for all the strains examined were very much higher than expected, which lead to the conclusion that DST agar is inappropriate for the sensitivity testing of fluoroquinolones. It appears to give accurate results for low-level ciprofloxacin but at higher concentrations the agar in some way inhibits the action of the antimicrobial, resulting in inflated MIC. This is also applicable to the sensitivity testing by breakpoint method that is employed by the SSRL, in that the breakpoint designations would result in false positives at the high-level ciprofloxacin concentrations.

On the basis of these findings the SSRL has changed the agar used in its breakpoint method for sensitivity testing and now uses Isosensitest agar.

Comparison between the MIC values for these antimicrobials and the source of the *Salmonella* isolate does not reveal any significant correlation between the two. MIC values were generally identical or within one doubling dilution on Mueller Hinton agar when isolates from different sources were compared within the same R-type category.

These MIC results for nalidixic acid and the fluoroquinolones complete the characterization of the strains of *Salmonella* by both the phenotypic methods of serotyping and phage typing and genotypic typing methods of plasmid profile analysis and PFGE. There is no apparent relationship between either the plasmid- or the pulsed field profiles and the MIC results, nor do they give any indication as to the mechanism of resistance.

In order to investigate the mechanism of resistance, this experiment (see chapter 4) was performed from pure cultures that were assumed to be populations of identical cells. It is possible that through spontaneous mutation, some cells within a susceptible population

acquire slightly elevated resistance to an antimicrobial even in the absence of the drug. Without selection pressure these mutants may co-exist within the larger susceptible population to the point of being undetected during routine sensitivity testing. Once selection pressure is applied by exposing the culture to an antimicrobial, these mutants are able to out-compete the susceptible cells which are inhibited whilst the mutants grow and multiply. In this instance it is useful to assess the presence of spontaneous mutants within ostensibly susceptible populations of *Salmonella* under the selection pressure of different fluoroquinolones and the frequency with which these mutants are selected.

Spontaneous mutants were selected on ofloxacin, norfloxacin and moxifloxacin, but no mutants were recovered on ciprofloxacin. Four strains of *S.* Typhimurium, one of *S.* Dublin and one of *S.* Virchow all yielded mutants with MICs more than twice those of their wild-type. The frequency with which these mutants were selected varied between $4x10^{-9}$ and $1x10^{-6}$ for the six strains; there was little difference between the rates of selection of the three fluoroquinolones (**Table 4:1**). Previous studies have suggested that it can be difficult to achieve an accurate assessment of mutation frequencies as many parameters such as antimicrobial agent, concentration of antimicrobial, bacterial stress and physiological conditions among others, need to be factored into the experiment (Martinez, 2000); one study reports that spontaneous mutants arise at the low frequency of 1 in 10^6 to 1 in 10^8 cells (Zhao & Drlica, 2001). Two of the strains exhibited lower frequencies than this but this may be due to the previously-mentioned difficulties such as concentration of antimicrobial, physiological conditions, bacterial stress etcetera.

Each of the spontaneous mutants examined in this experiment resulted in a single *gyrA* mutation (**Table 4:2**), which have been previously described (Griggs, *et al* 1996; Eaves *et al*, 2002). The resulting increases in MICs of these mutants, although demonstrative of

reduced susceptibility, are not highly resistant. It therefore seems reasonable to conclude that these are all first-step mutations. This may also explain the failure to select any spontaneous mutants on ciprofloxacin as previous studies have suggested that double mutations in *gyrA* or one additional mutation in another of the topoisomerase genes are required before ciprofloxacin resistance can be detected (Casin *et al*, 2003; Ling *et al*, 2003).

It was not possible to select any mutants exhibiting reduced susceptibility to ciprofloxacin from the majority of serotypes examined, including *S*. Enteritidis. This may be due to the selecting concentration of fluoroquinolones being too high as high-level fluoroquinolone resistance in clinical isolates of *Salmonella* is uncommon (Giraud *et al*, 2006); alternatively it may be that fluoroquinolone resistance mechanisms have a prohibitive fitness cost and thus limit the emergence of resistant strains (Lindgren *et al*, 2005; Giraud *et al*, 2006). This may also explain why there are so few published reports regarding spontaneous fluoroquinolone-resistant mutants.

The overall result of the inability to select spontaneous fluoroquinolone resistant mutants from the majority of strains coupled with the relatively low frequencies of those that were selected reinforces the previous conclusion that there was little fluoroquinolone resistance in strains of *Salmonella* isolated in Scotland in the period of this study.

To determine the capacity of the fluoroquinolones to select resistant mutants, fluoroquinolone-resistant mutants were selected by exposing susceptible isolates to numerous cultures on subinhibitory concentrations of antimicrobial and determining any accompanying decrease in susceptibility (see Chapter 5). As has been previously discussed, high-level fluoroquinolone resistance is rare in strains of *Salmonella* isolated in Scotland however; elsewhere treatment failures have been reported to occur during ongoing treatment with these drugs (Piddock *et al*, 1990; Boswell *et al*, 1997). Although an

in vitro experiment cannot replicate *in vivo* conditions accurately, these experiments attempted to detect and characterize the initial decrease in susceptibility of the isolate. The susceptible strains were exposed to subinhibitory concentrations of fluoroquinolones in two ways. Firstly, by exposure along an increasing concentration gradient of antimicrobial where only the growth at the highest concentration end was selected for further examination and secondly, by exposure to subinhibitory concentrations of antimicrobial where the entire plate population was repeatedly cultured on successive days. The former method only selected mutants with the highest resistance as they were taken from the growth at the highest concentration but the latter method repeatedly exposed the entire population to subinhibitory concentrations of the antimicrobial, thus potentially selecting mutants with only slightly reduced susceptibility in addition to the more resistant mutants.

One difference between these results and those of the previous experiment was that mutants were selected on ciprofloxacin. It may be that the lower concentrations of ciprofloxacin the strains were exposed to in these experiments, while inhibiting the susceptible cells, allowed mutants with slightly decreased susceptibilities to grow and increase resistance gradually whereas the higher concentrations of ciprofloxacin (0.5mg/L) used in the previous experiment may have been too high and inhibited the entire cell population.

From the 12 mutants examined only 5 *gyrA* mutations were detected (Table 5:4); with one, Asp87→Gly, being selected for the first time in this study although this has, like the other mutations, been described previously (Eaves *et al*, 2002). As in the previous section, only a single mutation was determined in each of the mutants. Although noteworthy increases in MIC were determined, none reached high-level resistance. Once again, first-step mutations appear to have been detected, although in the case of the mutants of *S*. Typhimurium

RDNC, an MIC of 1mg/L suggests that perhaps an additional mechanism of resistance such as an increase in active efflux, which has been shown to be a primary mechanism of resistance to ciprofloxacin in strains of *S.* Typhimurium (Giraud *et al*, 2000), may be involved.

The Thr57→Ser mutation detected in the *S.* Virchow mutant was the only *parC* mutation detected in any of the experiments. This is somewhat unusual in that *parC* mutations are usually second-step mutations in the acquisition of high-level resistance to fluoroquinolones in Gram-negative organisms (Bachoual *et al*, 1998) or first-step mutations in Gram-positive organisms. Ling *et al* found that the presence of Thr57→Ser in addition to a *gyrA* mutation resulted in higher resistance (Ling *et al*, 2003) and a previous study demonstrated that transformation of *parC* mutants with wild-type *parC* resulted in a partial reversal of ciprofloxacin resistance. It has also been reported that this single mutation in an *in vitro* mutant had no effect on the quinolone susceptibility of the resulting strain; although Bagel *et al* postulated that Thr57→Ser is a stabilizing mutation that allows high-level fluoroquinolone resistance to develop but does not itself confer a higher level of resistance (Bagel *et al*, 1999). In this instance the MIC of the mutant was determined to be 0.38mg/L, an increase from 0.016mg/L for the wild-type strain which suggests that, in the absence of a *gyrA* mutation, the Thr57→Ser mutation is a least partly responsible for the decrease in fluoroquinolone susceptibility exhibited by this mutant.

No correlation between the mutation selected and the fluoroquinolone on which it was selected could be found as, with the exception of Asp82→Asn which was selected only once on ofloxacin, more than one fluoroquinolone selected for the same mutation. Similarly, increased MIC could not be correlated to any one of the antimicrobials as different fluoroquinolones selected for the same mutation which resulted in the same

increase in MIC. Only the identical Ser83→Phe mutations selected on norfloxacin in a strain of *S.* Typhimurium DT104 and a strain of *S.* Dublin resulted in different increases in MIC; the MIC of the *S.*Typhimurium mutant was more than double that seen in the *S.* Dublin mutant.

Mutations at codon Asp87 were the most commonly detected but these resulted in three different amino acid changes amongst three different serotypes.

Overall, the frequently isolated serotypes of *S.* Enteritidis and *S.* Typhimurium developed a decrease in susceptibility to ciprofloxacin far more readily than less frequently isolated serotypes such as *S.* Kottbus, exhibiting increased MICs of 1mg/L and 1.5mg/L, with the latter only demonstrating an increased MIC of 0.25mg/L.

Plasmid-mediated mechanisms have resulted in resistance to most classes of clinically important antimicrobials, such as the sulphonamides, aminoglycosides and β-lactams (Normark & Normark, 2002); and in 1998, the 56kb plasmid pMG252 was implicated in the first report of plasmid-mediated resistance to quinolones. It was 2002 before the complete molecular mechanism of *qnr*-mediated resistance was determined. The 218 amino acid protein product of the gene *qnr* belonged to the pentapeptide repeat family (Tran & Jacoby, 2002). *In vitro* studies demonstrated that purified Qnr protected *E. coli* DNA gyrase from inhibition by ciprofloxacin, by binding to gyrase holoenzyme and its respective subunits GyrA and GyrB. This protection was shown to be proportional to Qnr concentration and inversely proportional to the concentration of ciprofloxacin (Tran & Jacoby, 2002).

qnr itself confers little resistance and strains carrying pMG252 would be classed as fluoroquinolone-susceptible (Rodríguez-Martínez *et al*, 2003); nevertheless its clinical importance lies in the fact that it facilitates the selection of higher level quinolone

resistance mutations that allow isolates to survive concentrations of antimicrobial that would be otherwise be inhibitory.

Early studies suggested that *qnr* plasmids might be limited and remain uncommon (Jacoby *et al*, 2003; Rodriguez-Martinez *et al*, 2003) however this has proved incorrect. The incidence of *qnr*-mediated resistance has continued to increase worldwide (Cheung *et al*, 2005; Jonas *et al*, 2005; Murray, 2008; Karlsson *et al*, 2009).

Previous studies have examined isolates resistant to nalidixic acid and reduced susceptibility to fluoroquinolones for *qnr*. However this study investigated a more unusual resistance phenotype- nalidixic acid susceptible isolates with reduced susceptibility to fluoroquinolones. This R-type has been previously described (Monoit-Ville *et al*, 1991; Hakanen *et al*, 2005) but until now, never investigated for the presence of *qnr* (see Chapter 6).

Seventy strains of *Salmonella* with reduced susceptibility to ciprofloxacin and nalidixic acid resistance (n=53) and resistance to cefotaxime (1mg/L) (n=17) were investigated for the presence of *qnr*. Thirty-four strains comprising 12 serotypes were found to harbour the genes; *qnrS1* (28), *qnrA1*(2), *qnrB1*(1), *qnrB2* (2) and *qnrB5* (1); of these serotypes, 7 have not previously been found to harbour *qnr* genes (Table 6:1). With the exception of one environmental and 3 veterinary isolates, all were of human origin, 58% of which were isolated from patients with a history of foreign travel.

The high incidence and wide range of serotypes harbouring *qnr* genes in this study confirmed that the prevalence of plasmid-mediated quinolone resistance is increasing. Recent studies have shown that new *qnr* genes, such as *qnrB10, qnrB12* and *qnrD,* are being continually described (Quiroga *et al*, 2007; Kehrenberg *et al*, 2008; Cavaco *et al*, 2009), with the most recent *qnrS4*, being described by Danish scientists (Torpdahl *et al*,

2009).

In addition to these new *qnr* genes, other plasmid-mediated resistance determinants have recently been described. *aac*(*6'*)-*Ib*-cr is a variant of the gene encoding an aminoglycoside acetyltransferase *aac*(*6'*)-*Ib* that is responsible for resistance to the aminoglycosides kanamycin, amikacin and tobramycin. The enzyme reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substituent (Robicsek *et al*, 2006). As in the case of *qnr*, the level of resistance conferred is small but has been shown to act additively with other plasmid-mediated resistance mechanisms such as *qnr*, and also facilitates the selection of more resistant mutants among a fluoroquinolone-exposed population of bacteria (Robicsek *et al*, 2006).

The *qepA* gene was identified in a plasmid, pHPA (Yamane *et al*, 2007), which encodes a 14-transmembrane-segment putative efflux pump that increases resistance to norfloxacin and ciprofloxacin and enrofloxacin by decreasing the accumulation of antimicrobial in the bacterial cell, whilst the MIC of other antimicrobials and dyes known to be substrates for efflux pumps remained unchanged (Périchon *et al*, 2007).

Previous experiments in this investigation have demonstrated that high-level resistance to fluoroquinolones was rare in strains of *Salmonella* isolated in Scotland from 1990-2000, however these new plasmid-mediated mechanisms of resistance which are easily transmissible, may pose the biggest threat to the future of this class of antimicrobial.

7:2 Limitations of this study

Despite several attempts, I was unable to gain access to the veterinary antimicrobial enrofloxacin. It would have been useful to include this fluoroquinolone in addition to the other fluoroquinolones used in the various investigations of this study. The results may have helped gain an insight into how the use of fluoroquinolones in veterinary medicine impacts on human medicine.

The number of mutations selected by either examining the entire population for spontaneous mutants or those selected upon exposure to subinhibitory concentration of fluoroquinolone were fewer than expected when considering the increased MIC of the mutant compared to the wild-type. It would therefore have been useful to examine all of the topoisomerase genes to determine whether any second-step mutations were present in *gyrB or parE*. It may also have been useful to examine the selected mutants for any increase in the levels of efflux to determine whether this was a factor in the increases in MIC determined. This would be especially important for those mutants with elevated MICs but no mutations detected in either the *gyrA* or *parC* genes. It would have helped if the mechanism of resistance in the MDR and FQR strains had been determined as this would have provided a means of comparison between mutants selected *in vivo* and those selected in this study.

Further investigations into the Thr57→Ser amino acid substitution detected in the *parC* gene of the strain of *S.* Virchow might confirm whether or not it was responsible for the increase in MIC exhibited by the mutant.

It may have been useful to perform growth studies on the mutants selected, to determine whether or not there was a fitness cost of increased resistance to fluoroquinolones.

Further investigations may have been made into the incidence of *qnr* genes in strains exhibiting resistance to nalidixic acid as many studies have done previously. This may have provided a more accurate assessment of the incidence of *qnr* in strains of *Salmonella enterica* in Scotland.

7:3 Final conclusions

The increasing reports of treatment failures with fluoroquinolones and the discoveries of new mechanisms of resistance, suggested that an investigation into the incidence of resistance in strains of *Salmonella enterica* isolated in Scotland was urgently required.

Comparison between MIC results obtained on DST agar and Mueller Hinton agar revealed that DST agar is unsuitable for use with fluoroquinolones. Determination of the MIC values of nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin and moxifloxacin for the strains examined demonstrated that although no clinical resistance to ciprofloxacin was present in any of the R-types selected, reduced susceptibility was exhibited by all but the FS group. Resistance to the other three fluoroquinolones was confined to strains exhibiting multi-drug resistances or those designated as resistant to 0.5mg/L ciprofloxacin by breakpoint method of susceptibility testing.

The isolates of *S*. Mbandaka all thought to be of clonal origin (see Chapter 2), exhibited MIC values within one doubling dilution, however plasmid and pulsed field profiles did not indicate any significant correlation between genotype, MIC value and source of isolate for any of the other serotypes studied.

Spontaneous fluoroquinolone-resistant mutants were selected for the serotypes *S.* Typhimurium and *S.* Dublin on each of the four fluoroquinolones, while only ciprofloxacin was capable of selecting for resistant mutants of *S.* Enteritidis. This contrasts with the population analysis study where ciprofloxacin failed to select any mutants. This suggests that resistance to ciprofloxacin requires more than the single first-step mutations characterized by sequence analysis of the mutants selected on the other three fluoroquinolones; and that these three fluoroquinolones, especially ofloxacin which selected mutants from different serotypes from different sources in the population analysis study, may be more capable at selecting for mutants during treatment.

There was no correlation between the mutation selected and the fluoroquinolone used. The increased MIC could not be correlated to any one of the antimicrobials as different fluoroquinolones selected for the same mutation which resulted in the same increase in MIC.

As spontaneous fluoroquinolone-resistant mutants were selected from less than 50% of the FS strains, it seems reasonable to conclude that the capacity to develop resistance to fluoroquinolones is not equal in all serotypes.

The prevalence of *qnr* genes was higher in strains of *Salmonella enterica* isolated in Scotland than had been demonstrated in previous studies in other parts of the world. There is also a wider distribution of these genes among different serotypes than has been previously demonstrated.

This study has demonstrated that for the period investigated, fluoroquinolone resistance in strains of *Salmonella enterica* isolated in Scotland reflected the findings of similar studies from other parts of the world in that, although resistance to nalidixic acid was common and

reduced susceptibility to fluoroquinolones was linked to this resistance, clinical resistance to fluoroquinolone was rare. With the relatively recent advent of plasmid-mediated quinolone resistance, this may not apply for much longer and surveillance of this class of antimicrobial is needed more than ever.

Appendices

Appendix I: Original breakpoint designations

Strains termed “MDR”

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19903865	Human	Sporadic	S.Bovismorbificans	-	S		R	R	S	S	S	R	S	S	S	S	R	S	
19910748	Human	Sporadic	S.Virchow	-	R		S	R	R	S	S	R	S	R	R	R	R	R	
19932001	Human	Sporadic	S.Oranienburg	-	R		R	R	S	S	R	R	R	R	S	R	R	R	
19932300	Human	Sporadic	S.Hadar	-	S		S	R	S	S	S	R	S	S	R	S	R	S	
19933344	Human	Sporadic	S.Typhimurium	204c	R		R	R	S	S	R	R	S	R	R	R	R	R	
19935442	Human	Sporadic	S.Hadar	-	S		S	R	S	S	S	R	S	S	R	R	R	S	
19940240	Human	Sporadic	S.Hadar	-	S		S	R	S	S	S	R	S	S	R	S	R	S	
19941463	Environmental		S.Typhimurium	204	R		R	R	S	S	R	R	S	S	R	R	R	R	
19950004	Human	Sporadic	S.Typhimurium	2	S		S	R	S	S	S	R	S	S	R	S	S	R	
19953743	Human	Sporadic	S.Virchow	-	R		S	R	R	S	S	R	S	S	S	S	S	S	

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19963399	Human	Sporadic	S.Brandenburg	-	S		R	R	R	S	R	R	S	S	S	S	R	S	
19966072	Bovine		S.Typhimurium	104b	R		R	R	S	S	S	R	S	S	S	R	S	R	
19970646	Human	Sporadic	S.Typhimurium	104	R		R	R	S	S	S	R	S	R	R	R	R	S	
19970917	Human	Sporadic	S.Typhimurium	104	R		R	R	S	S	S	R	S	R	R	R	R	S	R
19972895	Environmental		S.Typhimurium	104	R		R	R	S	S	S	R	S	R	R	R	R	S	R
19982602	Poultry		Monophasic group C1 Salmonella	-	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
19990031	Human	Sporadic	S.Typhimurium	104	R	S	S	R	S	S	S	R	S	R	R	R	S	R	R
19990095	Human	Sporadic	S.Hadar	-	S	S	S	R	S	S	S	R	S	S	R	S	R	S	R
19990515	Human	Sporadic	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	R	R	S	R
19990807	Not stated		S.Enteritidis	8	R	S	R	R	S	S	S	R	S	S	S	R	S	S	R
19990838	Human	Sporadic	S.Blockley	-	S	S	R	R	S	S	R	R	S	S	R	S	R	S	R
19990898	Poultry		S.Mbandaka	-	S	S	S	R	S	S	S	R	S	S	R	S	S	S	R
19990970	Poultry		S.Mbandaka	-	S	S	S	R	S	S	S	R	S	S	S	S	R	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19990983	Poultry		S.Virchow	-	S	S	S	R	S	S	S	R	S	S	S	R	S	R	R
19991026	Human	Tunisia	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R
19991711	Human	Egypt	S.Blockley	-	S	S	S	R	S	S	R	R	S	S	R	S	R	S	R
19991746	Poultry		S.Thompson	-	S	S	S	R	S	S	S	R	S	S	S	R	S	S	R
19991815	Human	Sporadic	S.Enteritidis	1	R	S	R	R	S	S	S	R	S	S	S	S	S	S	R
19991825	Pheasant		S.Enteritidis	4	S	S	S	R	R	S	S	R	S	S	S	S	S	R	R
19992689	Human	Sporadic	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R
19992820	Human	Spain	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	R	R	S	R
19993033	Human	Sporadic	S.Enteritidis	4	S	S	S	R	S	S	S	R	S	S	S	R	S	S	R
19993048	Human	Sporadic	S.Indiana	-	S	S	S	R	R	S	R	R	S	R	R	R	R	R	R
19995017	Human	Sporadic	S.Enteritidis	RDNC	S	S	S	R	S	S	S	R	S	R	R	S	R	R	R
20000283	Poultry		S.Mbandaka	-	S	S	S	R	S	S	S	R	S	S	S	S	S	R	R
20000673	Poultry		S.Senftenberg	-	S	S	S	R	S	S	R	R	S	S	S	S	S	S	R
20000721	Poultry		S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20000918	Human	Sporadic	S.Typhimurium	104b	R	S	R	R	S	S	S	R	S	R	R	R	R	S	R
20001058	Poultry		S.Mbandaka	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R
20002014	Human	Sporadic	S.Typhimurium	104b	R	S	R	R	S	S	S	R	S	R	R	R	R	S	R
20002058	Human	Sporadic	S.Typhimurium	104b	R	S	S	R	S	S	S	R	S	S	R	R	S	R	R
20002113	Human	Sporadic	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20002257	Human	Sporadic	S.Typhimurium	RDNC	R	S	R	R	S	S	R	R	S	R	R	R	R	R	R
20002399	Poultry		S.Hadar	-	S	S	S	R	S	S	S	R	S	S	R	S	R	S	R
20002427	Poultry		S.Java	- S		S	S	R	S	S	S	R	S	R	R	S	S	R	R
20002563	Environmental		S.Mbandaka	-	S	S	S	R	S	S	S	R	S	S	R	S	S	S	R
20003106	Human	Spain	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	R	R	S	R
20004010	Human	Sporadic	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R
20004028	Human	Spain	S.Hadar	-	R	S	S	R	S	S	S	R	S	S	R	S	R	S	R
20004661	Environmental		S.Mbandaka	-	S	S	S	R	S	S	S	R	S	S	S	R	S	R	R

Strains termed “FQR”

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19900377	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19900497	Poultry		S.Enteritidis	RDNC	S		S	R	S	S	S	R	S	S	S	S	S	S	
19900633	Poultry		S.Enteritidis	RDNC	S		S	R	S	S	S	R	S	S	S	S	S	S	
19900813	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19900828	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901024	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901059	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901127	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901135	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901221	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901257	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901259	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19901289	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901301	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901442	Poultry		S.Enteritidis	RDNC	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901527	Poultry		S.Enteritidis	RDNC	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901602	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901733	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901754	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19901928	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19902644	Human	Sporadic	S.Typhimurium	66	S		S	R	S	S	S	R	S	S	S	S	S	S	
19903116	Human	Sporadic	S.Enteritidis	RDNC	S		S	R	S	S	S	R	S	S	S	S	S	S	
19903993	Poultry		S.Enteritidis	Untypable	S		S	R	S	S	S	R	S	S	S	S	S	S	
19910646	Poultry		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19910665	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19911165	Poultry		S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19911537	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19912067	Poultry		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19921643	Poultry		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19933184	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19933339	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19933488	Environmental		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19934878	Poultry		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19935599	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19940784	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19940871	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19940880	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19941804	Poultry		S.Mbandaka		S		S	R	S	S	S	R	S	S	S	S	S	S	
19941864	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	
19943026	Human	Sporadic	S.Enteritidis	4	S		S	R	S	S	S	R	S	S	S	S	S	S	

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19964025	Human	Sporadic	S.Enteritidis	5a	S		S	R	S	S	S	R	S	S	S	S	S	S	
19965081	Human	Sporadic	S.Enteritidis	1	S		S	R	S	S	S	R	S	S	S	S	S	S	
19970269	Human	Sporadic	S.Enteritidis	7	S		S	R	S	S	S	R	S	S	S	S	S	S	
19990976	Poultry		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
19991308	Human	Sporadic	S.Enteritidis	4	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
19991700	Human	Spain	S.Enteritidis	4	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
19991722	Human	Gran Canaria	S.Enteritidis	4	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
19992252	Human	Sporadic	S.Enteritidis	1	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
19993983	Poultry		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20000635	Poultry		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20000821	Environmental		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20001054	Poultry		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20002524	Environmental		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20002550	Environmental		S.Mbandaka		S	S	S	R	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20004300	Human	Tenerife	S.Enteritidis	4	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20004594	Human	Sporadic	S.Enteritidis	1	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20004768	Feline		S.Enteritidis	1	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20005212	Human	Sporadic	S.Enteritidis	1	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R
20005300	Human	Sporadic	S.Enteritidis	1	S	S	S	R	S	S	S	R	S	S	S	S	S	S	R

Strains termed “NaCpL”

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19982854	Poultry		S.Liverpool		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19982886	Poultry		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19983032	Poultry		S.Senftenberg		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19983922	Canine		S.Binza		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19984026	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19990203	Poultry		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19990355	Canine		S.Newport		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19990380	Poultry		S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19990905	Human	Spain	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991305	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991378	Human	Family outbreak	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991459	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991601	Poultry		S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19991605	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991655	Human	Mediterranean	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991657	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991668	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991697	Human	Sporadic	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991754	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991760	Human	Diarrhoea since T in the Park	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991817	Human	Majorca	S.Enteritidis	7	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991915	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19991939	Human	Turkey	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992003	Human	Sporadic	S.Kottbus		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992070	Human	Spain	S.Hadar		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992196	Human	Family outbreak	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19992245	Human	North Africa	S.Enteritidis	Untypable	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992327	Human	Malta	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992365	Poultry		Non-motile group C4 Salmonella		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992434	Human	Majorca	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992465	Poultry		Non-motile group C4 salmonella		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992491	Poultry		S.Senftenberg		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992678	Human	Sporadic	S.Infantis		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992835	Poultry		S.Typhimurium	3	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19992934	Human	Sporadic	S.Hadar		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993007	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993056	Bovine		S.Dublin		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993084	Environmental		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993085	Environmental		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19993086	Poultry		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993115	Human	Sporadic	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993156	Human	Turkey	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993222	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993411	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993539	Human	Sporadic	S.Enteritidis	Untypable	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993553	Poultry		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993731	Poultry		S.Senftenberg		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993739	Human	Unknown	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993787	Human	Sporadic	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993795	Human	Spain	S.Enteritidis	RDNC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19993807	Environmental		S.Liverpool		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19994202	Poultry		S.Senftenberg		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19994385	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19994455	Human	Gran Canaria	S.Typhimurium	104b	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
19994609	Human	Morocco	S.Enteritidis	14b	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20000139	Bovine		S.Dublin		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20000466	Environmental		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20000672	Poultry		S.Liverpool		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20000923	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20001314	Human	Family outbreak	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20001505	Human	Lanzarote	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20001526	Human	Thailand	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002001	Human	Family outbreak	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002138	Human	Sporadic	S.Virchow		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002195	Human	Morocco	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20002216	Human	Spain	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002238	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002261	Human	South Africa	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002298	Environmental		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002308	Environmental		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002439	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002542	Environmental		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002590	Human	Sporadic	S.Enteritidis	RDNC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002618	Human	Family outbreak	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002621	Human	Sporadic	S.Enteritidis	6	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002660	Human	Ibiza	S.Enteritidis	1c	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002675	Human	Sporadic	S.Enteritidis	Untypable	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20002873	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20003149	Human	Turkey	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003165	Human	Family outbreak	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003184	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003270	Human	Lanzarote	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003558	Human	Sporadic	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003586	Human	Lanzarote	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003659	Human	Sporadic	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003924	Human	Gran Canaria	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20003957	Human	Sporadic	S.Kottbus		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004057	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004078	Human	Ibiza	S.Enteritidis	3	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004230	Human	Morocco	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004338	Human	Spain	S.Enteritidis	1c	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20004342	Human	Sporadic	S.Enteritidis	6a	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004592	Human	Sporadic	S.Enteritidis	RDNC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004730	Human	Lanzarote	S.Enteritidis	21	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004763	Human	Family outbreak	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004915	Environmental		S.Mbandaka		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004927	Human	Tenerife	S.Enteritidis	Untypable	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004928	Human	Tenerife	S.Antarctica		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004947	Poultry		S.Senftenberg		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20004972	Human	Spain	S.Enteritidis	RDNC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20005031	Human	Tenerife	S.Enteritidis	7	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20005084	Human	Java	S.Enteritidis	4	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20005141	Human		S.Enteritidis	Untypable	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R
20005226	Human	Lanzarote	S.Enteritidis	1	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20005319	Poultry		S.Thompson		S	S	S	S	S	S	S	R	S	S	S	S	S	S	R

Strains termed “FS”

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19982543	Poultry		S.Enteritidis	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19983155	Human	Sporadic	S.Dublin		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19983351	Canary		S.Typhimurium	RDNC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19993458	Human	Sporadic	S.Typhimurium	2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19983717	Human	Sporadic	S.Virchow		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19990341	Human	Family outbreak	S.Typhimurium	104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19990706	Poultry		S.Mbandaka		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19991035	Porpoise		S.Typhimurium	104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19991073	Human	Family outbreak	S.Brandenburg		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19991452	Pheasant		S.Binza		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19991870	Human	Sporadic	S.Enteritidis	5c	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
19991929	Human	Sporadic	S.Enteritidis	1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19992150	Human	Sporadic	S.Enteritidis	6a	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19993805	Bovine		S.Dublin		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19993849	Human	Sporadic	S.Typhimurium	104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19994076	Human	Family outbreak	S.Typhimurium	104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20000136	Bovine		S.Dublin		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20001178	Poultry		S.Typhimurium	104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20001735	Ovine		S.Dublin		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20001797	Bovine		S.Montevideo		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20003052	Bovine		S.Dublin		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20003940	Human	Sporadic	S.Typhimurium	40	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20003962	Human	Sporadic	S.Typhimurium	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20004447	Human	Ibiza	S.Enteritidis	21	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

SSRL No.	Isolate Source	Epidemiology	Organism Name	PT	Ap	Cx[1]	Cl	Cp	Fz	Gm	Ka	Na	Ne	Sp	St	Sx	Te	Tm	LCp[2]
20005189	Porcine		S.Typhimurium	170	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Antimicrobial abbreviations: Ap, ampicillin; Cx, cefotaxime; Cl, chloramphenicol; Cp, ciprofloxacin (0.5mg/L); Fz, furazolidone; Gm, gentamycin; Ka, kanamycin; Na, nalidixic acid; Ne, netilmicin; Sp, spectinomycin; St, streptomycin; Sx, sulphamethoxazole; Te, tetracycline; Tm, trimethoprim; LCp, ciprofloxacin (0.125mg/L).

[1] Sensitivity testing for cefotaxime began in 1998

[2] Sensitivity testing for reduced susceptibility to ciprofloxacin began in 1997

Appendix II: Preparation of working solutions for Plasmid profile analysis

TE 50:1

0.61g of Trizma base and 0.04g of EDTA was dissolved in 70ml of deionised water. The pH was adjusted to 8 and the volume was made up to 100mls. Stored at room temperature.

TBE buffer

500ml of 10X TBE buffer was added to 4500ml of deionised water.

Kado and Liu lysis buffer

3g of SDS and 0.6g of Trizma base were added to a beaker on a magnetic stirrer, containing approximately 70ml of deionised water. A probe from the Hydrus 300 pH machine from Fisherbrand was inserted into the beaker and monitored the pH. Sodium hydroxide solution was added slowly until a pH of 12.6 was achieved. The final volume was adjusted to 100ml. The buffer was stored at 4°C until needed, but as the SDS precipitates while being stored, it is necessary to place the buffer in a 56°C waterbath until the SDS has re-dissolved.

Ethidium Bromide (50mM stock solution)

10ml of Ethidium Bromide solution was added to 40 ml of deionised water, to make 50ml working solution and stored at 4°C. For staining, 0.5ml of this was added to 500ml of deionised water, which was sufficient to stain up to 20 gels.

Appendix III: Plasmid profiles

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
20004928	Antarctica		57
19983922	Binza		2.1
19991452	Binza		Plasmid-free
19990838	Blockley		5.2;4.6
19991711	Blockley		8.2;5.2
19903865	Bovismorbificans		Plasmid-free
19963399	Brandenburg		160;3.7
19991073	Brandenburg		Plasmid-free
19983155	Dublin		80
19993056	Dublin		Plasmid-free
19993805	Dublin		80
20000136	Dublin		80
20000139	Dublin		Plasmid-free
20001735	Dublin		80
20003052	Dublin		80
19900377	Enteritidis	4	57
19900497	Enteritidis	RDNC	57;2.1
19900633	Enteritidis	RDNC	57
19900813	Enteritidis	4	57
19900828	Enteritidis	4	57
19901024	Enteritidis	4	57
19901059	Enteritidis	4	57
19901127	Enteritidis	Untypable	57;2.1
19901135	Enteritidis	Untypable	57;2.1

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
19901221	Enteritidis	Untypable	57;2.1
19901257	Enteritidis	Untypable	57;2.1
19901259	Enteritidis	4	57
19901289	Enteritidis	Untypable	57;2.1
19901301	Enteritidis	Untypable	57;2.1
19901442	Enteritidis	RDNC	57;2.1
19901527	Enteritidis	RDNC	57;2.1
19901602	Enteritidis	4	57;2.1
19901733	Enteritidis	Untypable	57
19901754	Enteritidis	Untypable	57;2.1
19901928	Enteritidis	4	57
19903116	Enteritidis	RDNC	2.1
19903993	Enteritidis	Untypable	2.1
19910665	Enteritidis	4	57;2.1
19911165	Enteritidis	4	57
19911537	Enteritidis	4	Plasmid-free
19933184	Enteritidis	4	57
19933339	Enteritidis	4	57
19935599	Enteritidis	4	57
19940784	Enteritidis	4	57
19940871	Enteritidis	4	57
19940880	Enteritidis	4	57
19941864	Enteritidis	4	57
19943026	Enteritidis	4	57
19964025	Enteritidis	5c	57
19965081	Enteritidis	1	57

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
19970269	Enteritidis	7	57
19982543	Enteritidis	4	57
19984026	Enteritidis	1	Plasmid-free
19990380	Enteritidis	4	57;4.4
19990807	Enteritidis	8	57
19991308	Enteritidis	4	57
19991655	Enteritidis	21	57
19991657	Enteritidis	1	57
19991700	Enteritidis	4	57
19991722	Enteritidis	4	100;57
19991815	Enteritidis	1	57
19991817	Enteritidis	7	57
19991825	Enteritidis	4	57
19991870	Enteritidis	5c	57;2.8
19991929	Enteritidis	1	57
19992150	Enteritidis	6a	57;55
19992252	Enteritidis	1	120
19993033	Enteritidis	4	Plasmid-free
19993115	Enteritidis	4	57
19993539	Enteritidis	untypable	57
19994609	Enteritidis	14b	5.2
19995017	Enteritidis	RDNC	70;57;3.0;2.1
20001314	Enteritidis	4	57
20002195	Enteritidis	1	57
20002590	Enteritidis	RDNC	57
20002618	Enteritidis	21	57;4.9;4.6

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
20002621	Enteritidis	6	57
20002660	Enteritidis	1c	57
20004057	Enteritidis	1	57
20004078	Enteritidis	3	57
20004300	Enteritidis	4	45;6.0;4.5
20004338	Enteritidis	1c	57
20004342	Enteritidis	6a	110;57
20004447	Enteritidis	21	57;3.5
20004592	Enteritidis	RDNC	57
20004594	Enteritidis	1	Plasmid-free
20004730	Enteritidis	21	110;57
20004768	Enteritidis	1	57
20005031	Enteritidis	7	57
20005084	Enteritidis	4	57;4.8
20005141	Enteritidis	untypable	57;6.0;5.0;3.8
20005212	Enteritidis	1	57
20005300	Enteritidis	1	Plasmid-free
19932300	Hadar		6.1;3.3;2.1
19935442	Hadar		6.1;3.3;2.1
19940240	Hadar		6.1;3.2;2.1
19990095	Hadar		7.2;2.1
19990515	Hadar		15;7.4;3.2;2.1
19991026	Hadar		2.1
19992070	Hadar		2.1
19992689	Hadar		15;6.2;5.3;4.8;3.2;2.1
19992820	Hadar		15;4.8;2.1

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
20000721	Hadar		8.2;6.0;5.2;4.0;3.2;2.1
20002113	Hadar		8.2;3.2;2.1
20002399	Hadar		45;2.1
20003106	Hadar		10;4.2;3.8;3.2;2.1
20004010	Hadar		10;4.8;4.5;2.1
20004028	Hadar		10;3.2;2.1
19993048	Indiana		160;40;6.0;5.2;2.6
20002427	Java		20;4.5;2.0
19992003	Kottbus		2.1
20003957	Kottbus		8.0;3.2;2.1;2.0
19982854	Liverpool		5.1;4.3
19993807	Liverpool		5.1;4.3
20000672	Liverpool		5.1;4.3
19910646	Mbandaka		6.8;4.4;2.8
19912067	Mbandaka		4.8;3.4
19921643	Mbandaka		6.8;4.6;2.8
19933488	Mbandaka		4.6
19934878	Mbandaka		6.8;4.2
19941804	Mbandaka		6.8;4.2
19982886	Mbandaka		4.6;4.4
19990706	Mbandaka		90;3.6;2.8
19990898	Mbandaka		70;6.8;4.4
19990970	Mbandaka		90;6.8;4.4
19990976	Mbandaka		6.8;4.2
19993983	Mbandaka		6.8;4.8;3.6;3.4;2.1
20000283	Mbandaka		70;60;3.0;2.1

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
20000635	Mbandaka		70
20000821	Mbandaka		45;30;6.8;4.4
20001054	Mbandaka		70
20001058	Mbandaka		45;20;6.8;4.2
20002298	Mbandaka		4.6;4.4;3.0
20002524	Mbandaka		60;50;6.8;4.4;3.4
20002550	Mbandaka		50;6.8;4.4
20002563	Mbandaka		90;50;6.8;4.6;4.3;3.0
20004661	Mbandaka		90;50;6.8;4.6;4.3;3.0
19982602	Monophasic group C1		100;2.7
2000 1797	Montevideo		120
19932001	Oranienburg		160;147;120
19983032	Senftenberg		50;20;5.3;4.4;3.2;2.5
19992491	Senftenberg		50;2.1
20000673	Senftenberg		20;4.3;3.2
20004947	Senftenberg		50;5.3;4.4;3.2;2.5
19990203	Thompson		6.5;5.5;2.8
19991746	Thompson		7.2
19993085	Thompson		Plasmid-free
20005319	Thompson		Plasmid-free
19902644	Typhimurium	66	90
19933344	Typhimurium	204c	90;60
19941463	Typhimurium	204	160;90
19950004	Typhimurium	2	90
19966072	Typhimurium	104b	90;7.1
19970646	Typhimurium	104	90

SSRL Reference	Serotype	Phage type	Plasmid profiles (kb)
19970917	Typhimurium	104	90
19972895	Typhimurium	104	90
19983351	Typhimurium	RDNC	Plasmid-free
19983458	Typhimurium	2	90
19990031	Typhimurium	104	90
19990341	Typhimurium	104	90;50
19991035	Typhimurium	104	90
19992835	Typhimurium	3	90;2.8
19993849	Typhimurium	104	90
19994076	Typhimurium	104	90
20000918	Typhimurium	104b	110;90
20001178	Typhimurium	104	90
20002014	Typhimurium	104b	90
20002058	Typhimurium	104b	90;30;6.7;6.0
20002257	Typhimurium	RDNC	145;4.9;3.1;2.5;2.0
20003940	Typhimurium	40	Plasmid-free
20003962	Typhimurium	10	90
20005189	Typhimurium	170	Plasmid-free
19910748	Virchow		150
19953743	Virchow		100
19983717	Virchow		Plasmid-free
19990983	Virchow		6.8;4.4;2.1
19991601	Virchow		4.5;2.5;2.1;1.7
19992678	Virchow		Plasmid-free
20001526	Virchow		Plasmid-free
20002001	Virchow		Plasmid-free

Appendix IV: Preparation of working solutions for PFGE

0.85% Saline

4.25g of sodium chloride were dissolved in 500mlof distilled water and sterilized by autoclaving.

ES Buffer

18.6g of EDTA was added to a Pyrex beaker containing approximately 70ml of sterile deionised water, stirring on a magnetic stirrer. A probe from the Hydrus 300 pH machine from Fisherbrand was inserted into the beaker and monitored the pH. Sodium hydroxide solution was added to keep the pH above 8 to allow the EDTA to dissolve. Once all the EDTA was dissolved, the sodium hydroxide solution was added drop by drop until a final pH of 9.5 was achieved. The volume was adjusted to 100ml and stored at room temperature.

Proteinase K

0.5g of Proteinase K was dissolved in 20ml of sterile deionised water. Aliquots of 200µl were dispensed into sterile Treff tubes and frozen in the –20°C freezer. When needed, the required number of tubes would be removed from the freezer and allowed to thaw at room temperature.

TE (PFGE) Wash buffer

1.21g of Trizma base and 3.72g of EDTA were dissolved in approximately 900ml of deionised water. The pH was adjusted to 7.5. The volume was made up to one litre and sterilized by autoclaving. Store at room temperature.

0.5X TBE buffer

250ml of 10X TBE buffer was added to 4750ml of deionised water. Store at room temperature.

Appendix V: Pulsed Field Profiles

SSRL Reference	Serotype	Phage type	Pulsed field profile
20004928	Antarctica		AMANT1
19983922	Binza		AMBIN1
19991452	Binza		AMBIN2
19990838	Blockley		AMBLO1
19991711	Blockley		AMBLO2
19903865	Bovismorbificans		AMBOV1
19963399	Brandenburg		AMBRN1
19991073	Brandenburg		AMBRN2
19983155	Dublin		AMDUB1
19993056	Dublin		AMDUB1
19993805	Dublin		AMDUB1
20000136	Dublin		AMDUB1
20000139	Dublin		AMDUB1
20001735	Dublin		AMDUB2
20003052	Dublin		AMDUB1
19900377	Enteritidis	4	AMENT1
19900497	Enteritidis	RDNC	SENTXB.0001
19900633	Enteritidis	RDNC	SENTXB.0001
19900813	Enteritidis	4	AMENT3
19900828	Enteritidis	4	AMENT1
19901024	Enteritidis	4	SENTXB.0001
19901059	Enteritidis	4	AMENT2
19901127	Enteritidis	Untypable	SENTXB.0001
19901135	Enteritidis	Untypable	SENTXB.0001

SSRL Reference	Serotype	Phage type	Pulsed field profile
19901221	Enteritidis	Untypable	SENTXB.0001
19901257	Enteritidis	Untypable	SENTXB.0001
19901259	Enteritidis	4	SENTXB.0001
19901289	Enteritidis	Untypable	SENTXB.0001
19901301	Enteritidis	Untypable	SENTXB.0001
19901442	Enteritidis	RDNC	SENTXB.0001
19901527	Enteritidis	RDNC	SENTXB.0001
19901602	Enteritidis	4	SENTXB.0001
19901733	Enteritidis	Untypable	SENTXB.0001
19901754	Enteritidis	Untypable	SENTXB.0001
19901928	Enteritidis	4	SENTXB.0001
19903116	Enteritidis	RDNC	SENTXB.0014
19903993	Enteritidis	Untypable	AMENT7
19910665	Enteritidis	4	SENTXB.0001
19911165	Enteritidis	4	SENTXB.0001
19911537	Enteritidis	4	SENTXB.0014
19933184	Enteritidis	4	SENTXB.0001
19933339	Enteritidis	4	SENTXB.0001
19935599	Enteritidis	4	SENTXB.0001
19940784	Enteritidis	4	SENTXB.0001
19940871	Enteritidis	4	SENTXB.0001
19940880	Enteritidis	4	SENTXB.0001
19941864	Enteritidis	4	SENTXB.0001
19943026	Enteritidis	4	SENTXB.0001
19964025	Enteritidis	5c	AMENT4
19965081	Enteritidis	1	SENTXB.0040

SSRL Reference	Serotype	Phage type	Pulsed field profile
19970269	Enteritidis	7	SENTXB.0040
19982543	Enteritidis	4	SENTXB.0001
19984026	Enteritidis	1	SENTXB.0014
19990380	Enteritidis	4	SENTXB.0001
19990807	Enteritidis	8	SENTXB.0009
19991308	Enteritidis	4	SENTXB.0001
19991655	Enteritidis	21	SENTXB.0001
19991657	Enteritidis	1	SENTXB.0001
19991700	Enteritidis	4	SENTXB.0001
19991722	Enteritidis	4	SENTXB.0001
19991815	Enteritidis	1	SENTXB.0001
19991817	Enteritidis	7	SENTXB.0001
19991825	Enteritidis	4	SENTXB.0001
19991870	Enteritidis	5c	SENTXB.0001
19991929	Enteritidis	1	SENTXB.0001
19992150	Enteritidis	6a	SENTXB.0001
19992252	Enteritidis	1	SENTXB.0014
19993033	Enteritidis	4	SENTXB.0014
19993115	Enteritidis	4	SENTXB.0001
19993539	Enteritidis	untypable	AMENT5
19994609	Enteritidis	14b	AMENT6
19995017	Enteritidis	RDNC	SENTXB.0119
20001314	Enteritidis	4	SENTXB.0001
20002195	Enteritidis	1	SENTXB.0001
20002590	Enteritidis	RDNC	SENTXB.0001
20002618	Enteritidis	21	SENTXB.0001

SSRL Reference	Serotype	Phage type	Pulsed field profile
20002621	Enteritidis	6	SENTXB.0001
20002660	Enteritidis	1c	SENTXB.0001
20004057	Enteritidis	1	SENTXB.0001
20004078	Enteritidis	3	SENTXB.0001
20004300	Enteritidis	4	SENTXB.0001
20004338	Enteritidis	1c	SENTXB.0001
20004342	Enteritidis	6a	SENTXB.0001
20004447	Enteritidis	21	SENTXB.0005
20004592	Enteritidis	RDNC	SENTXB.0001
20004594	Enteritidis	1	SENTXB.0014
20004730	Enteritidis	21	SENTXB.0001
20004768	Enteritidis	1	SENTXB.0005
20005031	Enteritidis	7	SENTXB.0001
20005084	Enteritidis	4	SENTXB.0001
20005141	Enteritidis	untypable	SENTXB.0001
20005212	Enteritidis	1	SENTXB.0001
20005300	Enteritidis	1	SENTXB.0014
19932300	Hadar		AMHAD1
19935442	Hadar		SHADXB.0001
19940240	Hadar		SHADXB.0001
19990095	Hadar		SHADXB.0001
19990515	Hadar		SHADXB.0001
19991026	Hadar		SHADXB.0001
19992070	Hadar		SHADXB.0001
19992689	Hadar		SHADXB.0001
19992820	Hadar		SHADXB.0001

SSRL Reference	Serotype	Phage type	Pulsed field profile
20000721	Hadar		SHADXB.0001
20002113	Hadar		SHADXB.0001
20002399	Hadar		AMHAD2
20003106	Hadar		SHADXB.0001
20004010	Hadar		SHADXB.0001
20004028	Hadar		SHADXB.0001
19993048	Indiana		AMIND1
20002427	Java		SPTJXB.0003
19992003	Kottbus		AMKOT1
20003957	Kottbus		AMKOT2
19982854	Liverpool		AMLPL1
19993807	Liverpool		AMLPL1
20000672	Liverpool		AMLPL1
19910646	Mbandaka		AMMBA1
19912067	Mbandaka		AMMBA2
19921643	Mbandaka		SMBAXB.0012
19933488	Mbandaka		AMMBA3
19934878	Mbandaka		AMMBA3
19941804	Mbandaka		AMMBA3
19982886	Mbandaka		AMMBA3
19990706	Mbandaka		AMMBA4
19990898	Mbandaka		AMMBA11
19990970	Mbandaka		AMMBA3
19990976	Mbandaka		AMMBA5
19993983	Mbandaka		AMMBA3
20000283	Mbandaka		AMMBA3

SSRL Reference	Serotype	Phage type	Pulsed field profile
20000635	Mbandaka		AMMBA6
20000821	Mbandaka		AMMBA7
20001054	Mbandaka		AMMBA8
20001058	Mbandaka		AMMBA10
20002298	Mbandaka		AMMBA9
20002524	Mbandaka		AMMBA5
20002550	Mbandaka		AMMBA3
20002563	Mbandaka		AMMBA12
20004661	Mbandaka		AMMBA12
19982602	Monophasic group C1		AMMPC1/1
20001797	Montevideo		AMMVD1
19932001	Oranienburg		AMORA1
19983032	Senftenberg		AMSEN1
19992491	Senftenberg		AMSEN2
20000673	Senftenberg		AMSEN3
20004947	Senftenberg		AMSEN4
19990203	Thompson		AMTHO2
19991746	Thompson		AMTHO1
19993085	Thompson		AMTHO1
20005319	Thompson		AMTHO1
19902644	Typhimurium	66	AMTYM66/1
19933344	Typhimurium	204c	AMTYM204c/1
19941463	Typhimurium	204	AMTYM204/1
19950004	Typhimurium	2	AMTYM2/1
19966072	Typhimurium	104b	STYMXB.0001
19970646	Typhimurium	104	STYMXB.0001

SSRL Reference	Serotype	Phage type	Pulsed field profile
19970917	Typhimurium	104	STYMXB.0001
19972895	Typhimurium	104	STYMXB.0001
19983351	Typhimurium	RDNC	AMTYMRDN1
19983458	Typhimurium	2	AMTYM2/1
19990031	Typhimurium	104	STYMXB.0001
19990341	Typhimurium	104	AMSTYM104/1
19991035	Typhimurium	104	STYMXB.0001
19992835	Typhimurium	3	AMTYM3/1
19993849	Typhimurium	104	STYMXB.0001
19994076	Typhimurium	104	STYMXB.0001
20000918	Typhimurium	104b	AMTYM104b/2
20001178	Typhimurium	104	STYMXB.0001
20002014	Typhimurium	104b	AMTYM104b/1
20002058	Typhimurium	104b	STYMXB.0001
20002257	Typhimurium	RDNC	AMTYMRDN2
20003940	Typhimurium	40	AMTYM40/1
20003962	Typhimurium	10	AMTYM10/1
20005189	Typhimurium	170	AMTYM170/1
19910748	Virchow		AMVIR2
19953743	Virchow		SVIRXB.0001
19983717	Virchow		AMVIR1
19990983	Virchow		AMVIR3
19991601	Virchow		AMVIR3
19992678	Virchow		AMVIR2
20001526	Virchow		AMVIR4
20002001	Virchow		SVIRXB.0001

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