An investigation into clinical, epidemiological and genomic changes in epidemic Salmonella blood stream infection in Malawi

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

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The NTSPE study: "Nontyphoidal Salmonella Persistence and Evolution"

This thesis is the result of my own work. In some instances, work was done in conjunction with other colleagues and institutions. The roles of my co-workers and collaborators in this project are detailed below.

Collaborators & Co-workers

Activity	Responsibility
Screening of patients for recruitment	Jean Chikafa – field worker
Recruitment to bacteraemia study	MLW blood culture nurses: Margaret Chikopa, Harriet Chilonda, Beatrice Chilonda, and Nellie Manda
Assistance with recruitment & clinical procedures	Patrick Goodson – clinical officer
Data entry & abstraction	MLW Data Department, especially Malango Msukwa
Diagnostic bench microbiology	MLW Microbiology Department
Creation of PCR libraries, whole genome sequencing and post sequencing archiving of isolates	Wellcome Trust Sanger Institute Sequencing Pipeline
Instruction and collaboration in structural equation modelling	Dr B Faragher
Conversion of Omnilog data from Biolog phenotyping to log-fold change and principal component analysis	Dr Lars Barquist

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Dedication

To Helena, for coming to Malawi with me.

Key Words

Salmonella; *S*. Typhimurium; *S*. Enteritidis; *S*. Typhi; antimicrobial resistance; HIV; Africa; high-throughput nucleotide sequencing.

Abstract

Bloodstream infection (BSI) caused by nontyphoidal serotypes of Salmonella is one of the most important causes of morbidity and mortality in sub-Saharan Africa (SSA). Invasive nontyphoidal Salmonella disease (iNTS) is especially associated with HIV in adults and HIV, malaria and malnutrition in children in this setting. HIV-infected subjects are at particular risk of recurrent disease, both from recrudescence from a sanctuary site and re-infection. Whole-genome sequencing has revealed a novel sequence type (ST) of *S*. Typhimurium, ST313, is particularly associated with iNTS in SSA and that ST313 display the genomic signature of differential host adaptation. Little is known about African strains of *S*. Enteritidis.

The Malawi Liverpool Wellcome Trust Clinical Research Programme (MLW) has conducted BSI surveillance in adult and paediatric medical patients in Blantyre, Malawi since 1998, enabling long term trends in iNTS to be described. Paediatric iNTS disease has been placed in the context of malaria, malnutrition and seasonality using structural equation modelling. An observational cohort was recruited to describe changes in adult BSI cases following the roll-out of ART in Blantyre. A longitudinal cohort study with enhanced microbiological surveillance was undertaken to investigate the site of persistence of NTS in HIV infected adults and the effect of recurrence upon the emergence of antimicrobial resistance. Lastly whole-genome sequencing was undertaken to compare invasive, African strains of *S*. Enteritidis with a reference isolate and to construct a global phylogeny of this serotype.

There have been three epidemics of invasive Salmonella disease in Blantyre, all of which were preceded by the emergence of a multidrug resistant (MDR) strain. These were caused initially by *S*. Enteritidis which was then followed by *S.* Typhimurium, but most recently an epidemic of MDR *S.* Typhi has started. NTS has declined from epidemic levels, but remains an important cause of BSI in Blantyre and has been demonstrated to acquire cephalosporin

and fluoroquinolone resistance just once through an IncHI2 plasmid. The decline in paediatric malaria is complex and multi-factorial. It cannot be attributed solely to malaria control interventions as has been the case in other settings. All causes of BSI are falling in adults and this is likely to be attributable in part to the hugely successful HIV treatment programme in Blantyre. The rapid initiation of ART appears to rapidly confer protection against recurrence of iNTS and even though more adult patients are surviving into convalescence, in this cohort, no further emergence of extended resistance was seen. Just as there is a distinct African clade of *S.* Typhimurium, a distinct clade of *S.* Enteritidis has emerged in SSA, which also possess the genomic signature of differential host adaptation, a novel prophage repertoire and a novel, MDR plasmid.

In Blantyre, iNTS disease has declined from its epidemic peaks, but remains an important cause of BSI. The epidemiology of iNTS is more complex in Blantyre than other settings, but it seems likely that improved in-patient care and measures to control the HIV-pandemic impact on survival and recurrence. Two clades of NTS have emerged from host promiscuous serotypes, both of which have genomic degradation in genes governing potential host range and there is a critical need to understand the environmental niches and transmission pathways of these differentially adapted, invasive clades.

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Publications & Presentations Arising From Thesis

Peer reviewed journal articles

1. Feasey N, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. *Invasive Nontyphoidal Salmonella: an emerging and neglected tropical disease in Africa.* **Lancet** 2012 Jun 23;379(9834):2352-63
2. Okoro C, Kingsley RA, Quail MA, Kankwatira AM, Feasey N, Parkhill J, Dougan G, Gordon MA. *High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and re-infection in recurrent invasive non-typhoidal Salmonella Typhimurium disease*. **Clin Infect Dis** 2012 Apr;54(7):955-63
3. Feasey N, Healey P, Gordon MA. Invited review: *Investigation and management of diarrhoea in the HIV-positive patient.* **Aliment Pharmacol Therap** 2011 Sep;34(6):587-603.
4. Feasey N, Archer B, Keddy K & Gordon MA, *Age Distribution of Typhoid Fever and invasive Non-Typhoidal Salmonella in Sub-Saharan Africa.* **Emerg Infect Dis**. 2010 Sep;16(9):1448-51

Book Chapters

1. Feasey N, Gordon MA: Chapter 52 *Salmonella Infections* Manson's Tropical Diseases 23rd edition. Eds Lalloo, White, Farrar, White, Junghauss, Hotez, Kang. Elsevier, London 2013. In Press
2. Feasey N, Parry C: Typhoid Challenging Concepts in infectious diseases and microbiology: Typhoid Fever. 1st Edition, Eds Arnold, Griffin. In Press

3. Feasey N, Gordon MA: Chapter 49 Typhoid & Non-typhoid Salmonella Principles of Medicine in Africa. 4th edition, Eds Mabey, Gill, Parry, Weber, Whitty. Cambridge 2013.
4. Gordon MA, Feasey N, Graham S. Non-typhoid salmonella infection. Hunter's Tropical Medicine. 9th edition. Eds Magill, Ryan, Hill, Solomon. Elsevier 2013.

Oral Presentations

1. *"Three epidemics of Salmonella Blood Stream Infection"* American Society of Microbiology Salmonella Meeting, Boston, USA 2013
2. *"Bugs, drugs & HIV: bacteraemia & mycobacteraemia in Malawi."* MLW ASM, Chester 2012
3. *"Epidemiology of invasive Salmonella in Malawi"* MLW ASM, Malawi, 2011
4. *"Evolution of drug resistance in Invasive Salmonella in Malawi: summary of fellowship proposal."* MLW ASM, Warrington 2010

Posters

Three epidemics of Salmonella blood stream infection in Malawi. The 8th International Conference on Typhoid Fever and Other Invasive Salmonelloses, Dhaka, Bangladesh. March 2013.

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Abbreviations

µl	Microlitre
˚C	Degrees Centigrade
AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-retroviral therapy
ARV	Anti-retroviral
BSAC	British Society of Antimicrobial Chemotherapy
BSI	Blood Stream Infection
CDC	Centre for Control of Infection, Atlanta, USA
CVD	Centre for Vaccine Development, Maryland, USA
CI	Confidence Intervals
CoM	University of Malawi College of Medicine
COMREC	College of Medicine Research Ethics Committee
CPT	Cotrimoxazole prophylactic therapy
CRF	Case Report Form
CXR	Chest Radiograph
DCS	Diminished Ciprofloxacin Susceptibility
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
ESBL	Extended Spectrum Beta-Lactamase
FBC	Full Blood Count
g	Centrifuge gravitational force
GDP	Gross Domestic Product
GFI	Goodness of Fit Index
GPS	Global Positioning System
Hb	Haemoglobin

HIV	Human Immunodeficiency Virus
IDSA	Infectious Disease Society of America
iNTS	invasive Nontyphoidal Salmonella disease
IQR	Interquartile Range
IV	Intra Venous
Km	Kilometre
LSTM	Liverpool School of Tropical Medicine
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MLW	Malawi Liverpool Wellcome Trust Clinical Research Programme
MLST	Multi Locus Sequence Type
MTB	*Mycobacterium tuberculosis*
MLVA	Multi-locus Variable-Number Tandem-Repeat Analysis
n	Number
NEQAS	National External Quality Assessment Service (UK)
NSO	Malawi National Statistcis Office
NTS	Nontyphoidal Salmonella
NVGH	Novartis Centre for Vaccine Development, Siena, Italy
Od	Once daily
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PHE	Public Health England
PO	Per os – oral
QECH	Queen Elizabeth Central Hospital
Rx	Treatment
SSA	Sub-Saharan Africa

SNP	Single Nucleotide Polymorphism
SPI	*Salmonella* Pathogenicity Island
TB	Tuberculosis
UoL	University of Liverpool
VL	Viral load (copies/ml)
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WTSI	Wellcome Trust Sanger Institute
XLD	Xylose lysine deoxycholate agar

Glossary of Sanger "in-house" software and scripts

The following is a list of programmes written and available at WTSI which have been used in this thesis, but which are unpublished.

Programme	Author	Function
annotations_update.pl	Tim Carver	Transfers gene labels from an annotated to an unlabelled genome
bigger_blast.pl	Simon Harris	Creates file which compares 2 or more genomes that can be visualised using ACT (Carver)
get_sequence_alignment.pl	Andrew Page	In-silico MLST typing script
map_resistome.py	Simon Harris	Blasts sequence against database of resistance genes
multiple_mapings_to_bam.py	Simon Harris	"Wrapper" for automating mapping process on Sanger server, managing simultaneous submission of multiple large sets of sequence data
remove_blocks_from_ alignment.py	Simon Harris	Removes variable regions from alignment file
reportlabtests.py	Simon Harris	Maps metadata onto Newick-format tree and outputs pdf file
SMALT	Hans Ponstingl	Aligns reads against reference genome and creates consensus alignment file
snp_sites.pl	Andrew Page	Takes aligned genomes and removes sites where no snps are present, massively reducing the size of the alignment file prior to running maximum-likelihood modelling

Chapter 1: General Introduction

Summary

Invasive strains of non-typhoidal Salmonella (NTS) have emerged as a prominent cause of both endemic and epidemic blood stream infection in African adults and children, with an associated case-fatality of 20-25%. These strains are predominantly of serotype Typhimurium and Enteritidis and are commonly associated with multi-drug resistance. The clinical presentation of invasive NTS disease (iNTS) in Africa is diverse, with fever, hepatosplenomegaly, and respiratory symptoms being common, and features of enterocolitis being often notably absent. Recurrence is a key feature in HIV infected individuals. The most important risk factors for iNTS in Africa are HIV among adults, and malaria and malnutrition and HIVamong children.

A distinct genotype of *Salmonella* Typhimurium, ST313, has emerged as a novel pathovar in sub-Saharan Africa (SSA). Despite sharing a common serotype with Industrial-world diarrhoeal strains, it has undergone genomic degradation, acquired a different prophage repertoire and causes invasive disease in humans. This emerging pathogen has thus adapted to occupy an ecological and immunological niche provided by HIV, malaria and malnutrition in Africa.

Whether and how patterns of iNTS incidence, presentation and outcome have changed following the roll-out of anti-retroviral therapy and malaria control interventions is unknown. In contrast to *S*. Typhimurium ST313, little is known about the genetic characteristics of strains of *S*. Enteritidis causing invasive disease in Africa.

Search strategy and selection criteria

A formal systematic review was not undertaken, but to ensure that a comprehensive literature search was undertaken; all entries in MEDLINE, PubMed, and the Cochrane Library were searched from 2000 to 2013 to identify recent studies on invasive Salmonella including the pathogen, epidemiology, risk factors, pathogenesis and treatment. The main search terms were “Salmonella” in combination with “Typhi” or “nontyphoidal” or “Africa”. No language restrictions were applied. Important older reports were also included. Reference lists of articles identified by this search strategy were also reviewed. Review articles were cited where they provided comprehensive overviews beyond the scope of this literature review.

1.1 Introduction

The bacterial genus *Salmonella* is responsible for a huge global burden of morbidity and mortality. In relation to human disease, Salmonellae are traditionally grouped on the basis of their clinical behaviour into “typhoidal” serotypes (*S.* Typhi and *S*. Paratyphi A) which affect humans only and thousands of “non-typhoidal” Salmonella (NTS) serotypes which affect a range of species or are environmental. Not all NTS serotypes are equal, there is a spectrum; *S*. Dublin and *S*. Cholerasuis are far more likely to cause invasive disease than *S*. Typhimurium, a globally very common cause of enterocolitis (Jones et al. 2008). In industrialised countries, NTS predominantly cause a self-limiting diarrhoeal illness in healthy individuals, while bloodstream or focal infection is uncommon (Laupland et al. 2010) and mainly occurs in individuals with specific risk factors (Gordon 2008). In this setting, the strains most commonly associated with enterocolitis are *S*. Enteritidis and *S*. Typhimurium, and here, both of these serotypes have a low invasiveness index (Jones et al. 2008).

A sharp contrast is seen in sub-Saharan Africa (SSA), where NTS are consistently found to be the commonest bacterial bloodstream isolates in both adults and children presenting with fever (Gilks et al. 1990, Berkley et al. 2005, Gordon et al. 2008, Sigauque et al. 2009, Reddy et al.), associated with a case-fatality of 20-25%. Evidence to date from epidemiological studies, from the whole genome sequencing of one of the commonest invasive NTS serotypes in Africa, *S.* Typhimurium, and from discoveries about the host immunity at the cellular, humoral and mucosal level are coalescing to create a coherent picture of an emerging group of pathogens with a novel pathogenesis. This literature review will set the new findings in the context of what is known from other presentations of Salmonella disease, including the intriguing parallels and contrasts with Typhoid Fever.

1.2 A historical perspective

Salmonellae were first identified from pig intestines in the late nineteenth century by the USA Bureau of Animal Industry (Salmon 1885) and the genus was named after the director of the unit, Daniel Elmer Salmon by Theobald Smith. Most diarrhoeal Salmonella disease in the industrialised world is still considered to be zoonotic, due to a broad vertebrate host-range. Transmission is driven by commercial industrial production of meat, eggs, and processed food (e.g. chocolate (Gill et al. 1983), jalapeno peppers (Barton Behravesh et al. 2013) and peanut butter (MMWR 2009)). In industrial settings, *Salmonella enterica* remains a major cause of human gastrointestinal disease, with poultry the source of a worldwide epidemic of human infection (Barrow et al. 2012). The UK witnessed a rise in the incidence of NTS of >170% between 1981 and 1991, predominantly due to *S.* Enteritidis of phage type 4 (O'Brien 2013), although this epidemic has subsequently significantly declined through the introduction of poultry flock vaccination and hygiene programmes (O'Brien 2013). In

contrast to the picture with *S*. Enteritidis, rates of human cases of *S.* Typhimurium in the industrial world have shown less variation, but with this serotype, antimicrobial-resistance has emerged as a problem in both animal production and public health, in particular within atypical monophasic variants of *S*. Typhimurium (EFSA 2011). These are frequently associated with human enterocolitis (Trupschuch et al. 2010, Wasyl et al. 2012). Monophasic members of Salmonella predominantly belong to two main phage types, DT193 and DT104, and pigs are considered the predominant source (Hauser et al. 2010, Huehn et al. 2010, Wasyl et al. 2012), however it has recently been shown that strains of DT193 can have the potential to infect chickens (Parsons et al. 2013).

NTS were described as a common cause of paediatric bloodstream infection in Africa in several reports pre-dating the current HIV epidemic in the 1970s and 1980s (Duggan et al. 1975), and iNTS was first associated with malaria in children in 1987 (Mabey et al.1983). In 1983, shortly after AIDS was described, two reports of African adults with AIDS and *S*. Typhimurium bacteraemia emerged in the industrialised world (Clumeck et al. 1983, Offenstadt et al. 1983). In 1984, AIDS was identified in Zaire and Rwanda, and these reports also included cases of iNTS (Jonas et al. 1984, Piot et al. 1984). The first case-series of NTS bacteraemia was described in US AIDS patients in 1984 (Bottone et al. 1984), and the first epidemiological link between iNTS and AIDS was made in New Jersey (Profeta et al. 1985), followed by an age-stratified study in New York City, showing that iNTS infections were over-represented among registered AIDS patients by a factor of 198, and multiple-site infections by a factor of 305 (Gruenewald et al. 1994). Recurrent NTS bacteraemia was added to the CDC case-definition for AIDS in 1987. By 1990, NTS had been confirmed as a common HIV-related pathogen in Sub-Saharan African adults (Gilks et al. 1990).

In contrast, the “typhoidal” serotypes *Salmonella* Typhi and *S*. Paratyphi A are entirely host-restricted to humans, and cause invasive disease in immunocompetent hosts. Whilst they once represented a considerable public health problem in industrialised countries, improvements in sanitation have led to near-eradication, and most cases are imported. The few that are not are usually linked to recently imported cases (HPA 2012). There remains a large burden of typhoidal disease in the developing world, particularly Asia. The invasive systemic clinical syndrome, typhoid fever, also known as enteric fever, is typified by high fever and complicated by sepsis and shock, gastrointestinal bleeding or perforation, encephalopathy, and focal metastatic complications such as cholecystitis or hepatitis. The historical untreated case fatality is 20% (Bhan et al. 2005), although mortality is less than 1% with appropriate antimicrobial treatment (Parry et al. 2002). *S*. Typhi may persist in the reticuloendothelial system and biliary tract, from where bacteria are intermittently shed into the gastrointestinal tract, transmitting the disease to other humans, potentially over many years. Persistence of *S*. Typhi in the biliary tract is associated with an increased incidence of gall bladder carcinoma in endemic areas (Welton et al. 1979, Shukla et al. 2000).

1.3 Microbiology & Typing

The genus *Salmonella* consists of rod-shaped, Gram-negative, flagellated facultative anaerobes, and belongs to the family Enterobacteriaceae (Barrow G et al. 1993). Although closely related to *E. coli*, they are sufficiently genomically and biochemically distinct to warrant their own genus. Key differences include the acquisition of numerous Salmonella pathogenicity islands (SPIs), which encode numerous virulence determinants (Groisman et al. 1997). Currently, it is considered that, two species exist, *Salmonella enterica* and *Salmonella bongori*. The defining development in Salmonella taxonomy was the demonstration by DNA-DNA hybridisation in 1973 that a broad group of serotypes was

related at the species level (Brenner et al. 2000), however as this technique was applied to a broader group of strains, *S. bongori* was demonstrated to be a separate species (Reeves et al. 1989).

The species *Salmonella enterica* is further divided into 6 subspecies; *enterica* (I), *salmae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI). *S. bongori* was formerly classed as subspecies V. Almost all medically important serotypes are found in subspecies I whilst the members of the other subspecies are predominantly associated with cold-blooded animals and the environment.

The Salmonellae are further divided into more than 2500 serotypes on the basis of surface antigens, by use of a typing scheme initially devised by Kauffman (Kauffmann 1934). These antigens include the somatic (O) antigens, flagellar (H) antigens and surface (Vi) antigens. Serotypes have traditionally been named either for the syndrome they were thought to cause (eg, *Salmonella enterica* var Typhimurium [*S.* Typhimurium] was thought to cause a typhoid-like syndrome in mice) or for the location of their discovery (e.g. *Salmonella enterica* var Panama [*S.* Panama]).

In order to prevent confusion with the designation of species, serotypes are not written in italics and the first letter is capitalised. The full name of a serotype is therefore written:

"*Salmonella enterica* subspecies *enterica* serotype Typhi"

It is currently conventional to abbreviate it thus:

"*Salmonella* Typhi"

Or

"*S*. Typhi"

Salmonella serotypes may be sub-grouped on the basis of their O antigens. This gives rise to six groups; A, B, C1, C2, D and E. This approach also has problems as the consequent sub-groups often contain serotypes causing clinically distinct syndromes. For example, *S*. Enterica and *S*. Typhi are both group D, whilst *S*. Typhimurium and *S*. Paratyphi B are both group B.

Salmonellae are currently typed in reference laboratories by a combination of subgenomic and genomic methods. The features of a perfect typing system would include; reproducibility of results, portability between settings, ease of use, affordability and of course be highly discriminatory. Considered in those terms, all of the following methods have their advantages and disadvantages.

Pulsed field gel electrophoresis is a technique used for the separation of DNA molecules by applying to a gel matrix an electric field that periodically changes direction. DNA is first digested with restriction endonucleases. The methodology has been standardised and the results are reasonably reproducible, which has enabled its global roll-out and the formation of an organisation with the ambitious aim of surveying food borne disease globally (Pulsenet: www.pulsenetinternational.org). The technique is difficult to reproduce and expensive to set up and in that sense, not ideal for resource limited settings. It is also a sub-genomic technique and its discriminatory power is limited, for example it was not useful in making the important distinction between African strains of iNTS *S*. Typhimurium from diarrhoeal strains isolated in Europe (Kingsley et al. 2009).

In recent years a number of typing techniques have emerged which are based on investigation of the bacterial genome. Three genomic techniques in particular have started to gain traction. The first is multilocus variable-number tandem-repeat analysis (MLVA). Repeated sequences are common to all genomes (Kolpakov et al. 2003) and tandem-repeats represent a form of repeated sequence which produces uncharacteristic physical structures in DNA. These cause slippage of DNA polymerase and repeat amplification (Benson et al. 1999). These repeats may differ between different strains of a species and therefore enable highly sensitive discrimination. The increasing number of sequenced and annotated bacterial isolates with well described loci of repeat regions makes this technique ever easier to roll out. MLVA involves the amplification and fragment size analysis of DNA sequences known to contain tandem repeats.

The other two methods are both based on genome sequencing; multilocus sequence typing (MLST) and whole genome sequencing (WGS). The former involves the sequencing and comparison of housekeeping genes (Maiden et al. 1998). The largest collection of *S.* enterica MLST data is held at the University of Cork, Ireland (http://mlst.ucce.ie/mlst/dbs/Senterica) and is based on 7 such genes (*panB*, *fimA*, *aceK*, *mdh*, *icdA*, *manB*, *spaN*). As MLST resolves differences at the level of a single nucleotide within these genes, it is highly discriminatory and recognises evolutionary groupings of Salmonella isolates (Achtman et al. 2012). There are also increasingly inexpensive, PCR based methods of ascertaining an MLST type. Its principal limitation is that, unlike WGS, it only evaluates 7 housekeeping genes.

Lastly, in recent years the dramatic fall in the costs of high volume sequencing, coupled with advances in the processing power of computers and the development of software which enables the parsing and comparison of huge amounts of data, has made whole genome

sequencing a realistic prospect as perhaps the ultimate typing tool. The set up costs of this approach are such that the technology remains in the province of a few hubs at the moment, but the discrimination this technique offers has enabled researchers to trace the inter- and intra-continental transmission of a number different, recently emerged and closely related bacterial pathogens including *Salmonella* Typhimurium (Okoro et al. 2012 (1), Harris et al. 2010, Croucher et al. 2011 , Mutreja et al. 2011). Unlike other molecular typing methods, full sequence data in the context of a phylogeny can also be used to understand and explore the likely functional importance of genomic differences.

1.4 Burden and epidemiology of invasive Salmonella disease in Africa

In recent years, bacteraemia studies have suggested that NTS are among the commonest isolates from febrile presentations among adults and children across sub-Saharan Africa, especially where HIV prevalence is high (Reddy et al. 2010) (Figure 1.1). The patchy availability of high-quality or affordable diagnostic microbiology facilities throughout Africa makes an accurate description of the incidence of iNTS extremely difficult (Reddy et al. 2010). Under-appreciation of disease burden due to inadequate diagnostics and reporting is common to a variety of neglected tropical diseases (Feasey et al. 2010) including iNTS (Hotez et al. 2009). The total burden of invasive disease attributable to iNTS in Africa has not been measured, but is likely to be considerable, with an estimated annual incidence of 175-388/100,000 among children under the age of 3-5 years (Berkley et al. 2005, Enwere et al. 2006, Bassat et al. 2009, Agnandji et al. 2012), and 2000-7500/100,000 among HIV-infected adult cohorts (Gilks 1998, van Oosterhout et al. 2005, Gordon et al. 2008, Reddy et al. 2010). There is a marked bimodal age distribution of iNTS in Africa, in which children from 6-36 months (MacLennan et al. 2008, Milledge et al. 2005) and adults in their 3-4th decade are at greatest risk (Feasey et al. 2010). The majority of cases of iNTS across Africa

are due to either *S*. Typhimurium or *S*. Enteritidis (Mabey et al. 1983, Gordon et al. 2008, Reddy et al. 2010), although some sites report a smaller contribution from other serotypes including *S*. Isangi in South Africa (Wadula et al. 2006), *S*. Concord in Ethiopia (Beyene et al. 2011) and *S*. Stanleyville and *S.* Dublin (Tennant et al. 2010) in Mali. Several sites have noted a recent decline in iNTS incidence, and some have noted a temporal association with decreases in malaria infections (Mackenzie et al. 2010, Scott et al. 2011), although this association is not universally observed.

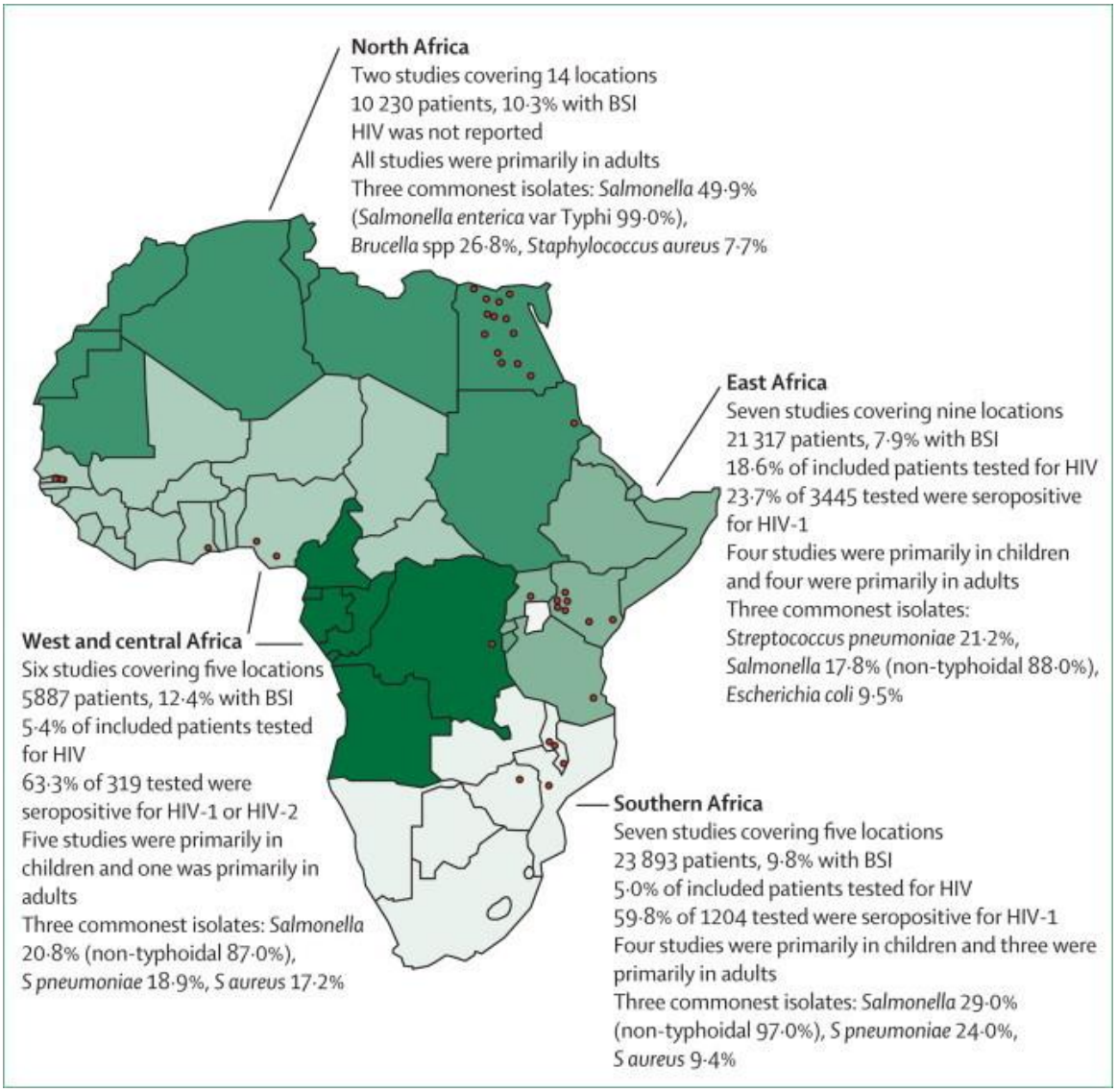


Figure 1-1: Map of Africa showing results of a meta-analysis of studies investigating the cause of BSI in febrile adults. Reproduced from Reddy et al (Reddy et al. 2010)

Whilst iNTS is underestimated, the incidence of typhoid fever in Africa has perhaps been overestimated, but there is still a lack of population-based data. The most recently published global burden of typhoid survey estimated a crude incidence in Africa of 50/100,000 persons/year (Crump et al. 2004), but this estimate was based on blood-culture data from vaccine trials from Egypt and South Africa in the 1970-80s (Wahdan et al. 1980, Klugman et al. 1987), and may have been inflated by outbreaks or pockets of disease. Review or meta-analysis of blood culture studies (Mweu et al. 2008, Reddy et al. 2010) has suggested that the overall burden of *S*. Typhi is lower than previously estimated in sub-Saharan Africa. *S*. Typhi is, however, the predominant invasive Salmonella serotype in North Africa (Reddy et al. 2010) where HIV is less prevalent, and there remain important foci or outbreaks of *S*. Typhi infection at some sub-Saharan African sites (Crump et al. 2011, Mtove et al. 2010, Keddy et al. 2010b). It has recently been reported in two studies that HIV may be protective against typhoid fever (Reddy et al. 2010, Crump et al. 2011).

In contrast to the findings in Africa, a recent multi-centre fever surveillance report in Asia found very little iNTS disease (Khan et al. 2010), compared to the dominance of typhoid fever (Ochiai et al. 2008), although there was poor sampling of the youngest and therefore most at-risk children at some study sites. The explanation for such disparity between Africa and Asia is unclear, although the lower prevalence of falciparum malaria and HIV in Asia may be relevant. This may change if HIV becomes more prevalent in Asia (Varma et al. 2010), although this will depend on access to ART. Reports from industrialised settings in the pre anti-retroviral therapy (ART)-era estimated incidences of NTS infections of approximately 400/100,000 p.a. among AIDS patients, greatly in excess of that in the general population (Gruenewald et al. 1994, Larsen et al. 2011) but there is evidence that the incidence fell following the introduction of ART (Larsen et al. 2011).

The contribution of NTS to diarrhoeal illness in Sub-Saharan Africa was, until recently, poorly described. Culture-based studies of diarrhoea in sub-Saharan Africa have found that NTS are isolated in 2-27% of culture-positive diarrhoeal illness (Obi et al. 1997, Germani et al. 1998 , Valentiner-Branth et al. 2003), but that they are also present in the stool of 2-7% of asymptomatic controls, making it difficult to attribute illness to stool positivity. A seroprevalence study of healthy children in Malawi revealed that 100% of healthy children had anti-Salmonella IgG antibodies by the age of 16 months, suggesting they have been universally exposed to either NTS or cross-reactive antigens at a very young age (MacLennan et al. 2008). The most recent global burden of NTS gastroenteritis study estimated that there were 2.5 million cases of disease and 4,100 deaths per year in Africa (Majowicz et al. 2010), but these data were extrapolated from returning travellers, which are unlikely to be representative of rural or low-income Africans. The biggest advance in our understanding of the epidemiology of paediatric diarrhoea comes from the recent Global Enteric Multicentre Study (GEMS), set in 7 countries, including Mali, Mozambique, the Gambia and Kenya, which did not find NTS to be a major cause of paediatric diarrhoea (Kotloff et al. 2013). In this study NTS was not amongst the top 10 causes of diarrhoea and amongst the African sites, was only described in Kenya.

It is not known whether the same strains of NTS cause both invasive and diarrhoeal disease or, if different, whether the modes of transmission are the same. Fundamental questions about the environmental reservoirs and host ranges of invasive strains in Africa remain unanswered. Studies of food quality have found NTS in home-cooked food (Taulo et al. 2008), fish from the great lakes of Africa and market food (Hang'ombe et al. 1999), but typing beyond serotype or genus level was not performed and their relevance to iNTS is uncertain. An important investigation into households of index cases of paediatric iNTS in Kenya showed that 6.9% of human contacts carried NTS, of which 66% were similar to the invasive strain in the index patient at molecular level (Kariuki et al. 2006). In contrast, only

unrelated strains of *S*. Typhimurium and *S*. Enteritidis and strains of *S*. Agona, *S*. Cholerasuis, *S*. Derby and *S*. Anatum were isolated from livestock or the household environment, raising the possibility that transmission primarily occurs between humans. There is an urgent need for a comprehensive investigation into the epidemiology of invasive and non-invasive NTS strains in Africa (Clemens 2009).

Published accounts of iNTS in Africa report that it is highly seasonal (Gordon et al. 2008). Peaks that occur during the rainy season among both adults and children coincide with increased incidences of malaria and malnutrition. iNTS has also been shown to occur in epidemics caused by sequential single serotypes among adults and children, lasting over several years, and these epidemics have been linked to the emergence of resistance to commonly used antimicrobials (Gordon et al. 2008). Figure 1.2 illustrates two previous epidemics of iNTS in Blantyre detected by BSI surveillance at the Malawi Liverpool Wellcome Trust Clinical Research Programme (MLW), the first caused by SEn and the second by STm. In both cases, the epidemics were preceded by a sharp rise in the proportion of isolates with an MDR phenotype. The resistance pattern of the SEn included newly-acquired resistance to amoxicillin, chloramphenicol, cotrimoxazole and doxycycline and that of STm, resistance to amoxicillin, chloramphenicol and cotrimoxazole. In the latter case, resistance to chloramphenicol was the sole additional drug resistance acquired. Of note, the MDR phenotype observed with SEn declined following the decline of the epidemic, whereas with STm, it persisted.

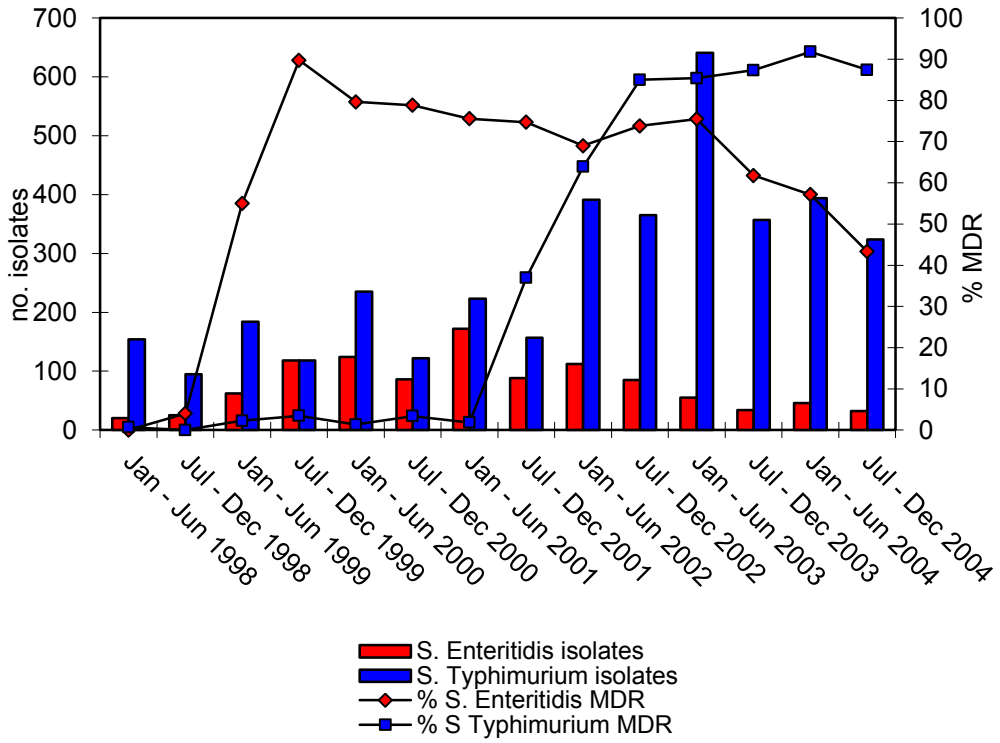


Figure 1-2: Epidemics of iNTS in Blantyre, Malawi (Gordon et al.)

BSI has been a common but under-appreciated and therefore neglected reason for adults to present to hospital in Sub-Saharan Africa (SSA), particularly in countries with a high HIV seroprevalence (Gilks et al. 1990, Petit et al. 1995, Ssali et al. 1998, Peters et al. 2004). This neglect results from the challenges of establishing high quality diagnostic microbiology systems in resource limited settings.

A recent meta-analysis of BSI in Africa found that the most frequent bacterial causes of adult BSI were Nontyphoidal Salmonellae (NTS), *Salmonella* Typhi and *Streptococcus pneumoniae,* and that except for *S.* Typhi bacteremia, HIV co-infection was a risk factor for BSI (Reddy et al. 2010). Prior to the introduction of antiretroviral therapy (ART), sentinel surveillance at Queen Elizabeth Central Hospital (QECH – see "Setting" in the methods chapter) established a 70% adult in-hospital HIV prevalence and a 38% case fatality among individuals with culture confirmed BSI (Gordon et al. 2001, Lewis et al. 2003). In addition to

NTS, in Malawi, the other major cause of BSI is *S. pneumoniae* (Gordon et al. 2001 , Peters et al. 2004).

In 2000, the overall estimated urban HIV prevalence in Malawi was 18%, which remained stable at 17.4% in 2010 (UNAIDS 2010). National roll-out of anti-retroviral therapy (ART) and cotrimoxazole preventive therapy (CPT) were commenced in 2004 and 2005 respectively (Weigel et al. 2012). Initially ART became available for patients presenting with WHO clinical stage III/IV disease and for those with a CD4 count of less than 200 cells/μL, which increased to 250 cells/μL in 2006, and 350 cells/μL in 2010. By the end of 2010, over 300,000 Malawians had started ART (MOH Malawi 2010), an estimated 46% of the eligible population based on the 2010 threshold (Weigel et al. 2013). As of the end of June 2010, 95% of ART patients were on CPT and a cumulative total of 339,000 patients (pre-ART and ART) had been entered in CPT registers (MOH Malawi, 2010). Whether and how BSI has changed in the era of rapid ART and CPT roll-out in Africa has not been well described.

1.5 Clinical Features of Salmonella disease in industrial settings

In the industrialised world NTS predominantly cause a self-limiting enterocolitis in immunocompetent individuals, leading to presentation with nausea, vomiting, profuse watery diarrhoea and abdominal pain (Hohmann 2001). Up to 5% will develop bacteraemia, but attributable mortality is probably quite low (1-5%) (Saphra et al. 1957, Cherubin et al. 1974, Todd et al. 1983, Hohmann 2001). NTS may persist in the gastrointestinal tract after diarrhoeal illness and the risk of this is increased by antimicrobial therapy (Sirinavin et al. 2000).

“Primary” NTS bacteraemia, without associated diarrhoea, occurs in at-risk groups, including patients who have immune-suppression due to HIV, steroids, malignancy, chronic renal or liver disease, diabetes, sickle-cell disease, and among the elderly and neonates (Gordon 2008). Mortality in adults was 12.2% in a hospitalised case-series from Spain (Galofre et al. 1994). Focal or metastatic disease also occurs in patients with structural abnormalities, such as valvular heart disease, aneurysms or atherosclerosis, biliary or urinary tract abnormalities, bony abnormalities or prostheses (Cohen et al. 1987).

iNTS is also a complication of several inherited immunodeficiencies, including Chronic Granulomatous Disease (Lazarus et al. 1975), in which phagocytes are unable to kill ingested organisms, sickle-cell disease, in which dysfunctional macrophages may cause susceptibility and B-cell deficiencies (Leen et al. 1986) in which the importance both of antibody and of the interplay between humoral and cellular systems (MacLennan et al. 2008) is apparent. Most strikingly, children with rare inherited deficiencies of components of the IL-12/IL-23 pathway have a particularly high incidence of invasive, recurrent iNTS disease (MacLennan et al. 2004), in keeping with the importance of these proinflammatory cytokines in the clearance of intracellular infections.

1.6 Clinical features of invasive NTS disease in sub-Saharan Africa

The clinical presentation of iNTS in Africa is typically of a febrile systemic illness (Graham et al. 2000, Gordon et al. 2002, Peters et al. 2004) resembling enteric fever, in which diarrhoea is often absent, and other clinical features are diverse and non-specific (Figure 1.3) (Gordon et al. 2002). An adult bacteraemia study in Malawi noted that a combination of high fever and splenomegaly was suggestive of iNTS, but that diagnosis cannot reliably be made without microbiology (Peters et al. 2004). In young children especially, there is problematic

clinical overlap with the presentations of pneumonia and malaria (Graham et al. 2000, Graham et al. , Nadjm et al. 2010), meaning that paediatric guidelines for empiric diagnosis and treatment in low-resource settings fail to distinguish and thus effectively treat iNTS. Apparent lower respiratory tract infection is commonly attributable to co-infection with other pathogens such as *M. tuberculosis* (Martinson et al. 2007) and *S. pneumoniae* (Gordon et al. 2002), although supporting microbiological data are lacking. Data from the pre-ART era suggest that even microbiologically confirmed iNTS, treated with appropriate antimicrobials has a case-fatality of 22-47% in African adults and children (Gordon et al. 2002, Gordon et al. 2008).

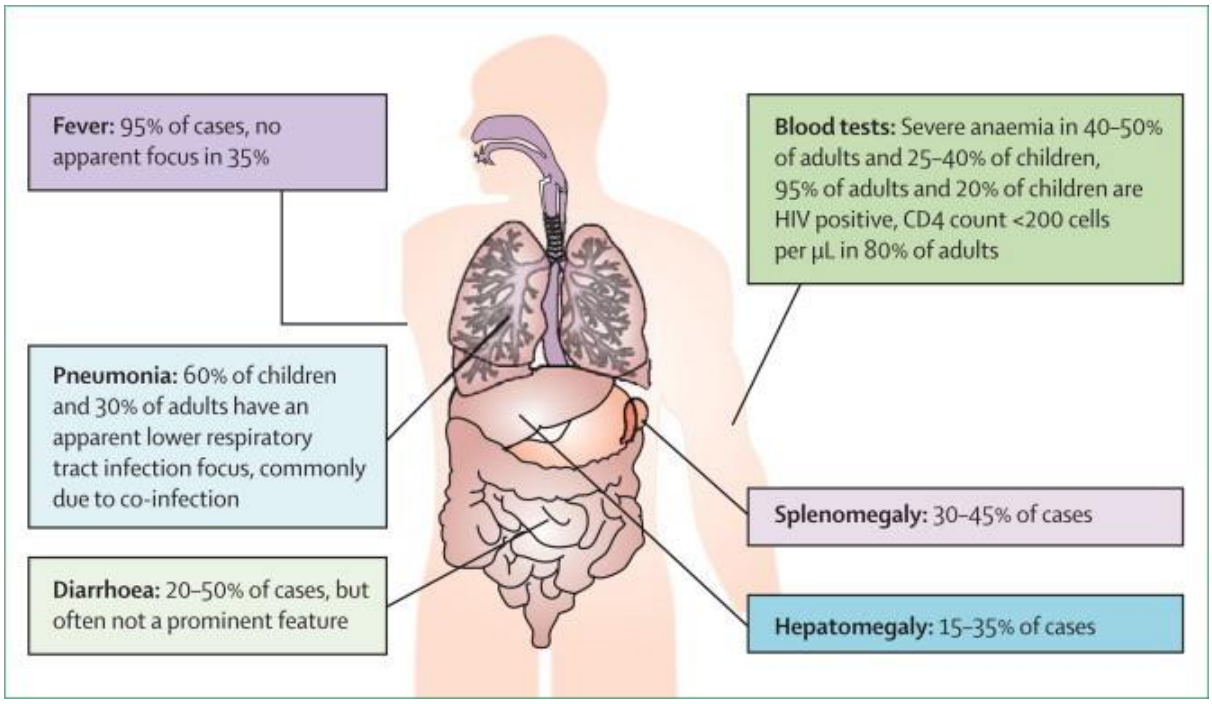


Figure 1-3: Homunculus demonstrating the clinical features of iNTS in adults and children in Africa (Feasey et al.)

Risk factors for iNTS in children include HIV infection, malnutrition (Graham et al. 2000), and a number of malarial syndromes, including; severe malarial anaemia (Bronzan et al. 2007, Mabey et al. 1987), acute severe malaria (Berkley et al. 2009) and recent malaria (Brent et al. 2006). As elsewhere, in countries such as Kenya where sickle-cell anaemia affects up to

6% of the population, it is also an important risk factor for iNTS among African children (Williams et al. 2009). iNTS predates HIV as an important clinical problem in children in Africa (Alausa et al. 1977, Wamola et al. 1981, Lepage et al. 1987, Mabey et al. 1983 , Green et al. 1993), and although HIV is independently associated with iNTS among febrile paediatric admissions (OR 2.6), only approximately 20% of African paediatric iNTS cases are HIV-infected (Brent et al. 2006, Gordon et al. 2008).

The principal risk factor amongst adults is undoubtedly advanced HIV infection. Case series typically show 95% of adult cases to be HIV-infected, of whom 80% have a CD4 T-lymphocyte count of under 200 cells/μl (Gordon et al. 2002). Among HIV infected adults, prior to the ART era, 20-40% of survivors experienced recurrence between 2 and 8 months of presentation even after appropriate antimicrobials (Gordon et al. 2002 & 2008). Despite re-treatment with antimicrobials, up to 25% of patients had multiple recurrences prior to the introduction of ART. Typing of index and recurrence strains at genomic level has suggested that both re-infection and recrudescence may cause recurrences, but that recrudescence with a highly similar isolate (less than 6 SNPs in the core genome) accounts for 80% of recurrences, despite multiple treatment courses with fluoroquinolones (Okoro et al. 2012 (2)). Figure 1.4 is taken from a WGS based investigation of recurrent isolates from Malawi and the phylogram reveals that whilst some isolates are almost indistinguishable, others sit on different branches of the tree (Okoro et al. 2012 (2)).

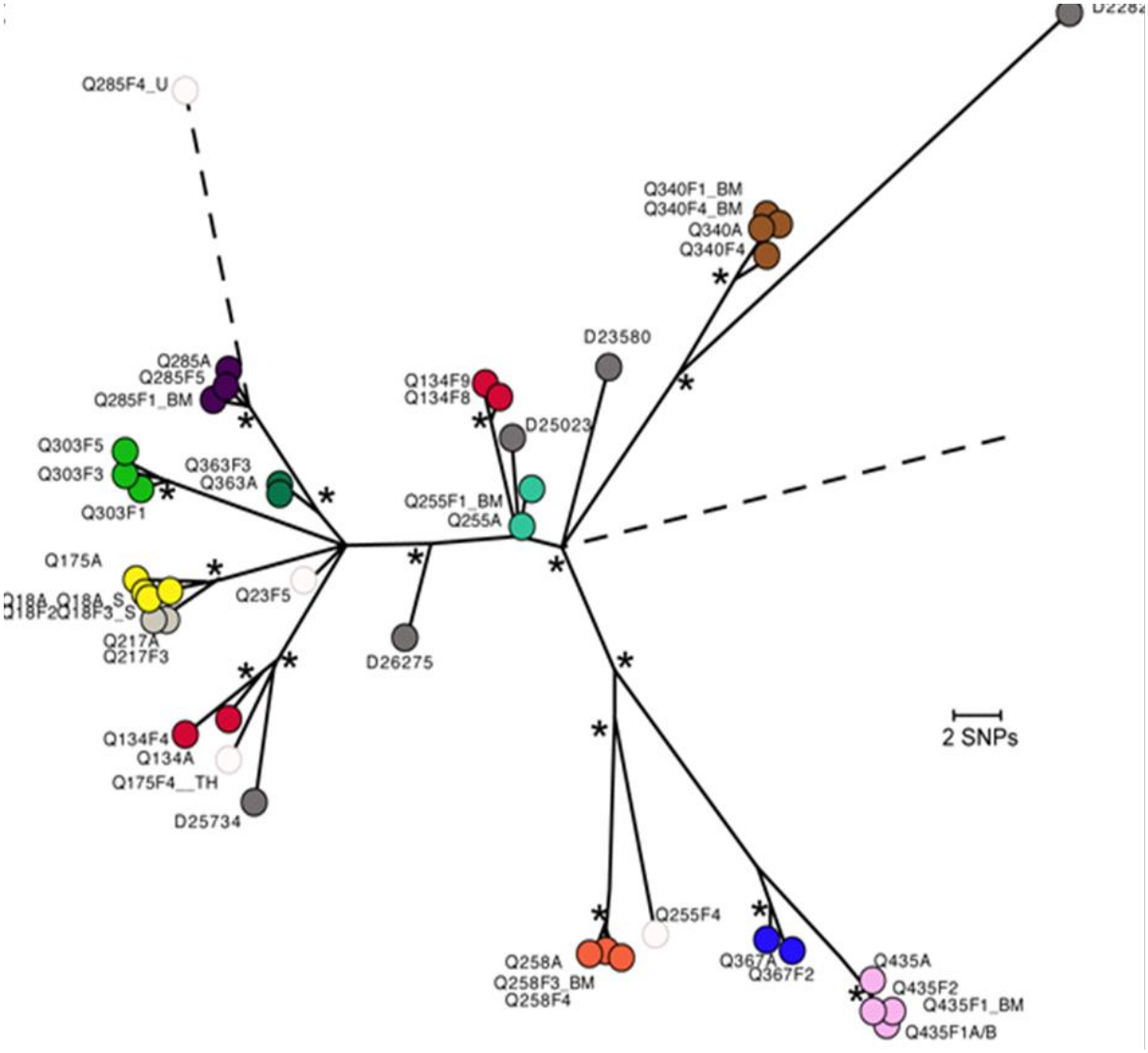


Figure 1-4: Radial phylogram showing relatedness of blood culture isolates of *Salmonella* Typhimurium at presentation (suffix A) and recurrence (suffix F1, F2, F3 etc). Isolates from the same individual are the same colour (Okoro et al 2012(2)).

In addition to sepsis, iNTS may seed to the meninges, especially in very young children. In Malawi, until recently, *S*. Typhimurium has been the second commonest cause of bacterial meningitis since the introduction of the *Haemophilus influenzae* B vaccine, accounting for 15% of culture proven meningitis in 2008 (unpublished data, MLW). Salmonella meningitis

carries 52% case-fatality among children (Molyneux et al. 2009) and 80% case-fatality among adults (Molyneux et al. 2009). Additionally, iNTS among African children is associated with active schistosomiasis (Neves et al. 1967, Rocha et al. 1971, Gendrel et al. 1984, Gendrel et al. 1990, Swindells et al.1996), as Salmonellae may adhere to the adult helminth's tegument (LoVerde et al. 1980, Melhem et al. 1984) , providing it with a sanctuary where it can evade antimicrobial treatment. Treatment with praziquantel kills the adult helminth and allows antimicrobials to eradicate the Salmonellae. Of note, Schistosomiasis has not been shown to contribute to susceptibility, death or recurrence of iNTS in HIV-infected adults (Dowling et al. 2002, Gordon et al. 2003).

1.7 Pathogenesis of diarrhoeal and invasive Salmonella Disease in immunocompetent hosts

The majority of clinical isolates of NTS can cause inflammatory enterocolitis and diarrhoea, and are competent to infect a broad range of potential vertebrate hosts. In contrast, the typhoidal strains evade the gut mucosal immune response to cause systemic disease, in a manner that is host-restricted to humans, and possibly more analogous to the clinical picture observed in iNTS in Africa. Considerable progress has recently been made in understanding the mechanisms underlying the spectrum of clinical syndromes observed, and their relevance to the host-range and transmission of different serotypes (Figure 1.5).

In relation to diarrhoeal disease, iNTS can exploit the gut mucosal inflammatory response that accompanies infection in immunocompetent individuals, and gain a selective advantage over the resident gut microbiota in the inflamed gut lumen (Stecher et al. 2007). There are likely to be multiple mechanisms underpinning this mode of pathogenesis. For example most Salmonellae harbour an *iro* gene-cluster (*iroN iroBCDE*), which can confer resistance to the

host antimicrobial peptide lipocalin-2 (Fischbach et al. 2006), by encoding a lipocalin-resistant siderophore that supplies iron in the inflamed gut. Salmonellae are also equipped to out-compete the microbiota in an inflammatory environment. For example, they can stimulate host-driven production of an electron acceptor that allows the pathogen to use respiration to compete with fermenting gut microbes (Winter et al. 2010, Godinez et al. 2011, Rivera-Chavez et al. 2013). Thus, the symptoms of NTS diarrhoeal gastroenteritis are caused by inflammatory mechanisms that are also key to transmission of the luminal pathogen.

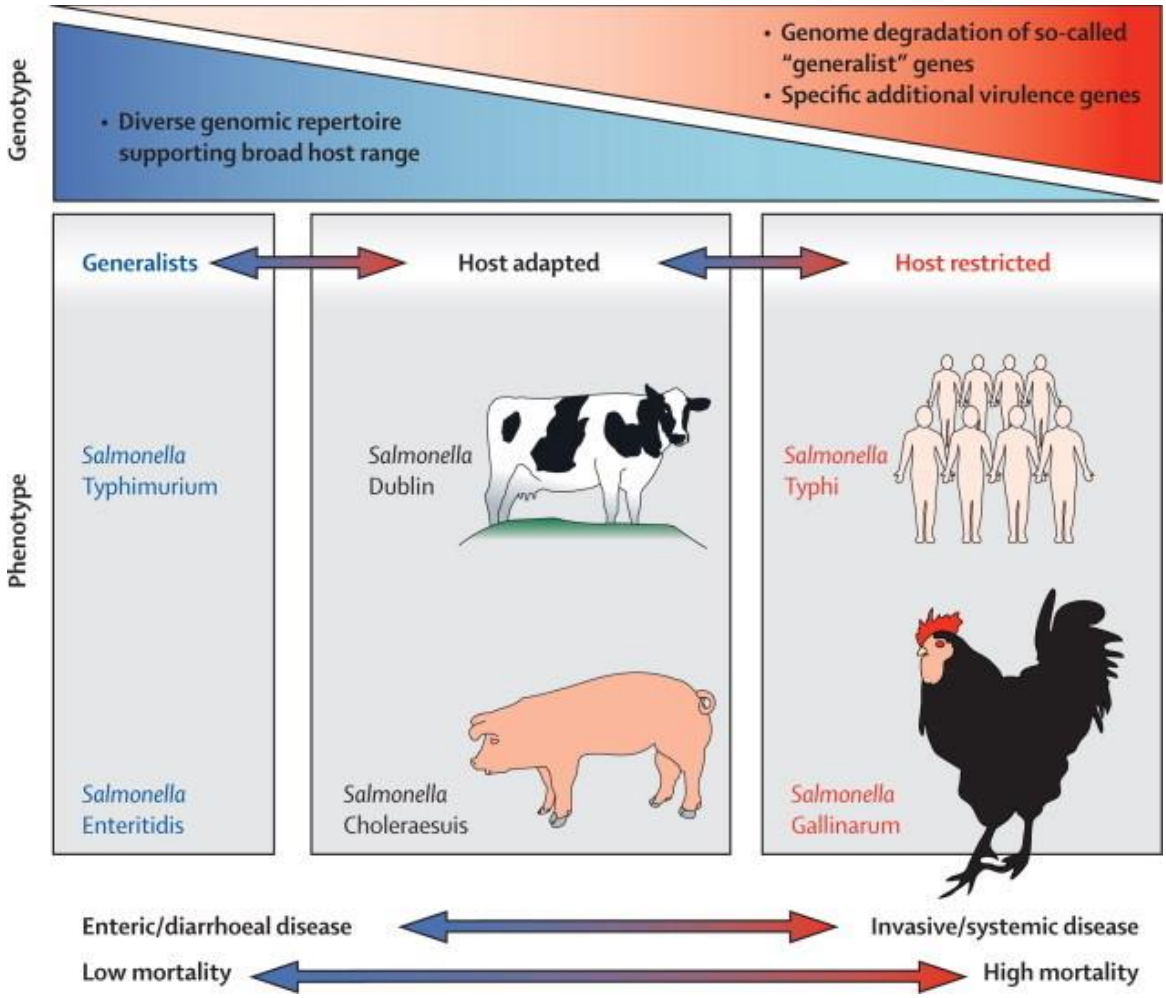


Figure 1-5: The features of host adaptation among Salmonellae, and their impact on the clinical syndrome in the host (Feasey et al. 2012).

S. Typhi, by contrast, generally avoid triggering a dominant primary gut mucosal inflammatory response through evasion mechanisms involving virulence-associated adaptations such as the expression of the immunomodulatory Vi capsule and the accumulation of an inactivated gene repertoire that may favour entry through a non-inflammatory pathway. *S*. Typhi also target small-intestinal microfold (M) cells, which are epithelial cells overlying Peyer's patches, that sample the antigenic content of the gut (Jepson et al. 2001). M-cells direct *S*. Typhi to underlying phagocytes such as dendritic cells and macrophages in the lamina propria, which favour intracellular dissemination to the lymphatics, the blood stream and viscera (Mittrucker et al. 2000, Hutchinson et al. 2011). The bacteria persist in the reticulo-endothelial system where they replicate prior to being shed back into the circulation (precipitating symptoms of enteric fever), and shed into the gastrointestinal tract in infected bile. This alternative invasive strategy of typhoidal Salmonellae ensures high-level shedding by a small number of individuals, securing the onward transmission of this human-restricted organism.

1.8 Three key immunological defects contributing to iNTS in HIV-infected adults

Three key immunological defects have been recently described, which may contribute to the invasive pathogenesis of NTS in HIV-infected adults in Africa (Figure 1.6). Firstly, an important defect has been identified in the gut mucosa. The gastrointestinal tract is a site of early and profound CD4 T-cell depletion in HIV infection (Brenchley et al. 2008), especially IL-17-producing T-cells (Th17 cells). Th17 cells and their associated family of cytokines, including IL 17, 21, 22 and 26, co-ordinate mucosal defence through several mechanisms. They are critical to the integrity, repair and maintenance of the epithelial mucosal barrier, and induce epithelial cell expression of antimicrobial peptides such as β-defensins and lipocalin. In addition, Th17 T-cells also play an important role in stimulating innate immune

responses, by mediating neutrophil chemo-attraction and function (Dandekar et al. 2010). A study in SIV-infected macaques, using a ligated ileal-loop model, showed that SIV-infected animals had depleted IL-17-producing mucosal cells, blunted cellular Th17 responses, and reduced expression of genes involved in the maintenance of the epithelial barrier (Raffatellu et al. 2008). This was associated with translocation across the gut mucosa and systemic dissemination of Salmonellae. A key role for Th17 responses in protection against Salmonella dissemination was confirmed using a streptomycin pre-treated $IL17^{-/-}$ mouse model, which also demonstrated reduced neutrophil influx, and increased *S*. Typhimurium invasion (Raffatellu et al. 2008). These data demonstrate that loss of gut mucosal IL-17 cells in HIV is likely to be a key mechanism by which Salmonellae disseminate from the gut to cause invasive disease in HIV. Loss of neutrophil chemoattraction may also explain the apparent lack of enteritis and diarrhoeal disease during HIV-associated iNTS disease (Gordon et al. 2002).

Secondly, dysregulated cytokine production during intracellular infection appears to permit persistence and recurrence of iNTS. Intracellular replication and persistence is a key pathogenic competence for Salmonellae causing invasive disease. Quantitative blood and bone-marrow cultures taken from HIV-infected adults with iNTS revealed a similar quantity of NTS in both compartments at index presentation, but 6-fold concentration of NTS in bone-

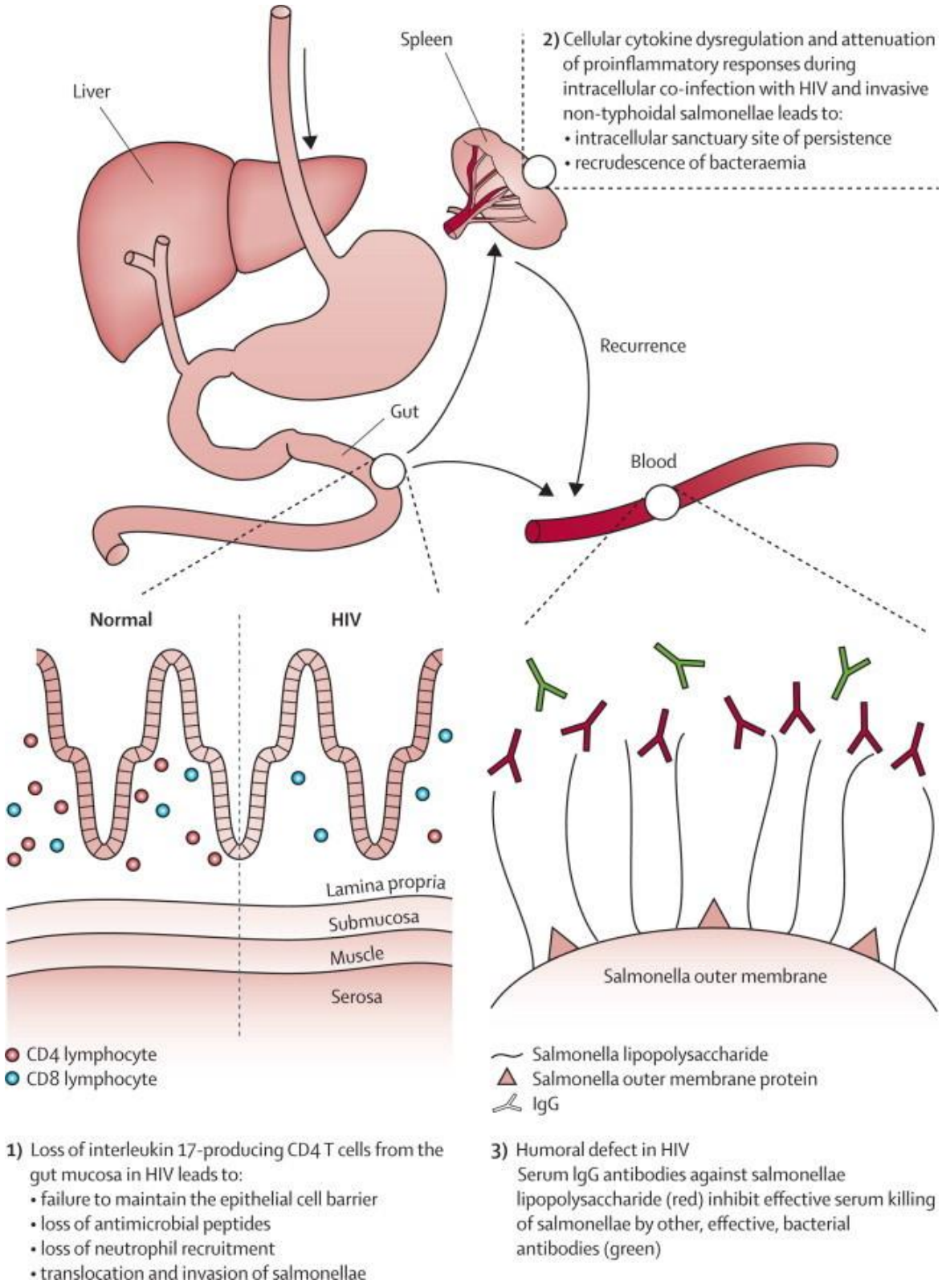


Figure 1-6: Three recently-described key defects that contribute to the pathogenesis of iNTS in HIV (Feasey et al. 2012)

marrow compared to blood at relapse episodes, suggesting persistence and replication. Furthermore, a significant proportion of bacteria in both blood and bone-marrow were intracellular during iNTS. Bacterial load at relapse negatively correlated with cytokine levels and CD4 cell count, suggesting that failure of immunological control permitted escape from the intracellular compartment and symptomatic relapse(Gordon et al. 2010). Investigation of intracellular killing and cytokine production by *ex-vivo* macrophages from HIV-infected adults did not show an intrinsic defect of macrophage killing, but instead demonstrated a highly dysregulated cytokine milieu after *S.* Typhimurium challenge. TNFα, IL-10 and IL-12 were produced in excess during early HIV-disease, but markedly reduced in late HIV-disease, when HIV-infected adults are most susceptible to iNTS disease (Gordon et al. 2007). Profound attenuation of pro-inflammatory cytokine responses is confirmed by transcriptional analysis of whole blood responses during iNTS disease (Schreiber et al 2011). Cytokine responses are critical for control of intracellular Salmonella infection (Dougan et al. 2011) and the dysregulation and attenuation seen in HIV are likely to explain intracellular persistence of NTS, failure of immunological control, and episodes of relapse of iNTS seen in advanced HIV infection.

Thirdly, based on murine models and epidemiological data from young children, there is increasing recognition that antibodies are likely to be important for both serum killing and intracellular oxidative killing of invasive Salmonellae (MacLennan et al. 2008, Gondwe et al. 2010, Dougan et al. 2011). A novel humoral defect has been described in HIV-infected Malawian adults, a proportion of whom had impaired serum killing of NTS strains. This impairment, paradoxically, was associated with the presence, rather than absence of IgG antibodies directed against Salmonella. The antibodies, directed against Salmonella LPS, were demonstrated to impair serum killing by blocking or competing with co-existing effective bactericidal antibodies directed against Salmonella outer membrane proteins (MacLennan et al. 2010). Although the clinical significance of these antibodies is still not proven in terms of a

relationship to disease susceptibility or patient outcomes, this finding may have some implications for the choice of targets for potential vaccines.

1.9 Immunological defects in African children

In children, other conditions are important risk factors for iNTS as well as HIV, especially severe malarial anaemia and malnutrition.

The mechanism through which malaria pre-disposed to iNTS was thought to be predominantly due to macrophage dysfunction consequent upon iron release following haemolysis, but two recent studies using mouse models suggest a more complex interplay between NTS and malaria, involving both cytokine dysregulation and haem and haem oxygenase-dependent dysfunctional granulocyte mobilization (Roux et al. 2010, Cunnington et al.2012). While homozygosity for sickle-cell anaemia is a risk factor for iNTS (Williams et al. 2009), heterozygosity (HbAS) is protective against all bacteraemias, including iNTS, probably due to the strong protection afforded against malaria (Scott et al. 2011). In contrast to the situation described in HIV-infected adults, African children between 4-16 months have poor serum killing of Salmonella associated with a relative early deficiency of anti-Salmonella IgG and an excess of iNTS disease (MacLennan et al. 2008). Furthermore iNTS infection is less common in the first 3-4 months of life, consistent with temporary protection afforded by transplacental protective antibodies, by antibodies in maternal colostrum, or by avoidance of environmental exposure during exclusive breast-feeding. Together, these findings suggest that antibody plays an important role in protection against iNTS in children.

There is an epidemiological association between malnutrition, infection in general (Pelletier et al. 1995) and iNTS (Graham et al. 2000 & personal communication C Maclennan), however the mechanism through which malnutrition predisposes to NTS is unclear and has

not been explored. The interaction is likely to be complex as malnutrition, malaria and HIV are overlapping phenomena. HIV predisposes to malnutrition and malaria (Osterbauer et al. 2012), malnutrition may predispose to malaria (Deen et al. 2002) and are most prevalent in Malawi during the same season and malaria and HIV have numerous interactions including within infants and upon the human placenta (Steketee et al. 1996).

1.10 Evolution and host-adaptation of *S.* Typhimurium in Africa

In order to understand the pathogenesis of iNTS, it is important to appreciate both human-host immunity and the considerable genetic diversity of the genus Salmonella. Host-restricted Salmonellae such as *S.* Typhi, which are traditionally associated with systemic disease, have evolved a more restricted genomic repertoire, compared to Salmonellae which maintain a broad host range and predominantly cause enteritis (Figure 1.5). The loss of functional gene capacity among invasive serotypes such as *S.* Typhi and *S.* Paratyphi A is at least partly attributable to the inactivation of genes that facilitate persistence of more promiscuous serotypes in the intestinal lumen as well as the ability to target and manipulate host cells (Pitter et al. 2007, Holt et al. 2008). Loss of functional gene capacity, with genome degradation from a progenitor with broader host-range, has been observed in a number of other human-restricted pathogens such as *Mycobacterium leprae* (Cole et al. 2001), *Yersinia pestis* (Thomson et al. 2006), *Bordetella pertussis* (Parkhill et al. 2003), and Rickettsia (Andersson et al. 1999).

The Salmonella serotypes which most commonly cause iNTS in SSA are *S.* Typhimurium and *S.* Enteritidis, and are usually associated with a broad host range and with enteric disease (Mermin et al. 2005). Recent whole genome sequencing and PCR analysis, performed on a series of invasive isolates of *S.* Typhimurium from Malawi and Kenya,

identified a dominant regional genotype of *S*. Typhimurium, multi-locus sequence type ST313 which is uniquely found in Africa, and which has multiple genetic differences compared to other strains of this serotype (Figure 1.7) (Kingsley et al. 2009). Although other genotypes of *S*. Typhimurium such as ST19 (which includes classical gastrointestinal strains such as DT104) can clearly cause iNTS in Africa, ST313 isolates are dominant in many sub-Saharan regions. ST313-like isolates have been retrospectively identified from Central Africa as early as the 1980's (personal communication, M. Al-Mashhadani and C.M.Parry).

An important characteristic of ST313 isolates is a degraded genome capacity, in the form of pseudogenes and deletions. Strikingly, 60% of this degraded genomic repertoire is also degraded in *S*. Typhi and S. Paratyphi. Some of these genes have known functions relating to pathogenesis, and some are of unknown function, but the convergence between ST313 and *S*. Typhi, despite the distinct phylogenetic lineages of these Salmonella species (Roumagnac et al. 2006) Figure 1.7 raises the possibility that ST313 has adapted to occupy a unique niche in Africa, undergoing convergent micro-evolution to become differentially adapted to industrial-setting strains (Kingsley et al. 2009).

A recent landmark study which built on these insights revealed that the vast majority of *Salmonella* Typhimurium isolates associated with iNTS disease in SSA comprised just two highly conserved lineages of MLST type ST313 (Okoro et al. 2012 (1)). This study demonstrated that transmission of STm ST313 into a new location is a discrete event, which is followed by subsequent dissemination. Independent acquisition of a Tn21 element bearing multiple drug resistance genes by both lineages may have further facilitated their successful transmission across the sub-continent, especially resistance to chloramphenicol, a component of the empirical therapy for sepsis across much of the continent (Okoro et al. 2012(1)).

It is not currently known if these ST313 strains cause an appreciable burden of gastroenteritis in Africa. However, Salmonella isolates of an identical MLST type were recovered from healthy people cohabiting with index cases of iNTS disease in Kenya, suggesting that asymptomatic carriage may be important in the transmission of these pathogens(Kariuki et al. 2006). Molecular epidemiological analysis of invasive and diarrhoeal strains of *S*. Typhimurium and *S*. Enteritidis will be valuable in understanding and interrupting the transmission of invasive ST313 strains in Africa. However, it is important to note that the immunological and genetic make-up of the host may still be the dominant factor influencing susceptibility to iNTS.

1.11 Management of Salmonella BSI

Salmonellae were once susceptible to a broad range of affordable and effective antimicrobials, but multi-drug-resistant (MDR) strains (Gordon et al. 2008, Kingsley et al. 2009) have emerged. Guidelines for the management of uncomplicated Salmonella enterocolitis are based on a Cochrane review (Sirinavin et al. 2000) which recommends supportive therapy only. The next section therefore focuses on what is known about the management of iNTS in Africa and draws parallels with what is known about management of the clinical syndrome caused by the classically invasive serotype of Salmonella, *S*. Typhi.

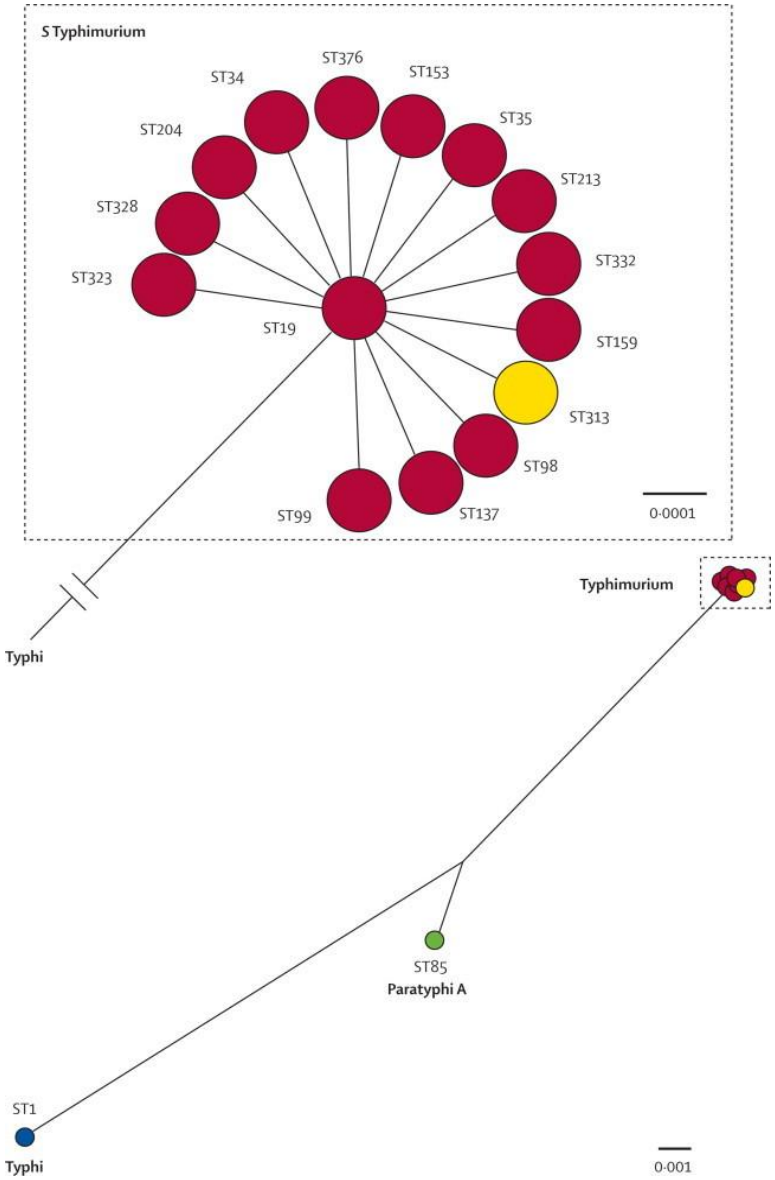


Figure 1-7: Radial phylogram showing the phylogenetic relationships of *S. enterica* serotype Typhimurium sequence types (STs).

In this figure, the *S*. Typhimurium STs (red or yellow circles) are rooted to *S*. Typhi (blue circle) and *S*. Paratyphi A (green circle). Genetic distance is calculated as the number of single nucleotide polymorphisms (SNPs) per base and is indicated as a solid black line. The length of this line is proportional to the degree of genetic divergence. *S.* Typhimurium STs from various worldwide locations outside sub-Saharan Africa SSA (Red) and from SSA (yellow) are indicated (Kingsley et al. 2009).

1.11.1 Typhoid

Chloramphenicol was first used to treat typhoid fever in 1948 (Woodward et al. 1948). It was effective in accelerating recovery to a week or less and reduced the incidence of complications and death. Relapse and chronic faecal carriage were a problem with chloramphenicol therapy, but less so if therapy was given for two to three weeks. The haematological side-effects of chloramphenicol were a concern although appeared to be uncommon in endemic areas. Chloramphenicol resistance appeared in the early 1970s but co-trimoxazole and amoxicillin were equally effective alternatives (Mirza et al. 1996).

Plasmid-mediated resistance to chloramphenicol, ampicillin and co-trimoxazole (referred to as multidrug resistant or MDR phenotype) emerged rapidly in the late 1980s in the Indian subcontinent and parts of South-East Asia in some areas causing large outbreaks (Taylor et al. 1985 , Mirza et al. 1996). Although the proportion of strains with the MDR phenotype has subsequently declined in many areas (Rahman et al. 2002) in only very few have they disappeared. Extended spectrum cephalosporins, fluoroquinolones and azithromycin have been the main alternatives in the face of resistance to the older agents (Parry et al. 2002). Drugs such as the aminoglycoside gentamicin are active in vitro but not in the patient because they lack intracellular penetration (BSAC 2009).

Oral ciprofloxacin and ofloxacin were found to be very effective for treating typhoid; based on studies of fever clearance they offer short recovery times (3–5 days) and low rates of relapse and post-treatment faecal carriage (White et al. 1996, Cao et al. 1999, Girgis et al. 1999). Oral ciprofloxacin and ofloxacin at 10-20mg/kg daily yield peak levels and an area under the plasma concentration curve (AUC) values considerably above the minimum inhibitory concentration (MIC) of against wild-type *S.* Typhi and *S.* Paratyphi A of ≤ 0.03 µg/ml. Furthermore, the antibiotics are bactericidal against the bacteria and are concentrated in the

intracellular location of the bacteria in the host. Even very short courses of therapy of five days or less at a dose of 10 mg/kg were found to be acceptably effective in non-severe disease and potentially employable in outbreak situations (Parry et al. 2002).

Unfortunately, after two to three years of widespread use of ciprofloxacin and ofloxacin as empiric therapy for non-specific febrile illness, typhoid isolates with decreased susceptibility to ciprofloxacin and ofloxacin emerged, particularly in South and South East Asia. Isolates with decreased ciprofloxacin (and ofloxacin) susceptibility (DCS) have MICs ranging between 0.1 and 0.5 μg/ml and are usually nalidixic acid resistant (Wain et al. 1997, Crump et al. 2003). These are usually spontaneous mutants that are naturally present within the wild-type population, which have been selected for by widespread antibiotic use. These strains have single point mutations at position 83 and 87 of the *gyrA* gene encoding amino-acid changes that lead to impaired binding of ciprofloxacin or ofloxacin to DNA gyrase (Wain et al. 1997). Ciprofloxacin or ofloxacin treatment of infection with these strains is associated with prolonged fever clearance times, high clinical failure rates and increased post-treatment faecal carriage when compared with the response to infections with wild type strains. These differences are particularly evident when lower doses and short durations of the antibiotics are used.

The new generation fluoroquinolone gatifloxacin remains effective for such infections (Dolecek et al. 2008). In three randomized controlled trials conducted in areas where the proportion of DCS strains is greater than 80% the cure rates have been > 90% with fever clearance times of 3-4 days and very low relapse rates. The drug is retains its activity because of binding with topoisomerase IV, usually not modified in DCS strains, as well as DNA gyrase. Gatifloxacin has been associated with dysglycaemia in the elderly and those being treated with corticosteroids and this has led to restrictions on its use in some countries (Lewis et al. 2008). Although the population at risk of typhoid, children and young adults, is

different from the group at risk of this side-effect, glucose levels should be monitored if this drug is employed.

The parenteral extended spectrum cephalosporins are the principle choice for patients sick enough to require hospital admission (Parry et al. 2002). Cefotaxime (1g IV tds) and ceftriaxone (2g IV od) retain useful activity against *S*. Typhi and other salmonellae. Reports of resistance to this group of drugs are currently rare in *S.* Typhi and *S.* Paratyphi A, although are emerging in NTS. Treatment with these antibiotics is associated with slow recovery times and relapse and must be given for a sufficient duration which is probably at least ten days (Wasfy et al. 2002). Second-generation cephalosporins are also not effective either orally or intravenously (Parry et al. 1991).

Azithromycin, an azalide antibiotic, is an effective oral option in typhoid fever (Parry et al. 2007, Dolecek et al. 2008). It is concentrated within cells, making it ideal for the treatment of this intracellular infection. It has fever clearance times of less than a week and very low rates of relapse and re-infection (Parry et al. 2007). Resistance appears to be an uncommon event, although there are anecdotal reports of increasing resistance in South Asia. As yet there are no clearly defined disc susceptibility breakpoints so the laboratory is unable to report antibiotic susceptibility to the clinician. BSAC and Eucast comment that wild-type isolates of *S.* Typhi should have an MIC ≤ 16 μg/ml (BSAC 2009). In the randomized clinical trials that have included azithromycin the dose used has varied between 10 and 20 mg/kg and the duration of treatment varied between 5, 7 and 10 days (Chinh et al. 2000). There is no clear evidence as to which is best although many recommend a dose of 20mg/kg/day for seven days.

There have been no comparative antibiotic trials for the management of severe and complicated typhoid fever. Third generation cephalosporins are often started when patients

present with severe sepsis and before a diagnosis is established. In a confirmed, fully susceptible infection whether a fluoroquinolone would be better than an extended spectrum cephalosporin is not known and the role of combination therapy is also undefined. Azithromycin should be avoided as a single agent in severe disease. In patients with intestinal perforation, surgery is necessary with a widening of the antibiotic spectrum to cover the gastrointestinal flora. In the subset of patients with an altered conscious level (delirious, obtunded, stuporose or comatose) or shock (defined as a systolic blood pressure of less than 90 mmHg in adults or less than 80 mmHg in children), with evidence of decreased skin, cerebral, or renal perfusion, where the mortality can be up to 50%, adjunctive therapy with high-dose dexamethasone (initially 3 mg/kg body weight, followed by eight doses of 1 mg/kg 6-hourly) significantly reduced mortality in a single placebo controlled randomized controlled trial conducted in the 1980's. Limitations of this study were that patients were treated with chloramphenicol rather than more modern agents and the number included was small (Hoffman et al. 1984).

The extended spectrum cephalosporins remain a suitable choice for the empiric therapy of typhoid when patients are sick enough to require admission to hospital. When the diagnosis is confirmed and the patient is beginning to improve an oral step down to azithromycin or ciprofloxacin (if the isolate is susceptible) is a reasonable strategy. Oral azithromycin or ciprofloxacin (if the isolate is susceptible) can be used from the outset if the disease is non-severe and can be managed as an out-patient. Gatifloxacin is a further option in the presence of DCS strains although it is unclear if it will remain active against strains, increasingly reported from cities in the Indian sub-continent, with full ciprofloxacin resistance (MIC ≥ 1.0 μg/ml).

The proportion of strains with the MDR and/or DCS phenotype varies in different endemic areas and has varied over time. The data are incomplete and depend on the availability of

quality assured microbiology data. In some areas such as Indonesia and The Philippines, chloramphenicol susceptible strains remain the norm. In most of the South and South East Asia strains with DCS are common and although the proportion with MDR has declined they have not disappeared. Surveillance data from Africa is limited to a few centres, but there have recently been a number of reports of Typhoid outbreaks from southern and Eastern Africa and both MDR and DCS infections have been reported in Kenya and South Africa (Keddy et al. 2010).

1.11.2 iNTS in Africa

No intervention studies have been run to investigate the optimal management of iNTS in Africa. Guidance is based on experience and expert opinion guided by the local susceptibility of the organism where available.

In Malawi, epidemics of MDR iNTS have been documented (Gordon et al. 2008). The first was caused by *S*. Enteritidis resistant to amoxicillin, chloramphenicol, cotrimoxazole and doxycycline, but the mechanisms of resistance in this setting are unknown. An association between invasive isolates of *S.* Enteritidis from African patients presenting to healthcare facilities in Europe has recently been made (Rodriguez et al. 2011, Rodriguez et al. 2012). This group found two drug resistance regions within the virulence plasmid; a class 1 integron (carrying dihydrofolate reductase (dhfr) A7, dihydropteroate synthase (sul) 1, chloramphenicol acetyltransferase and tet A and a second fragment ß-lactamase (bla) TEM-1, strA and B (streptomycin resistance) and dihydropteroate synthase 2 (Rodriguez et al. 2011).

WGS of *S.* Typhimurium ST313 strains isolated from the blood of patients in Malawi, revealed a composite element encoding MDR genes located on a virulence-associated plasmid (pSLT-BT), potentially linking antimicrobial resistance to virulence (Kingsley et al. 2009). In this case, the antimicrobial resistance genes identified were sul1 and II, strA and strB and blaTEM-50, dhfrI, aminoglycoside resistance gene aadAI, and chloramphenicol acetyltransferase.

A combination of these two epidemics and the non-specific nature of iNTS disease have necessitated the widespread use of antimicrobials including 3rd generation cephalosporins and fluoroquinolones such as ciprofloxacin for empiric management of sepsis, which the poorest health systems in the world can ill afford, and which if used widely for empirical therapy may promote the development of further antimicrobial resistance.

IDSA guidelines for the management of iNTS in HIV-infected adults recommend 6 weeks of fluoroquinolone therapy in order to prevent recurrence, however there are no intervention studies to inform the best combined antimicrobial and ART regimen to treat acute infection and prevent relapse. Furthermore, fluoroquinolones are also important in the management of drug-resistant TB, and since the two organisms may co-infect the same HIV-infected patient this might promote further TB drug-resistance. As azithromycin has been used with great success in the management of typhoid fever, it may represent an attractive alternative antimicrobial. Ceftriaxone is probably an appropriate first-line intravenous treatment for patients unable to take oral medication, but aminoglycosides do not penetrate intracellularly and so are not considered appropriate for treatment.

Recurrent iNTS in the context of HIV has been shown to decline following initiation of antiretroviral therapy (ART) in individuals outside Africa (Hung et al. 2007) and the impact of

roll-out of ART on iNTS disease in Africa has been assessed in Chapter 7 (Larsen et al. 2011).

1.12 Concluding remarks & Aims & Objectives of Thesis

In SAA, there is a large and underappreciated burden of disease attributable to iNTS, a neglected tropical disease. Studies of both the host and the pathogen have led to an understanding of the ways in which immunosuppressed populations with HIV, malaria and malnutrition in Africa have provided an ecological niche for the emergence of iNTS as opportunistic pathogens. In Malawi there have been two reported epidemics of Salmonella BSI, firstly due to *S*. Enteritidis and secondly due to *S*. Typhimurium, although a third, caused by *S*. Typhi began in mid 2011. It has been demonstrated that it was a novel strain of *S*. Typhimurium that filled the immune-suppressed human niche, becoming apparently more human-adapted in its genome. The possibility that a comparable phenomenon has occurred with blood stream invasive *S*. Enteritidis will be addressed in chapter 8. The acquisition of multidrug resistance has probably further enabled its rapid spread and evolution across the region.

Research Hypothesis

Sequential serotypes of differentially adapted NTS have rapidly evolved to occupy the evolutionary niche created by a combination of HIV and extreme poverty across SSA. As the HIV pandemic starts to decline in Africa following the roll-out of ART, so too will NTS as a cause of BSI.

Aims

- Describe trends in BSI in adults in Blantyre since the roll-out of ART
- Describe trends in iNTS disease in adults and children Blantyre, Malawi following:
    - The rapid and extensive roll-out of ART
    - The scale up of malaria control investigations
    - Improvement in food security
- Investigate the site of iNTS persistence in HIV infected adults and whether persistence contributes to microevolution of resistance
- Provide a genetic analysis of isolates of both fully susceptible and MDR *S.* Enteritidis from the Malawi epidemic to isolates from epidemics in the UK to investigate whether a distinct pathotype of SEn is responsible for BSI in Africa as is the case with STm

See Figure 1.8 for thesis outline

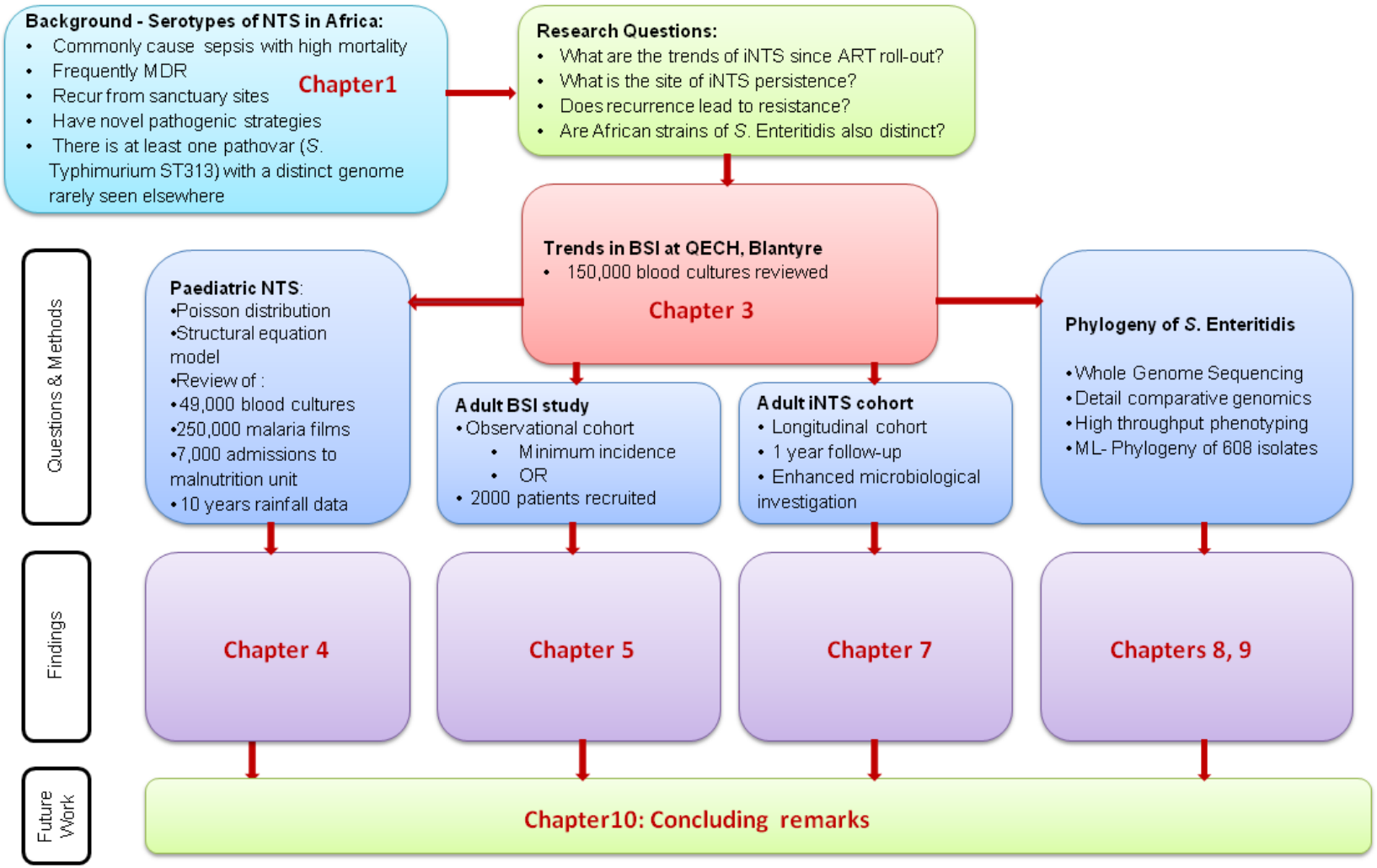


Figure 1.8: Thesis outline

Chapter 2: Setting & Methods

2.1 Overview

In order to answer the questions set out in the preceding chapter, a combination of approaches were used. These included; an extension of previous work undertaken using MLW's long-term bacterial surveillance, recruitment of new cohorts to answer specific questions about blood-stream infection and iNTS in Blantyre, Malawi and an investigation of iNTS Enteritidis in Africa using WGS and comparative genomic techniques.
MLW has conducted high quality BSI surveillance on febrile adult and paediatric medical patients presenting to QECH since 1998 as part of its core activities. The setting, criteria for culturing blood and laboratory methodology for routine diagnostic microbiology are described in section 2.1.

Patients were recruited, cared for and followed up to discharge from the study by a study team based at QECH and MLW. Two clinical studies were undertaken, the first to survey clinical outcomes from BSI and the second to look in detail at clinical outcomes from iNTS in the post ART era. The latter study required invasive diagnostic procedures and diagnostic microbiological methods with were new to MLW. The methods are described in sections 2.2 - 2.5.

The final phase of the study took place at WTSI, at which time WGS data from sequenced *Salmonella* isolates was evaluated in order to investigate the genomic architecture and phylogeny of these isolates. Accordingly, methods for DNA extraction, whole genome sequencing and WGS data analysis are described in sections 2.6 and 2.7.

2.2 Study Site

2.2.1 Malawi

Malawi is small country located in sub-Saharan Africa to the South East of Zambia and the South West of Tanzania, whilst the Southern borders are surrounded by Mozambique (Figure 2.1). It is 118,000 km^2 in area, although approximately 20% of this is lake surface-area. It is a sub-tropical country with one long rainy season from November to March, and its climate favours the production of maize as the staple crop of subsistence farmers .
It is consistently rated as one of the 10 poorest countries in the world. Most of its foreign earnings from trade are derived from tobacco, tea and sugar, although a uranium mine has recently opened. The largest single contributor to GDP however is overseas aid, which accounts for 42% of earnings . The estimated per capita income in 2009 was $833, 90% of people however lived on less than $2/day (source World Bank: http://www.worldbank.org/en/country/malawi).

The population at the time of the last census in 2008 was approximately 13 million, giving it a population density of 139/km^2, however with a fertility rate of 5.3, sharp falls in under 5 mortality and increasing adult life expectancy the most recent estimate (July 2013) is 16.7 million (Central Intelligence Agency, USA: https://www.cia.gov/library/publications/the-world-factbook/geos/mi.html). Blantyre is the principle city in the southern region, and Blantyre district has a population of approximately 1 million. The national prevalence of HIV is estimated at 11.9%, but in Blantyre it was estimated at 17.9% in 2010 (MacPherson et al. 2012). The 2010 Malawi national guidelines for the treatment of HIV infection in adults (http://www.hivunitmohmw.org) recommend commencing ART in patients with a CD4 count of 250 cells/mm^3 or who present with a WHO stage III or IV condition , although at the start of the study the CD4 count threshold was 350 cells/mm^3. iNTS is a WHO clinical stage IV disease. Due to the cost of more modern agents, patients commence on stavudine, lamivudine and nevirapine, unless co-infected with TB, despite WHO recommendations to

change to lamivudine, tenofovir and efavirenz. The guidelines recommend cotrimoxazole prophylactic therapy (CPT) for all patients with symptomatic HIV (WHO stage 2 or above) or a CD4 count <500 cells/mm^3.

Malawi is a malaria endemic country with seasonal peaks; the malaria season commences shortly after the start of the rains and lasts until May-June.

2.2.2 Queen Elizabeth Central Hospital (QECH)

QECH is the largest government hospital in Malawi and serves Blantyre district and the Southern Region. QECH provides free health care to around 10,000 adult (age>15 years) and 50,000 paediatric medical in-patients a year of whom an estimated 70% of adults are HIV-infected (Lewis et al. 2003) Most adults with severe febrile illness that are willing and able to seek health care in urban Blantyre are referred or self-present to QECH. There are also smaller numbers of secondary and tertiary referrals from outside of Blantyre.
Nursing levels are low (approximately 150 for the entire hospital in 2010) and whilst nurses are able to give intravenous drugs or fluids, oral medication, food and personal care is delivered by family members or close friends, so-called “guardians.” Guardians are critical not just to the delivery of patient care, but also the process of giving consent to care. If a patient is unable to give consent to enter a study due to confusion or severity of illness, it is considered acceptable and appropriate as part of a COMREC approved protocol to seek informed, written consent to enter a study from a guardian. This is confirmed when the study participant becomes well enough to give consent.

Figure 2-1 Map of Africa and Malawi

2.2.3 Participating Laboratories

The work was split between 2 laboratories, WTSI and MLW. All clinical procedures were performed or supervised by trained individuals. Local anti-septic precautions were taken for the study subject and barrier protection in the form of disposable gloves and aprons were used by the investigator. All chemicals and reagents were obtained from Sigma or BDH unless indicated otherwise.

The Malawi Liverpool Wellcome Trust Clinical Research Programme (MLW)

MLW is one of the Wellcome Trust's 5 major overseas programmes (http://www.mlw.medcol.mw/). It was created in 1995 in partnership with the University of Malawi College of Medicine, University of Liverpool and Liverpool School of Tropical Medicine. MLW is situated in the grounds of QECH. It has offered a quality controlled blood culture service since 1998. Blood cultures are routinely obtained from any adult patient with

an axillary temperature over 37.5°C or with clinical suspicion of sepsis. Blood cultures are obtained on febrile children admitted to the hospital who: (i) have had a thick-film which is negative for malaria parasites and have no obvious focus of infection, (ii) are considered to be critically ill with sepsis regardless of the thick-film result or (iii) fail to respond to treatment for malaria. The criteria for both admission and blood culture have not changed in the department of medicine or paediatrics since the surveillance was introduced in 1998.

Most microbiological investigations were undertaken at the MLW laboratories, and all virology, biochemistry and haematology. The one exception was TB culture which was carried out in the linked TB laboratory situated within CoM. In addition to advanced diagnostic microbiology facilities, there is a molecular laboratory at MLW, where DNA extraction was carried out. The MLW laboratory is externally quality controlled by the United Kingdom National External Quality Assessment Service (UK NEQAS).

Wellcome Trust Sanger Institute (WTSI): WTSI (Cambridge, UK: www.sanger.ac.uk) operates a high-throughput WGS pipeline based on Illumina HiSeq 2500 machines. WGS work described in this thesis, from creation of PCR libraries through to archiving of raw sequence data as fastq files, was done by the WGS pipeline team. In addition, all plasmid work (laboratory bench-work and analysis) was carried out at WTSI.

2.3 Observational Cohort Study: Blood Stream Infection at QECH following the roll-out of ART

2.3.1 Summary & Timeline

In this study, MLW's routine BSI surveillance as enhanced in consecutive patients presenting with clinical suspicion of BSI (anyone meeting the criteria for a blood culture)

between 8am and 5pm on weekdays from June 2009 - June 2010. Surveillance was enhanced by collection of demographic, clinical, laboratory and in-hospital outcome data on a standard CRF (Appendix 1.1).

2.3.2 Criteria for entry

All patients referred or presenting to the adult medical admission ward (4B) at QECH Blantyre with an axillary temperature >37.5°C were eligible. Patients were excluded if they had already been in hospital for more than 72 hours and might therefore have had a hospital-acquired infection, or if they of their guardians refused or were unable to give consent to record clinical information. Patients unwilling or unable to give consent were investigated by blood culture as usual, they were simply not recruited to the study.

2.3.3 Study Team

This study was conducted by a fieldworker (Jean Chikafa), who screened the patients for recruitment, and a team of trained study nurses; Margaret Chikopa, Harriet Chilonda, Beatrice Chilonda, and Nellie Manda. These nurses worked between 8am and 5pm Monday to Friday and had been trained to take sufficient volumes of blood (7-10mls) using aseptic technique, and the impact of the introduction of this team has been described (Mtunthama et al. 2008). Contamination rates are monitored and are consistently low at 5-10% for the entire department of adult medicine (personal communication, Brigitte Denis).

2.3.4 Protocol

All study patients were classified as suspected BSI. In addition to a blood culture, they were consented for an HIV test and had blood drawn for a malaria test during the rainy season.

Following the blood-culture result, they were further classified as either culture-confirmed BSI (patients with a significant pathogen) or culture-negative (patients with a negative/contaminated culture, as defined below). CD4 counts were not routinely checked during the study period, but were done by the clinical teams when indicated according to national guidelines. Patients were followed up until discharge and their clinical outcome was recorded.

2.4 Longitudinal Cohort Study: Outcomes from iNTS in HIV infected adult patients in the ART era

2.4.1 Summary & Timeline

Adult patients with a blood culture positive for non-typhoidal Salmonella were recruited on the day of diagnosis. Patients were followed up on a monthly basis for 3 months and again at 12 months. Study patients were prescribed IV ceftriaxone (2g od) until their fever settled and were then changed to oral ciprofloxacin (500mg bd) in order to complete 10 days of effective antimicrobial therapy. They were commenced on ART at this time. During follow up, patients had bone marrow, stool and bile sampled for selective Salmonella culture in order to attempt to determine the site of persistence of NTS, as described below. Recruitment for this longitudinal cohort study was initially conducted as a pilot study from January - June 2010 and subsequently from October 2012 to March 2012. The final 1 year follow-ups were concluded in April 2013. Data was entered onto a standard CRF (Appendix 1.2).

2.4.2 Study Team

In addition to the PI, the study team included a fieldworker, Jean Chikafa, who screened patients and a clinical officer, Mr Patrick Goodson, who assisted with recruitment, follow-up

and clinical procedures. Between these three individuals, there was always a team member on site between 8am and 5pm Monday to Friday in order to review patients.

2.4.3 Criteria for entry

Inclusion criteria

- HIV positive adults (over 16 years)
- Competent to give informed consent, if necessary with the assistance of a guardian
- Microbiologically confirmed iNTS
- Domicile in Blantyre, or within 30km of QECH

Exclusion criteria

- Individuals who are terminally ill
- Individuals who have undergone upper GI surgery
- Women who are pregnant, or who suspect they might be pregnant

  Children under the age of 16 years
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives

2.4.4 Study visits and patient sampling

Following diagnosis with iNTS, informed written consent to enter the study was obtained and blood was drawn for Hb, CD4 count, HIV VL and stool obtained for selective Salmonella culture (Figure 2.2). The patients were commenced or continued on IV ceftriaxone (2g od) until deemed well enough to switch to oral ciprofloxacin (500mg bd) to complete a 10 day course of therapy modelled on treatment regimens for *S*. Typhi (for which there is a greater evidence base, but still no large RCTs to guide treatment duration). Patients were encouraged to register with an ART clinic at diagnosis and to commence ARVs within 2

weeks of diagnosis. They were discharged as soon as well enough to go home and followed-up 1 month after commencement of treatment.

Visit 1

The 1 month follow-up was the principle time point for investigation of persistence of NTS. At this time, blood, bile, faeces and bone marrow aspirate (BMA) were collected (see detailed description below for how specimens were handled and processed). A maximum of 20ml of blood was obtained which represented less than 2% of the circulating volume of the subjects recruited. Blood culture was repeated to ensure that a positive BMA (if present) represented true persistence in the bone marrow and not (in the case of a positive blood culture) ongoing or recurrent circulating systemic infection. 2ml was saved for batched HIV viral load testing, 2ml for Hb and 2ml for CD4 count.

Enterotest

An Enterotest™ capsule (HDC Diagnostics, San Jose, California), also known as a “hairy string capsule” was used to capture bile for selective Salmonella culture. This is a weighted gelatin capsule containing a 140cm absorbent nylon string. One end was pulled from the capsule and taped to the side of the mouth (Figure 2.3) and then the patient swallowed the capsule. For this test, the patient is required to fast overnight, then remained ambulatory but fasting for the next 4-6 hours. The string was then retrieved after 4-6 hours by tipping the patient’s head back and gently withdrawing the capsule, by which time the capsule had been carried to the duodenum and dissolved, as evidenced by bile-staining of the string. The bile stained portion of the string was then cultured overnight in selenite enrichment broth, before being inoculated onto XLD agar (see below for full microbiological methods). The procedure causes mild discomfort, but no complications have been described (12), and none were seen in this study.

Bone Marrow Aspiration

In order to sample the intracellular compartment, bone marrow fluid was aspirated (Gordon et al. 2010). This semi-sterile procedure involved regional antisepsis, careful local anaesthesia infiltration, and aspiration of 2-3ml using a disposable bone marrow needle inserted at the posterior superior iliac crest, with the patient in the left lateral position (Figure 2.4). Aspiration of larger volumes produces dilution of the specimen with circulating blood. As peripheral blood would already have been adequately sampled, an increased volume of diluted bone marrow would not have added to the sensitivity of the diagnostic technique.
If the subject experienced undue discomfort, the procedure was abandoned. Possible complications are bleeding or infection, but this has not been seen in over 100 procedures at MLW under study conditions (Gordon et al. 2010). Furthermore the procedure might have detected persisting NTS, which might then be treated prior to a symptomatic relapse, a result which would directly benefit patients. Subjects found to have asymptomatic persistence on bone marrow-culture were intended to be offered appropriate secondary antibiotic treatment to prevent bacteraemic relapse.

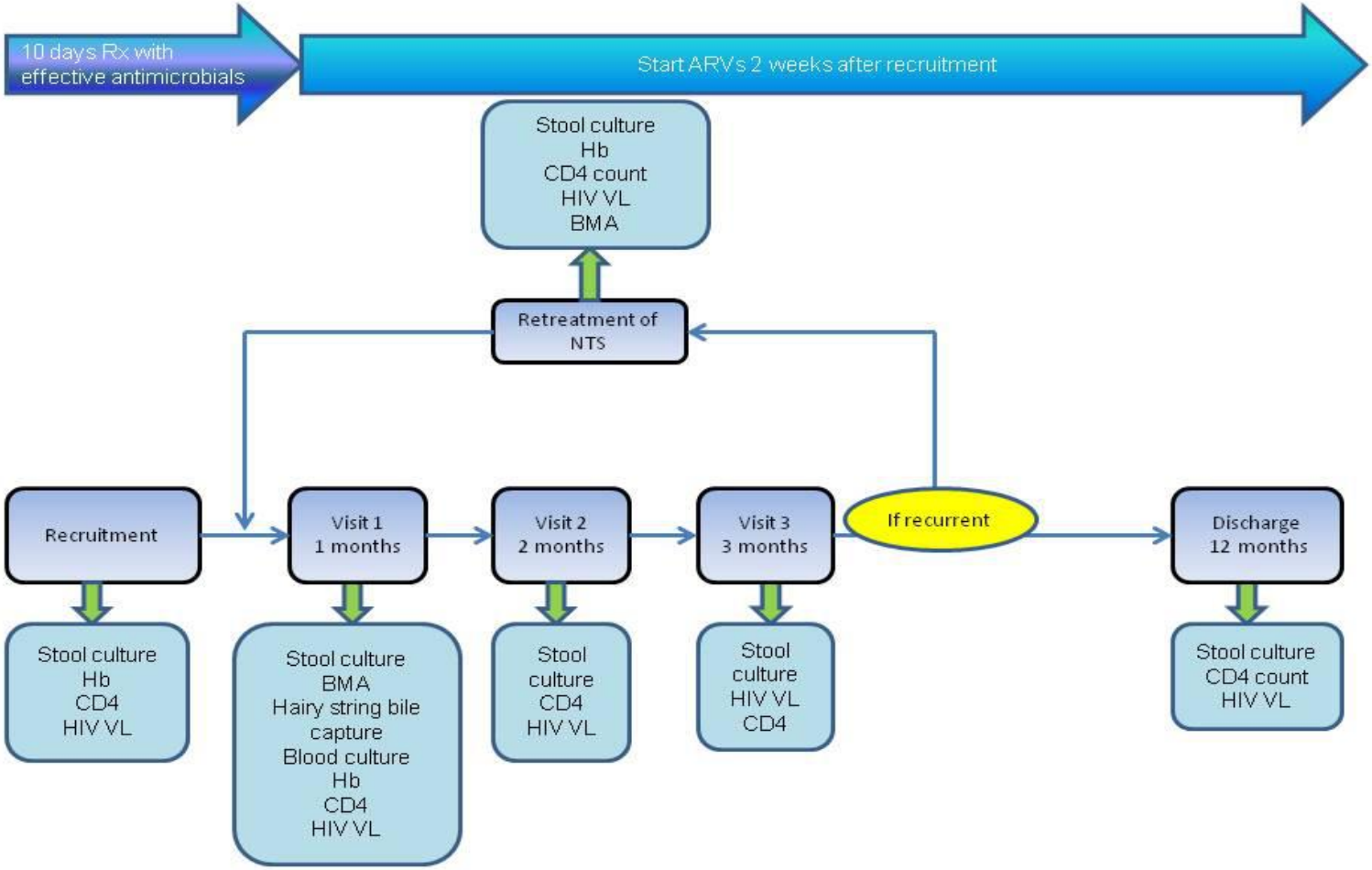


Figure 2-2 Study Flow: Outcomes from iNTS in HIV infected adult patients in the ART era

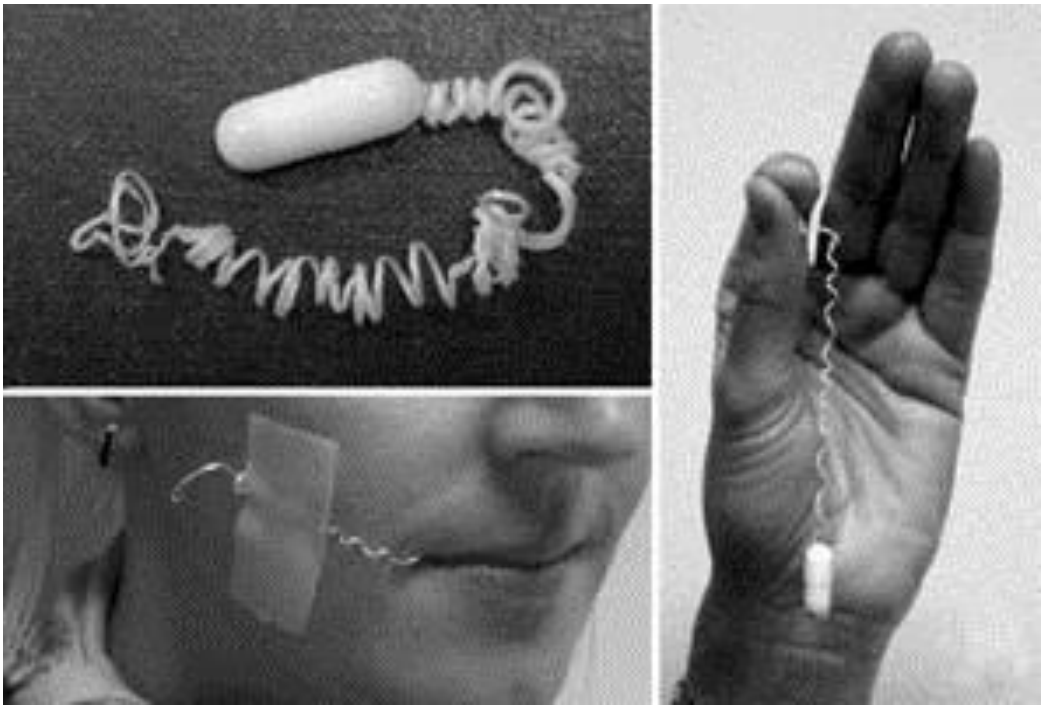


Figure 2-3: Enterotest capsule: Top left and right, string extending from capsule, bottom right, string attached to patient's cheek (Leodolter et al. 2005)

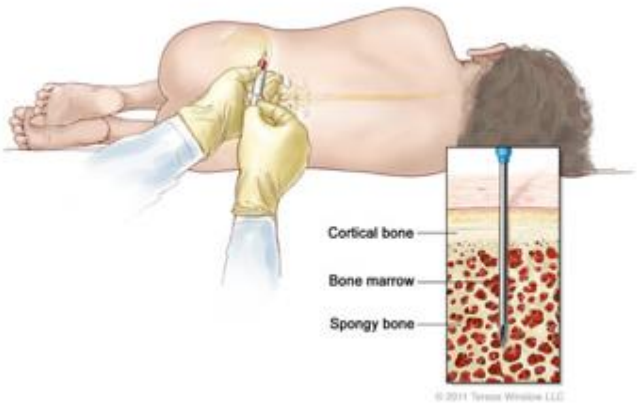


Figure 2-4: Procedure for bone marrow aspiration

Visits 2 & 3

The second and third visits were scheduled at 2 and 3 months after recruitment. At this time blood, for Hb, CD4 count and HIV VL was taken, and a further stool sample for selective Salmonella culture. This visit offered the opportunity to track the clinical progress of patients early in the course of immune reconstitution and at their most vulnerable to further OI, IRIS or death.

Tracking between visits

Patients were given the mobile phone number of the field worker, Mrs Jean Chikafa who is fluent in Chichewa and so were able to contact the study team whenever required. The study team recorded the patient's phone numbers or the number of a friend that they could be contacted on. Details of how to find their homes were also recorded. After the third visit, patients were contacted on a monthly basis to ensure they were still in receipt of ART and still alive until the final, 12 month follow-up.

Recurrence

Recurrence was clinically defined as any repeat episode of iNTS, following appropriate antibiotic treatment, within the 1 year period of follow up. During the study, blood culture results were monitored to detect study patients with recurrent disease. Discrimination between relapse and re-infection by iNTS is possible by comparative genomic analysis of WGS data from sequenced isolates (Okoro et al. 2012(2)). In the event of recurrence, patients were consented to have a further marrow, stool and bile culture, and blood drawn

for CD4, HIV VL, and serum and plasma. They then returned to “Visit 1” on the study flow chart.

Visit 4 & discharge

Patients were booked in for review at 12 months, at which time blood was drawn for CD4 count, HIV VL and stool taken for selective Salmonella culture. Following this visit they were discharged from the study.

2.5 Diagnostic Laboratory Procedures

2.5.1 Blood culture

Blood culture was undertaken by sampling 7-10mls of blood, using an aerobic bottle (BacT/Alert bioMérieux, UK). All isolates were identified using standard diagnostic techniques as previously reported (Barrow G et al. 1983). Salmonella serotyping follows from what is known about common *Salmonella* serotypes isolated at MLW, and antisera are kept to identify Salmonellae as one of *S*. Typhimurium, *S*. Enteritidis, *S*. Typhoid or *Salmonella* sp. The following antisera are kept: PSO, PSH, O4, O9, Hi, Hm, Hg, Vi, Hd. If an isolate is PSO and PSH positive, but otherwise negative, it is recorded as *S. sp*. For the purpose of these studies, organisms that are routinely found as part of the normal skin or oral flora were considered to be contaminants, including; coagulase-negative *Staphylococci* (CNS), bacillus spp. and diptheroids. Alpha-hemolytic *Streptococci* (AHS) other than *S. pneumoniae* were also considered as contaminants when there was no clinical suspicion of endocarditis.

2.5.2 Selective Salmonella Culture from Faeces

When screening for Salmonella carriage in the GIT of an otherwise asymptomatic patient (specifically without diarrhoea), faecal samples were collected. Once received in the lab, the stool was processed under a laminar flow cabinet. A loop-full of the sample was added to a labelled 10ml aliquot of Selenite F broth and incubated in air at 37˚C overnight. On day 2, a loop-full from the top of the Selenite F broth was onto inoculated onto XLD agar and incubated in air at 37˚C overnight. If there was no growth suggestive of Salmonella overnight, the plates were discarded. If hydrogen sulphide producing (black) colonies grow on the XLD plate, a single colony was picked and inoculated onto a triple sugar agar slope, a urea slope and a blood agar (BA) plate and incubated in air at 37˚C overnight. If the triple sugar slope was positive (black streak) and the urea slope was negative (no colour change), then colonies on the BA plate were used to set up an API 10S (bioMérieux), DST plate and to perform Salmonella serology. On the final day, the API and sensitivity plate were read, the results recorded and all confirmed Salmonella were stored at -80˚C.

2.5.3 Bile culture

The entire hairy string was immersed in a labelled 10ml aliquot of Selenite F broth and incubated in air at 37˚C overnight and the above protocol for selective culture of Salmonella from faeces was followed from this stage onwards (Barrow G et al. 1983).

2.5.4 Bone marrow aspirate fluid culture

Marrow aspirate was inoculated into an aerobic bottle (BacT/Alert bioMérieux) and from this stage the standard culture procedure for blood was followed.

2.5.5 Antimicrobial susceptibility testing

DST was performed by disc diffusion according to British Society of Antimicrobial Chemotherapy standards (BSAC 2009). Penicillin resistance in *S. pneumoniae* was defined as a zone size of ≤19mm against a 1µg oxacillin disc on iso-sensitest agar supplemented with 5% sheep blood incubated overnight in 5% CO_2. All isolates of Salmonella were further tested to derive a precise MIC of ciprofloxacin by E-test® (bioMérieux, UK).

2.5.6 Other diagnostic tests

Thick blood films were examined for Plasmodium parasites by trained MLW technicians. HIV testing was performed according to the Malawi national HIV rapid antibody testing protocol, using Determine^{TM} HIV-1/2 tests as the first test in a serial testing algorithm. All positive test results were confirmed by Uni-Gold^{TM}.

Full blood counts were tested by one of three machines (SYSMEX KX-21N, ABX MICROS 60, Beckman Coulter Ac-T 5diff CP). All three machines were routinely serviced, calibrated and results were quality controlled (see below). Haemoglobin (Hb) levels were classified following WHO recommendations (anaemia: < 13.0 g/dl in men or <12.0 g/dl in women; severe anaemia: Hb <8.0g/dl in men and women) (WHO 2011). CD4 counts were performed on a Beckman Coulter EPICS (Indianapolis, USA).

2.5.7 HIV Viral load

RNA for HIV VL was extracted from 1 ml of blood using the Qiagen Universal Biorobot® (Limburg, Netherlands) and Qiagen All-for-one ® extraction kits. HIV viral load was assayed using the Abbott m2000rt.

2.6 Laboratory Quality Control at MLW

Laboratory external quality assurance at MLW was monitored by subscription to the United Kingdom National External Quality Assessment Service (UK NEQAS) schemes for the entire period of these studies.

2.7 Data storage and statistical analysis

Microbiology results for all samples processed at MLW (both routine and study) since the inception of the service have been recorded in databases. From 1998-2010 results were manually transcribed into books. Data from this era were double entered into a secure, Microsoft Access database and discrepancies were checked. During 2010, an electronic laboratory information management system (LIMS) was introduced at MLW and from October 2010, all specimen request forms and laboratory worksheets have been scanned and all microbiology results directly entered into the LIMS.

All CRFs were constructed in Microsoft Word, printed onto paper and filled in by hand. All research staff received training on how to complete the CRFs prior to the study. All completed CRFs were stored in the MLW archive and will be held for 10 years.

Completed CRFs from the BSI study were sent to the MLW data entry clerks at the end of each calendar month during the course of the study. Data were double entered into a secure Microsoft Access database.

Unless otherwise stated, all statistical analysis was conducted using STATA for windows software (version SE/11; 4905; Stata corp; College Station, Texas 77845 USA). Details of specific statistical methodology are provided in subsequent chapters. Statistical significance is reported throughout this thesis at the level <0.05.

The primary aim of the thesis was to detect either phenotypic fluoroquinolone resistance, or the emergence of a genomic signature of incipient fluoroquinolone resistance. During the process of study design, the issue of performing a power calculation to inform study design was considered and I was advised by collaborators at WTSI that attempting to detect the emergence of resistance in real time had not been attempted before, and therefore that a power calculation would not be possible

2.8 Molecular methods

2.8.1 DNA extraction for whole genome sequencing

DNA extraction for whole genome sequencing was run on the Qiagen Universal Biorobot® (Limburg, Netherlands) using Qiagen All-for-one ® extraction kits. Salmonellae grow so well in culture that there is a risk of blocking the filters on the machine by submitting too many cells. The following protocol was used to produce optimal specimens for DNA extraction:

Day 1: Streak out bacteria onto Luria-Bertani (LB) Agar. Incubate overnight in air at 37°C.

Day 2: Dispense 800µl aliquots of nutrient broth into cells of 96-well plate. Pick a single colony and inoculate into nutrient broth (either LB or brain heart infusion (BHI). Seal plate and incubate overnight in air at 37°C.

Day 3: Centrifuge box: first weigh & prepare counter-balance, check plate holders are matching/balanced and then centrifuge at 10,000 g, at room temp, for 5 minutes. Discard supernatant by invert into bucket containing Virkon™ (seal and leave bucket overnight), then tap residual onto blotting paper.

The pellet was then re-suspended in 800 µl of sterile saline, sealed and vortexed and the split down into 2x200µl aliquot libraries and 1x400µl aliquot library. The first 200µl library was used for DNA extraction, the second was sealed and stored in a refrigerator and the last was sealed and frozen.

2.8.2 Preparation and use of DNA extraction robot

The manufacturer's instructions (http://www.qiagen.com/products/catalog/automated-solutions/sample-prep/biorobot-universal-system) were followed, with a pause factored in after addition of buffers in order to centrifuge at 10,000 g for 2mins. Products of bacterial lysis formed a pellet at the bottom. Further pauses were included after each vacuum step to check if plate had emptied, if not, a sterile needle was used to unblock the filter as per the manufacturer's suggestion. The final output volume was set to be 110µl.

2.8.3 Proof of success of DNA extraction

Two µl of DNA was mixed with loading dye and this and a DNA ladder were run on a 0.8% agarose gel stained with ethidium bromide. The gels were run at 90 mAmp for 30 minutes, then photographed under UV lamp using a Gel-Doc (Bio-Rad, California, USA).

2.8.4 Dispatch to WTSI

The first 50 µl was sent to WTSI in labelled, Parafilm™ sealed ependorfs and the remaining 50-60 µl stored in the fridge in case of loss during shipping. DNA was shipped with a photograph of the gel and a manifest.

2.8.5 Plasmid DNA extraction

Two methods were used for plasmid DNA extraction. The first (Kado & Lui) was used as a screen for the presence of plasmids, but the product needs to be used immediately. The second method (Alkaline Lysis) enables the storage of extracted DNA in a refrigerator; freezing would, however, destroy the plasmid DNA.

Kado & Lui

To analyse bacterial plasmid content and sizes, the method of Kado and Liu was performed (Kado et al. 1981) with minor modifications (suggested by Dr Derek Pickard at WTSI). The protocol is as follows:

Reagents:

Normal strength E Buffer:

- 40mM Tris
- 2mM Na2EDTA
- pH adjusted to 7.9 with Glacial Acetic Acid

Lysis Buffer: Make up precisely.

1. Weigh out 0.6055 grams of Tris base and dissolve in 40mls of distilled water. Add 30mls of 10% SDS to this.

2. Make up fresh, 100mls of 2M NaOH. Use a 100ml volumetric flask for accuracy. Can be stored for use in Kado and Liu for up to 3 weeks maximum.

3. To bring the SDS/Tris solution to pH12.60, add 3.2mls of the 2M NaOH solution. Bring to 100mls with water using a volumetric flask for accuracy once again. Use this lysis solution on the day of preparation only.

Phenol/Chloroform: Ultrapure phenol/chloroform (1:1 ratio) saturated with Tris pH8.0

5 X Final Sample Buffer: 100ml volume, 30% Glycerol plus 50mM DiSodium EDTA (pH8.0), 10mM Tris (pH8.0) and 0.25% Bromophenol blue

Kado and Liu method for Plasmid Screening

1. Take 4 colonies of bacteria off a streaked plate and add to 3ml of L-broth culture. Leave at 37^{O}C overnight in a shaking incubator.
2. Centrifuge down to recover pellet, drain and resuspend in 150ul E buffer
3. Add 300ul of lysis buffer and gently mix to observe clearing.
4. Heat sample at 55^{O}C for 60 minutes precisely. Add up to 600ul of phenol/chloroform and gently mix. Centrifuge for 30 minutes to separate layers. Remove approximately 35 to 45ul of sample from top layer and add 8ul FSB.
5. Load samples onto a 0.7% E buffer gel. If available, run samples down a vertical gel apparatus to enhance banding resolution.

6. Run gel at 10 volts per cm of gel length. Circulate buffer between compartments if possible when using vertical gels.
7. When blue dye is near bottom of the gel (or run longer if necessary), remove the gel to a sandwich box containing Ethidium Bromide (add 10ul of a 10mg/ml stock to 200mls of water)
8. Leave to stain for 30-45 minutes and then photograph using the Gel-Doc**.**

Alkaline Lysis

Plasmid DNA was extracted using a second alkaline lysis method (Sambrook et al. 1989).

1. 1.5 ml of overnight culture was pelleted by centrifugation at 30,000 g for 30 secs.
2. The pellet was re-suspended in 100µl of ice-cold buffer (Solution 1: 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8)
3. 200 µl of freshly prepared lysis solution (Solution 2: 0.2 M NaOH, 1% SDS) was mixed in by gentle inversion.
4. The tube was kept on ice whilst 150 µl of ice-cold precipitation solution (Solution 3: potassium acetate; 5 M with respect to potassium and 3 M with respect to acetate) was added, the tube inverted and then held on ice for 5 minutes.
5. After centrifugation at 30,000 g for 5 minutes the supernatant was transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (of proportions 25:24:1) was added, mixed by vortexing, then centrifuged for 2 mins at 30,000 g.
6. Double-stranded DNA was precipitated from the supernatant using 2 volumes 70% ethanol and mixed by brief vortexing.
7. This was allowed to stand 2 mins at room temperature, then centrifuged for 5 mins at 30,000 g.
8. The supernatant was removed and the pellet washed with 1 ml of ice-cold 70% (w/v) ethanol, spun and centrifuged as before.

9. The pellet was re-disolved in 30 µl TE buffer containing RNase A (20 µl/ml) and stored at –20°C. For more concentrated plasmid DNA samples, for example those used for restriction endonuclease digestion (see 2.4.2), up to 12 samples from the same culture were extracted separately, and then pooled.

2.8.6 Whole Genome Sequencing

Following DNA extraction, PCR libraries were prepared from 500ng of DNA as previously described (Quail et al. 2012). Isolates were sequenced using Illumina HiSeq2500 machines (Illumina, San Diego, CA, USA) and 150 bp paired-end reads were generated.

2.9 Bioinformatics

2.9.1 Comparative Genomics and Phylogeny

Two approaches were used to investigate sequenced genomes. Firstly, simple phylogeny using a small strain collection and based on single nucleotide polymorphism (SNP) differences between core regions of bacterial genomes was constructed to look for evidence of large differences suggestive of different clades. On the basis of any differences, representative examples of these clades were then annotated to create “enhanced” draft sequences. Once a rationale for further investigation had been derived, detailed phylogeny based on SNP differences between core regions of bacterial genomes was created using large collections of isolates.

2.9.2 De-novo assembly, alignment and comparison of individual genomes

Isolates were assembled using Velvet (Zerbino et al. 2009) and aligned against reference strains using Abacas (Assefa et al. 2009). Annotations were then transferred from the reference to the aligned genome using annotations-update. Contigs that failed to align were considered to be potential components of mobile genetic elements, and were investigated using gene-prediction models (Glimmer3 (Salzberg et al. 1998)). Predicted genes were curated using FASTA (Pearson et al. 1998). Non-aligned segments were then concatenated to the aligned genome and the entire genome was compared to the reference by BLAST and visualized using the Artemis Comparison Tool (ACT) (Carver et al. 2005).

The ACT comparison file was then inspected in 2 stages. Firstly differences on a large scale (insertions, deletions and rearrangements) and their impact upon individual genes were noted. In the second pass, on a much smaller scale, genes were inspected one by one to look for SNPs and, if present, whether they caused synonymous or non-synonymous mutations. This approach was taken because genomic differences at a structural level are evaluated at a different magnification of the genome sequence to SNPs.

2.9.3 Creating a phylogeny

In this approach, graphically represented in Figure 2.5, paired-end reads were mapped straight onto a reference genome and the phylogeny was estimated using maximum-likelihood modelling. Only the core regions of the reference genome were used. This is because the inclusion of mobile genetic elements such as prophages and plasmids, which may transfer horizontally, would confound an evolutionary model based on the assumption that strains have evolved from a point source.

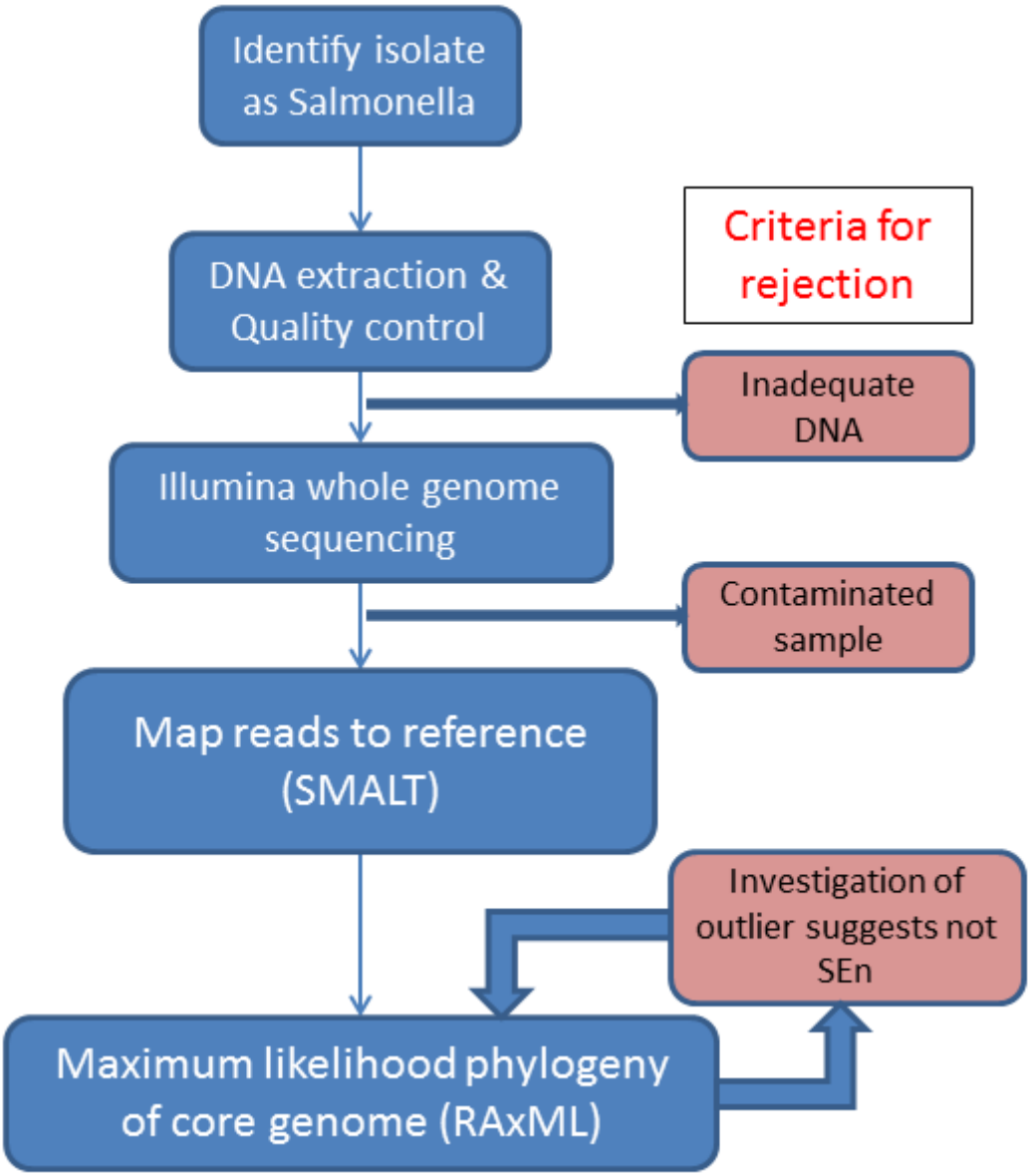


Figure 2-5: Phylogeny pipeline

2.9.4 Mapping

Paired-end reads in a fastq format were mapped against a reference multifasta using SMALT via a mapping pipeline at WTSI created by Dr Simon Harris. The output included BAM files for each genome and a single combined alignment file.

2.9.5 Curation of alignment

It is necessary to remove variable regions of the genome, including plasmids, prophage regions and insertion elements as these mobile genetic elements can by nature move in and out of genomes and therefore do not necessarily reflect the long term evolution of the organism. In order to achieve this, a script called “remove blocks from alignment” (SR Harris) needs was used. The “blocks” are the variable regions of the reference genome which need to be specified in a table file of these regions. Variable regions of the sequences under investigation that are not present in the reference genome will not have been mapped anyway.

Alignment files are often huge and it is helpful first to reduce their size then to curate them before attempting to construct a phylogenetic tree. In order to reduce the file size to one usable at a reasonable speed by phylogeny-generating software and to manually quality-control data, it is useful to use a programme that can find SNP sites from an alignment file. In this study, the WTSI in-house programme “snp_sites” (A. Page) was used.

Once this had been done, the alignment was visualized using SeaView (Gouy et al. 2010) a graphical user interface for multiple sequence alignment. Sequences that appeared to have exceptionally different alignment from the rest were then individually investigated before making a decision about whether to retain them in the analysis. For example, NTS were selected on the basis of serotype, which at best has 95% reproducibility. Isolates which were significant outliers were individually curated by in-silico PCR (“primers_to_tab” [S.R. Harris]) using serotype specific primers (Levy et al. 2008, Tennant et al. 2010) and in-silico MLST (“get_sequence_type” [A. Page]) and if found to be of both the wrong sero-type and wrong MLST group, were removed at this stage.

2.9.6 Generation of Phylogenetic Tree

Phylogenetic trees were generated using the maximum likelihood method. This is a character state method which considers a nucleotide position as a character and the nucleotide at each position as a "state". The previous methodology generated a single file of SNPs aligned in columns, with individual isolates in rows (Lemey et al. 2009). Maximum Likelihood (ML) methodology aims to find a tree topology for the alignment which can be explained with the smallest number of character changes. ML algorithms search for the tree that maximizes the probability of observing the character states. This process is very computationally demanding. In this study, Randomized Accelerated Maximum Likelihood (RAxML) was used, a program for sequential and parallel Maximum Likelihood-based inference of large phylogenetic trees which employs several heuristics to drastically reduce likelihood search times (Stamatakis et al. 2005, Stamatakis 2006).

2.10 Ethical approval & consent

Ethical approval from the University of Malawi College of Medicine Research Ethics Committee was prospectively applied for. The study "Blood Stream Infection at QECH following the roll-out of ART" was granted approval under the COMREC number P.05/08/672 and the study "Outcomes from iNTS in HIV infected adult patients in the ART era" was granted approval under the COMREC number P.07/09/808 (see Appendix 3).

Informed written consent was obtained from all patients at the time of recruitment. A patient information sheet was provided in both English and Chichewa, which made it clear that patients were free to withdraw at any time. The consent form conformed to GCP and was approved by COMREC and LSTM.

Chapter 3: Trends in Salmonella BSI at Queen Elizabeth Central Hospital, Blantyre 1998-2013

3.1 Summary

MLW has conducted quality controlled routine blood culture surveillance of febrile paediatric and adult medical patients presenting to QECH since 1998. This huge dataset has been reviewed to evaluate changes in Salmonella BSI at QECH over the last 15 years.

There have been three sequential epidemics of Salmonella BSI, all preceded by and closely associated with the emergence of the same multidrug resistant phenotype; resistance to amoxicillin, chloramphenicol and cotrimoxazole. The first was an epidemic of *S*. Enteritidis from 1999-2003, following which not only the number of cases, but also the MDR fraction declined. The second was an epidemic of *S*. Typhimurium that began in 2000 and which is currently still declining, but in which the MDR phenotype has persisted. iNTS has dramatically declined from its peak in 2003, but still remains an important cause of BSI. Recently, however an ongoing epidemic of MDR *S*. Typhi began in Blantyre.

Despite the extensive use of cephalosporins and fluoroquinolones and the emergence of ESBL-producing and fluoroquinolone resistant *E. coli* and *Klebsiella sp.* in Blantyre, similarly extensive drug resistance has only been detected once among invasive Salmonella isolates.

Review of the age distribution for these invasive pathogens confirmed a discretely bimodal age distribution of iNTS in adults and preschool children, whereas presentation of *S*. Typhi was across a broader distribution of children and young adults. It also suggests a changing

epidemiological picture for paediatric *S*. Typhimurium, with substantial reductions children under the age of 1 year.

These data point to the need for several avenues of future research to explain these observations. Important questions posed by these data include:

1. What factors may have led to emergence of sequential epidemics of iNTS and Typhoid?
2. What factors have contributed to the overall decline is iNTS in children (chapter 4) and adults (chapter 5 & 7)?
3. What were the mechanisms of cephalosporin and fluoroquinolone resistance in the single extensively drug resistant isolate of *S*. Typhimurium (chapter 6)?
4. What, if any, is the nature of the genomic and phenotypic difference between drug susceptible and MDR strains of *S*. Enteritidis (chapter 8 & 9)?

3.2 Introduction

From 1998-2004, NTS were the most common blood culture isolates from patients at QECH and 2 epidemics of MDR iNTS were described in this period (Gordon et al. 2008). In contrast, *S*. Typhi was an uncommon cause of BSI (Figure 3.1), and the isolates have predominantly been drug susceptible.

A previous analysis of the age distribution of patients with Salmonella BSI presenting at the peaks of the epidemics in Malawi revealed a bimodal age distribution (Figure 3.1), with peaks in children under the age of 2 and adults between 20 and 40 years, reflecting the role of HIV as a risk factor for adult iNTS disease (Feasey et al. 2010). There was a significant gender difference in median age of infection amongst adults (median age 33 years for

women vs. 37 years for men; p<0.001) reflecting the different age at which HIV infection is most prevalent in SSA (WHO 2010).

Since 2004, numerous changes have occurred in Malawi which might impact upon the incidence of Salmonella BSI. There has been rapid and progressive roll-out of: antiretroviral therapy (ART) – including prevention of mother-to-child transmission (PMTCT) therapy, cotrimoxazole prophylactic therapy (CPT) and malaria control interventions (see chapter 2.1 setting). Furthermore there has been a nationwide fertiliser subsidy programme leading to improved nutrition, and an increase in access to improved drinking water and improved sanitation facilities . In-patient care at QECH has been improved by the increased in the number of trained medical staff and the introduction of broad spectrum antimicrobials, ciprofloxacin in 2002 and ceftriaxone in 2004 (personal communication, M. Molyneux). During this time period, admissions to QECH have been roughly stable (Everett et al. 2011).

In light of these changes, the trends in isolation of Salmonella from the blood of febrile patients presenting to QECH from 1998-2013 have been described in order to evaluate, for the major serotypes:

1. Total numbers
2. Age distribution
3. Drug susceptibility profile

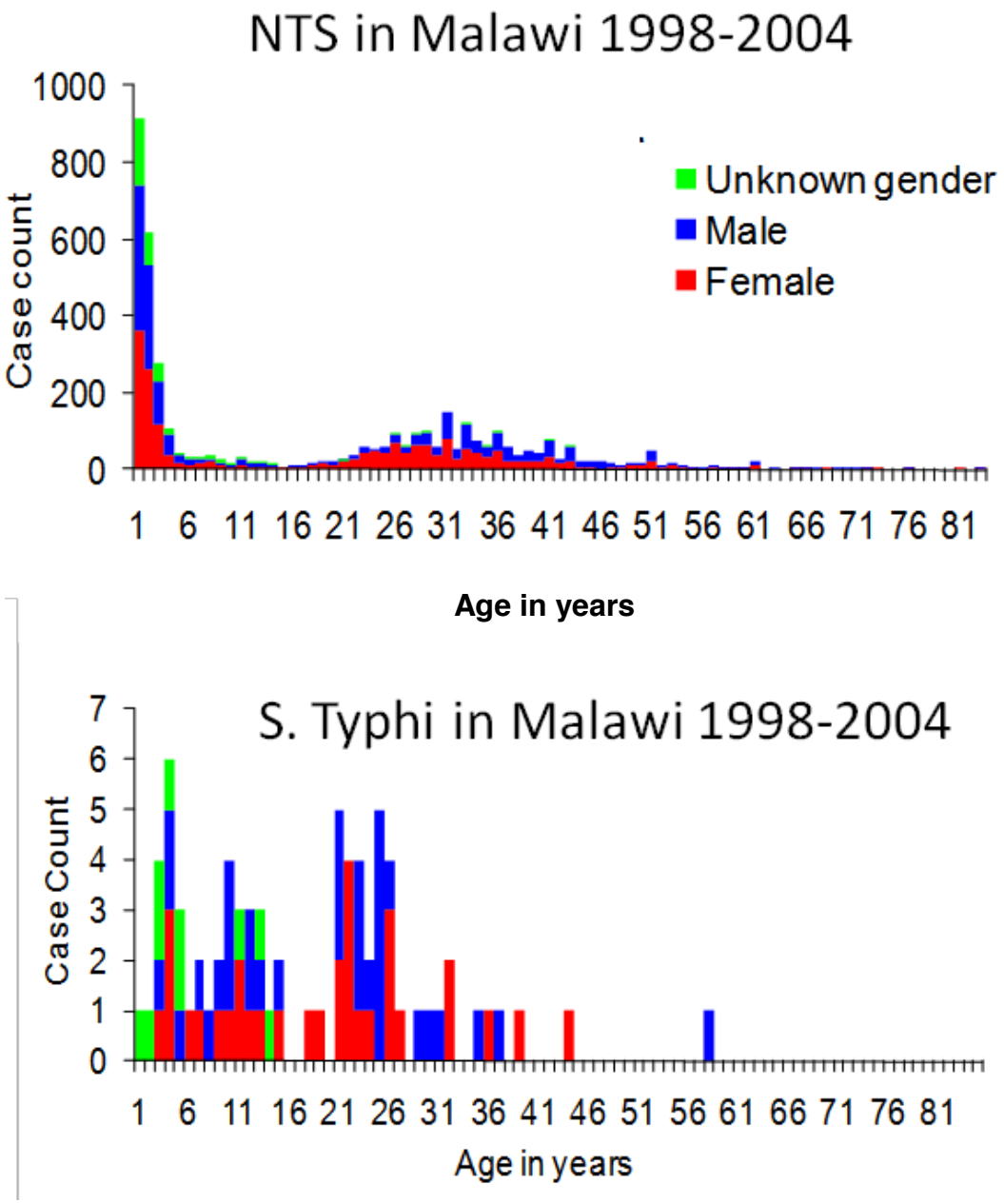


Figure 3-1 Age and gender distribution of patients with Salmonella BSI in Blantyre, Malawi, 1998–2004

3.3 Methods

The setting, criteria and methods for taking blood cultures, the automated blood culture system and the methods for processing positive blood cultures and data handling are described in chapter 2.

At the time of writing, fully-validated data entry of the entire MLW archive from the original ledgers is underway, but incomplete. The data have therefore been drawn from four sources:

1. MLW Laboratory Information Management System; all blood culture results have been entered into this digital system in real-time from October 2010 onwards.
2. The MLW archive; this is an ongoing double-entry data exercise of all blood culture results including negative cultures, complete to the dates listed below
    a. Adult blood cultures: 2004 - Oct 2010
    b. Paediatric blood cultures: 2009 - Oct 2010
3. A previous data entry exercise of positive blood cultures – paediatric Salmonellae 2005 - 2008 (inclusive) and *S. pneumoniae* 2005 - 2008 (inclusive).
4. MD thesis of Dr M Gordon: all fully validated results from 1998-2004

Sources 1, 2 and 4 were considered to be of high quality as they include all blood culture results. Source 1 was validated in real time and 2-4 were double entered from the original ledgers. Source 3 was an exercise conducted in 2009, and was also double-entered, however the staff who conducted the exercise have left MLW, therefore this data-set was spot-checked for accuracy against the ledgers by checking three consecutive weeks/year from this database against the original ledgers. Total blood culture numbers and total *S. pneumoniae* numbers were used to put the figures into context.

The dataset was analysed using stata 12 (version SE/12; Stata corp, Texas USA). The full dataset was analysed by year to describe trends and then subdivided according to age. Age strata used were ≤1 year, age >1 - <16 and ≥16 to denote adults. In order to investigate whether there has been a change in age distribution, median age with IQRs was calculated. Medians were compared using the Kruskal-Wallis equality of populations test. As iNTS in this setting has previously been reported to have a bimodal age distribution, age distribution of patients with *S*. Typhimurium BSI was described for adults over 16 and children under 16, whereas cases with *S*. Typhi were described as one group.

In order to describe trends in drug resistance, isolates were categorised according to combination of drug resistance displayed and were re-coded as MDR if resistant to 3 or more antimicrobials and trends in the proportion of isolates over time which were MDR have been presented.

3.4 Results

3.4.1 Data spot check

In total, 3/249 (1.2%) pathogens had been omitted. The 98.8% accuracy from the spot check suggested that the previous data entry exercise was of an acceptable standard and the data not covered by the other data entry exercises was abstracted from this set.

3.4.2 Trends in Salmonella BSI at QECH in Blantyre 1998-2013

A total of 153,365 blood cultures were taken from patients presenting to QECH and processed at MLW in nearly 15 years between 1st January 1998 and 30th November 2013; 84,699 from children <16 and 68,930 from adults ≥16 (Table 3.1). Salmonella was isolated from blood 10,132 times (6.6%). In comparison, the second most common bloodstream isolate, *Streptococcus pneumoniae,* was cultured on 3,692 occasions (2.4%). Figure 3.2 A-C summarises the number of cultures and annual trends in rates of Salmonella and Pneumococcal isolation and puts the trends in context of total number of blood cultures taken. Figure 3.2D describes the changes in the yield of Salmonella from blood cultures.

Table 3.1: Trends in Salmonella isolation at MLW from 1998-2013

		1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Totals
All	*S.* Typhimurium	253	298	382	454	847	1,031	704	718	562	508	472	279	200	183	118	111	7,120
	S. Enteritidis	48	186	199	240	137	95	77	76	49	47	44	36	33	22	19	12	1,320
	S. sp.	10	34	51	35	72	62	60	34	16	9	12	18	20	8	4	7	452
	S. Typhi	24	13	5	4	4	9	17	19	18	10	16	19	18	67	186	811	1,240
	All Salmonella BSI	335	531	637	733	1060	1197	858	847	645	574	544	352	271	280	327	941	10,132
	S. pneumoniae	220	297	247	131	214	290	268	560	396	305	161	142	121	176	107	57	3,692
	Total BC	8545	7823	8052	7653	8978	11174	10653	12933	10123	9167	8628	7991	8507	9890	10433	12815	153,365
	Salmonella yield (%)	3.9	6.8	7.9	9.6	11.8	10.7	8.1	6.5	6.4	6.3	6.3	4.4	3.2	2.8	3.1	7.3	6.6
Children <16	*S.* Typhimurium	24	129	213	204	337	383	285	280	221	175	237	108	76	70	40	55	2,837
	S. Enteritidis	5	104	121	142	75	26	29	29	15	12	20	15	16	15	4	3	631
	S. sp.	2	17	23	6	58	8	40	8	7	1	2	6	11	5	2	5	201
	S. Typhi	3	2	4	1	1	5	10	10	7	3	10	9	5	39	96	493	698
	All Salmonella BSI	34	252	361	353	471	422	364	327	250	191	269	138	108	129	142	556	4,367
	S. pneumoniae	91	160	120	46	92	108	133	147	86	75	57	37	33	85	43	16	1,329
	Total BC	5839	4709	5090	4130	4409	5270	5750	6817	4554	4275	4860	4186	4528	5813	6398	8071	84,699
	Salmonella yield (%)	0.6	5.4	7.1	8.5	10.7	8.0	6.3	4.8	5.5	4.5	5.5	3.3	2.4	2.2	2.2	6.9	5.2
Adults ≥16	*S.* Typhimurium	229	169	169	250	510	648	419	438	341	333	235	171	124	113	78	56	4,283
	S. Enteritidis	43	82	78	98	62	69	48	47	34	35	24	21	17	7	15	9	689
	S. sp.	8	17	28	29	14	54	20	26	9	8	10	12	9	3	2	2	251
	S. Typhi	21	11	1	3	3	4	7	9	11	7	6	10	13	28	90	318	542
	All Salmonella BSI	301	279	276	380	589	775	494	520	395	383	275	214	163	151	185	385	5,765
	S. pneumoniae	129	137	127	85	122	182	135	413	310	230	104	105	88	91	64	41	2,363
	Total BC	2706	3114	2962	3523	4569	5904	4903	6116	5569	4892	3768	4069	3979	4077	4035	4744	68,930
	Salmonella yield (%)	11.1	9.0	9.3	10.8	12.9	13.1	10.1	8.5	7.1	7.8	7.3	5.3	4.1	3.7	4.6	8.1	8.4

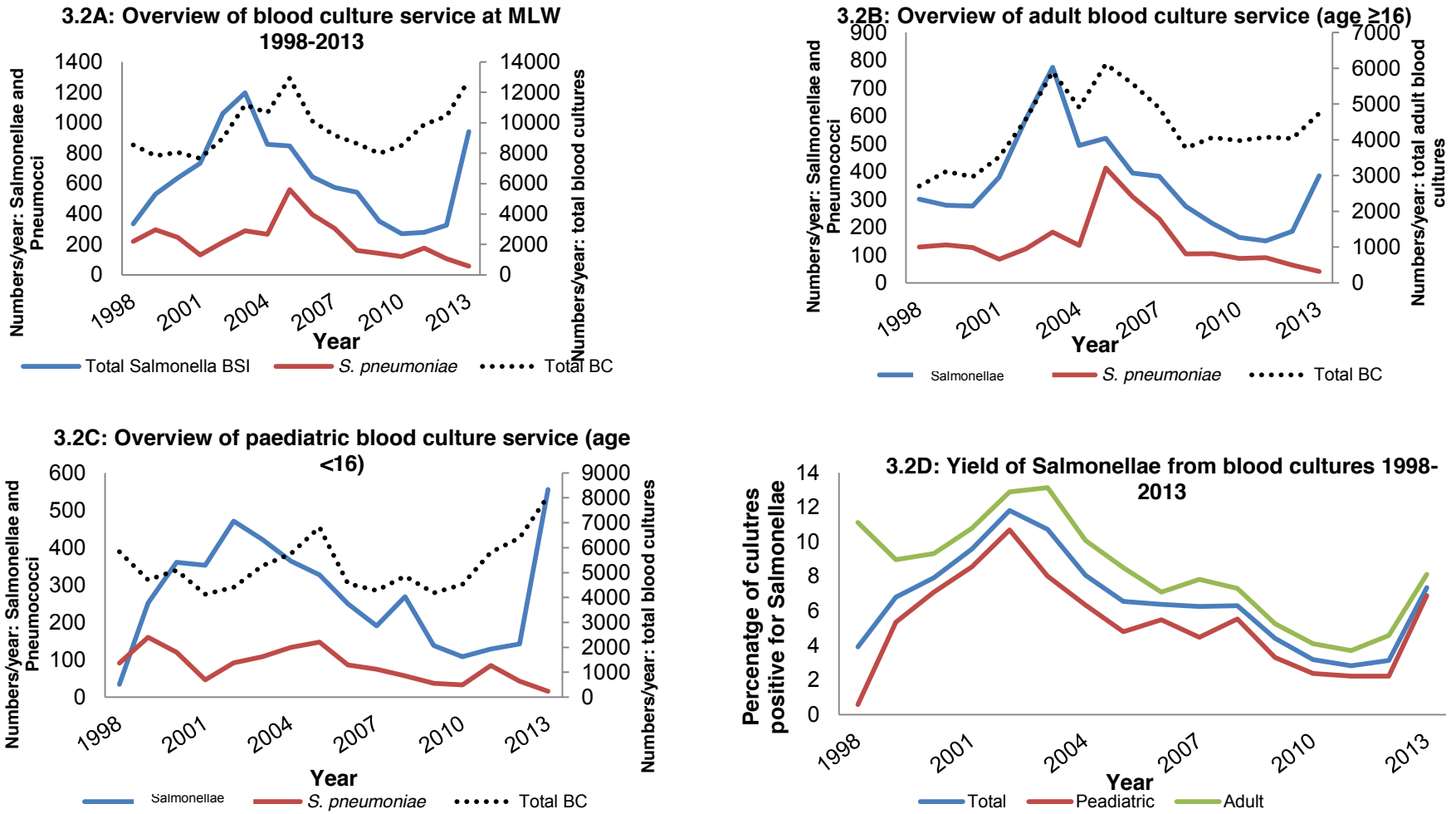


Figure 3-2: Overview of MLW blood culture service, 1998-2013. A: All blood cultures taken. B: Adult blood cultures. C: Paediatric cultures. D: Yield of Salmonella from blood cultures. Dotted line represents total blood cultures taken, not total positive.

3.4.3 Trends in Salmonella BSI by serotype

Annual trends in isolation of Salmonella from blood at MLW from 1998-2013 are depicted in Figure 3.3. *S.* Typhimurium accounted for 7,120/10,132 (70%) Salmonella isolates; 4,283/5,765 (74%) in adults and 2,837/4,367 (65%) in children. *S.* Typhi was isolated 1,240 times, comprising 12% of all Salmonella isolates (9% of adult isolates, and 16% of paediatric isolates). *S.* Enteritidis was isolated 1320 times, comprising 13% of all Salmonella isolates (12% in adults, and 14% in children) and of the remainder, which were not further serotyped at MLW, Salmonella sp. were isolated 452 times, 4% of all Salmonellae (4% in adults, and 5% in children).

As the data for 2013 are only complete to November 30th at the time of writing, the following analysis is based on data to the end of 2012 unless stated. There have been three epidemics of Salmonella BSI in Blantyre, Malawi. The first two have previously been described (Gordon et al. 2008). There was an 88% fall in the rate of NTS BSI/year from a peak of 1188 cases in 2003 to 141 cases in 2012. Most of this fall was explained by the fall in *S.* Typhimurium cases (peak in 2003 of 1031 cases falling to 118 in 2012 – 89%), although all NTS have fallen; *S.* Enteritidis from a peak of 240 cases in 2002 to 19 in 2012 (92%) and *S. sp.* from a peak of 72 cases in 2001 to 4 in 2012 (94%). This is summarised in Table 3.1 and Figure 3.3.

In 2011, an epidemic of Typhoid fever (*S.* Typhi) began in Blantyre, when the number of cases increased from a 13-year mean of 14/year to 67 in 2011, then 186 in 2012 and 811 by the end of November 2013. The evolution of the epidemic is displayed in terms of monthly cases since January 2011in Figure 3.4.

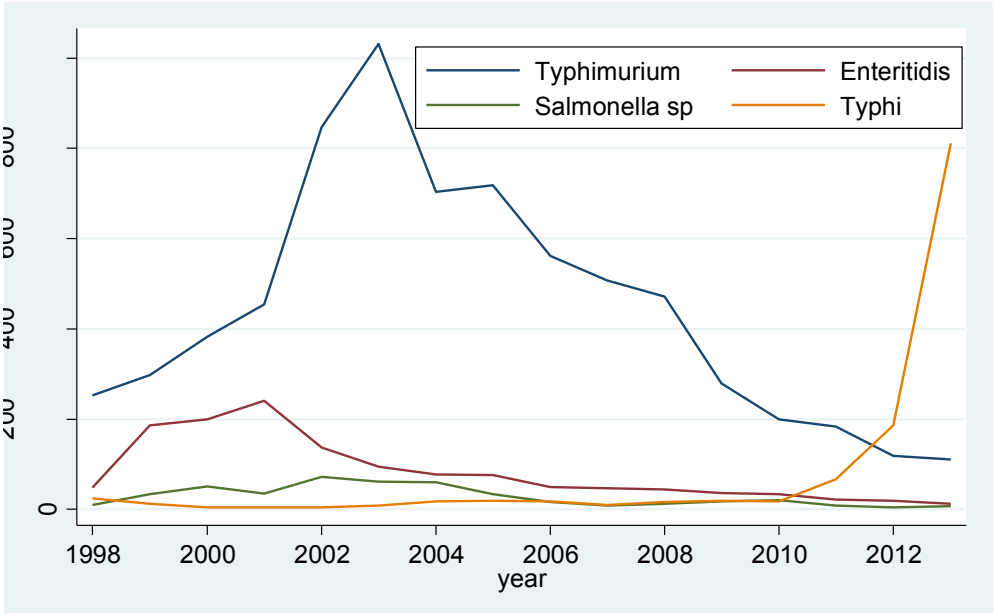


Figure 3-3: Annual trends in all Salmonella BSI 1998-2013

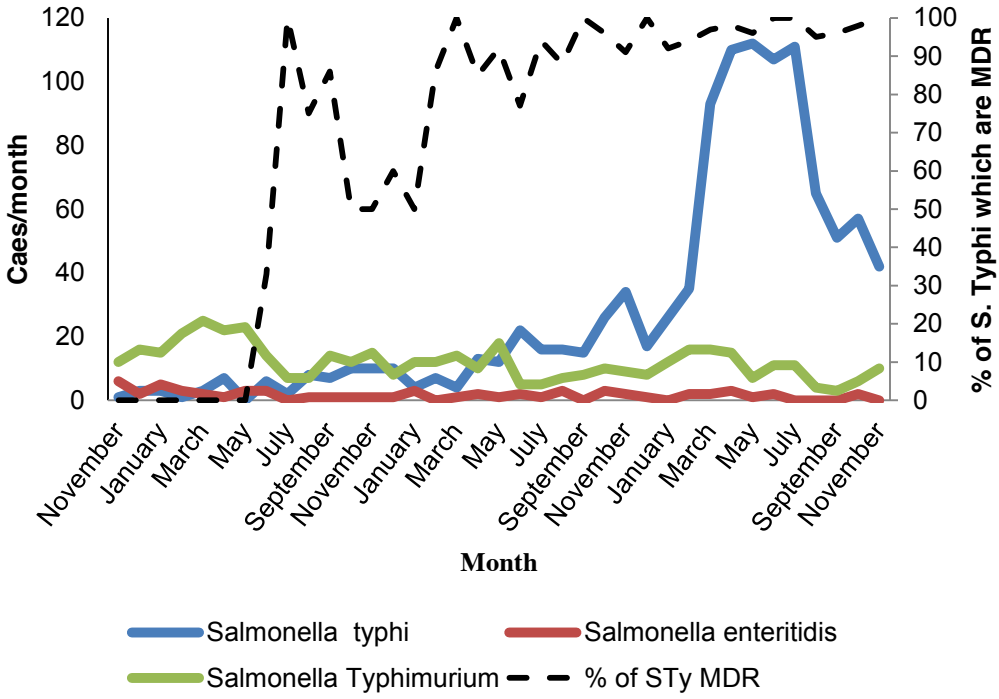


Figure 3-4: Monthly Salmonella cases since November 2010 reveals the emergence of an epidemic of MDR S. Typhi since mid-2011. The MDR phenotype become common 12 months before monthly case numbers doubled

3.4.4 Age distribution of Salmonella serotypes

The previous analysis of the age distribution of Salmonella BSI from 1998-2004 (Feasey et al. 2010) was repeated. The previous overall finding of a bimodal age distribution of iNTS was confirmed for the two most common types of NTS (*S.* Typhimurium and *S.* Enteritidis) and for *Salmonella sp.* The majority of cases occur in children under 1 year of age and iNTS becomes uncommon in children older than 3 years of age, there is a second peak in adulthood, between the ages of 20-50. This reflects the current age distribution of adult HIV in Malawi (Figure 3.5 & Feasey et al. 2010). The continuous distribution of Typhoid cases that was previously described was also maintained in this dataset which included many more epidemic cases, with the majority of cases occurring in children and young adults.

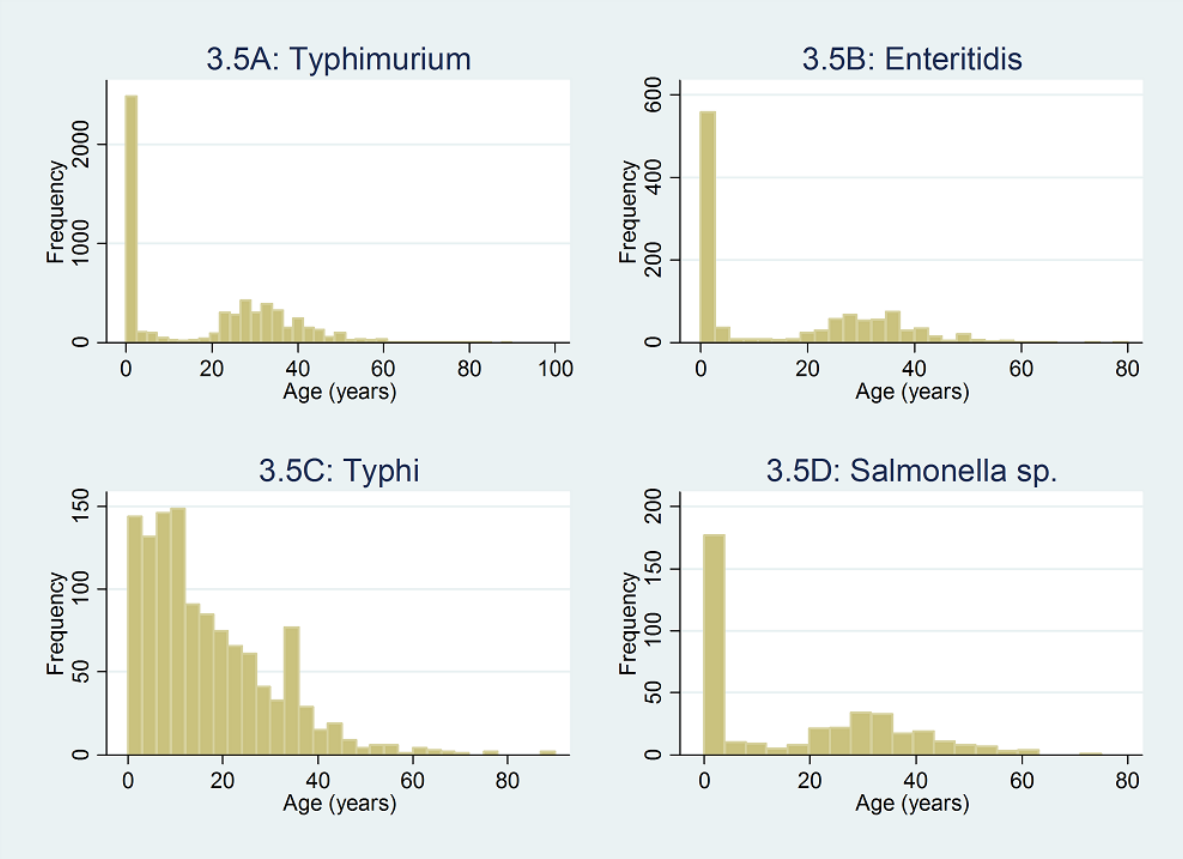


Figure 3-5: Histogram showing age distribution of Salmonella BSI cases from 1998-2013 divided into serotypes: A: *S.* Typhimurium, B: *S.* Enteritidis, C: *S.* Typhi: D:

Salmonella sp. The three NTS serotypes follow the same bimodal pattern, whereas Typhoid is most common in children and early adulthood

In view of the three age distribution divisions described above, the rates of the different serotypes per year have also been presented in three age strata (Figure 3.6), adults (age >16 years), children (aged ≥1 - <16 years) and infants (age <1 year). The decline in iNTS is evident in all 3 age groups, but is perhaps most marked in children <1. The burden of the Typhoid epidemic has fallen most heavily upon children aged 1-16 years. The factors associated with the decline of iNTS among children are explored further in chapter 4, and changes in the incidence, presentation and mortality of iNTS among adults in the post-ART era are are further described in chapters 5 and 7.

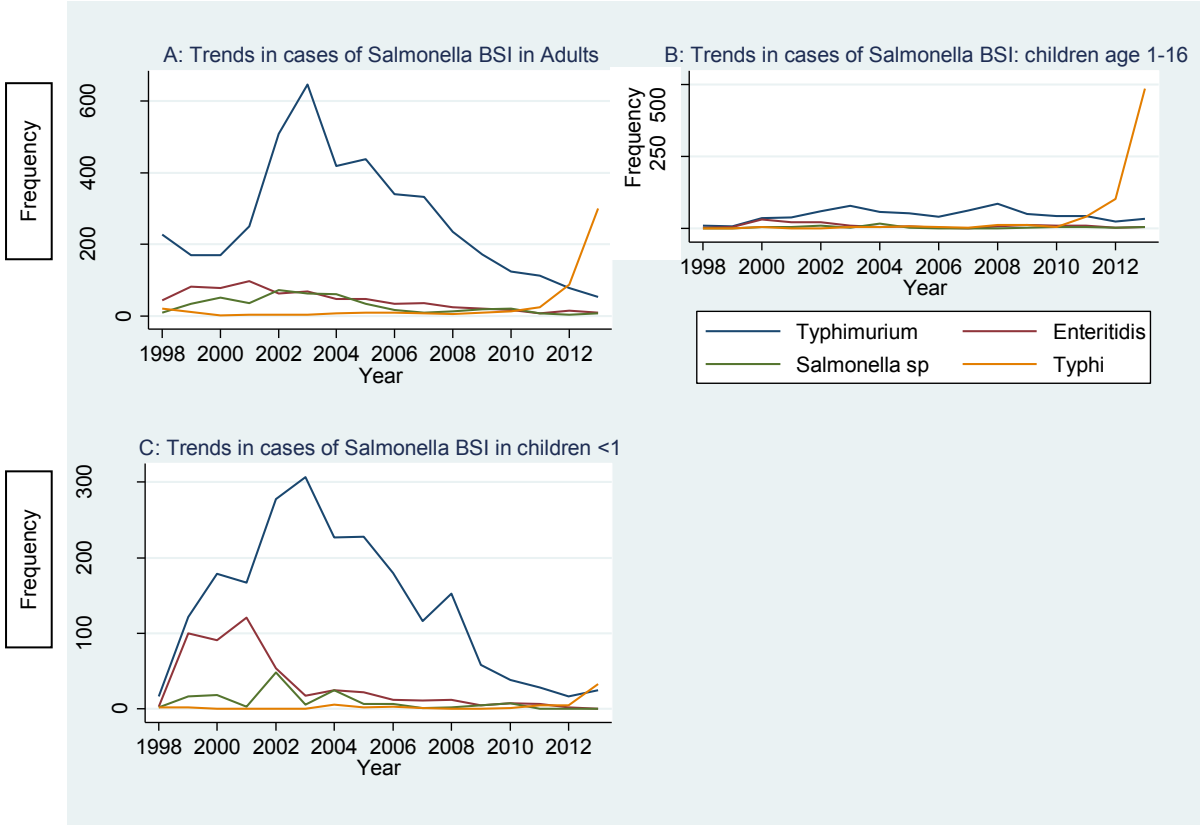


Figure 3-6: Trends in Salmonella BSI by serotype and age category reveals a decline in iNTS across all age groups and the epidemic of Typhoid amongst children Figure A: Adults ≥16 years, B: children 1-16 years and C: children <1 year

In light of changes in the incidence of Salmonella BSI, the median age and Interquartile range of cases of the 2 dominant Salmonella serotypes, *S*. Typhimurium and *S*. Typhi have been plotted (Table 3.2 and Figure 3.7). Cases of *S*. Typhimurium have been split into adults ≥16 and children <16. The median age at which adults present with iNTS disease has significantly increased from 32 to 35 during the study period ($p < 0.001$). Similarly, the median age of paediatric disease has increased, from a median age of 7 months (IQR 3-12 months) to 15 months (IQR 12- 48 months) ($p < 0.001$). There has been no significant change in the median age at which patients present with *S*. Typhi ($p=0.28$), despite the emergence of an epidemic (Figure 3.7).

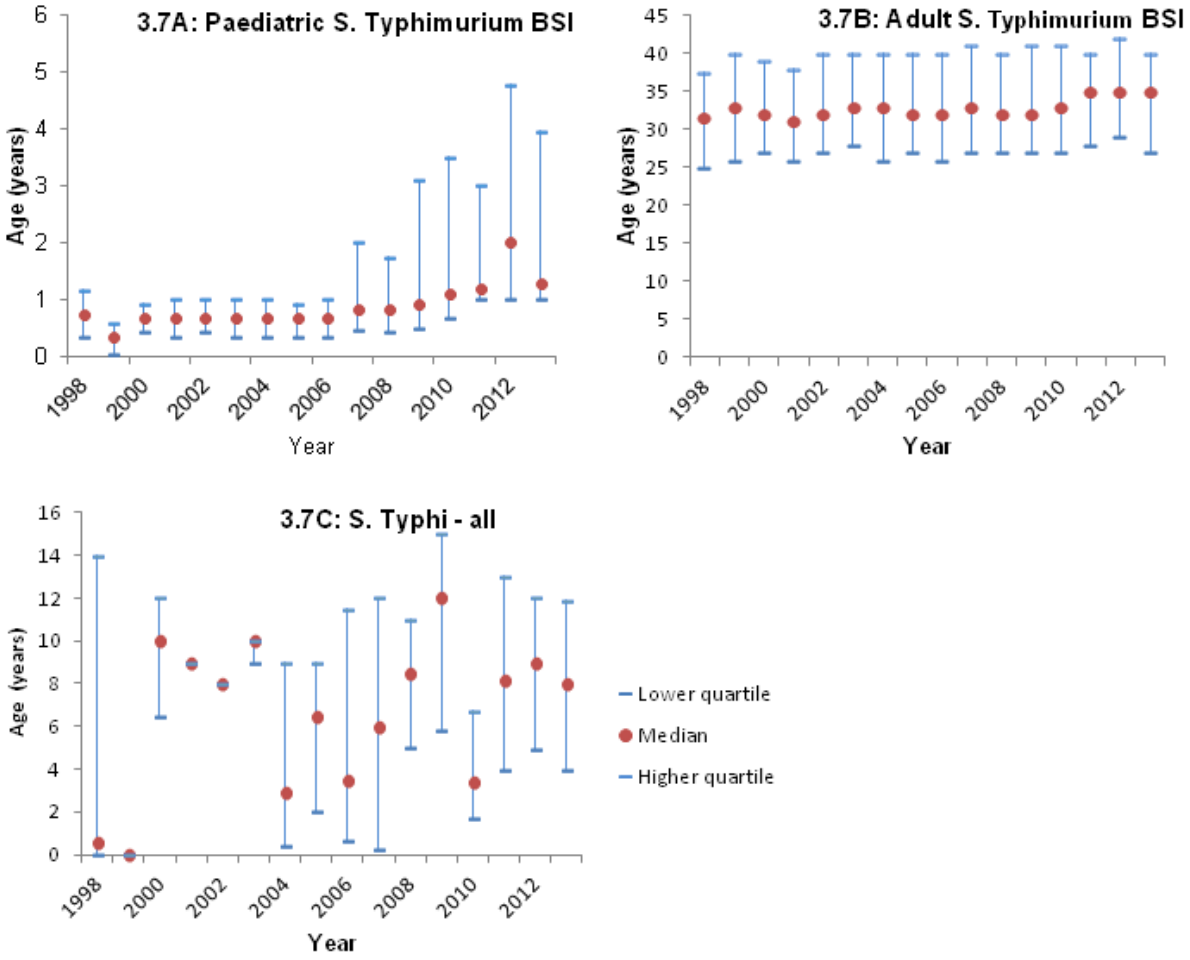


Figure 3-7: Change in median age of Salmonella BSI cases reveals a rising median age of paediatric iNTS. Figure A: Paediatric *S.* Typhimurium, Figure B: Adult *S.* Typhimurium, Figure C Typhi

Table 3.2: Change in median age of Salmonella BSI 1998-2013

	Paediatric *S.* Typhimurium			Adult *S.* Typhimurium			*S.* Typhi - all		
Year	Lower quartile	Median	Higher quartile	Lower quartile	Median	Higher quartile	Lower quartile	Median	Higher quartile
1998	0.33	0.75	1.17	25	32	38	0.0	0.6	14.0
1999	0.04	0.33	0.58	26	33	40	0.0	0.0	0.0
2000	0.43	0.67	0.92	27	32	39	6.5	10.0	12.0
2001	0.33	0.67	1.00	26	31	38	9.0	9.0	9.0
2002	0.42	0.67	1.00	27	32	40	8.0	8.0	8.0
2003	0.33	0.67	1.00	28	33	40	9.0	10.0	10.0
2004	0.33	0.67	1.00	26	33	40	0.4	2.9	9.0
2005	0.33	0.67	0.92	27	32	40	2.0	6.5	9.0
2006	0.33	0.67	1.00	26	32	40	0.6	3.5	11.5
2007	0.47	0.83	2.00	27	33	41	0.3	6.0	12.0
2008	0.42	0.83	1.74	27	32	40	5.0	8.5	11.0
2009	0.50	0.91	3.10	27	32	41	5.9	12.0	15.0
2010	0.67	1.10	3.50	27	33	41	1.7	3.4	6.7
2011	1.00	1.20	3.00	28	35	40	4.0	8.2	13.0
2012	1.00	2.00	4.79	29	35	42	4.9	9.0	12.0
2013	1.00	1.27	3.97	27	35	40	4.0	8.0	11.9

3.4.5 Antimicrobial resistance

All three epidemics of Salmonella BSI have been preceded by the emergence of an MDR phenotype, seen in the year before the epidemic began (Figure 3.8).

There are a number of combinations of antimicrobial resistance patterns, all of which continue to circulate (Figure 3.9 and Appendix 4 Tables A1-A4). In the case of *S.* Typhimurium, the most common resistance pattern, prior to the emergence of the MDR clone which caused an epidemic, was to cotrimoxazole and amoxicillin. This clone was a clade of ST313 and was almost completely replaced by the MDR ST313 clone from 2002 (Kingsley et al. 2009). Fully susceptible *S.* Typhimurium isolates have been uncommon throughout the study period.

The picture with *S.* Enteritidis contrasts with that seen in *S.* Typhimurium. In this case, an MDR phenotype emerged and caused an epidemic starting in 1999, which had largely declined by 2005. Overall the MDR clone has been more successful over the total study period than the susceptible one, causing 764/1320 (58%) of *S.* Enteritidis BSI, as compared to 414 (31%) susceptible cases, and peaking during the epidemic. In contrast to *S.* Typhimurium, emergence of the MDR-type, however, was not associated with a fall in absolute numbers of the fully susceptible strains (Figure 3.9) and for the last 3 years, as the total number of *S.* Enteritidis isolated has fallen, fully susceptible strains have been a more common cause of *S.* Enteritidis BSI from a trough of 22% to a figure of 50% in 2013. This trend is described in Figure 3.10, which reveals a clear picture of fairly sustained transmission of the fully susceptible, but non-epidemic phenotype, and an epidemic of an MDR phenotype superimposed. Both clearly show an eventual decline following the epidemic. This is explored in chapters 8 and 9.

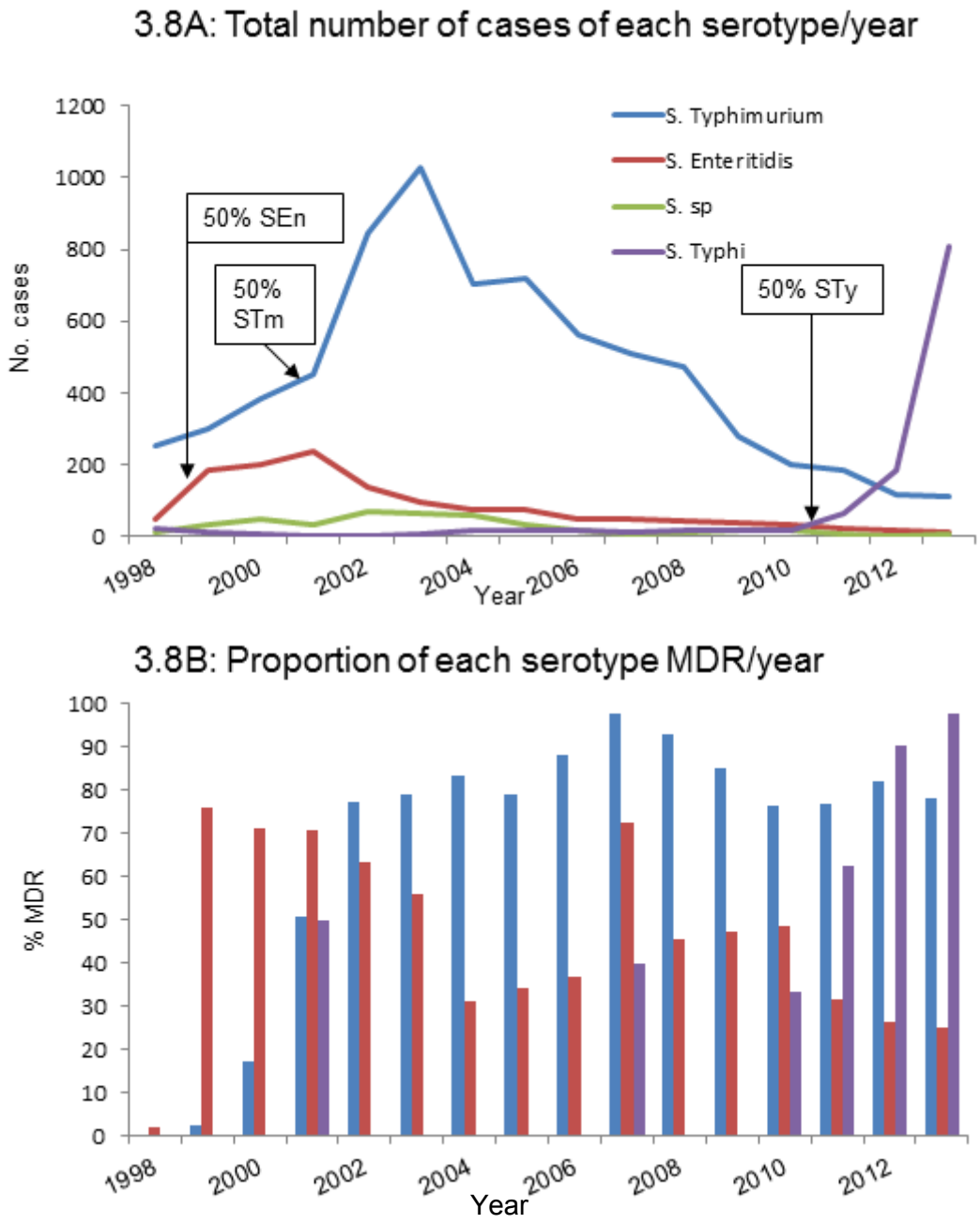


Figure 3-8: Change in MDR status amongst key invasive Salmonella isolates. A: Total cases/year and B: proportion each serotype that is MDR in each calendar year (*S. sp.* excluded for clarity). In each case, the MDR phenotype emerges the year before the epidemic and the point at which 50% of isolates of each serotype were found to be MDR is highlighted in the arrowed boxes.

The diversity of Salmonella sp. over the study period is currently unknown. An MDR phenotype become common from 2000 (25%) and 2010 (65%), however absolute numbers have been in decline since 2005 and the MDR phenotype has become less common (14-25% over the last 3 years). These isolates should be investigated further to see if the drug resistance elements are shared with the *S*. Enteritidis and *S*. Typhimurium isolates. This work has begun and it was identified that 46/324 iNTS isolates from 1998-2004 were of serotype *S*. Bovismorbificans (Bronowski et al. 2013).

Lastly, in the case of S. Typhi, there has been rapid emergence of an epidemic of MDR disease since 2011. Of note, the number of susceptible cases has remained constant during the epidemic and during the period between 2009 and 2011 preceding the epidemic, while the diversity of antimicrobial resistance types increased amongst *S*. Typhi isolates early in the course of the epidemic.

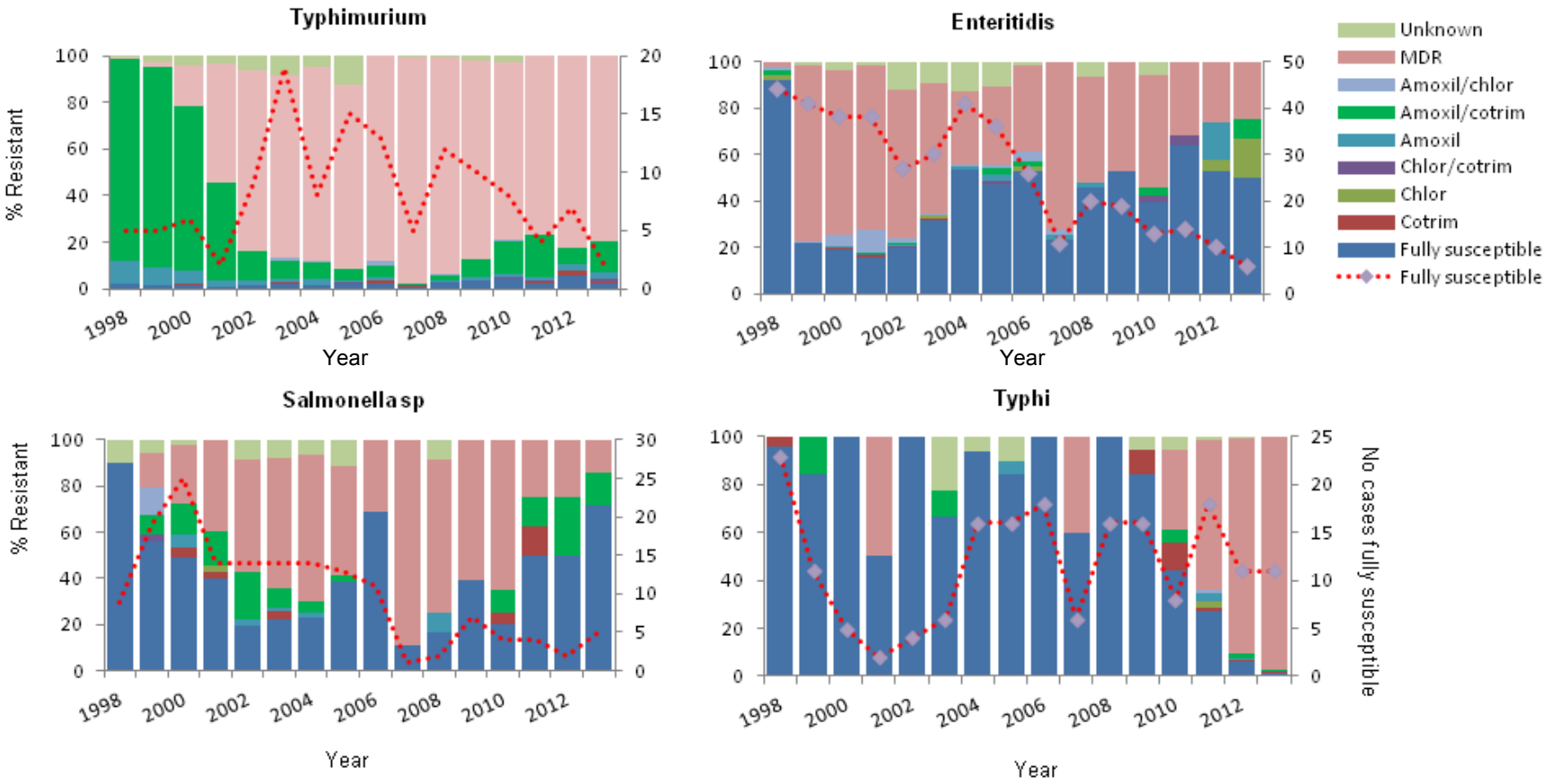


Figure 3-9: Proportion of each resistance pattern for each year and serotype. The dotted red line describes the number of isolates which remain fully susceptible

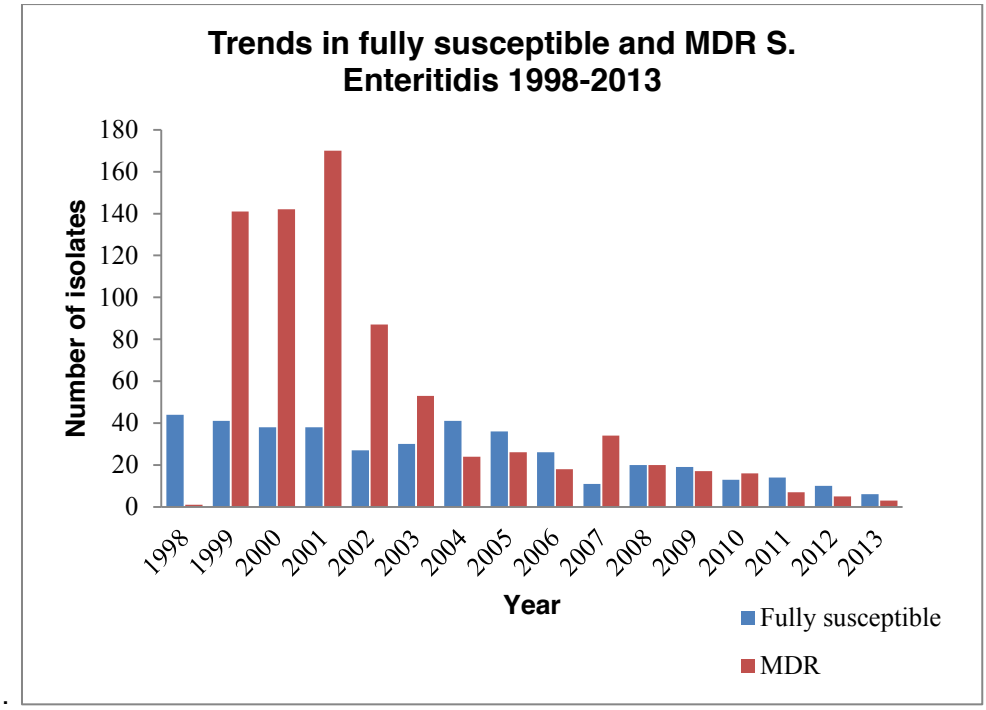


Figure 3-10: Trends in fully susceptible and MDR phenotypes of *S.* Enteritidis

3.4.6 Fluoroquinolone and cephalosporin resistance

There has been just one Salmonella isolate with confirmed ceftriaxone and ciprofloxacin resistance. This isolate came from a patient who presented with MDR *S.* Typhimurium BSI in March 2009 and re-presented one month later with an isolate of *S.* Typhimurium which was additionally resistant to both ceftriaxone and ciprofloxacin. This pattern of resistance has not been seen before or since, but given its potential importance, this isolate is explored in depth in chapter 6.

3.5 Discussion & Conclusions

There has been a decline in all types of NTS at QECH and in all age groups. This has occurred at the same time as a drop in Pneumococcal BSI (Everett et al. 2011), which is of considerable interest as NTS are transmitted via the faecal-oral route and have a strong epidemiological link to malaria prevalence, whereas Pneumococcal BSI is transmitted via the respiratory route and does not have the same association with malaria (Scott et al. 2011). In a recent meta-analysis of BSI in Africa, HIV was found to be a risk factor for any form BSI except Typhoid (Reddy et al. 2010) and given that ART has been rapidly and successfully rolled out in Blantyre since 2005, this might be the explanation of the decline in BSI. In contrast, there has been an epidemic of Typhoid. Whether and how recent changes in the availability of ART, malaria control interventions and improved nutrition are affecting the incidence of BSI in Malawi is a key question (addressed in chapter 4 & 5).

The median overall age of *S*. Typhimurium BSI is unchanged, consistent with ART reducing the number of susceptible adults as a key driver of the fall (see chapter 5). The increase in the median age of paediatric iNTS has been driven by a fall in the number of children under 1 year of age developing iNTS. This may reflect malaria control interventions, roll-out of PMTCT or improved nutritional status and is explored in chapter 4.

The emergence of MDR phenotypes in Malawi preceded the epidemics of all three Salmonella serotypes. In the case of *S*. Typhimurium, an MDR ST313 replaced a dual resistant ST313. Both of these clades of ST313 have evidence of genomic degradation consistent with narrowing of host range, novel virulence determinants and a virulence plasmid containing drug resistance elements (Kingsley et al. 2009 , Okoro et al. 2012(1)). In the case of *S*. Typhi, this serotype is already host restricted to humans and MDR strains dominate around the world. The epidemic of *S*. Enteritidis is intriguing since in contrast to *S*.

Typhimurium, the emergence of the MDR phenotype did not displace susceptible *S*. Enteritidis from its niche (chapter 8). Cases of drug susceptible *S*. Enteritidis continued to circulate in fairly constant numbers throughout the MDR epidemic, and have now become a more common cause of BSI than MDR *S*. Enteritidis following the waning of the epidemic strain.

Just one ESBL-producing and ciprofloxacin resistant *S*. Typhimurium isolate has emerged. This is surprising as by 2013, approximately 20% of community acquired *E. coli* and 80% of *Klebsiella sp.* BSI isolated at MLW were resistant to both agents. The fact that it has occurred at all in what remains an important pathogen is of considerable importance, especially as this has also occurred five times in Nairobi, Kenya (personal communication Dr S. Kariuki).

3.6 Future Work

1. Further describe the apparent decline in adult BSI, including iNTS, at QECH and put into the context of clinical outcomes and ART use (chapter 5)
2. Investigate the decline of iNTS in children and analyse in the context of the changing picture of malaria and nutritional status in Blantyre (chapter 4)
3. Explore the mechanism of cephalosporin and fluoroquinolone resistance in the extensively drug resistant isolate of *S*. Typhimurium from 2009 (chapter 6)
4. Recruit a longitudinal cohort of adult NTS patients (chapter 7) to investigate
    a. Whether recurrent iNTS favours emergence of extensive drug resistance
    b. Site of persistence of disease in-between presentation and recurrence
    c. Impact of ART on recurrence and outcome

5. Investigate the genomic differences between drug susceptible and drug resistant Enteritidis to explore whether the difference in behaviour (endemic vs epidemic) is explicable (chapter 8)

Chapter 4: A decline in the incidence of paediatric non-typhoidal Salmonella bloodstream infection at a large teaching hospital in Malawi

4.1 Summary

Invasive Nontyphoidal Salmonellae (iNTS) are responsible for a huge burden of paediatric morbidity and mortality in Sub-Saharan Africa and have been the most common isolates from the blood of febrile children in Malawi for over ten years. Recently there have been reports of a decline in childhood iNTS from both Kenya and the Gambia associated with malaria control. In Malawi, however risk factors for iNTS additionally include both HIV and malnutrition.

Trends in isolation of paediatric iNTS from blood culture at QECH have been reviewed and put in context of paediatric malaria slide positivity, admissions to the QECH nutritional rehabilitation unit and monthly rainfall from 1st January 2001 to 31st December 2010. Analysis of these very large data sets reveals a significant ($p < 0.001$) decline in iNTS at QECH, in Blantyre without a comparable or sustained decline in the malaria slide positivity rate since that described between 2001 and 2003. Poisson regression and structural equation models (SEM) were fitted to the data to explore relationships between the outcome and predictor variables. During the study period, 49,030 paediatric blood cultures were taken, which yielded 3,105 NTS isolates. NTS fell by 11.8% annually. Allowing for a 1 month lag effect of rainfall, the SEM revealed significant and independent effects of rainfall and malaria slide positivity, but not malnutrition on prevalence of iNTS. The decline in paediatric malaria cases from a peak in 2001 to the beginning of a plateau in 2003 has contributed to the observed fall in iNTS, but cannot fully explain the observed decline in iNTS. Rainfall is likely

to play a role which is difficult to model because of potentially complex temporal relationships with other risk factors.

4.2 Introduction

Blood stream infection (BSI) caused by non-typhoidal Salmonella (NTS) is a major cause of morbidity and mortality in children across Sub-Saharan Africa, especially those aged between 6 and 30 months. The strong epidemiological association between malarial anemia and iNTS has been documented in several African countries (Mabey et al. 1983, Bronzan et al. 2007, Feasey et al. 2012). Furthermore, there is increasing evidence from mouse models that multiple malaria-induced immune defects, including iron release from haem, impaired neutrophil function and a reduction in IL12 production predispose to iNTS.(Roux et al. 2010, Cunnington et al. 2012) There are many other factors which may influence host susceptibility to iNTS among children, including inadequate protective antibody (MacLennan et al.), malnutrition (Amadi et al. 2001) and the impaired cell-mediated immunity caused by HIV infection (Graham et al. 2000, Gordon et al. 2002, Gordon et al. 2010).

Recently a temporal association between a decline in the incidence of malaria and a falling incidence of iNTS has been reported both from both The Gambia and from Kenya (Mackenzie et al. 2010, Scott et al. 2011). In Kenya, HbAS genotyping as part of a longitudinal study of children with blood stream infection showed that HbAS afforded reduced protection against blood stream infection (particularly with Gram negative organisms including NTS) as the incidence of malaria fell. The fact that sickle-cell trait, which protects patients against malaria also protected against BSI *until* the incidence of malaria began to drop suggests that malaria was mechanistically responsible for blood stream infections, not simply temporally associated with BSI incidence. The authors concluded that 62% of blood stream infections were attributable to malaria.

In Malawi large-scale implementation of malaria control interventions began in 2007 (Chapter 2.1: Setting). These interventions have yet to impact on the incidence of mild and severe malaria, which has not yet significantly declined since a fall between 2001 and 2003.(Roca-Feltrer et al. 2012). In contrast, there has been a highly-effective roll-out of antiretroviral therapy in Malawi since 2005, and by 2010 46% of eligible adults and 47% of pregnant, HIV-infected women were in receipt of ART (MOH Malawi 2010) In addition, a programme to subsidize fertilizer for subsistence farmers, which began in 2005, has contributed to reductions in all measures of malnutrition between 2004 and 2010, with prevalence of stunting (-11%), wasting (-33%) and underweight for age children (-24%) all falling.

In light of these changes in public health in Malawi, trends in iNTS in paediatric admissions to Queen Elizabeth Central Hospital (QECH) from 2001-2010 have been reviewed.

4.3 Methods

4.3.1 Study site and population

The study site is detailed in full in Chapter 2.1: Setting. Data from children attending QECH, from January 2001 until December 2010 were reviewed. The primary route of admission for all children is the paediatric Accident and Emergency (A&E) Unit and all children presenting with a febrile illness have blood obtained for a thick blood film malaria parasite examination. Ordinarily, children are only ever treated for malaria on the basis of a positive smear.

4.3.2 Data abstraction

A retrospective analysis to establish monthly numbers of *P. falciparum*-positive blood-slides recorded at the A&E paediatric unit at QECH from January 2001 to December 2010 has previously been undertaken (Roca-Feltrer et al. 2012). To relate trends of malaria infection to indices of iNTS, all blood cultures collected during the same period from paediatric admissions were reviewed. To describe seasonality patterns of both malaria and iNTS infections, complete daily rainfall data (mm) were obtained for the Blantyre District from the Department of Climate Change and Meteorological Services, Malawi and data were averaged from the two main district stations (Chichiri and Chileka) by month. As national nutritional data were only available for two time points, monthly admission numbers to the Moyo House Nutritional Rehabilitation Unit (NRU) at QECH from 2006-2010 were used as a surrogate of malnutrition in the community. Admission policy, which did not change across the study period for the NRU is that all children with one or more of the following admission criteria should be admitted: a median upper arm circumference (MUAC) < 11.5 cm, nutritional oedema, or weight for height < 70% predicted. No other surrogate of nutrition was available aside from the previously cited DHS data, from just two time points.

4.3.3 Statistical methods

The total numbers of iNTS cases, malaria cases and admissions to the NRU were computed, along with the total rainfall (in mms), for each year of the study period (Table 4.1, Figure 4.1). The arithmetic means for these four measures were also computed by calendar month, along with their 95% confidence intervals (to indicate year-on-year variation) (Table 4.2); robust standard error estimates were used for the iNTS, malaria and NRU admission counts to allow for extra-Poisson variation (i.e. for their positive skewed distributions).

A series of Poisson regression models were then fitted to the data collected monthly over the study period (2001 to 2010 inclusive) at QECH. The outcome (dependent) variable in each model was number of paediatric iNTS cases; the predictor (independent) variables were total rainfall (mm), number of malaria cases recorded, year and calendar month. Number of admissions to the NRU was also included in some of the models, but these data were available only for the period 2006 to 2010 inclusive.

Rainfall could have multiple possible effects in relation to iNTS. There could be a direct transmission effect resulting from surface water and inadequate sanitation drainage, which might have an impact on transmission within days. Rainfall was therefore initially fitted assuming that any effect of this variable on iNTS cases occurred in the same calendar month; however as it is probable that the effects of rainfall occur sometime after a change in rainfall, in particular with regard to mosquito numbers and therefore incidence of malaria, rainfall was re-fitted allowing for a more realistic one month lag effect. It is additionally possible that rainfall has much longer-term and indirect impacts, over a growing season, on crop yield and nutrition and susceptibility to infection at a local or national level. These more complex climatic relationships were not modelled.

As the estimated effect sizes for unit changes in rainfall, malaria prevalence and admissions to the NRU were very small, these variables were re-scaled: rainfall was converted to a unit of 10mm and admissions to the NRU were also converted to a unit of 10 admissions, while malaria prevalence was re-scaled to a unit of 100 cases.

The Poisson regression models did not permit any consideration of possible important relationships between the predictor (independent) variables; rainfall, malnutrition and malaria prevalence. As these variables are known to be statistically and mechanistically related, a series of structural equation models (SEMs) were fitted to the data, which enabled a more complex set of hypothesised inter-relationships to be explored between the variables (Figures 4.3 and 4.4). SEMs were constructed using the IBM SPSS Amos (release 20.0.0) software package. This was done in collaboration with statistician Dr B. Faragher at the Liverpool School of Tropical Medicine.

4.4 Results

A total of 49,093 blood cultures were taken between January 2001 and December 2010. Of these, 21% (n=10,265 blood cultures) yielded isolates of clinical significance and 7,244 (15%) yielded organisms retrospectively classed as likely contaminants. NTS were isolated from 3,105 blood cultures, 30% of the total number of significant blood culture isolates (Table 4.1 and Figure 4.1). Monthly data from the paediatric A&E unit were available for the same period, except from October to December 2004 as laboratory records for this period were missing. A total of 242,953 slides were taken for malaria between January 2001 and December 2010 and 61,320 (~25%) of these were found to be positive.

4.4.1 Poisson regression models

Monthly rainfall levels, iNTS cases, malaria cases and admissions to the NRU are summarised in Figure 4.1. Totalled by year (Table 4.1), there were marked variations in the average rainfall levels over the study decade but no evidence of any systematic trend, either upwards or downwards (p=0.927); however, iNTS incidence fell on average by 11.8%

annually (p<0.001), malaria incidence fell on average by 5.7% per year (p = 0.002) and admissions to the NRU fell on average by 7.1% per year (p = 0.053).

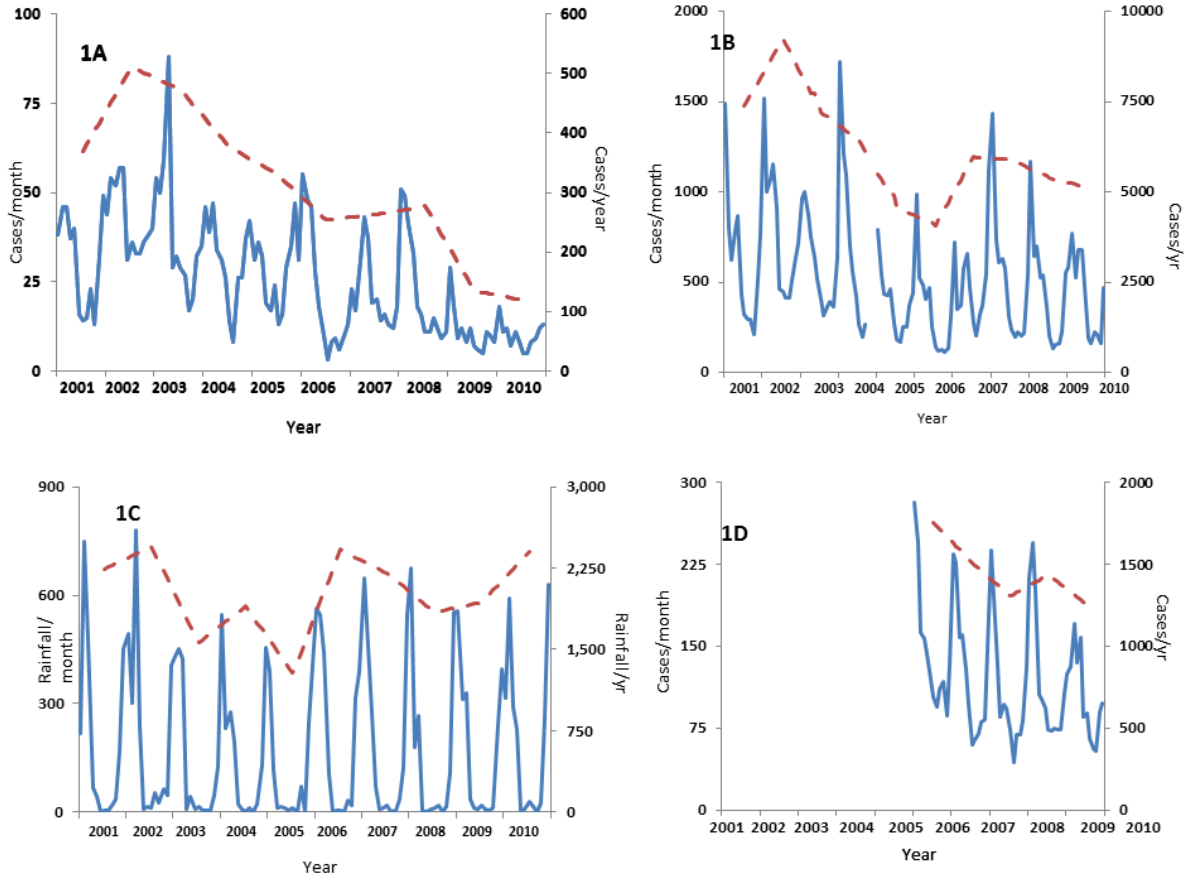


Figure 4-1: Trends in paediatric iNTS case numbers (1A). paediatric malaria case numbers (1B), rainfall in mm (1C) and admissions to the Moyo NRU (1D). The Blue line (left axis) is by month and the red line (right axis) by year.

Table 4-1: Total iNTS cases, malaria cases, annual rainfall and Salmonella admissions by year

	Year									
	2001	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
iNTS cases reported	369	511	472	376	330	254	263	277	135	119
Malaria cases reported	7392	9251	7217	8572*	4591	4062	5969	5903	5374	5132
Total rainfall (mms)	2240	2449	1565	1897	1282	2424	2202	1843	1935	2397
Admissions to NRU	---	---	---	---	---	1752	1508	1307	1434	1255

*based on extrapolation of data from Jan to Sep inclusive – malaria cases not recorded for Oct, Nov and Dec 2004

Averaged by month (Table 4.2 and Figure 4.2), there were marked and statistically very significant variations in the monthly rainfall levels, iNTS incidence, malaria incidence and admissions to the NRU ($p < 0.001$ for all variables) – but these trends were cyclical, with the smallest values in the middle of the calendar year. These monthly trends in the three clinical variables were all very significantly correlated with rainfall ($r = 0.375$ for Salmonella incidence, $r = 0.599$ for malaria incidence and $r = 0.689$ for admissions to the NRU; $p < 0.001$ for all variables).

Allowing for a one month lag between rainfall levels and clinical events, these monthly trends became even stronger for Salmonella incidence and NRU admissions, and did not change for malaria ($r = 0.506$ for Salmonella incidence, $r = 0.602$ for malaria incidence and $r = 0.837$ for admissions to the NRU; $p < 0.001$ for all variables), supporting our use of a rainfall time-lag in later models

Table 4-2: Mean monthly total rainfall, iNTS admissions and malaria cases by month (with 95% confidence intervals)

Month	iNTS cases reported	Malaria cases reported	Total rainfall	Admissions to NRU
January	38.9 (31.9 : 47.5)	1137.7 (931.3 : 1389.9)	483.6 (398.1 : 569.1)	217.8 (176.7 : 268.5)
February	36.8 (28.6 : 47.4)	756.6 (618.1 : 926.2)	393.7 (276.1 : 511.3)	208.4 (174.1 : 249.5)
March	37.5 (29.0 : 48.6)	677.4 (542.9 : 845.3)	357.2 (241.0 : 473.3)	164.8 (146.7 : 185.2)
April	35.8 (24.3 : 52.7)	662.9 (548.1 : 801.8)	96.9 (43.2 : 150.5)	128.6 (105.4 : 156.9)
May	26.6 (19.0 : 37.2)	635.8 (552.3 : 731.9)	15.7 (6.7 : 24.6)	125.6 (106.5 : 148.1)
June	19.4 (15.0 : 25.1)	397.3 (346.2 : 456.0)	7.9 (5.6 : 10.1)	97.6 (87.7 : 108.6)
July	15.2 (10.1 : 22.8)	265.8 (212.9 : 331.9)	12.3 (7.1 : 17.5)	79.8 (67.7 : 94.1)
August	14.3 (9.8 : 20.9)	219.9 (171.7 : 281.6)	12.1 (2.5 : 21.6)	67.8 (54.9 : 83.8)
September	18.1 (13.3 : 24.6)	264.0 (216.9 : 321.3)	16.2 (3.2 : 29.1)	76.0 (61.7 : 93.7)
October	18.1 (12.8 : 25.7)	269.4 (201.1 : 361.0)	23.6 (12.5 : 34.6)	79.2 (62.6 : 100.3)
November	23.9 (16.7 : 34.3)	352.2 (256.6 : 483.6)	168.0 (113.7 : 222.4)	83.0 (78.5 : 87.8)
December	26.0 (18.5 : 36.5)	616.8 (487.3 : 780.7)	436.4 (357.0 : 515.8)	122.6 (108.2 : 139.0)

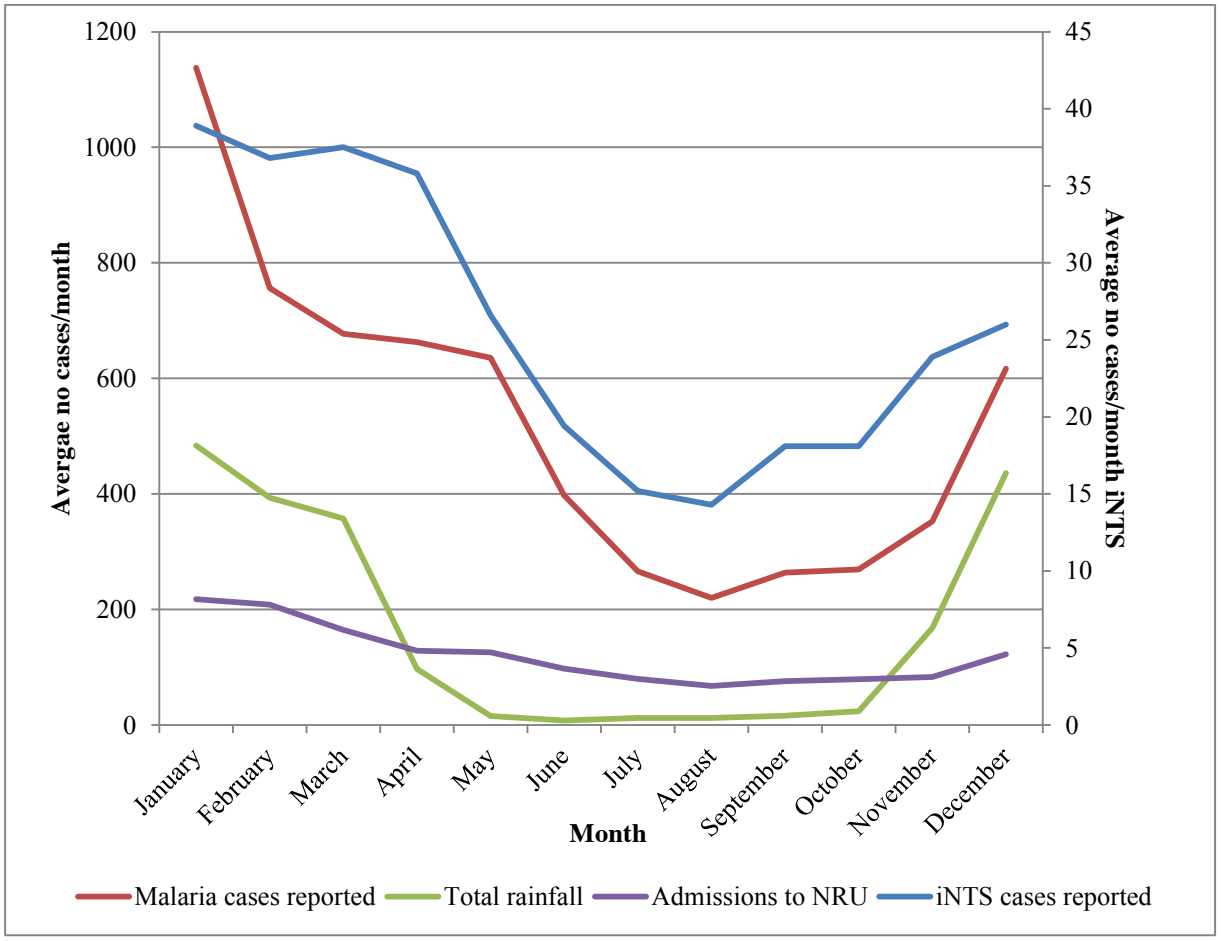


Figure 4-2: Mean monthly total rainfall, iNTS admissions and malaria cases by month illustrating seasonal association of all variables

Fitting the data from the whole of the study period using Poisson regression (Table 4.3), both malaria prevalence and year were significantly and independently related to iNTS prevalence, but rainfall was only significantly related to iNTS when a one month lag was allowed for. Adding admissions to the NRU to the model (and hence restricting the model to the years 2006 to 2010 inclusive), malaria prevalence and year remained statistically highly significant, whilst neither admissions to the NRU nor rainfall (even with a one month lag allowed for) were significantly related to iNTS prevalence.

Table 4-3: Rate ratio estimates for unit increase in iNTS admissions

Predictor	Rate ratios (95% confidence intervals)	
	Model 1 (2001 – 2010)	**Model 2 (2006 – 2010)**
No rainfall lag		
Rainfall (cms)	0.999 (0.840 : 1.188) [p=0.989]	0.912 (0.610 : 1.362) [p=0.651]
Malaria incidence (x100)	5.349 (2.051 : 13.951) [p=0.001]	8.422 (1.866 : 38.003) [p=0.006]
Admissions to Nutritional Unit (x10)	---	0.718 (0.227 : 2.269) [p=0.718]
Year	0.073 (0.037 : 0.142) [p<0.001]	0.020 (0.004 : 0.102) [p<0.001]
1 month rainfall lag		
Rainfall (cms)	1.181 (1.010 : 1.381) [p=0.037]	1.223 (0.804 : 1.860) [p=0.347]
Malaria incidence (x100)	6.188 (2.623 : 14.597) [p<0.001]	9.377 (2.511 : 35.018) [p=0.001]
Admissions to Nutritional Unit	---	0.546 (0.167 : 1.781) [p=0.316]
Year	0.069 (0.036 : 0.134) [p<0.001]	0.017 (0.004 : 0.083) [p<0.001]

Rate ratios for individual calendar months not shown

4.4.2 Structural equation modelling

As the monthly variation in iNTS cases, malaria cases and rainfall were seasonal only, these were not adjusted for in the SEM analyses.

The SEM (Figure 4.3A) for the whole of the study period with no lag time for rainfall indicated statistically significant direct effects of malaria prevalence (a positive effect) and year (a negative effect) on iNTS prevalence. The effect of rainfall on iNTS was indirect only and appeared to be mediated through its own significant effect upon malaria prevalence and the significant association between malaria and iNTS prevalence levels.

Allowing for a 1 month lag effect of rainfall (Figure 4.3B), however, the influence of rainfall on iNTS became both direct and indirect (again through its own significant effect on malaria prevalence and the significant association between malaria and iNTS prevalence levels observed in model 2A).

In model 2A, the goodness-of-fit index (GFI) statistic was 0.603, indicating that approximately 60% of the variance in this data set was explained by the factors included in the SEM model; the corresponding figure in model 4.3B was 0.581 (approx. 58%) indicating that both models fitted the data almost equally well (Table 4.4). Based on the relative magnitudes of the standardised regression coefficients in the two SEM models (Figures 4.3A and 4.3B), fitting a one month rainfall lag indicated that just under 40% of the apparent impact of malaria incidence on iNTS incidence may actually have been due to the previous month's rainfall levels; the relative effect of time was the same in both models.

It is unlikely that time in itself is influencing the incidence of iNTS cases; the magnitude of the time variable more probably indicates that there were other unmeasured factors that vary

annually that were also affecting iNTS, over and above that which is explained by the other factors in these two models.

Table 4-4 Estimated effect of the role of variables in the SEM upon paediatric iNTS as a percentage

	Model 1: no nutrition data		Model 2: admissions to NRU included	
	No lag (Figure 4.3A)	**Rain lag (Figure 4.30B)**	**No lag (Figure 4.4A)**	**Rain lag (Figure 4.4B)**
GFI statistic	0.603	0.581	0.502	0.464
% of variation in iNTS cases accounted for by*:				
Malaria	48	30	38	31
Rainfall	9	28	8	31
Malnutrition	NA	NA	27	9
Time	44	42	27	29

* based on SEM standardised regression coefficients

The SEM (Figure 4.4A) for the period 2006 to 2010 only, with no lag time for rainfall, indicated statistically significant direct effects of malaria prevalence, admission rate to the NRU and year on iNTS prevalence, but (as in Figure 4.3A) rainfall did not appear to have a direct influence. In addition, year was found be significantly negatively related to admissions to the NRU (suggesting additional factors causing a decrease, which were not included in the model), and there was a highly significant correlation between rate of admission to the NRU and malaria prevalence. Rainfall did however indirectly influence iNTS prevalence through observed direct effects on both malaria prevalence and rate of admissions to the NRU – the independent relationship between rainfall and admission to NRU was the strongest observed in the model.

In this model, the GFI statistic was 0.502, indicating that approximately 50% of the variance in this data set was explained by the factors included in the SEM model. Although Figure 4.4A is more complex than Figure 4.3A, there is a common fundamental flaw in that neither model takes into account the period of time over which rainfall might act.

Allowing for a hypothesised 1 month lag effect of rainfall (Figure 4.4B), the SEM changed in that rainfall did appear to directly, independently (and significantly) influence iNTS prevalence. In this model, year and malaria slide positivity continued to have a direct effect upon iNTS, whereas malnutrition was no longer directly associated with iNTS; this latter finding would suggest that any apparent direct relationship between malnutrition and iNTS prevalence was due a confounding and very strong effect of the previous month's rainfall. The association (statistical correlation) between malaria and malnutrition persisted.

In this model, the GFI statistic was 0.464, again indicating that approximately 46% of the variance in this data set was explained by the factors included in the SEM model.

Figure 4.3A: Structural equation model for 2001 to 2010 – no lag for rainfall

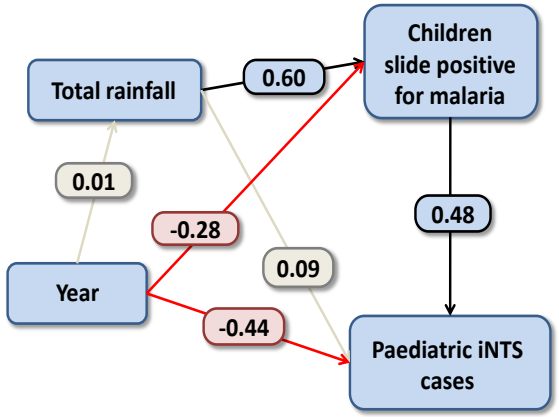


Figure 4.3B: Structural equation model for 2001 to 2010 – 1 month lag for rainfall

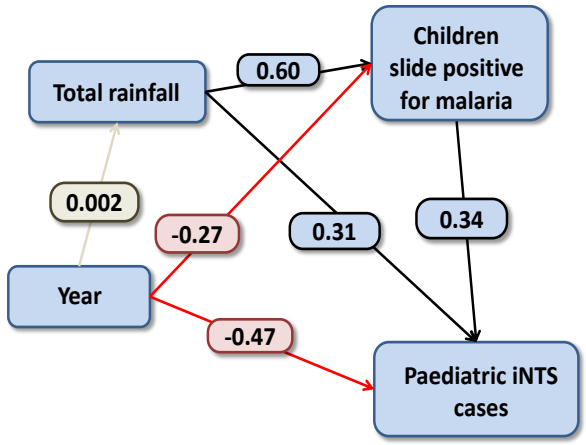


Figure 4-3: Structural equation modelling of the period 2001 – 2010, with no nutritional data available

N.B. Numbers in boxes are standardised regression coefficients from SEM model fits. Numbers in blue boxes indicate statistically significant ($p<0.05$) *positive* relationships; numbers in red boxes indicate statistically significant ($p<0.05$) *negative* relationships; numbers in grey boxes indicate statistically *non*-significant ($p\geq0.05$) relationships.

Figure 4.4A: Structural equation model for 2006 to 2010 – no lag for rainfall

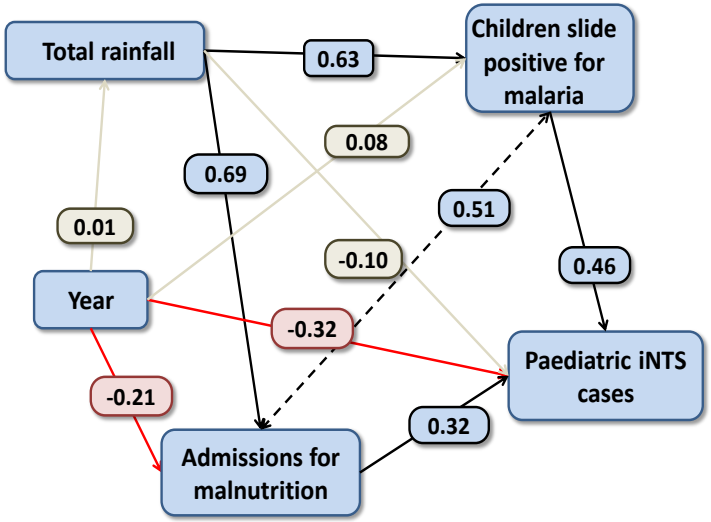


Figure 4.4B: Structural equation model for 2006 to 2010 – 1 month lag for rainfall

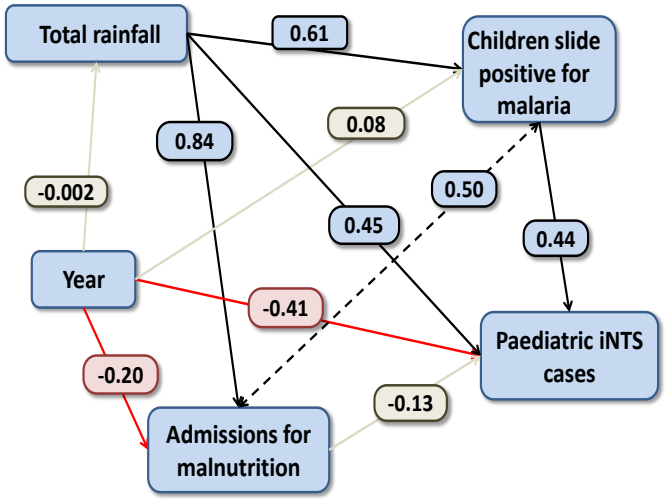


Figure 4-4 Structural equation modelling of the period 2006 – 2010, with nutritional data available

N.B. Numbers in boxes are standardised regression coefficients from SEM model fits. Numbers in blue boxes indicate statistically significant ($p<0.05$) *positive* relationships; numbers in red boxes indicate statistically significant ($p<0.05$) *negative* relationships; numbers in grey boxes indicate statistically *non*-significant ($p\geq0.05$) relationships.

4.5 Discussion

Analysis of these very large data sets reveals a significant decline in iNTS at QECH, in Blantyre following an epidemic in 2002-4. There has not been a comparable or sustained decline in the malaria slide positivity rate since that described between 2001 and 2003 (Roca-Feltrer et al. 2012), a finding which is corroborated across Malawi by WHO reports (Malawi DHS 2010). It is therefore hypothesized that the decline in paediatric malaria cases from a peak in 2001 to the beginning of a plateau in 2003 had contributed to the observed fall in iNTS, but could not explain all of it.

In this study, both conventional (Poisson) regression and SEM analytical methods were evaluated. It is clear from the inferences possible from the two methods that, in this instance, Poisson regression was an inadequate model as it did not take any account of important inter-relationships between the predictor variables. It was considered likely that such inter-relationships would be important in this study and the SEM models proved this to be so.

The relative merits of the two analytical methods can be seen from Figures 4.5A and 4.5B. In a conventional regression model (Figure 4.5A), it is assumed that all of the predictor variables only influence the outcome, in this case iNTS prevalence, directly – and do so independently of one another. A SEM model (Figure 4.5B), however, allows for inter-relationships between the predictor variables, opening up the possibility of an indirect association between a predictor variable and the outcome variable through a second predictor variable (i.e. allowing for mediating and confounding effects to be present).

In this study, the use of SEM methods enabled relations between the variables under investigation to be estimated and tested using a combination of statistical data and assumptions based on what is known about iNTS. The SEM has produced a useful model that postulates causality by enabling an estimate of variance of paediatric iNTS based on a more realistic set of assumptions about the factors in the model. These models highlight the complexity of the epidemiology of not only iNTS, but also the interactions between the factors which coalesced to enable the emergence of epidemic iNTS in Blantyre. Poisson modelling, as depicted in 4A assumes that malaria, rainfall time and malnutrition are all acting independently upon iNTS. This is biologically implausible for many reasons: people at the hungriest during the rainy season and nutritional levels only start to improve 2-3 months after the harvest, so nutrition and rainfall are associated. Rainfall also crates the stagnant water mosquitoes need to breed, which facilitates the malaria season. The maximum likelihood mathematics underpinning SEM (4B) allow interaction between all the variables.

The simpler of the two SEM models apparently provided a better statistical fit to the study data, but this finding may be illusory. Theoretically, adding data on malnutrition admissions should have improved the overall model fit – and would have done so had data on these admissions been available for the whole of the 10 year study period. Unfortunately, however, malnutrition admission data were available only for the last 5 years (i.e. for only half) of the study period. As malaria prevalence appeared to be falling over the first 5 years but then levelled off, it is possible that this fact influenced the fit of the data. For certain, however, models 3a and 3B were based on only half the number of data points available for models 2A and 2B, so direct comparisons of the respective model fits should be made with caution.

Figure 4.5A: Poisson regression model assumes total independence of variables

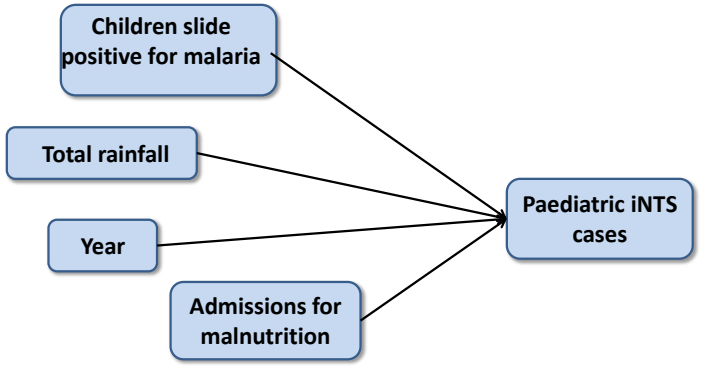


Figure 4.5B: Structural equation model estimates inter-relatedness of all variables

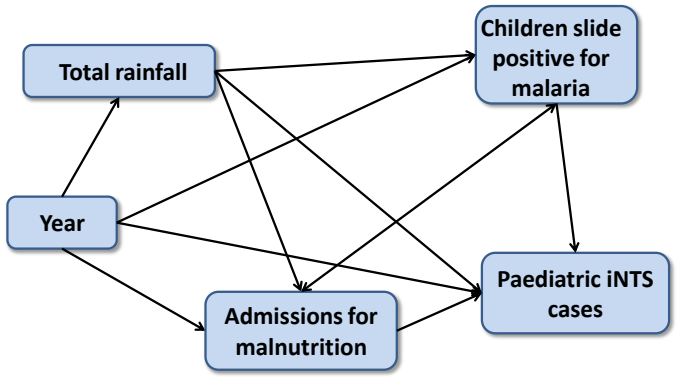


Figure 4-5: Poisson regression modelling of dependent and independent variables relies on the assumption that independent variables are zero related (4A). The SEM (4B) demonstrate that this is not the case in this study and that the assumptions of the Poisson model are too simplistic.

Malaria undoubtedly contributed at least in part to the decline in iNTS, and this was consistently seen across all our models, with a similar magnitude of effect. The Blantyre picture is thus consistent with the experiences reported in the Gambia and Kenya (Mackenzie et al. 2010, Scott et al. 2011), where a causal association has been proposed between a decline in the incidence of malaria and a decline in paediatric iNTS. Our exploratory modelling, however, also clearly demonstrated that other factors apart from malaria are independently associated with reductions in iNTS disease, and this is in keeping with experiences in South Africa where reported cases of iNTS are in decline (personal communication Karen Keddy), but which is largely free of malaria. Of note, in addition to the decline in iNTS, there has been a striking decline in invasive pneumococcal disease over the same time period. This has been the second most common blood culture isolate from children admitted to QECH (Everett et al. 2011), but has not been associated with malaria and is not transmitted by the same route as iNTS. Of note, reduction in gram positive blood stream infections in Kenya, including pneumococcal BSIs, were not associated with reduced protection from HbAS, suggesting a lack of relationship to malaria (Scott et al. 2011).

There are several possible explanations for a decline in paediatric iNTS aside from changes in the incidence of malaria parasitaemia. Malnutrition has previously been associated with iNTS in children (Graham et al. 2000 , Mandomando et al. 2009), and decline in malnutrition may have contributed to a reduction in the incidence of iNTS in children. Malawi has made considerable progress in improving the provision and security of food supplies during the period of this study. The prevalence of underweight for age children in Malawi has declined from 25% in 2000 (Malawi DHS 2003) to 14% in 2007 (Malawi Development Report 2007). Structural equation modelling of improved nutrition using NRU admissions as a surrogate suggests that improved nutrition has complex and strong interactions with both rainfall and malaria which are difficult to model, but that in Blantyre, malnutrition may not be directly

contributing to the burden of iNTS, if a 1 month lag for the effect of rainfall is taken into account. A simple short-term lag may, however, be over-simplistic in relation to malnutrition, given the length of the growing season.

In both of the rainfall-lag models, seasonal rainfall is exerting a significant effect, both directly and through its effect on Malaria prevalence. It is possible that rainfall washes faecal waste into water supplies and also possible that the environmental conditions during the rainy season facilitates environmental growth of NTS as has been observed in industrial settings (Wilkes et al. 2011, Strawn et al. 2013), and these might explain the direct effects of rainfall seen in 2B and 3B.

The consistent negative association with time confirms that there were other factors outwith the model that contributed to the decline in iNTS incidence. During the period of this study there has also been improvement in access to sanitation and hygiene facilities and to potable water in Malawi (source: UNICEF) which might have reduced the risk of iNTS, although the mode of transmission of iNTS remains uncertain.

iNTS has been associated with HIV in 20% of childhood cases (Amadi et al. 2001) and HIV may be declining as a risk factor for iNTS in Blantyre. Data on the prevalence of HIV in under-3s are unavailable, but the prevalence of HIV in pregnant women is estimated to have fallen from 19% in 2000 to 14% in 2010. Several developments may have reduced the prevalence of HIV infection among young children, firstly anti-retroviral therapy (ART) has been rapidly and extensively rolled-out since 2005. Secondly there has been a national program of peripartum anti-retroviral therapy to reduce mother-to-child transmission of HIV and the proportion of HIV infected pregnant women receiving ART has increased from less

than 5% in 2004 to 47% in 2010 (UNAIDS 2010). Thirdly a policy has been implemented for all children born to HIV-infected mothers to receive cotrimoxazole prophylaxis throughout infancy which although resistance is the norm, may have a protective effect in vivo.

4.6 Limitations

This represents a study from a single centre within Blantyre only and may not represent what is happening in the community with complete accuracy. We are confident that the number of iNTS cases detected are not simply the "tip of the iceberg" as neither ciprofloxacin nor cephalosporins are available in the community, so patients still need to attend QECH to get effective treatment, although some patients are likely to have died in the community. Although the stipulated criteria for taking blood slides at the outpatient clinic have not changed over the last decade, these may have been applied differently by different clinicians and/or may have been subject to operational aspects (e.g. the number of children attending the outpatient clinic during the rainy months can be overwhelming). Changes in health seeking behaviour over time may have affected the data of this retrospective hospital-based analysis and introduced some selection bias, but this factor is unlikely to have affected iNTS and malaria infections very differently. Although these potential biases may have partially obscured a decline in slide positivity rates over time, it is unlikely that a decline in malaria slide positivity of sufficient magnitude to mirror the marked decline in iNTS culture positivity would have been masked. Comprehensive data on HIV infection and malnutrition in children are lacking.

A further limitation is climate data. There are only two weather stations in the whole of Blantyre that collect daily rainfall data and none that collect 3 hourly data as would be the norm in the United Kingdom. The position of the stations 20km apart does not enable fine resolution of the microclimate in different parts of the city, however this spacing is consistent

with the U.K Synoptic network which measures rainfall using stations not more than 50km apart (http://badc.nerc.ac.uk/data/ukmo-midas/ukmo_guide.html).

4.7 Conclusions

There was an average annual decline in iNTS of 11.8% in Blantyre, Malawi, between 2001 and 2010, however it remains a common and important cause of BSI. The interaction between NTS, the human host and extrinsic risk factors remains incompletely understood. These data suggest that this relationship is complex and that the observed decline was multi-factorial and cannot all be explained in its entirety by a decline in the prevalence of malaria. The SEM models are strong indicators of causality and implicate independent contributions of rainfall and malaria, but additionally, the low GFIs for the models indicate that a considerable proportion of the variance within the models is not explained by either rainfall or malaria. This supports the hypothesis that malaria incidence is not the sole explanation for trends in iNTS in Malawi, They also highlight complex and independent interactions between rainfall, malaria and malnutrition. Studies are urgently needed to understand the environmental reservoirs, transmission mechanisms and epidemiology of iNTS in SSA. Meanwhile improved infectious disease surveillance across the region is required in order to monitor the impact of control programs on the burden of malaria, malnutrition and HIV.

Chapter 5: A reduction in blood stream infection and in-patient case fatality in adults at a large African teaching hospital following roll-out of antiretroviral therapy

5.1 Summary

Blood-stream infection (BSI) is one of the principal determinants of the morbidity and mortality associated with advanced HIV infection, especially in sub-Saharan Africa. Over the last 10 years, there has been rapid roll-out of anti-retroviral therapy (ART) and cotrimoxazole prophylactic therapy (CPT) in many high HIV prevalence African countries. A prospective cohort of adults with suspected BSI presenting to Queen's Hospital, Malawi was recruited between 2009 and 2010 to investigate whether and how ART and CPT have affected BSI. Comparison was made with a cohort pre-dating ART roll-out. Malawian census and Ministry of Health ART data were used to estimate minimum incidence of BSI in Blantyre district. 2,007 patients were recruited, 90% were HIV infected. Since 1997/8, culture-confirmed BSI has fallen from 16% of suspected cases to 10% ($p<0.001$) and case fatality rate from confirmed BSI has fallen from 40% to 14% ($p <0.001$). Minimum incidence of BSI was estimated at 0.03/1000 years in HIV uninfected vs. 2.16/1000 years in HIV infected adults. The estimated incidence rate-ratio for BSI compared to HIV negative patients was 80 (95% CI:46-139) in HIV-infected/untreated adults, 568 (95% CI:302-1069) in patients in the first 3 months of ART and 30 (95% CI:16-59) in patients after 3 months of ART. Following ART roll-out, the incidence of BSI has fallen and clinical outcomes have improved markedly. Nonetheless, BSI incidence remains high in the first 3 months of ART despite CPT. Further

interventions to reduce BSI-associated mortality in the first 3 months of ART require urgent evaluation.

5.2 Introduction

Bloodstream infection (BSI) is a common reason for adults to present to hospital in sub-Saharan Africa (SSA), particularly in countries with a high HIV seroprevalence (Gilks et al. 1990, Petit et al. 1995, Ssali et al. 1998, Peters et al. 2004). Prior to the introduction of antiretroviral therapy (ART), sentinel surveillance at a large hospital in Malawi established a 70% adult in-hospital HIV prevalence, whilst a previous BSI study in the same setting from 1997/8 revealed a 16% yield of pathogens from blood culture and a 38% case fatality among individuals with culture-confirmed BSI (Gordon et al. 2001, Lewis et al. 2003). A recent meta-analysis of BSI in Africa found that the most frequent bacterial causes of adult BSI in SSA were NTS and *Streptococcus pneumoniae,* and that except for *S.* Typhi BSI, HIV co-infection was a risk factor for any cause of BSI (Reddy et al. 2010).

A fall in HIV-associated BSI has been described in industrial settings (Hung et al. 2007), but whether and how BSI has changed in the era of rapid ART and CPT roll-out in Africa has not been well described (Arthur et al.2001). We have therefore used active routine surveillance for BSI amongst adult patients at a large government hospital in Malawi to prospectively evaluate aetiology and outcome of suspected BSI episodes over a 1-year period. We have compared these findings with those from BSI studies performed in the same setting and using the same methodologies that pre-date ART and CPT roll out.

5.3 Methods

5.3.1 Setting

Most adults with severe febrile illness attending QECH from urban Blantyre are referred (approximately 10%) or self-present (approximately 90% - personal communication Dr M Nyirenda). At the time of both studies, patients were triaged through a clinic room, rather than an Emergency Department (see Chapter 2 for full details).

5.3.2 Study Team

Three blood culture nurses and one field worker (see Chapter 2 for full details).

5.3.3 Study Design: 2009/10 cohort

Aerobic blood cultures were obtained as per previously declined criteria (see chapter 2: general methods). Mycobacterial culture, serological and molecular diagnostics were unavailable to either study. Blood culture is taken at the time IV access is gained, therefore intravenous antimicrobials are only administered after blood culture is obtained.

In the present study, we prospectively recruited between 8am and 5pm on weekdays from June 2009 - June 2010 and collected demographic, clinical (including prior use of antimicrobials), laboratory and in-hospital outcome data on a standard case record form

(CRF, see Appendix 2). All study patients were classified as suspected BSI and further classified as either culture-confirmed BSI (patients with a pathogen) or culture-negative (patients with a negative/contaminated culture).

Patients from outside the Blantyre area were excluded and blood cultures for febrile illness after hospital admission were not taken as part of this study, and so nosocomial infection would not have been detected.

5.3.4 Recruitment and management of historical, comparator cohort in 1997/1998

There have been two previous adult BSI cohorts recruited at QECH (see Table 5.1). The first study ran for a full year from 1st December 1997 and collected basic demographic and clinical outcome clinical data retrospectively from all adult admissions to QECH who had had blood drawn for culture (Gordon et al. 2001). As the criteria for drawing blood have not changed, the recruitment criteria were very similar. The second cohort was prospectively recruited from February-May 2000, but also specifically recruited patients in shock or with a history of fever in the preceding 4 days (Peters et al. 2004). Patients were only recruited 2 days per week and patient numbers were smaller, furthermore, recruitment was undertaken at the peak of both the malaria and “hungry” season when patients were likely to be at their most susceptible to BSI. Consideration was given to the recruitment strategy of the two studies and it was felt that the earlier study was comparable to the 2009/10 study, whereas the later one was not.

Comparison of microbiological diagnosis and clinical outcome was therefore made to a comparable cohort of febrile adults recruited in 1997/8 (Gordon et al. 2001). In the

intervening 11 years, criteria for admission and blood culture have remained the same. Numbers of trained medical practitioners at QECH have increased and the once daily broad-spectrum IV antimicrobial ceftriaxone has replaced chloramphenicol and benzyl-penicillin in the empirical management of sepsis.

Table 5.1: Comparion of study design of the 3 adult BSI cohorts from Blantyre

	1997/8 (Gordon 2001)	2000 (Peters 2004)	2009/10
Design	Retrospective	Prospective	Prospective
Time span	Full Year/all patients	Feb-May 2 days/week	Full year/working hrs
Details recorded			
Patient History	No	Yes	Yes
Examination	No	Yes	Yes
Clinical outcome	Yes	Yes	Yes
Laboratory tests undertaken			
FBC	No	Yes	Yes
Malaria slide	No	Yes	Yes
HIV test	No	Yes	Yes
Blood culture result	Yes	Yes	Yes

5.3.5 Blantyre district demographics

As QECH is the only hospital in the Blantyre district where treatment is freely available, it was possible to estimate a minimum incidence of BSI for the whole area. Study patients were categorized as HIV negative; HIV infected, but non-treated; HIV infected on ART for ≤ 3months; and HIV infected and treated for > 3 months. The difference between the date at which a patient began ART and date of presentation was used to assign ART period. The numerators for minimum incidence calculations were estimated from BSI cases observed in the study population and proportionately adjusted upwards based on the total number of culture confirmed BSIs in adult patients at QECH during the study period. We have assumed that the proportion of specific patient groups was the same in the study and non-study BSI suspects.

In order to estimate the denominator figures, the adult population of the Blantyre district was estimated from the 2008 Malawi census projections (Malawi NSO 2008), whilst adult HIV prevalence in urban Blantyre was estimated at 17% in 2010 (MacPherson et al. 2011). The total adult HIV infected and uninfected population of Blantyre was estimated using these data and stratified by age. Public and private ART clinics are required to document numbers of patients starting and receiving ART, and those who default treatment, transfer to another clinic or die whilst on ART. These data are reported to the Department of HIV and AIDS, Malawi Ministry of Health (MOH). A recent external data quality audit found that monitoring and evaluation of the ART programme in Malawi was well organized and produced data of very high quality (Weigel et al. 2012). Person-years of observation were reduced in proportion to expected deaths during the observation period, recently estimated in Malawi at 37% during the first 3 months of ART and 8%

thereafter (Weigel et al. 2012). Deaths were assumed to be evenly distributed over the observation period.

5.3.6 Statistical Analyses

Statistical analyses were done using STATA for windows software (version SE/11; Stata corp, Texas USA). Where medians are used, interquartile range (IQR) is presented. Statistical testing was considered significant at the 5% significance level. Z-tests were used to compare differences in proportions between blood culture yield and mortality between the 2 cohorts. Incidence rates (IR) and incidence rate ratios (IRR) with their corresponding 95% confidence intervals were used to describe incidence of BSI in the 2009-10 cohort. Univariate and multivariate logistic regression analysis were performed to identify potential factors associated with case-fatality and reported as odds ratios with 95% confidence intervals.

5.3.7 Ethical approval

The study was given prospective ethical approval by the University of Malawi College of Medicine Research Ethics Committee (COMREC no. P.05/08/672 Appendix 3). All patients or their relatives gave informed written consent prior to recruitment into this study.

5.4 Results

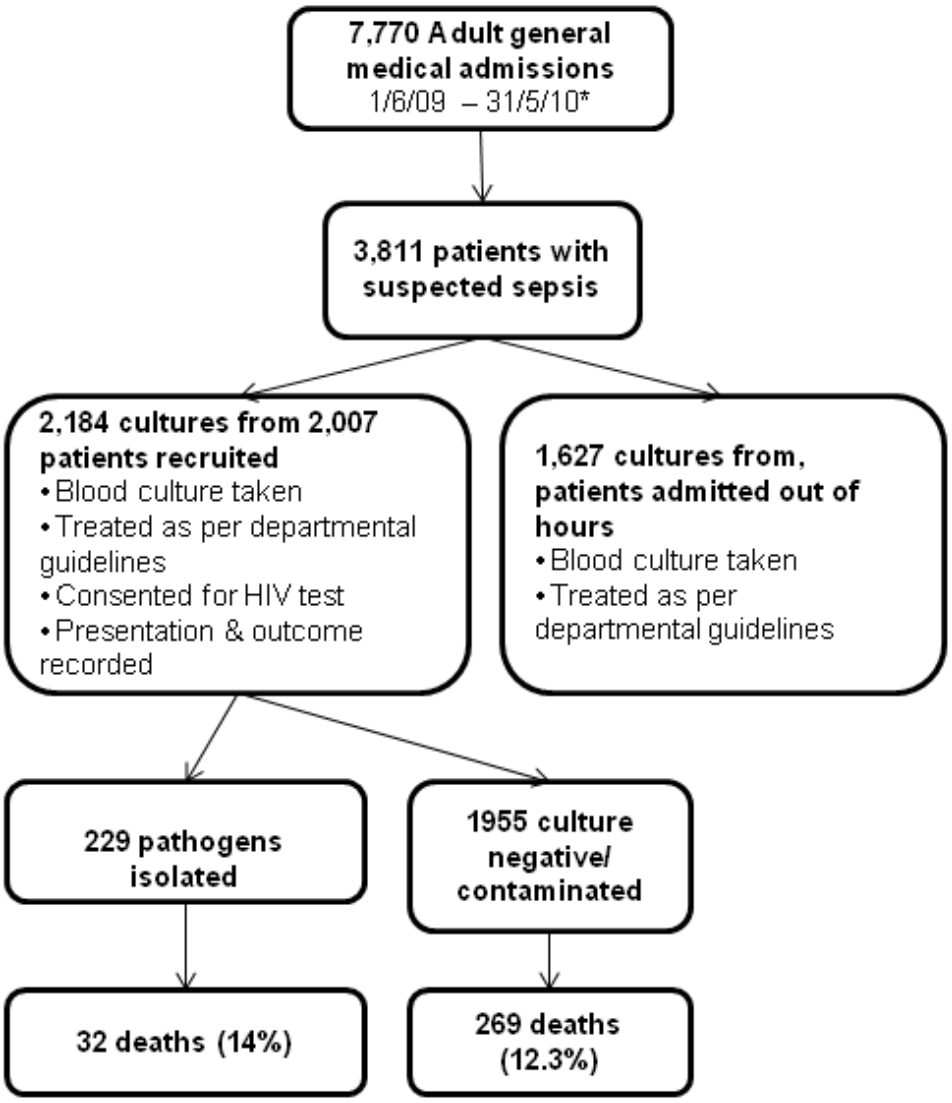
5.4.1 General Characteristics of 2009/10 cohort

In total, MLW processed 3,811 blood cultures from adult patients between June 2009 and July 2010, and 449 pathogens were detected. In this study, a total of 2184 cultures (1/patient/visit) were collected including 177 blood-cultures for repeat admissions (see Figure 5.1). Thus a total of 2,007 adults with clinically suspected BSI were recruited between July 2009 and June 2010. 908/2,007 (45%) were male. Only 126 patients (6%) had been admitted to hospital in the previous year (Table 5.1). 1902/2007 (95%) patients consented to HIV testing and 1717 (90%) were HIV-infected. 623/1717 (36%) were taking CPT and 485/1717 (28%) patients were taking ART at presentation. 212/485 (44%) were taking ART for less than 3 months. 357/2056 (17%) of patients had been prescribed antimicrobials before presentation to QECH, although they were no less likely to yield a pathogen from blood culture (OR 0.96 [95% CI: 0.6-1.4]).

The syndromic diagnoses were notably similar, although not formally comparable to those reported in a similar study conducted in 2000 (Peters et al.), with lower respiratory tract infection (376 cases) and non-focal sepsis (669 cases) remaining the most common. In 1686/2,007 (84%) of episodes, intravenous (IV) ceftriaxone was commenced at presentation; this was unavailable at QECH in 2000 when empirical first line antibiotic therapy was IV crystalline penicillin and chloramphenicol.

The median Hb was 10.0 g/dl (IQR 7.6-12.0). Of the 367/1717 (21%) patients with a documented CD4 count, 275 (75%) had a count less than $200x10^9$ cells/l (median $100x10^9$ cells/l [IQR 33-199]).

Among the 1,400/2,007 (70%) study patients tested for malaria, 272 (15%) were slide positive for *P. falciparum*, of whom 14 (6%) were also bacteraemic.



5-1: Recruitment to study

Table 5-1: Basic description of clinical and laboratory data

	Number	%
Male Gender	908/2,007	45.2
Age Mean(sd)	34.4 (12.6)	
Clinical features suggestive of HIV at presentation*	1169/2,007	58.2
HIV status		
Total HIV infected	1717/1902	90.0
Taking ART	485/1717	28.2
0-3 months	212/485	43.7
>3 months	273/485	56.3
Taking cotrimoxazole prophylactic therapy	623/1717	36.3
Clinically suspected focus of infection		
Respiratory	376/1228	30.6
Central Nervous Syndrome	78/1228	6.4
Gastrointestinal	22/1228	1.8
Non focal sepsis	669/1228	54.4
Other	83/1228	6.8
Median Haemoglobin (g/dl, IQR)	10.0 (7.6-12.0)	
Median CD4 (x10^9 cells/l, IQR)	100 (33-199)	

*At least one of Kaposi's sarcoma, oral candidiasis, wasting, generalized lymphadenopathy or herpes-zoster

5.4.2 Causes of BSI 2009/10 and comparison to historical cohort

Of 2184 episodes of suspected BSI, 229 (10%) yielded a pathogenic organism, a significant decrease when compared to 449/2789 (16%; p <0.001) in the 1997/8 study (Table 5.2 & Figure 5.3). A further 157 cultures (7%) were contaminated. Amongst the

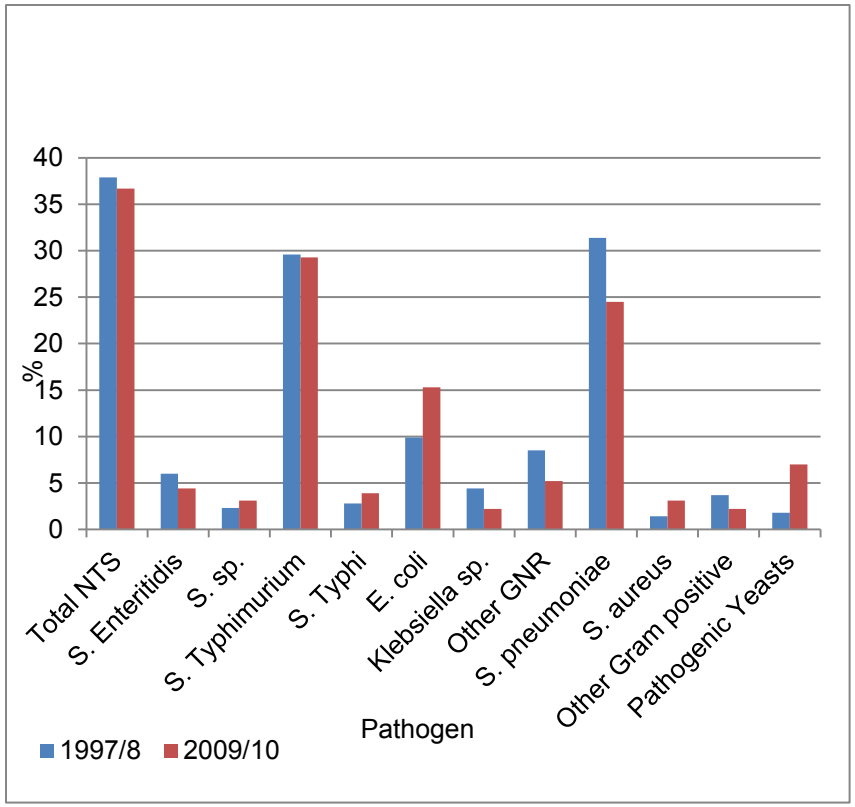
non-study blood cultures, 220/1627 (14%) grew a significant isolate and 51% (229/449) of the total yield of pathogens during the study period, were from study participants. One patient had 2 pathogens isolated from a single blood-culture.

The most common culture-confirmed cause of BSI remained NTS (84/229 [37%]). Between the periods 1997/8 and 2009/10 the proportion of all samples yielding NTS declined significantly from 6% to 4% (p=0.0008), reflecting the overall reduction in BSI. In 1997/8 100% of NTS were susceptible to chloramphenicol. In the present study, 66/84 (79%) of NTS isolates were MDR (resistant to amoxicillin, chloramphenicol and cotrimoxazole) and 73/84 (87%) were resistant to cotrimoxazole. Cephalosporin and ciprofloxacin resistance were not observed.

Table 5-2: Comparison of bacterial pathogens isolated from blood culture in 1997/98 and 2009/10. NTS are evaluated in total and by serotype. *All figures are % fall, unless indicated by "+" *C. albicans* or *C. neoformans***

	1997/8 (Gordon 2001)			2009/10			Change in % of total cultures from 1998-2010 (%)*	Z test P value
	Number	% of significant isolates	% of total cultures	Number	% of significant isolates	% of total cultures		
Gram negatives								
Total NTS	164	37.9	5.9	84	36.7	3.8	35.6	<0.001
S. Enteritidis	26	6.0	0.9	10	4.4	0.5	44.4	0.10
S. sp.	10	2.3	0.4	7	3.1	0.3	25.0	0.56
S. Typhimurium	128	29.6	4.6	67	29.3	3.1	32.6	0.004
S. Typhi	12	2.8	0.4	9	3.9	0.4	0.0	1.0
E. coli	43	9.9	1.5	35	15.3	1.6	+6.7	1.0
Klebsiella sp.	19	4.4	0.7	5	2.2	0.2	71.4	0.02
Other Gram Negative Bacilli	37	8.5	1.3	12	5.2	0.5	61.5	0.004
Gram positives								
S. pneumoniae	136	31.4	4.9	56	24.5	2.6	46.9	<0.001
S. aureus	6	1.4	0.2	7	3.1	0.3	+50.0	0.48
Other Gram Positive pathogens	16	3.7	0.6	5	2.2	0.2	66.7	<0.001
Pathogenic Yeasts**	8	1.8	0.3	16	7.0	0.7	+133.3	0.042
Total pathogens	433	100	15.5	229	100	10.4	32.9	<0.001
Total blood cultures	2789			2184				

A



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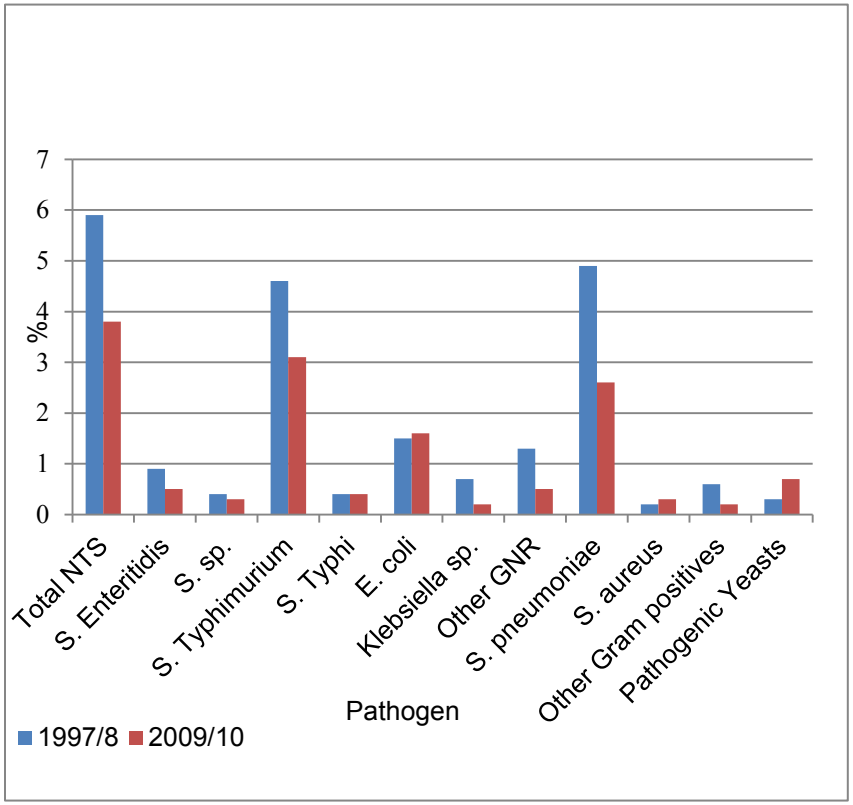

Figure 5-2: Proportion (%) of bacteria isolated from blood culture in 1997/8 and 2009/10: A: As a proportion of all pathogens isolated and B: As a proportion of blood cultures taken

S. pneumoniae was still the second most frequent BSI isolate (56/229 isolates [25%]), however between 1997/8 and 2009/10 the proportion of all cultures yielding *S. pneumoniae* fell from 5% to 3% (p <0.0001). The proportion of *S. pneumoniae* resistant to penicillin by disc testing was 12/54 (22%), an increase from 11% in 1997/8, whilst 54/57 (95%) were resistant to cotrimoxazole.(Everett et al. 2011). There was 1 cephalosporin resistant strain of *S. pneumoniae* in 2009/10.

E . coli remained the third most commonly isolated pathogen. There were 35/229 (15%) *E. coli* isolates, which did not represent a significant change from 1997/8 (43/433 [10%] isolates, p=0.95), whereas *Klebsiella spp* were identified in only 6/229 (2%) isolates compared with 19/433 (4%) in 1997/8 (p= 0.02). 34/35 (97%) *E. coli* and 5/6 (83%) *Klebsiella* were resistant to cotrimoxazole.

Broader spectrum antimicrobials, ciprofloxacin (2002) and ceftriaxone (2004), were introduced at QECH in between these studies. Despite this, Methicillin-resistant *Staphylococcus aureus* (MRSA, 1 isolate only) and extended spectrum beta-lactamase (ESBL) producing Enterobacteriaceae, 4 *E. coli* (also resistant to ciprofloxacin), were isolated rarely. There were 3 ciprofloxacin resistant isolates in the study (1 *E. coli*, 1 *K. pneumoniae* and 1 *C. freundii*

In addition to the bacterial pathogens, *C. neoformans* was isolated in 15/229 (7%) cases of BSI, 0.7% of all cultures and *Candida albicans* was isolated once. This represents a rise since 1997/8 when *C. neoformans* was isolated in 8/433 (2%) cultures whilst *C. albicans* was not isolated at all in the previous study.

5.4.3 Clinical outcomes

The median duration of admission was 3 days (IQR 1-7), but was not reported for the 1997-8 study. Mortality among all 2184 episodes of suspected BSI (with or without culture confirmed BSI) declined from 18% in 1997 to 12% in 2010 (p-value<0.001)(Table 5.3 and Figure 5.4). Among 229 episodes of culture confirmed BSI, case fatality was14% in 2010, significantly decreased from 39% in 1997 (p<0.001) (Table 5.4). Most of this reduction represents improvements in outcome from culture-confirmed BSI; the fall in mortality among febrile patients with a negative blood culture from 331/2356 (14%) in 1998 to 240/1971 (12%) in 2010 was not significantly different (p=0.066).

Table 5-3: Comparison of case fatality rates 1997/98 & 2009/10

	1997/8 [6]		**2009/10**		
	n	%	n	%	z- test (p-value)
All-cause mortality	502/2789	18.0	269/2184	12.3	<0.001
Blood culture-confirmed BSI	171/433	39.5	32/229	14.0	<0.001
Nontyphoidal Salmonellae	54/164	32.9	9/84	10.8	<0.001
S. pneumoniae	49/136	36.0	4/56	8.9	<0.001
Blood-culture negative/contaminated	331/2356	14.0	237/1955	12.1	0.066

In the present cohort, male sex (OR 1.7: 95% CI 1.3-2.2), anemia (OR 2.4: 95% CI 1.5-3.8) and HIV status (OR 2.3: 95% CI1.3-4.1) were identified as risk factors for increased

mortality among febrile admissions by univariate analysis (Table 5.5). Neither a positive blood culture, nor any of the individual common bloodstream pathogens were associated with a significantly increased risk of death (Table 5.6). Following multivariate analysis, anaemia (OR 3.0 [95% CI: 1.7-5.3]) and male gender (OR 1.7 [95% CI: 1.2-2.5]) remained independent risk factors for mortality.

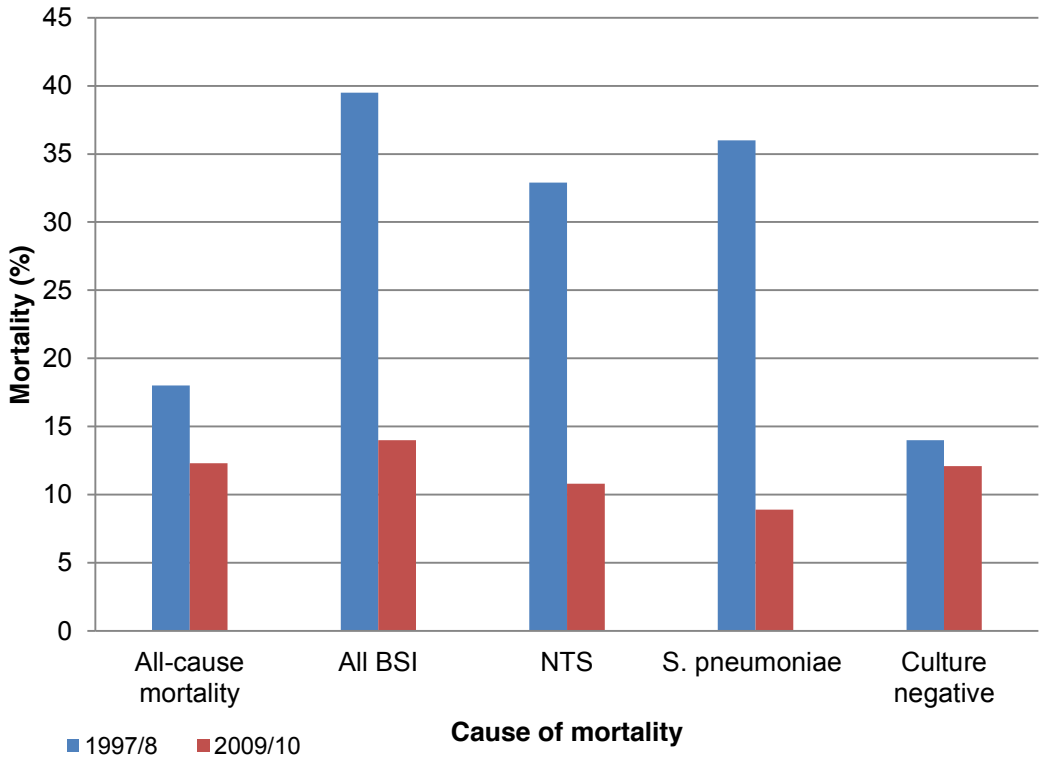

Figure 5-3: Difference in mortality between 1997/8 and 2009/10 by blood culture result

Table 5-4: Factors associated with death among all patients admitted with clinically suspected BSI in 2009/10

Variable	Univariate Analysis			Multivariate Analysis		
	OR	[95% CI]		Adjusted OR	[95% CI]	
Gender	1.7	1.3	2.2	2.0	1.4	2.9
HIV infection	2.3	1.3	4.0	1.4	0.7	2.9
Anaemia (WHO criteria(WHO)						
Mild anaemia	1.4	0.8	2.3	1.6	0.9	2.7
Moderate anaemia	1.5	0.9	2.3	1.6	1.0	2.7
Severe anaemia	4.3	2.8	6.7	4.5	2.7	7.6
Blood stream infection						
Malaria	0.6	0.4	1.0	0.8	0.5	1.5
iNTS	0.8	0.4	1.6	0.6	0.2	1.4
Other Enterobacteriaceae	1.8	0.9	3.4	1.1	0.4	3.0
S. pneumoniae	0.5	0.2	1.4	0.4	0.1	1.8

HIV infected patients were analysed separately, enabling ART status and cotrimoxazole to be included in the model (Table 5.6). In this analysis, male gender (OR 1.7 [95% CI: 1.3-2.2]) and severe anaemia (OR 4.3 [95% CI: 2.8-6.7]) were again significant risk factors for mortality.

Table 5-5: Factors associated with death among HIV infected patients admitted with clinically suspected BSI in 2009/10

Variable	Univariate Analysis			Multivariate Analysis		
	OR	[95% CI]		OR	[95% CI]	
Gender	1.9	1.4	2.4	2.0	1.4	3.0
Anaemia (WHO criteria(WHO)						
Mild anaemia	1.7	1.0	2.9	1.6	0.9	2.8
Moderate anaemia	1.7	1.0	2.7	1.6	1.0	2.8
Severe anaemia	4.7	2.9	7.7	4.5	2.6	7.7
BSI						
Malaria	0.6	0.4	1.0	0.9	0.5	1.6
NTS	0.7	0.4	1.4	0.4	0.1	1.2
Other Enterobacteriaceae	1.6	0.8	3.1	1.1	0.4	3.2
S. pneumoniae	0.5	0.2	1.4	0.2	0.0	1.5
Therapy						
CPT	1.2	0.9	1.6	1.0	0.6	1.8
No ART	0.5	0.1	5.0	1.2	0.6	2.5
ART < 3/12	0.6	0.1	5.7	1.4	0.5	3.9
ART >3/12	0.5	0.0	4.6	1.2	0.5	3.3

5.4.4 Co-trimoxazole preventive therapy

There was no significant difference in mortality due to BSI between HIV infected patients on CPT and those who were not (OR 1.87 [95% CI: 0.88, 3.99]). HIV infected patients with suspected BSI and taking CPT were no less likely to have a positive blood culture than if those were not (OR 0.86, 95% CI 0.61-1.20). Patients taking CPT were

significantly more likely to have BSI from a cotrimoxazole resistant organism (OR 7.0 [95% CI 1.6-31.2]), but significantly less likely to be slide-positive for malaria parasites than those who were not (OR 0.35 [95% CI 0.24,0.53]).

5.4.5 Impact of ART on presentation with and mortality from suspected BSI

The minimum incidence of culture-confirmed BSI was estimated at 0.06/1000 person-years in the HIV uninfected adult population. Taking into account the number of blood cultures taken out of study hours and the proportion with culture confirmed BSI, this figure was adjusted upwards by a factor of 100/51 to 0.12/1000 to reflect the total BSI burden. Crude incidence rates calculated from the study population and adjusted figures are shown in Table 5.7. Minimum adjusted incidence among the HIV infected adult population not on ART was estimated at 9.6 and at 68.4 in those on ART< 3 months (4.3 amongst HIV infected patients overall). Incidence declined to 1.8 in those on ART >3 months (Table 5.7). Rate of proven BSI was significantly lower amongst patients who had been on ART for >3 months than those not on ART (p=<0.0001) or on ART <3 months (p=<0.0001). We calculated estimated incidence rate ratios (e-IRR) of BSI using HIV-uninfected patients as the comparator group and stratified these results by age. HIV infected adults not on treatment had a crude e-IRR of BSI of 80 (95% CI: 46,139), for those on ART for less than 3 months the e-IRR was 568 (95% CI: 302,1069), while for those on ART >3 months it was 30 (95% CI: 16,59).

Amongst patients on ART for <3 months, there were 30 BSIs, including 13 iNTS, 5 *S. pneumoniae*, 8 other bacterial infections and 4 *C. neoformans*. The relative frequency of these pathogens was similar to that seen at other stages of HIV treatment and no single

agent was over represented during the first 3 months of ART. The median time from commencing ART to presenting with BSI was 33 days (IQR 9, 66).

The minimum case fatality rate (CFR) from suspected sepsis per 1,000 adults per year in Blantyre based on study participants was 0.03 amongst HIV uninfected patients, 2.84 in HIV infected patients not on therapy, 18.04 in patients in the first 3 months of ART and 1.37 in patients on ART for greater than 3 months. If the estimate was adjusted to include the number of suspected sepsis cases presenting out of study hours, whilst adjusting for repeat cultures, it gave a minimum estimated CFR of 0.05 in HIV uninfected patients, 4.8 in HIV infected patients not on treatment, 30.7 in those in treatment for less than 3 months and 3.3 in those on treatment for more than 3 months. This assumes that case fatality was similar by clinical group in the out of hours and study hours recruitment periods. Mortality risk ratios when compared to HIV uninfected patients were 93.9 (95% CI: 54.4-161.8) for untreated HIV patients, 586.6 (95% CI: 312.6-1100.7) for those on treatment for less than 3 months and 45 (95% CI:24.5-84.2) for those on treatment more than 3 months.

Table 5-6: Minimum incidence of BSI and death in the Blantyre district

HIV Status	Age group (years)	Culture confirmed BSI		Estimated population in Blantyre district 2009/10	Estimated person years of observation	Minimum incidence BSI/1000 patient yrs		Incidence rate ratio relative to HIV negative	
						Crude	Adjusted	(95% CI)	
HIV uninfected	all	14/171	8.2	475,214	463,334	0.06	0.12		
	<45	13/154	8.4	403,932	393,834	0.06	0.12		
	≥45	1/29	3.4	71,282	69,500	0.02	0.04		
HIV infected, no ART	all	148/1180	12.5	65,763	60,831	4.86	9.72	80.3	(46.4,138.9)
	<45	113/961	11.8	57,026	55,600	4.06	8.12	66.6	(36.7,120.7)
	≥45	31/208	14.9	11,405	11,120	5.58	11.16	193.2	(26.4,1415.2)
HIV + ART <3/12	all	30/212	14.2	9,124	1,718	34.92	69.84	568.0	(301.7,1069.3)
	<45	26/161	16.1	7,570	1,425	36.5	73	542.9	(279.5,1054.3)
	≥45	4/32	12.5	1,554	293	27.3	54.6	948.8	(106.4,8463.4)
HIV + ART >3/12	all	24/273	8.8	27,298	26,206	1.84	3.68	30.3	(15.7,58.5)
	<45	18/203	8.9	22,261	21,370	1.68	3.36	25.5	(12.4,52.0)
	≥45	5/55	9.1	5,037	4,836	2.06	4.12	71.8	(8.4,614.3)
Total				587,270	552,088				

5.5 Discussion

A highly effective public health approach has been used to roll-out ART in Malawi, a high HIV prevalence, resource-poor country. Benefit in terms of a reduction of mortality has previously been demonstrated at population level (Jahn et al. 2008, Mwagomba et al. 2010). Our study suggests that this benefit is likely in part to have derived from the prevention of BSI in patients on longer-term ART. This is consistent with data from autopsy results from Northern Malawi that indicated that AIDS related infections were the most common causes of mortality prevented with progressive ART scale up (Chihana et al. 2012).

The clinical and laboratory findings confirm that patients presenting to QECH with suspected BSI frequently have advanced HIV disease. Whilst it is likely that improved recognition of BSI, better clinical care and greater availability of broad spectrum antibiotics (i.e. ceftriaxone) all contributed to the observed improvement in survival, the marked reduction in the eIRR of BSI amongst patients well established on ART when compared to HIV-infected patients on ART for less than 3 months or not at all is consistent with ART as one of the primary drivers of these changes. This conclusion is supported by fact that mortality amongst patients established on ART for >3 months and presenting with suspected sepsis was also significantly lower when compared to HIV-infected patients not established on ART or only established for less than 3 months.

We were not able to perform viral load assays to address the possibility that poor outcomes represent ART failure in some of our patients well established on ART. Reports from Malawi suggest that significant primary ART resistance is as yet uncommon (Wadonda-Kabondo et al. 2012), but it is likely that either ART failure or non-compliance were issues issue in some of the patients who reported that they had been on ART for >3 months. It is likely that BSI

would have been even lower in patients who were demonstrably successfully established on ART for >3 months as evidenced by CD4 count or fully suppressed HIV-viral load.

CPT has been shown to be effective in reducing mortality in Zambian children aged 1-14 years and in Ugandan adults (Chintu et al. 2004, Mermin et al. 2004). In our study, CPT use was not associated with a reduction in the detection of BSI or decreased case fatality, but only with protection from malaria, in keeping with previous reports from the DART study based in Uganda and Zimbabwe (Walker et al. 2010). Whilst this study was not designed to evaluate the efficacy of CPT, we found nearly universal resistance to cotrimoxazole amongst key pathogens, which could render CPT less effective against *S. pneumoniae* and NTS. We therefore suggest that the utility of cotrimoxazole should be re-evaluated and the potential of alternative prophylactic agents, such as azithromycin tested.

It is noteworthy that in this high HIV prevalence population, 88% of deaths occurred amongst blood culture negative patients. Although it is likely that part of the mortality was due to BSI caused by fastidious organisms or masked by prior antibiotics, we and others have shown that unrecognised TB explains some of these deaths (Monique van Lettow et al. 2012, Feasey et al. 2013). Formal evaluation of novel diagnostics to more rapidly identify and treat smear negative TB and TB BSI is required (Boehme et al. 2010).

5.6 Limitations

The measured incidence rates of BSI have several potential sources of error, principally linked to this being a single centre in-patient study; individuals accessing out-patient care at other health centres, or who died before reaching QECH could not be sampled. Consequently, this study is likely to have underestimated BSI and therefore these data represent minimum estimates of the disease burden. It was necessary to make a correction

to allow for sampling only on working days of the week, which may have introduced error, particularly due to the assumption that HIV treatment stages were distributed equally amongst patients presenting out of hours. Our denominator data are derived from Malawian census, Malawi DHS report (2010) and Malawi MoH data, all of which are audited and which are believed to be robust and accurate (Weigel et al. 2012)..

There are very few laboratories conducting robust long-term surveillance for BSI in large urban populations in sub-Saharan Africa and therefore the data presented are restricted to a single centre in Malawi. Evidence from earlier BSI studies in Malawi and other populations throughout Eastern and Southern Africa would suggest similarities between these sites and therefore these data are likely to be generalizable to other African settings (Gordon et al. 2001, Reddy et al. 2010). Although diagnostic yield from aerobic cultures at QECH has previously been optimised (Mtunthama et al. 2008), the microbiological surveillance was limited by lack of mycobacterial and anaerobic culture facilities and both serological and molecular diagnostics. A further limitation was the lack of CD4 count and HIV viral load testing, both of which would have helped to identify which among the patients on ART for > 3 months had HIV-treatment failure.

Follow-up in the present study was confined to in-hospital outcomes and therefore it is possible that there was an unrecognised high out-of-hospital early mortality following discharge (Monique van Lettow et al. 2012). However, the improved survival seen in this study is consistent with nationally collected ART outcome data (Weigel et al. 2012).

Retrospective studies have several limitations; in general terms, there is the potential for significant selection bias and misclassification/information bias as a result of the retrospective design. In this study, the later cohort was prospectively recruited, whereas in the original study the data were collected retrospectively. Despite this, the outcome events,

(BSI and death) were not possible to misclassify and the differences between the two cohorts were so marked that they are likely to have been real.

5.7 Conclusions

Following the successful roll-out of ART, the incidence of BSI has fallen and clinical outcomes from BSI have improved significantly in a high HIV prevalence African adult population. Nonetheless, BSI incidence remains high in the first 3 months of ART despite cotrimoxazole prophylaxis. To ensure the continued success of ART programmes throughout sub-Saharan Africa, approaches to the intensification of BSI prophylaxis during the initiation of ART require further evaluation. In particular since this study demonstrated widespread cotrimoxazole resistance among key organisms responsible for BSI, including *S. pneumoniae* and NTS. Additional interventions to reduce the considerable BSI-associated early mortality in the first 3 months of ART require urgent evaluation.

5.8 Future Questions

1. What is the role of Mycobacteria, other fastidious or slow growing bacteria (including genus Leptospira, Rickettsia and Brucella) and viruses (i.e Influenza and Chikungunya) in febrile BSI/non-focal infection in Blantyre?
2. What is the optimal empirical antimicrobial regimen for patients presenting with severe sepsis and septic shock to QECH? In light of recent work on TB BSI (Feasey et al. 2013), is there a role for empirical anti-tuberculosis therapy?
3. Does nosocomial infection occur at QECH and if so, what form does it take, which pathogens are involved and what is the extent of antimicrobial resistance?
4. What are the long-term trends in isolation of all key pathogens and ART roll-out

Chapter 6: Acquisition of extended spectrum ß-lactamase and fluoroquinolone resistance via an IncHI2 plasmid in recurrent blood-stream invasive *Salmonella* Typhimurium infection in Malawi

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6.1 Summary

Extended-spectrum cephalosporins and fluoroquinolones have become key drugs for the empirical management of sepsis and culture-confirmed MDR iNTS in Malawi. Resistance to these agents has already become more common in *E. coli* and *Klebsiella sp*. We have identified the first case of recurrent MDR iNTS with extended spectrum resistance to cephalosporins and ciprofloxacin in an HIV-infected adult in Malawi. The index and recurrence isolates were investigated by WGS and the plasmid by conjugation experiments, plasmid profiling and PCR-typing.

The core genomes of the pair of isolates were indistinguishable, suggesting recrudescence of the original isolate. Fourteen contigs of sequence, totaling 287kb of novel sequence was identified in the recrudescent isolate by WGS. Separation of plasmid DNA by gel-electrophoresis and PCR amplification of plasmid DNA suggested a single IncHI2 plasmid carrying multiple resistance determinants including a *bla*CTXM-15 ESBL-resistance gene and a *qnrB1* fluoroquinolone resistance gene. Additionally a *mobC* gene was identified in sequence novel to this family of plasmids which perhaps

explains why this large plasmid was able to transfer within the patient. Should this pattern of resistance become widespread in Africa, it would render iNTS untreatable with available antimicrobials.

6.2 Introduction

The two epidemics of MDR iNTS have necessitated the widespread use of 3rd generation cephalosporins in the empirical management of sepsis at QECH and ciprofloxacin for the treatment of culture-confirmed iNTS (chapter 1 & 2). Given that 90% of febrile adult medical patients admitted to QECH are HIV infected (chapter 5) and at high risk of iNTS, up to 85% of such patients receive empirical ceftriaxone at presentation to QECH (chapter 5). Resistance to cephalosporins and fluoroquinolones is already common amongst isolates of *Escherichia coli* (approx 20% isolates to both classes) and *Klebsiella sp.* (~90% ESBL and ~50% ciprofloxacin resistant) from QECH (unpublished data, MLW), but these pathogens represent less than 10% of BSI. Should this pattern of antimicrobial resistance spread to iNTS, there would be no affordable treatment for one of the most common causes of sepsis in the region. We have investigated the mechanisms of the extensive antimicrobial resistance and transmission of this resistance pattern in this isolate.

6.3 Methods

The index and subsequent *S.* Typhimurium strains were investigated by whole genome sequencing (chapter 2). Plasmid mobility was investigated using plasmid conjugation

and profiled using DNA extraction and PCR (chapter 2). The plasmid was then compared to previously sequenced members of the same plasmid-incompatibility group using maximum-likelihood modelling (see chapter 2).

6.3.1 Strains

S. Typhimurium isolates A54285 (index) and A54560 (recurrence) were isolated from the same patient at index presentation and at recurrence 1 month later. Phenotypic drug susceptibility testing was undertaken by the disc diffusion method (BSAC 2009). The isolate cultured at presentation was resistant to amoxicillin, chloramphenicol and cotrimoxazole, whilst the isolate cultured at recurrence was additionally resistant to tetracycline, ciprofloxacin and ceftriaxone. *S.* Typhimurium D23580, a sequenced and annotated strain of MLST type ST313 (Kingsley et al. 2009), was used as a reference for sequence comparison. A rifampicin resistant mutant of *E. coli* C600 containing no plasmids was used as a recipient for conjugation.

6.3.2 Sequencing and comparative genome analysis

Genomic DNA from isolates A54285 and A54560 was prepared by Promega Wizard® genomic DNA purification kit and sequencing libraries were prepared from 500ng of DNA as previously described (Quail et al. 2012). Isolates were sequenced using Illumina HiSeq2000 (Illumina, San Diego, CA, USA) and 150 bp paired-end reads were generated. Reads were mapped to D23580 using SMALT (Ponstingl - unpublished) and

core regions of the genome were investigated for the presence of single nucleotide polymorphisms (SNPs) within the chromosome using Seaview (Gouy et al. 2010).

Isolates were assembled using Velvet (Zerbino et al. 2008) and aligned against the reference strain D23580 and its associated plasmids using Abacas (Assefa et al. 2009). Contigs that failed to align with D23580 were considered as potential mobile genetic elements, and were further characterised using BLASTn analysis and in-silico PCR in order to identify genes that predict plasmid incompatibility group (Inc-group).

The novel plasmid was aligned against a finished IncHI2 plasmid, R478. Annotations from corresponding regions of R748 were transferred (annotations_update.pl) and Glimmer3 was used to predict genes in the remaining contigs. These were annotated firstly by using a FASTA search (Pearson et al. 1988) and then manually curated using Artemis (Rutherford et al. 2000). The raw-reads from A54560 were mapped to the alignment to assess whether reads mapped across contig boundaries. If they did, this would have confirmed that the alignment was correct. Failure to map across contig boundaries would indicate that the assembly remained incomplete and the plasmid sequence in a draft form.

Assembled sequence data from a predicted IncHI2-plasmid was compared to other sequenced IncHI2 plasmids (R478 Accession number: BX664015 (Gilmour et al. 2004), pEC-IMP Accession number: EU855787, pEC-IMPQ Accession number: EU855787 (Chen et al. 2009) and pK29 Accession number: EF382672 [Chen et al. 2009]) and used to create a phylogenetic tree of the plasmids based on single nucleotide polymorphisms (SNPs) using RAxML (Stamatakis 2006).

6.3.3 Plasmid isolation and analysis

Plasmid DNA was isolated from Salmonella strains using 2 different alkaline lysis methods , firstly the Kado & Lui method (Kado et al. 1981) and visualized by gel-electrophoresis using a 0.8% agarose gel (see chapter 2 for full method). A second alkaline lysis protocol optimized for large plasmids was performed on the Salmonella strain and the transconjugant (Cain et al. 2012).

Plasmids were recovered following conjugal transfer from *S.* Typhimurium A54560 to *Escherichia coli* K12 recipient strain c600 (Rif^R). Donor and recipient strains were mated by mixing 100 µl of overnight cultures on Luria broth (LB) agar plates and incubated overnight at 26°C. Cells were recovered by re-suspending in phosphate buffered saline and serially diluted to obtain cell counts. Dilutions from 10^{-1} to 10^{-7} were plated onto MacConkey agar containing rifampicin (100 µg/ml) and ceftriaxone (4µg/ml) to select for transconjugants (TCs) and to ensure TCs were *E. coli*. Cells were further plated on rifampicin to enumerate the donor cells and the transfer frequency was calculated as number of TCs per donor. The resistance phenotype of TCs was checked using the disc diffusion method (BSAC 2009), and all resistances from the donor strain transferred.

Transconjugants were also screened by PCR-based replicon typing (Carattoli et al. 2005) to confirm the incompatibility group suggested by in-silico plasmid typing.

PCR was run on a Bio-Rad DNA Engine Tetrad 2 Peltier Thermal Cycler. PCR reactions were performed using Invitrogen Platinum® PCR super-mix kit as per the manufacturer's instructions with approximately 20ng DNA template. Reactions were run with an initial incubation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 minutes, and then a final incubation at 72°C for 5 min. DNA fragments were resolved by gel electrophoresis on 0.8% agarose gel, stained with ethidium bromide (5 µg/ml), and visualized using a GelDoc image analysis station. Primers used in this study to screen for plasmid incompatibility group were TTTCTCCTGAGTCACCTGTTAACAC (forward) and GGCTCACTACCGTTGTCATCCT (reverse), which generate a 646 bp fragment (Carattoli et al. 2005).

6.4 Results

6.4.1 Case Description

In 2009 MLW isolated an MDR *S.* Typhimurium with both ciprofloxacin and ceftriaxone/extended-spectrum ß-lactamase resistance. It was isolated from the blood of a 40-year-old, female patient who initially presented on 1st March 2009 with MDR *S.* Typhimurium BSI. The patient was HIV infected and not taking ART. The patient was treated with ceftriaxone (2g IV od) and discharged on ciprofloxacin (500mg bd). The patient was readmitted 1 month later on 2nd April. On this occasion she was found to have MDR *S.* Typhimurium BSI with additional resistance to ceftriaxone and ciprofloxacin. In the absence of a locally available effective antimicrobial, such as a carbapenem, tigecycline or azithromycin, she was prescribed ceftriaxone, gentamicin and high dose ciprofloxacin, but she died shortly after her second presentation.

6.4.2 Strains

Strains A54580, A54560 and D23580 were resistant to ampicillin, chloramphenicol and cotrimoxazole. A54560 had acquired ceftriaxone, ciprofloxacin and tetracycline resistance.

6.4.3 Whole genome sequencing

Isolates A54285 and A54560 were extremely similar to reference *S.* Typhimurium ST313 D23580 - the core genome of these isolates differed from D23580 only by 34 SNPs. However, no core genome sequence differences between the two isolates from this patient were found. This suggests that the episode of iNTS caused by A54560 represented recrudescence of the original infection caused by A54285 rather than infection with a new strain (Okoro et al. 2012(2)).

D23580 contains 4 plasmids pBT1, pBT2, pBT3 and pSLT-BT, the *S.* Typhimurium virulence plasmid (Kingsley et al. 2009). Only 2 of these were present in A54285 or A54560: pSLT-BT, the *S.* Typhimurium virulence plasmid with an MDR cassette and pBT3. However, after alignment of A54560 against D23580, an additional 280kb of DNA was found separated into in 14 contigs, which was unique to A54560. BLASTn nucleotide comparisons revealed significant similarities (78-79% over total length with 99% identity over the alignment) between the published IncHI2 plasmids pK29, R478 and pEC-IMP and pEC-IMPQ. In-silico PCR using primers described by Carottoli (Carattoli et al. 2005) detected only IncHI2 regions. Accordingly we based subsequent

experiments on the hypothesis that there was one single large plasmid present in A54560.

6.4.4 Plasmid architecture

The novel plasmid, here named pSTm-BTCR, was first aligned against IncHI2 plasmid R478, however six small contigs did not align against R478, but were most closely related to a segment of pKOX2, a plasmid found in a carbapenemase producing strain *Klebsiella oxytoca* (Huang et al. 2013). This plasmid was used to align these contigs. Mapping reads from A54560 against this assembly failed to confirm the alignment of any of the 14 contigs, therefore the current assembly remains in draft format.

The draft assembly is presented in Figure 6.1. This image demonstrates several features of interest. Firstly there is a contig break across R478 gene SMR088, a putative helicase. Mapping raw sequence data to the assembly should have demonstrated reads mapping across the gene, the fact that in addition to a break in the assembly the raw sequence data stop on either side of this break (i.e. the break is predicted by two different models) suggests that it is likely to be a site of an insertion. Secondly, genes from R478 SMR 96-157 are predominantly, but not completely deleted. This region contains numerous antimicrobial resistance genes and heavy metal resistance genes. The presence of fragments of mercuric and copper resistance genes as pseudogenes on the novel plasmid suggests their former presence and subsequent replacement. Lastly there is the deletion of a tn10 from the novel plasmid with respect to R478 like element. This region also contributes antimicrobial resistance genes to R478.

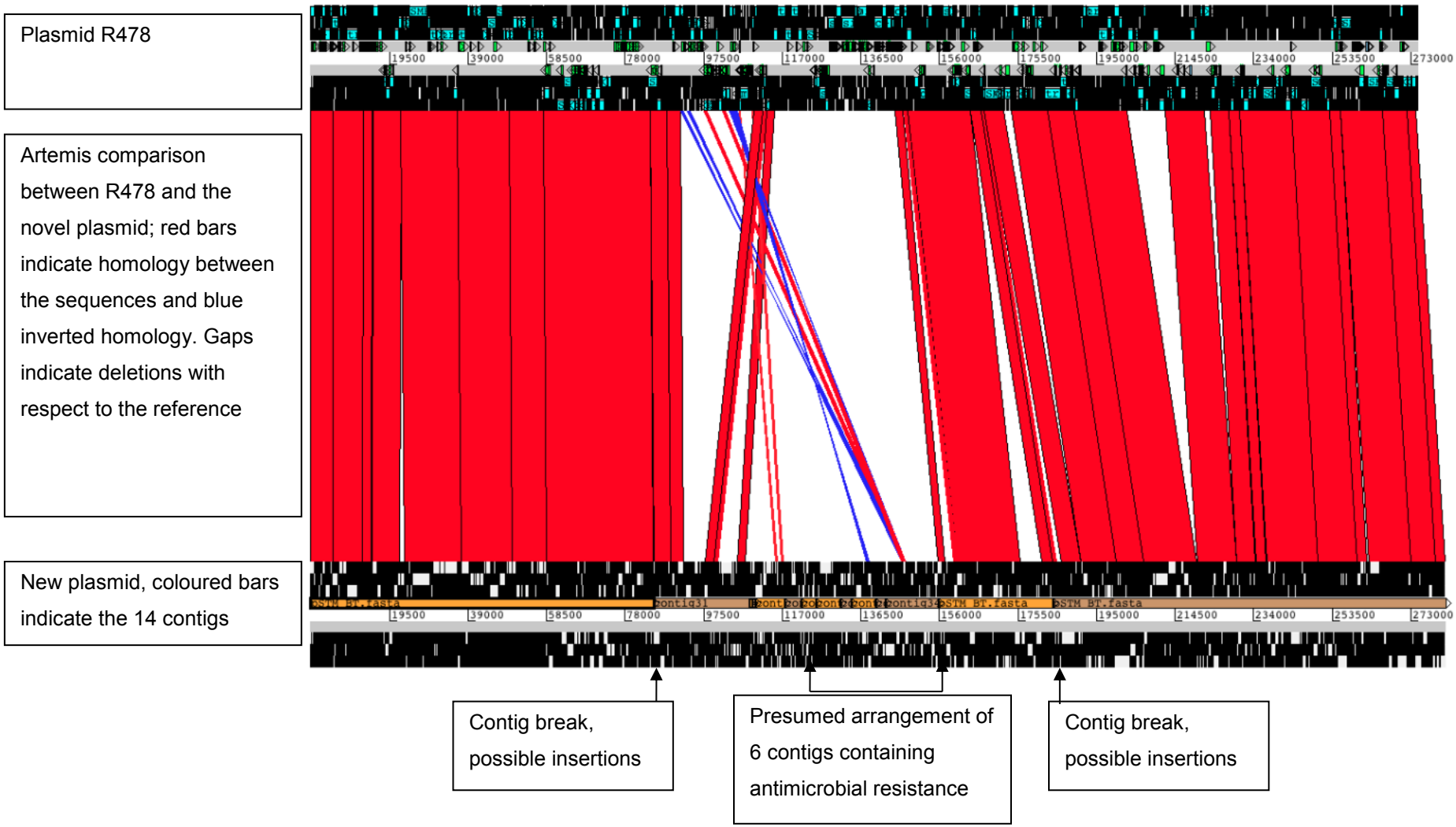


Figure 6-1 ACT comparison of the architecture of IncHI2 plasmid R478 and the novel IncHI2 plasmid: Red indicates colinearity of sequence, gaps indicate regions where sequences differ

6.4.5 Plasmid genome content

Following annotation transfer from R478 and gene prediction and curation of the remaining contigs, 298 predicted coding regions were identified (Table 6.1). The plasmid backbone contained genes required for replication, conjugative transfer and partition. It also contained a toxin/antitoxin addiction system.

Table 6-1: Functional categories of predicted genes in pSTm-BTCR

Functional Category	Number
Replication, transfer and partition	75
Metabolic	27
Virulence/Resistance	36
Membrane associated	25
Hypothetical	124
Pseudogene	11
Total predicted coding regions	298

This sequence that did not align with R478 was screened by blasting the DNA sequence against a “resistome” database under construction at WTSI, which contains all published antimicrobial resistance genes using the script map_resistome.py (SR Harris). The results are described in Table 6.2.

Table 6-2: Resistance genes detected by blasting against Sanger "resistome" database

Resistance Gene	Start	End	Strand	Length	SNPs	Match (%)	Length match (%)
blaTEM-1	534	1395	-	861	0	100.0	100
blaCTXM-15	5044	5920	+	876	0	100.0	100
Aac-3-IIa	1784	2645	-	861	34	96.1	100
DHFR A14	84	558	+	474	2	99.6	100
Aac-6-Ibcr	133	733	+	600	0	100.0	100
blaOXA1	863	1694	+	831	0	100.0	100
Tet-A	1606	2806	+	1200	61	94.9	100
QnrB1	4486	5131	+	645	0	100.0	100
DHFR-A1	175	649	-	474	0	100.0	100

The repertoire of antimicrobial resistance genes was confirmed by manual curation. This approach additionally identified the presence of *sul1, strA, strB, catB3* and *tetR.* In total there were 14 antimicrobial resistance genes, which encoded resistance to: tetracycline (*tetR*, *tetS*), ESBLs (*bla*CTX-M15, *bla*TEM-1, *bla*OXA-1), chloramphenicol (*catB3*), aminoglycosides (*strA*, *strB*, aminoglycoside 6 acetyl transferase and Aac-3-IIa), ciprofloxacin (*qnrB1* and aminoglycoside 6 acetyl transferase) and sulphonamides (dihydrofolate reductase A14 and A1 and *sul2*). Although the alignment was not confirmed, the presumptive arrangement based on pKOX-2 is depicted in Figure 6.2.

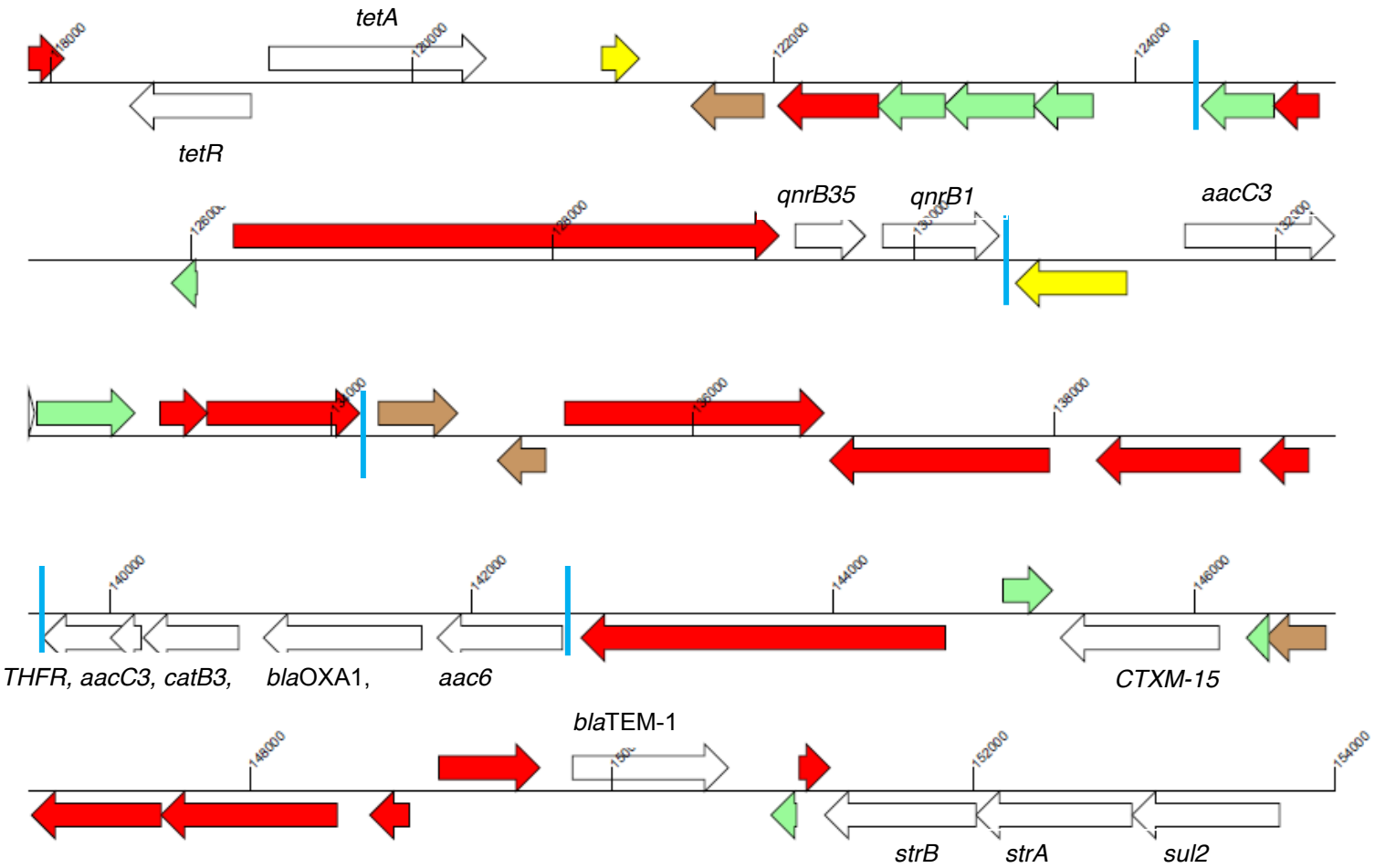


Figure 6-2: Presumed arrangement of antimicrobial resistance elements. The 5 contig boundaries are represented by vertical bars and antimicrobial resistance genes are labeled

In addition there were three families of genes encoding resistance to heavy metals, including arsenate and arsenite (*ars* genes B,C,R, and H), tellurite (*ter* A-F and W-Z) and mercuric ions (*merD, B, E* and *R2)*. The mercuric resistance set was not complete and genes encoding silver resistance, present on R478, were not present.

In addition to the resistance elements, the process of assembly and alignment against a reference IncHI2 and annotation revealed the presence the *mobC* gene. Knock-out of this gene has been associated with a 50-fold reduced frequency of conjugal mobilization of plasmids if donor cells lack this gene (Zhang et al. 1997).

6.4.6 Plasmid isolation and profiling

Kado and Lui plasmid extraction and gel-electrophoresis revealed the presence of plasmids corresponding to pSLT-BT (118kb) in A54528 and A54560, but not pBT1 or 2(87kb), compared to D23580 (Figure 6.3). This was predicted by the WGS data. There was no evidence of the suspected 280kb plasmid in A54560, but this was not surprising as a plasmid of that size was unlikely to be resolved using standard gel electrophoresis; however it confirmed that there are no smaller plasmids that might explain the observed pattern of resistance.

6.4.7 Plasmid conjugation

A54560: There was evidence of conjugative transfer with growth of E. coli resistant to ceftriaxone visible on MacConkey enriched with rifampicin and ceftriaxone at a

conjugation frequency of 6.5 x 10^{-2} transconjugants per donor. Transconjugation of an IncHI2 plasmid was confirmed by PCR for the IncHI2 region. An IncHI1 plasmid (pHCM1) was used as a negative control and serial dilutions of A54560 and the recipient *E. coli* were tested. All dilutions of the test strains were positive (Figure 6.4).

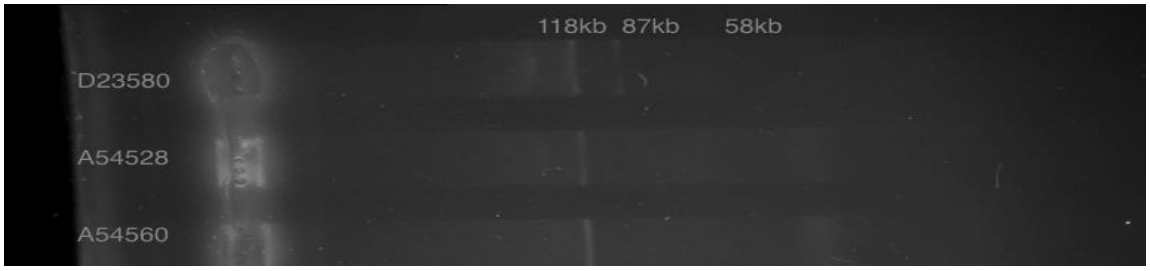


Figure 6-3: Gel-electrophoresis of extracted plasmid DNA reveals approximate sizes through comparison to those presented in the finished *S.* Typhimurium, D23580. Of note pBT1 (87kb) was not present in either of the strains under investigation. A band is present at ~118kb which represents pSLT-BT, the MDR virulence plasmid. There is no band corresponding to a 280kb plasmid

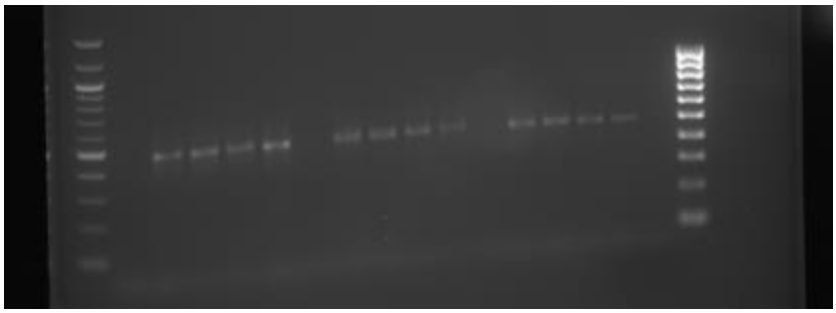


Figure 6-4: Gel electrophoresis of PCR products reveals IncHI2 defining region is present in all transconjugants. DNA in 3 groups of 4 with negative control between ladder on left and first group

6.4.8 Plasmid phylogeny

The novel plasmid together with fully sequenced IncHI2 plasmids R478, pK29, pEC-IMP and pEC-IMPQ (Figure 6.5) were compared to the core sequence of the inc-HI2 plasmid family. The tree reveals a conserved back-bone, based on 85 informative SNPs, with 2 SNP-dense regions of recombination.

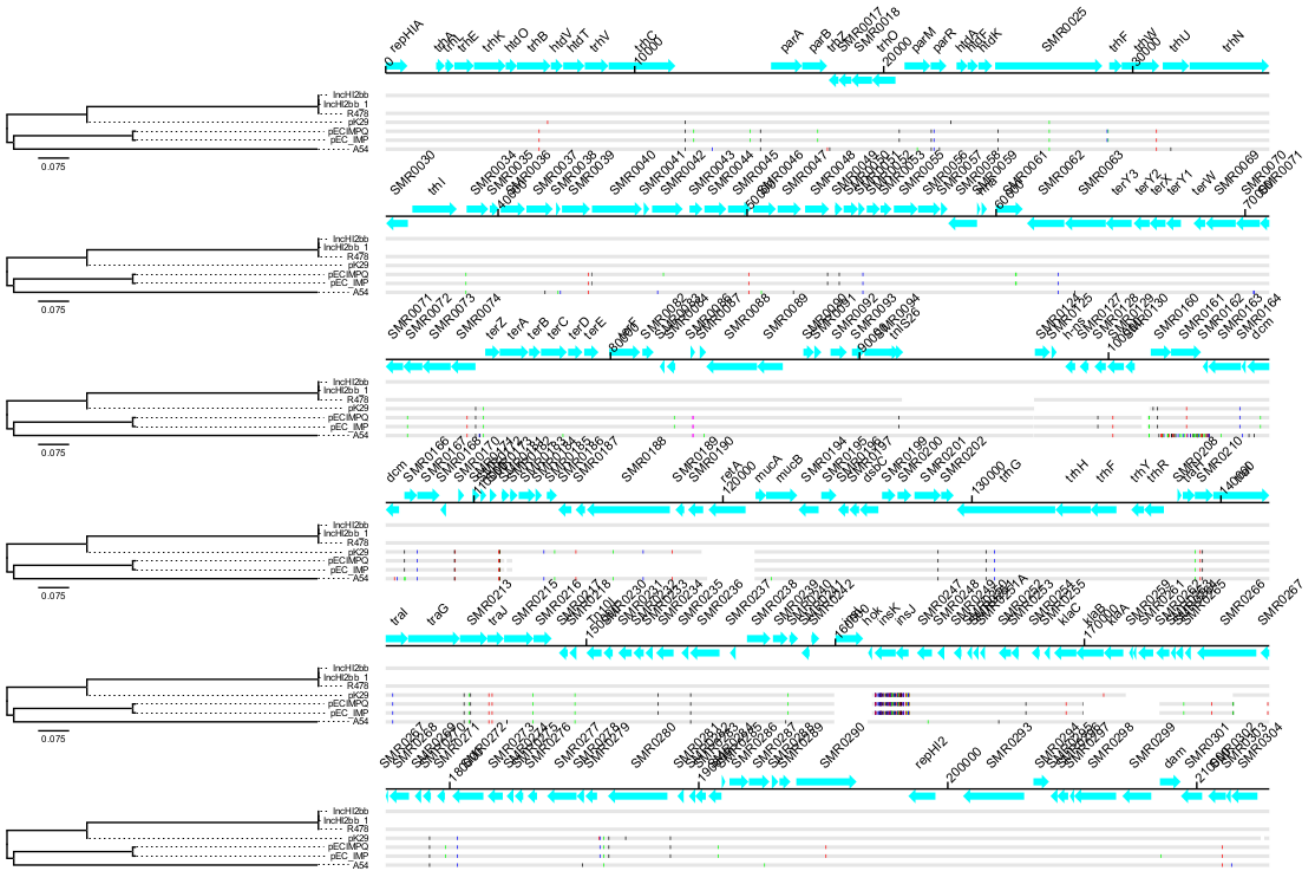


Figure 6-5: Phylogeny of sequenced IncHI2 plasmids reveals close similarity to other sequenced IncHI2 plasmids. The grey bars represent regions of R478 to which the other plasmids map, whereas an absence of grey indicates no mapping is present. The coloured lines represent single nucleotide SNPs with respect to R478.

6.5 Discussion

We present a case of recrudescent iNTS in a female HIV infected adult patient in whom the organism acquired an IncHI2 plasmid conferring ESBL and fluoroquinolone resistance in-between episodes of sepsis. The consequent pattern of resistance rendered the patient's sepsis untreatable with locally available antimicrobials and she died.

The core-genomes of the two bacterial isolates were identical, strongly suggesting that this was a case of recrudescence/relapsing sepsis, following incomplete clearance of the first infection rather than re-infection with a new strain (Okoro et al. 2012(2)). This makes it highly likely that acquisition of the plasmid occurred within the patient and we have demonstrated that this plasmid has the ability to transfer itself by conjugation. Partial decolonization of the patient's gastrointestinal tract by ceftriaxone and fluoroquinolone antimicrobial therapy may have rendered it a receptive environment for colonization by an ESBL-producing organism. This organism may then have transferred the plasmid to the *S*. Typhimurium.

This remains the only case of ESBL and fluoroquinolone resistant iNTS detected in Malawi, which is surprising as a diverse range of ESBL genotypes were observed in other enterobacteriaceae in Blantyre within a year of ceftriaxone coming into common usage there (Gray et al. 2006). IncHI2 plasmids are generally thought to transfer most efficiently below 30^{o}C, a lower temperature than found in the human gastrointestinal tract (Taylor et al. 1980), which might explain why the transfer of this particular resistance element has not been observed in this setting before or since, despite the on-going antimicrobial selection pressure. Of note, however, the novel plasmid contains gene *mobC* which has previously been reported to facilitate plasmid transfer (Zhang et al.1997) and its presence might explain how this plasmid was apparently able to transfer to the NTS strain in a patient.

The patient was given appropriate antimicrobial therapy. It is impossible to know whether conjugative transfer of this IncHI2 plasmid occurred in spite of this, or if the patient did not take the medication as prescribed. Certainly the antimicrobial therapy was necessary and antimicrobial stewardship could not have prevented this event. Instead, this is an example of HIV creating an environment that facilitated the spread of antimicrobial resistance through making the human host susceptible to gram-negative infection and thus hospitalization. The best method of preventing recurrence of this scenario is early diagnosis and treatment of HIV. In a resource rich environment, this patient would have been isolated in a side-room and barrier nursed. No such facility existed at QECH, where there are no staff or resources to support an infection control strategy.

The spread of mobile genetic elements (MGEs) that confer antimicrobial resistance amongst gram-negative organisms is a global cause for concern. WGS based methods offer the opportunity to track and analyse these elements, however as this study reveals, it is not possible to investigate them using a bioinformatic approach alone. The combination of laboratory and in-silico analysis presented in this chapter offer one solution, however it is too labour-intensive to represent a feasible approach to the large-scale, routine investigation of the epidemiology of MGEs. One solution to this problem is offered by newer sequencing machines, such as the Pacific Bio that can produce extremely long contigs (5-10 Kbp as opposed to 120 bp), to extract and sequence of large numbers of plasmids, which might be used as scaffolding for improved plasmid alignment.

6.6 Conclusions

We present a case of recrudescent iNTS disease, in which the pathogen acquired both extended spectrum ß-lactamase and ciprofloxacin resistance encoded by an IncHI2 plasmid. This pattern of resistance rendered one of the most common bloodstream pathogens in sub-

Saharan Africa effectively untreatable in this setting. Paucity of diagnostic microbiology laboratories in this region makes the detection of this resistance pattern almost impossible. There is an urgent need both to investigate the transmission of this pathogen and to build diagnostic capacity in Africa. Prompt diagnosis and treatment of HIV in Africa could be important to reduce the risk of emergence and spread of antimicrobial resistance.

6.7 Future work

- Describe prevalence and diversity of antimicrobial resistance among gram negative pathogens contained in the MLW archive
- Sequence other genera of ESBL producing and fluoroquinolone resistant gram negative bacteria from the MLW archive in order to describe the diversity of MGEs responsible for antimicrobial resistance in Malawi for further investigation by:
    - Illumina WGS
    - Plasmid profiling
    - Plasmid sequencing by PacBio system

Chapter 7: Clinical outcomes from iNTS in adults in the era of ART roll-out

7.1 Summary

iNTS is one of the most important causes of adult sepsis at QECH, Blantyre. It is associated with epidemics of MDR disease, high mortality, and frequent relapse. Relapsing iNTS necessitates multiple courses of antimicrobials, which may facilitate the emergence of further antimicrobial resistance. Previous studies have shown that relapse is likely to be caused by both recrudescence from a sanctuary site and re-infection, and that one possible site of persistence is the intra-cellular compartment of the macrophage.

A longitudinal cohort of HIV-infected adult patients presenting with their first culture-confirmed case of iNTS was recruited at QECH from 2010-2012. Patients were treated with IV ceftriaxone and then oral ciprofloxacin followed by initiation of ART within a month of presentation, and followed-up for 1 year or until death. Patients were reviewed on an out-patient basis and bile, stool and bone marrow were sampled for selective Salmonella culture to investigate the site of persistence.

63 HIV infected adults with iNTS were recruited. In-patient mortality in this cohort was 10% (10/99) as compared to 47% in a previous cohort (1999/2000) and 1-year mortality was 39% (22/57) as compared to 77% in the pre-ART era. Recurrence had also significantly fallen following the roll-out of ART (OR of recurrence 0.26 [95% CI: 0.09 - 0.76] in patients taking ART). Further emergence of fluoroquinolone or cephalosporin resistance was not observed.

Bile and bone marrow cultures were consistently negative, and the site of persistence was thus not established.

Clinical outcomes from iNTS in HIV-infected adults have significantly improved in the post-ART era, however it remains an important cause of sepsis in the large pool of profoundly immune-suppressed patients that exists in Blantyre. There is a need to intensify efforts to diagnose and promptly treat HIV earlier in the course of disease to prevent both new cases of iNTS and recurrent iNTS disease. It remains the case that most iNTS disease in Blantyre is resistant to cotrimoxazole, therefore there is an urgent need to investigate the role of alternative prophylactic therapy to CPT. In addition to patient-centred approaches to prevention of iNTS, there is a pressing need to investigate environmental reservoirs of NTS to prevent transmission of this pathogen in the community.

7.2 Introduction

It has previously been reported that iNTS is one of the most common causes of BSI at QECH, with high mortality (47% in-patient case-fatality and 77% case-fatality at one year) and frequent recurrence in those that survived admission (43% - Gordon et al. 2002). This study was undertaken in 1999-2000, prior to the roll-out of ART and at a time when 95% *S.* Typhimurium isolates were susceptible to chloramphenicol. Whole genome sequencing of the paired isolates from this and a subsequent study suggested that recurrence was caused by both recrudescence from a sanctuary site (80%) and re-infection (20%) (Okoro et al. 2012(2)), and also suggested that microevolution did not significantly occur between presentation and recurrence, suggesting either a non-replicative dormancy or a highly protected state of persistence. Following previous work (Gordon, M.A. 2010) the likely site of persistence of iNTS was thought to be bone marrow, but this contrasts with all other Salmonellae which have been found to persist in either the biliary tree (*S.* Typhi) or the colon

(other NTS). Understanding the site of persistence is of importance to guide eradication in individuals in order to prevent recurrent iNTS disease. Furthermore, if the site of persistence is the gastrointestinal tract, gastrointestinal shedding will occur and it is of public health importance to understand this in order to prevent onward transmission.

In 2002, an epidemic of MDR *S.* Typhimurium BSI began in Blantyre, necessitating the introduction of extended spectrum antimicrobials. In 2002, ciprofloxacin was introduced and became widely used for the empirical management of sepsis if patients failed to respond to chloramphenicol and in culture-confirmed iNTS. In 2004, ceftriaxone replaced combined benzylpenicillin and ciprofloxacin in the empirical management (see chapter 2, setting). Between 1998 and 2004, in-patient mortality declined year on year from to 29% in 1998 to 20% in 2004 (Gordon et al. 2008). This gradual improvement in in-patient mortality may have been influenced by a combination of improved recognition and early treatment of sepsis, improved clinical staffing and the introduction of ciprofloxacin in 2002, however ART was not rolled-out in Malawi until 2005.

Findings from the 1999/2000 cohort revealed the importance of recurrence as a clinical problem in Blantyre (Gordon et al. 2002), whilst the sequencing of paired isolates lend considerable weight to the theory that recurrence, is more frequently caused by recrudescence from a sanctuary site than by new infections (Okoro et al. 2012(2)). The description of recurrence in a second cohort recruited after the introduction of ciprofloxacin demonstrated recurrence of iNTS in 30% of patients even after 10 days of ciprofloxacin. It was also observed that there was a 6-fold higher concentration of NTS in bone marrow, compared with blood samples, at recurrences. This concentration was in the intracellular compartment, indicated by a higher yield from lysed compared to unlysed blood and bone marrow samples, which suggested systemic intracellular replication (Gordon et al. 2010) and lead to the hypothesis that the intracellular compartment is the site of persistence.

7.2.1 Research questions

1) What are the clinical outcomes of adult iNTS, in the era of ART roll-out?

2) Where do NTS persist in adults, and what are the potential consequences of the site of persistence for transmission?

3) Do repeated courses of ciprofloxacin for recurrent iNTS lead to fluoroquinolone resistance among iNTS and other major pathogens?

7.3 Methods

See chapter 2 for setting (2.2), and methods (2.4) including summary (2.4.1) study team (2.4.2) criteria for entry (2.4.3) and study visits and patient sampling (2.4.4).

7.3.1 Clinical outcome measures and statistical analysis

In-patient outcomes were defined based on death or live discharge from QECH. Outpatient outcomes were calculated based on survival or death in the group of patients that survived admission to QECH and excluded those patients that died during their first admission to QECH. Recurrence and clinical outcomes from the 2010-13 cohort were compared to a previous cohort recruited in the same setting by Dr M. Gordon in 1999/2000 (Gordon et al. 2002) using ORs.

Medians have been used to summarise non-parametric data. Medians are presented with inter-quartile ranges (IQRs) except for small datasets (<10 data points), where the range has been used. Univariate analysis of risk factors for mortality was calculated using Mantel-

Haenszel odds ratios (ORs) using the Stata command “mhodds” and has been presented with 95% confidence intervals (CI) and p-values. If a p value was less than <0.1, the risk factor was additionally included in multivariate analysis by logistic regression.

7.4 Results

During the pilot phase, from Jan 2010 - June 2010, 13 adult patients with iNTS were recruited. The study was then broadened to include more comprehensive clinical and microbiological surveillance. This phase began in October 2010 and ran for 18 months until the end of March 2012 (Figure 7.1). During this period there were an estimated 11,550 adult medical admissions to QECH (unpublished data, MLW Surveillance Programme of IN-patients and Epidemiology [SPINE]). A total of 6,000 blood cultures yielded 126 patients with a first culture-confirmed presentation with iNTS, of whom 50 (40%) were recruited to the study. In total 99/126 (78%) had a known in-patient outcome. 10/99 (10%) died compared to an overall in-patient mortality of 15% at QECH during a similar period (SanJoaquin et al. 2013). In total 63 patients were recruited, including 58 cases caused by *S.* Typhimurium, 3 by *S.* Enteritidis and 2 by *Salmonella sp.* 57 (90%) cases were successfully followed up for 1 year or until death.

7.4.1 Characteristics of the clinical cohort

The median age in the cohort was 36 years (IQR 34.2-38.6) and 37/63 (59%) were male. All were HIV infected. Patients reported feeling unwell for a median of 11 days; however this varied considerably (IQR 5-220 days). There was no single dominant mode of presentation (Table 7.1); 97% patients reported feeling feverish, 56% reported cough, 34% acute diarrhoea, 23% headache and 12% symptoms of urinary infection. 42/63 (67%) patients

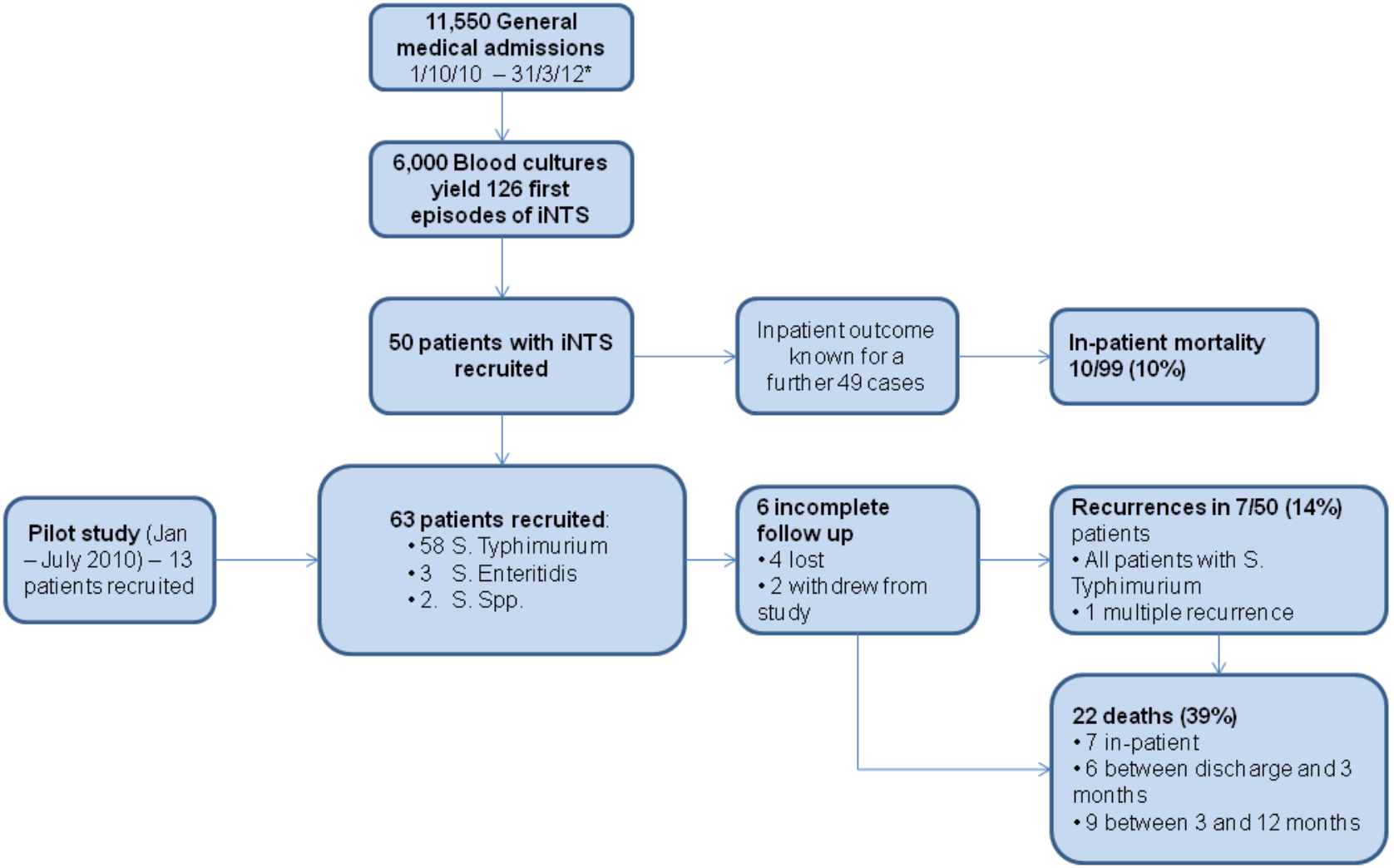


Figure 7.1: Recruitment and follow up of adult iNTS cohort. *MLW-SPINE

gave a history of health problems for > 3 months or AIDS defining conditions, of whom 22 were already known to be HIV infected. 48% patients already knew their HIV status and 18 had commenced ART; 7 had, however, only been taking ART for <1 week. Seven had started very recently (within a week of presentation) and 7 had been taking ART for ≥ 90 days. 21 (33%) patients were taking CPT.

On examination, 78% of patients were febrile and 32% of patients had evidence of severe sepsis, as defined by a positive blood culture, and hypotension (systolic blood pressure <90 mm Hg) (Dellinger et al. 2013). 34 (54%) patients had signs suggestive of HIV (one or more of; wasting, jaundice, oral thrush, Kaposi Sarcoma, lymphadenopathy or shingles scarring). 49% of patients had abnormal GI examination, including 40% with splenomegaly, whilst 30% had chest signs and 5% had cardiac murmurs, although none of these patients had echocardiographically confirmed endocarditis.

A high proportion of patients had advanced HIV (Table 7.3). The median haemoglobin was 7.4 g/dl (IQR: 5.8-8.6), the median CD4 count was 87 cells/l (IQR 27-139) and the median viral load was 600,000 copies/ml (IQR 1,200-6.6million).

Table 7-1: Clinical features at presentation: history from patients

Clinical Feature	Number*	Denominator	Proportion (%)
Age (median [IQR])	36.4 (34.2-38.6)		
Male gender	37	63	59
History of presenting complaint			
Duration of illness days (median [IQR])	11 (5-220)		
Feverish	61	63	97
Cough	35	62	56
Chest pain	10	49	20
Dyspnoea	18	49	37
Acute diarrhoea	34	61	56
PR bleed	4	48	8
Vomiting	23	63	37
Abdominal pain	25	49	51
Dysuria	2	49	4
Urinary frequency	6	50	12
Headache	23	51	45
Past medical history, AIDS defining illness or chronic symptoms [> 3 months])			
Self-reported weight-loss	25	60	42
Diarrhoea	9	61	15
Fever	8	50	16
Cough	4	51	8
Shingles	12	62	19
Candidiasis	13	51	25
Cryptococcal Meningitis	4	63	6
Dysphagia	2	49	4
Kaposi sarcoma	4	63	6
Neurological disability	1	63	2
Known to be HIV-seropositive	30	63	48
Drug History			
Prior antimicrobials†	12	63	19
Prior CPT	21	63	33
Prior ART	18	63	29
Median duration in days of ART (IQR)	7 (7-90)		

* Unless otherwise specified † 6 amoxicillin, 1 erythromycin, 1 ciprofloxacin, 4 patient did not know

Table 7-2: Clinical features at presentation: findings on examination

Findings on examination	Number	Denominator	Proportion
Median temperature (°C)	38.9 (37.5-39.7)		
Proportion febrile	42	54	78
Median pulse (bpm)	115 (102-135)		
Median systolic BP(mmHg)	98 (88-110)		
Severe sepsis	16	50	32
Signs of HIV*	34	63	54
Wasting	22	58	38
Jaundice	4	58	7
Oral thrush	12	58	21
Kaposi Sarcoma	6	55	11
Lymphadenopathy	6	48	13
Zoster scar	5	54	9
Cardiac murmur	3	63	5
Chest signs	17	63	30
GI signs	31	63	49
Splenomegaly	23	58	40

* Any of wasting, jaundice, oral thrush, KS, lymphadenopathy or zoster scar

Table 7-3: Laboratory results at presentation

Laboratory results at presentation	Median	IQR	
Haemoglobin g/dl (IQR)	7.4	5.8	8.6
CD4 count cells/µl (IQR)	87	37	139
HIV seropositive (%)	63	63	100
HIV viral load copies/ml (IQR)	600,000	1,200	6,600,000

Blood culture results at presentation indicated that the iNTS MDR phenotype remains common, with 53 (84%) isolates being MDR. 6 (10%) isolates, all of which were *S.*

Typhimurium, were susceptible to chloramphenicol (Table 7.4) and only 4 (6%) were fully susceptible.

Table 7-4: Blood culture results at presentation

Serotype	MDR	Amoxicillin/ Cotrimoxazole	Drug Susceptible	Total
***S.* Typhimurium**	51	6	1	58
***S.* Enteritidis**	1	0	2	3
Salmonella Sp.	1	0	1	2

7.4.2 Clinical management of cohort

61/63 patients were initially prescribed IV 2g ceftriaxone o.d. for 2-4 days, and those that survived to discharge completed 10 days total treatment with oral ciprofloxacin 500mg bd. 2/63 patients were treated with IV benzylpenicillin and chloramphenicol as the hospital pharmacy briefly ran out of ceftriaxone and ciprofloxacin in late September 2011. Despite the fact that both isolates were MDR, one patient survived admission without effective antimicrobial therapy. All patients that survived admission registered for antiretroviral therapy prior to discharge.

7.4.3 Clinical outcomes from iNTS in the ART-era

56/63 patients from the cohort survived admission and 50 were successfully followed-up until death or survival at 1 year, through an overlapping combination of out-patient follow-up,

surveillance of ward admissions and telephone consultations. 15 patients died after discharge, 6 in the first 3 months and 9 in the subsequent 9 months.

The in-patient outcome was known for 99 patients, and 10/99 (10%) died in hospital, which was not significantly different from the published overall mortality for adult medical admissions at the time of 15% (0.6128 [95% CI: 0.26 to 1.42]) (SanJoaquin et al. 2013), but significantly better than the 1999/2000 cohort, at which time iNTS patients were significantly more likely to die (OR 8.42 [95% CI: 3.33 - 21.32]).

Overall, the 1-year mortality was 38% (Figure 2), this figure is significantly worse than the 15% 1-year mortality of all patients in the their first year of ART in Malawi (OR 3.58 [95% CI: 2.10 - 6.10]) (Weigel et al. 2013), but it compares favourably to the previous clinical cohort recruited in the same setting from 1999-2000, which reported 1 year mortality of 77% (OR with respect to 2009/10 cohort 5.5 [95% CI: 2.7-11]) (Gordon et al. 2002). If the cohorts are only analysed with respect to post-discharge survival to 1-year, there has still been significant improvement in outcome, with OR of out-patient death in the pre-ART era of 3.60 (95% CI: 1.58 - 8.23) compared to the current cohort.

Of the 9 late deaths (≥ 3/12 after presentation), 2 patients died from cryptococcal meningitis, one from Guillain–Barré syndrome (GBS), 1 from Hepatitis-B related hepato-cellular carcinoma and one died after an episode of recurrent iNTS, with disseminated TB and HIV treatment failure. The remaining 4 patients were reported by their families to have died at home. Only one of these 9 attended out-patient follow-up clinics and their outcomes are known either due to readmission or through telephone consultation with their families.

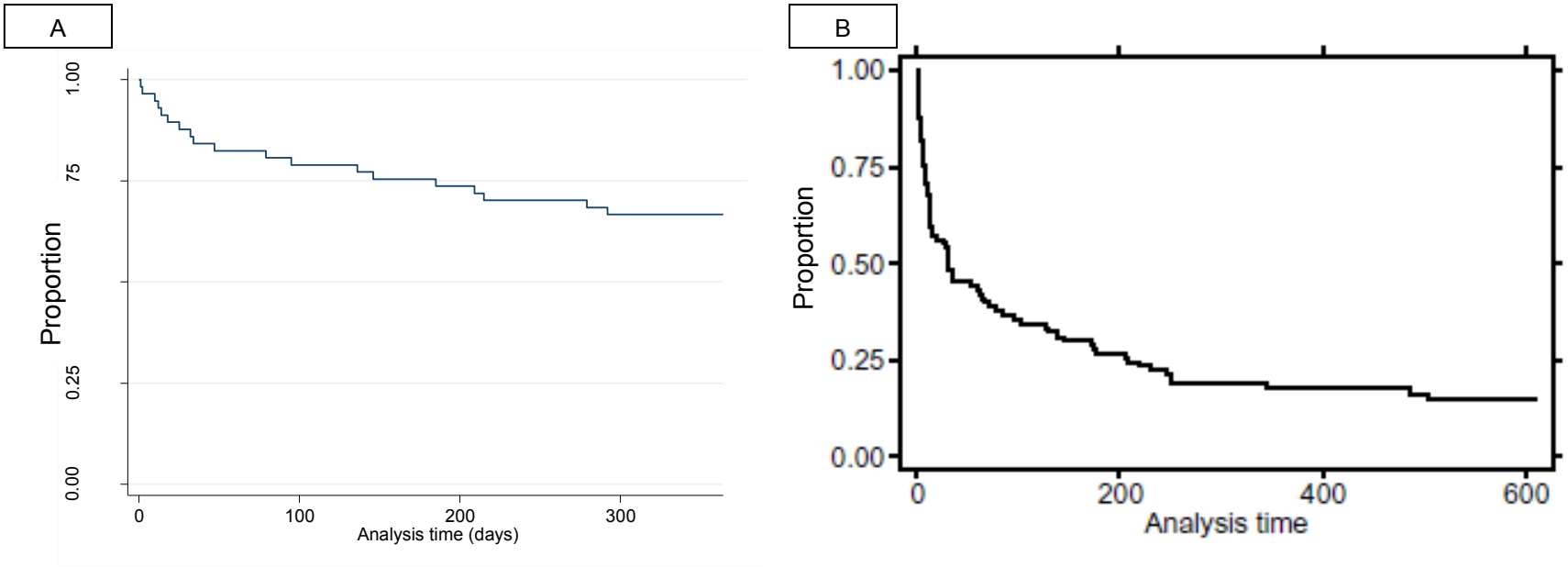


Figure 7-2: Kaplan-Meier survival estimates in days of (A) 2010-12 adult iNTS cohort and (B) the 1999/2000 cohort (Gordon et al. 2002)

Univariate analysis of risk factors for death revealed only one significant risk factor for mortality in the cohort, which was a history of dyspnoea (OR 6.86 [95% CI: 1.49-35.55]). Headache, presentation with a fever >37.5oC and splenomegaly found to be significantly or borderline significantly protective (Table 7.5). When these factors were analysed by multivariate analysis, none were found to be statistically significant (Table 7.6).

Table 7-5: Univariate analysis of risk factors for death in the year after diagnosis with iNTS (20010-13 cohort)

	OR	95% CI		P-value
Age	1.04	0.98	1.10	0.19
Male gender	1.31	0.43	9.98	0.63
Presenting complaint				
Median duration of illness (days)	1.02	0.99	1.05	0.19
Cough	3.00	0.90	10.00	0.06
Chest pain	1.52	0.35	6.60	0.58
Dyspnoea	6.86	1.49	31.55	0.00
Acute diarrhoea	0.81	0.27	2.46	0.71
PR bleed	0.00	0.00	0.00	0.23
Vomiting	0.70	0.22	2.18	0.53
Abdominal pain	1.22	0.35	4.25	0.76
Urinary frequency	3.60	0.50	26.10	0.17
Headache	0.32	0.09	1.20	0.07
Confusion	1.94	0.11	34.11	0.64
Weight loss	1.59	0.51	4.92	0.42
Diarrhoea	2.43	0.47	12.50	0.27
Fever	0.67	0.12	3.86	0.64

Cough	2.31	0.28	18.89	0.42
Known to be HIV seropositive	1.08	0.32	3.66	0.90
Drug History				
Prior antimicrobials	1.18	0.32	4.35	0.81
Prior CPT	0.63	0.20	2.06	0.45
Prior ART	1.08	0.32	3.65	0.90
Findings on examination				
Febrile	0.22	0.05	1.00	0.03
Severe sepsis	0.86	0.25	2.94	0.80
Signs of HIV	1.47	0.48	4.46	0.49
Chest signs	NA			
GI signs	NA			
Splenomegaly	0.77	0.61	0.99	0.36
Laboratory results at presentation				
Haemoglobin per g/Hb	1.22	0.84	1.77	0.29
CD4 count per cell/µl	0.99	0.98	1.00	0.15
HIV viral load per copy/ml	0.99	0.99	1.00	0.24

Table 7-6: Multivariate risk factors for death

Multivariate analysis (if p < 0.1 by univariate analysis)	**OR**	**[95%**	**CI)**
Dyspnoea	3.85	0.84	17.75
Headache	0.21	0.04	1.07
Febrile	0.88	0.50	1.56
Splenomegaly	0.33	0.07	1.60

7.4.4 Site of persistence of Salmonella

In total, 28 patients consented to have follow-up investigations on their return to the out-patient clinic. At the time of the first follow-up visit, none of the patients were taking ciprofloxacin or ART, but all were taking CPT: 1/28 (4%) stool samples were positive for *S*. Typhimurium, but all 21 bile samples and 11 bone marrow aspirates (BMAs) were culture negative at the first visit (Table 7.7). Two of the patients who consented to BMA subsequently presented with recurrent iNTS disease. At subsequent visits, 1 patient who originally presented with *S*. Typhimurium BSI was stool culture positive for *S*. Typhimurium at visit 3 having previously been stool culture negative on visits 1 and 2, and another patient who originally presented with *S*. Typhimurium BSI was stool culture positive for *S*. Enteritidis at the 1 year time point. It is interesting to note that both of the 2 patients who presented with *S*. Typhimurium BSI and positive stool culture were stool culture positive for different Salmonella serotypes at presentation (1 *S*. Typhi and 1 *S. sp.*), as this is in contrast to findings in a previous study (Gordon et al. 2010). This might reflect the fact that the earlier study was done during a period of epidemic transmission of a single NTS serotype. Of the 4 patients with a positive Salmonella stool culture, 3 were positive for a different serotype to that causing their index presentation, which suggests people were being exposed to multiple serotypes of Salmonella in the community at the time of this study. This investigation did not demonstrate the site of persistence of NTS between episodes of index and recurrent iNTS.

Table 7-7: Microbiology results at routine follow up

	Recruitment	14 -28 days	2 months	3 months	1 year
Selective Salmonella Stool Culture	2/23	1/28	0/20	1/13	1/12
Serotype isolated*	1x *S. sp.* 1x *S.* Typhi	*S.* Typhimurium		*S.* Typhimurium	*S.* Enteritidis
Bile culture	NA	0/21	NA	NA	NA
Bone marrow culture	NA	0/11	NA	NA	NA

* All 5 patients initially presented with *S.* Typhimurium BSI

7.4.5 Recurrent iNTS disease

Patients had routine follow-up blood cultures at the same time as BMA, otherwise they were only re-cultured if febrile (≥ 37.5^{o}C). In total, 28 blood cultures were taken from 22/50 patients who survived discharge; 11 at the time of BMA and 17 due to recurrent fever. During the study period there were 8 cases of recurrent iNTS disease in 7 patients. 3/7 patients were recruited at index presentation, although one declined to attend follow-up, however 4/7 patients initially declined to take part in microbiological follow-up, but gave subsequent consent to follow-up after presentation with a symptomatic recurrence. 3 patients had already been commenced on ART at presentation: 1 for 7 days, one for 65 and one for 350. The median time from first presentation to recurrence was 59 days (range 26-93 days) and 6/7 patients had been started on ART at the time of recurrence. In these 6 patients, the median time between commencing ART and recurrence was 83 days (range 14-370 days). One patient had 2

recurrences and was additionally stool culture positive for *S.* Typhimurium at the first episode of recurrence. 5/7 patients survived to 1 year after follow-up, including the patient with multiple recurrences. Of the 2 that died, 1 had virological evidence of ART failure (HIV VL 490 copies/ml after 1 month ART and 15,000 after 3 months), plus disseminated TB and the other did not engage with follow-up after discharge. When the current cohort was compared to a previous cohort from the same setting recruited in 2000 that pre-dated the roll-out of ART, OR of recurrence in the pre-ART era was 3.8 (95% CI: 1.32 - 10.97). There was no emergence of resistance to ciprofloxacin or ceftriaxone between presentation and recurrence in this cohort.

Table 7-8: Recurrence

Recurrence	Number	Denominator	Proportion
Number of patients with recurrence	7	50	14%
Time to recurrence (days) median (range)	59 (43 – 82)		
Median CD4 cells/μl (range)	71 (4 – 186)		
On ART at first presentation	3	7	43%
On ART at re-presentation	6	7	86%
Median duration ART at recurrence (days) median (range)	84 (26 – 93)		
Died by 1 year	2	7	29%

7.5 Discussion

7.5.1 What are the clinical outcomes of adult iNTS, in the era of ART roll-out?

In this study there was a 10% in-patient mortality, not significantly lower than the overall 15% for all adult medical admissions to QECH during the same period (SanJoaquin et al. 2013), but significantly lower than the 47% cited for iNTS patients in the 2000 cohort (Gordon et al. 2012). In-patient mortality from iNTS gradually fell from 29% in 1998 to 20% in 2004 (Gordon et al. 2008) in the pre-ART era. This gradual fall suggests a role for multiple in-patient factors in improving in-patient outcomes including choice of antimicrobial (ceftriaxone as a once daily IV drug is more likely to be administered than chloramphenicol qid), increased medical staffing and greater awareness of the importance of the condition. There is no suggestion that the cohort of patients was any less immune-suppressed at the point of presentation with iNTS in 2010-12 than 2000 as a consequence of ART, the median CD4 count was 99 cells/μl in the earlier cohort and 68 cells/μl in the 2010/12 cohort.

Not only was the 1 year mortality, at 39% also significantly lower than seen with the 2000 cohort, but the out-patient mortality, adjusting for improvements in in-patient management was also significantly lower. Furthermore, recurrences of iNTS were significantly less frequent in the 2010/12 cohort, even at the first visit before ART was commenced. This strongly suggests that the combination of ciprofloxacin and early initiation of ART combine to protect against both recurrence of iNTS disease and death.

The multivariate analysis did not detect any statistically significant risk factors for death in the 2010-13 cohort, although the cut-off for inclusion in this analysis at a p-value of 0.1 by univariate analysis was highly stringent for a study with limited numbers. The study was not, however specifically designed to investigate risk factors for a poor outcome from iNTS disease

7.5.2 Where do NTS persist in adults, and what are the potential consequences of the site of persistence for transmission?

Patients attended follow-up for microbiological investigation between 2 weeks and 4 weeks after presentation. At this time, patients should have completed their course of ciprofloxacin therapy and not have started ART, but they should been taking CPT. Culture of 28 stool samples revealed 1 which was positive for *S*. Typhimurium. 21 patients consented to hairy string bile capture for bile culture, all of which were negative and 11 consented to bone-marrow aspirate. The fact that all of these cultures were negative, including 2 complete sets from 2 patients who ultimately developed recurrence, could be explained by several possibilities:

1. The culture methodology was inadequate, which is unlikely as standard methodology for Salmonella culture was used.
2. Patients were sampled too closely to the end of their course of ciprofloxacin to detect NTS by culture based methods. Although a possible explanation, this approach was based on experience with a previous ciprofloxacin-treated cohort, in which 14% had symptomatic recurrent disease within a month (Gordon et al. 2010) and 27/210 (13%) were BMA culture positive at symptomatic relapse.

Certainly it would have been unethical to delay initiation of ART any longer to accommodate later culture.

3. Uptake of and adherence to CPT was considerably higher in the later cohort, and could have suppressed infection to a level below that which could be cultured, despite high levels of *in vitro* resistance.
4. The intracellular compartment of cells contained in the bone marrow was not found to be the site of persistence. This may be the case and it is interesting to consider two cases of recurrent iNTS. In the case described in chapter 6, a recurrence strain which was chromosomally indistinguishable from the conserved region of the index strain had acquired an ESBL producing plasmid by the time of recurrence, presumably by conjugative transfer, perhaps from another enteric pathogen. This suggests the possibility that the gastrointestinal tract might be the site of persistence. In the second case, the single patient with multiple recurrences of iNTS was also stool culture positive at the first recurrence, and unusually re-presented with acute diarrhoea in addition to sepsis 12 days after commencing ART, perhaps suggesting a gastrointestinal immune-reconstitution inflammatory syndrome.

Subsequent rounds of stool culture at routine follow-up visits yielded only 2 isolates, 1 of which was discordant with the presentation isolate. Of the 5/96 positive stool cultures from presentation or follow-up, 3 yielded serotypes which were discordant with their blood-culture results, perhaps suggesting that this group of patients are continually exposed to a variety of Salmonellae. It is also possible that enteric exposure and persistence of NTS becomes relatively more important than intracellular persistence in relation to recurrence after successful initiation of ARVs.

7.5.3 Do repeated courses of ciprofloxacin for recurrent iNTS lead to fluoroquinolone resistance among iNTS and other major pathogens?

There was no suggestion of the emergence of resistance. All of the isolates remained susceptible to ciprofloxacin and ceftriaxone and there was no change in the antimicrobial susceptibility pattern amongst the seven patients with recurrent disease. This is perhaps unsurprising for several reasons. Firstly there has been a sharp decline in the frequency with which NTS is isolated at MLW. Secondly recurrence in those patients that do develop iNTS has significantly declined. Thirdly, on the one occasion that ESBL production and ciprofloxacin resistance was observed, it was caused by a family of plasmids which do not have the capacity to transfer in-vivo (chapter 6). Lastly, WGS-based analysis of the strain collection of paired index and recurrence isolates from the 2 previous cohorts suggested that chromosomal microevolution occurs too slowly for chromosomal resistance mutations to emerge during asymptomatic, quiescent persistence within a single individual (Okoro et al. 2012(2)). In 5 years from 2009-13 there was just one case of resistance amongst 2171 iNTS isolates or 1/2171 (binomial exact confidence intervals: 0.00001-0.00237). As these data predict only 1 highly resistant case of iNTS per 421-85,470 cases this study should not be repeated in this setting at this time.

7.6 Limitations

Recruiting a cohort of patients with iNTS in Blantyre proved extremely challenging. The most obvious reason for this was the decline in iNTS which occurred during the study

period which led to the recruitment of small numbers of index cases of iNTS (chapter 3). Investigating the site of persistence and demonstrating that recurrence promotes emergence of resistance was dependant on obtaining cultures from patients during convalescence and prior to recurrence, however initiation of ART rapidly prevents recurrence of iNTS and therefore emergence of resistance through multiple exposures to antimicrobials. Whether or not recurrence might eventually have contributed to the emergence and sustained transmission of extensively resistant NTS could not be answered by this study. It is however as important to demonstrate that ART rapidly protects against recurrence.

Admission to hospital in Malawi is frequently perceived to be associated with death in Malawi and there is a reluctance to return unless there is a direct benefit. Despite a financial incentive to return for follow-up, study patients were reluctant. The same study team recruited two additional HIV-infected adult cohorts, one with TB-BSI (Feasey et al. 2013) and a second with cryptococcal meningitis (Rothe et al. 2013) during the same period with much greater success. One difference is that patients from both of these cohorts had to return to QECH for medication (quadruple anti-tuberculosis chemotherapy and fluconazole respectively), but another was perhaps the inconvenience of having the clinical procedures which were entailed in follow-up, especially the BMA.

7.7 Conclusions

Clinical outcomes from iNTS have significantly improved both at presentation and during convalescence and immune reconstitution. The use of once-daily IV ceftriaxone followed

by oral ciprofloxacin, plus improved training and staffing levels are likely to have contributed to improved in-patient mortality to enable survival to commence ART. Recurrent iNTS is now uncommon following the prompt initiation of ART, and in keeping with this, a site of persistence was not demonstrated. Improvements in inpatient mortality are likely to reflect a number of improvements in in-patient care, whilst improvements in 1 year mortality and the decline in recurrence are both likely to be attributable to the roll-out of ART, and possibly also to increased uptake and adherence to CPT. Although the incidence of iNTS has declined following a period of single-serotype epidemics, it remains a common cause of febrile presentation to QECH, and there remains a large cohort of patients in Blantyre with profound immune-suppression at risk of this condition. General measures to reduce the incidence of iNTS include public health measures to prevent the transmission and to facilitate the early diagnosis and treatment of HIV. Specific measures should include the early initiation of ART to prevent recurrence.

7.8 Future Work

There is strong evidence of differentially adapted strains of *S.* Typhimurium causing epidemics of BSI in Africa. It is unclear whether these are truly host restricted, and how they are transmitted. There is an urgent need for an investigation into the environmental reservoirs and transmission of these strains in order to devise public health measures to further reduce transmission. This is especially important in view of the diversity of faecal Salmonella strains detected in this study.

Chapter 8: The phylogeny of *S.* Enteritidis in Malawi reveals two clades with contrasting virulence determinants

8.1 Summary

Salmonella Enteritidis is second only to *S.* Typhimurium as cause of iNTS in Africa (Reddy et al. 2010). An epidemic of MDR *S.* Enteritidis was reported in Malawi in 1999-2001, preceding a separate epidemic of *S.* Typhimurium, which began in 2002 (Gordon et al. 2008). Whether and how *S.* Enteritidis have evolved to cause invasive disease in SSA, and if there is any commonality with the evolution of iNTS *S.* Typhimurium ST313 is unknown, however a pilot study looking at phylogeny of 41 isolates of *S.* Enteritidis isolated in Malawi from 1998-2006 suggested the existence of 2 clades of *S.* Enteritidis, both causing BSI over the same time period.

A detailed analysis of two representative isolates of each of the two clades was made through comparison to the closed, reference genome of *S.* Enteritidis PT4 strain P125109 (Thomson et al. 2008) using the Artemis Comparison Tool (ACT). Differences between them and predicted variable and conserved regions of the reference were described.

Comparative genomic analysis revealed that isolate A1636, which clustered with P125109, was highly similar in both variable and conserved regions of the genome. In contrast, the second clade represented by isolates D7795 and A7830, was strikingly

different. These isolates had a genomic signature suggestive of a change in host adaptation, novel prophage regions and an MDR virulence plasmid containing several likely additional virulence determinants. The pattern of gain and loss of mobile genetic elements suggests that they diverged from a common ancestor, not that one evolved from the other.

The genotypic changes were then corroborated with the phenotype using the Biolog™ phenotyping microarray and visualized using Pathway Tools. This high-throughput phenotyping confirmed the loss of metabolic pathways in the second clade which would be predicted by the genome (e.g. glycerol and fuculose) and highlights disruption to pathways not predicted by the genome (e.g. butyric acid metabolism).

These data were used as the rationale for a broader look at the phylogeny of *S*. Enteritidis to investigate whether there genuinely is an “African” clade of *S*. Enteritidis, whose emergence has paralleled that of the “African” *S*. Typhimurium epidemic of ST313 (Chapter 9).

8.2 Introduction

In industrialised settings, *S*. Enteritidis is a major cause of epidemic gastroenteritis associated with intensive poultry farming and egg production. It is a host generalist and has a low human invasiveness index (Jones et al. 2008). *S*. Enteritidis has been in decline in this setting, following a number of interventions in the farming industry involving both hygiene and vaccination (O'Brien 2013).

S. Enteritidis has also been a major cause of BSI in SSA. The first epidemic of iNTS to be described in Malawi was caused by MDR *S.* Enteritidis and began in 1999 (see Chapter 1 and 3) (Gordon et al. 2008). *S*. Enteritidis was subsequently reported as being the second most common cause of iNTS across SSA (Reddy et al. 2010). Although *S*. Enteritidis has declined as a cause of BSI in Malawi (chapter 3), it remains an important pathogen in numerous sites across Africa (personal communication from Jan Jacobs [Congo], Karen Keddy [South Africa] and Myron Levine [Mali]).

Also focused on SSA, a previous investigation of epidemic strains of MDR *S*. Typhimurium revealed a novel pathotype of MLST type ST313, which differ from strains that cause enterocolitis in industrialised settings by showing patterns of genomic degradation likely to be associated with more invasive disease and differential host adaptation (Kingsley et al. 2009, Parsons et al. 2013). This was followed by a pilot investigation by Dr Robert Kingsley into 41 strains of *S*. Enteritidis, selected on the basis of temporal (2000-2006) and antimicrobial diversity. The DNA was extracted in Malawi by Dr C. Msefula and sequenced at WTSI using Illumina HiSeq 1000. This pilot study had already suggested the presence of 2 clades of *S*. Enteritidis (fig 8.1).

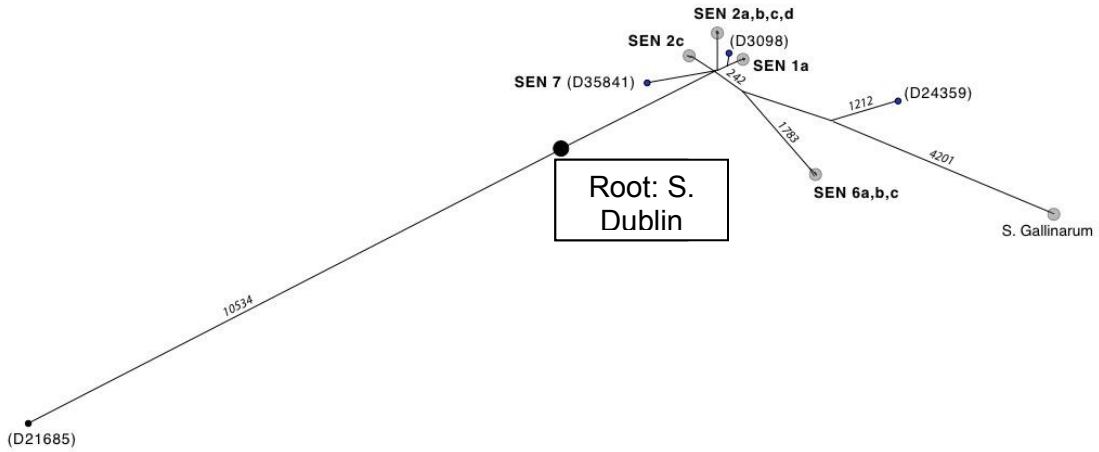


Figure 8-1: Phylogram from pilot investigation of the phylogeny of *S.* Enteritidis in Malawi. SNPs are indicated by numbers associated with lineages. Groups of strains that cluster within particular PFGE-types (bold type) are indicated (grey circles). D21685 transpired to be *S.* Typhi.

The phylogram depicted in Figure 8.1 was created from Illumina sequenced strains of *S.* Enteritidis isolates cultured from the blood of adults and children with iNTS in Blantyre from 1998-2006, which were mapped to the reference (P125109 – see below) and constructed using RAxML. It reveals that most isolates cluster in two clades of PFGE-types SEN 1 and SEN 2c. Those from SEN 1 cluster with P125109 and were all drug susceptible, whilst those in SEN2c were MDR. This unpublished pilot study provides the rationale for a more extensive investigation into the genomic differences between pre-epidemic and epidemic strains of *S.* Enteritidis in Malawi and across Africa.

It is critical to know if there are essentially two ecotypes of *S.* Enteritidis that circulate both in the developed and developing world with manifestly different aetiologies and

likely routes of spread. It is of important from a public health perspective to understand whether a single clade of *S*. Enteritidis causes both iNTS in Africa and enterocolitis in industrial settings as consumption of eggs acquired from industrial-scale egg production is becoming more common in SSA. If not, the epidemic of *S*. Enteritidis which emerged in industrial settings in the latter part of the twentieth century may yet spread to poorly regulated farming markets in emerging economies. It is equally important to understand if there is an "African" clade of *S*. Enteritidis, which has a distinct genome, is differentially host-adapted and is more intrinsically invasive as has been observed with *S*. Typhimurium ST313 (Kingsley et al. 2013). A detailed description of another NTS serotype which has evolved to cause invasive disease in the ecological niche created by immune suppressed patients would lend weight to the argument that the current traditional distinction between "typhoidal" and "nontyphoidal" Salmonellae (Chapter 1) is fundamentally flawed and over-simplistic in relation to human disease.

Aims

1. Investigate and describe in fine detail the genomic differences between African and "classical" *S*. Enteritidis (Chapter 8A)
2. Investigate whether genotypic changes are having an effect on phenotype using high-throughput microarray phenotyping of strains (Chapter 8B)

8.3 Methods

8.3.1 Strain Selection

Salmonella Enteritidis P125109 (referred to as P125109 from now on) was used as a reference for both comparative genomic work and for the construction of the phylogeny. This strain was isolated from a poultry farm during a human epidemic of enterocolitis in the UK in 1988. The strain was of phage type 4 and contributed by Professor Paul Barrow. It was assembled, annotated and finished at WTSI (Thomson et al. 2008), at which time variable and conserved regions were predicted. The annotated sequence is publically available (EMBL accession no. AM933172).

The 41 Malawian isolates from the pilot study were assembled by Velvet (Zerbino et al. 2008) and assembly quality was assessed manually using the n50 statistic, or median contig length. Two high-quality whole genome sequence assemblies of *S*. Enteritidis isolates were selected following the pilot study for in-depth analysis, one from each major cluster; SEN 1(susceptible) and 2c (MDR) of the phylogram depicted in Figure 8.1. During this study, additional strains were being sequenced with far greater depth of coverage (x70 -100 on recent sequencing runs compared to x20 previously) and far longer reads (100-120bp/read vs 50bp previously). This provided a large collection of sequenced isolates with far more robust sequence data.

8.3.2 Comparative Genomics: assembly, alignment and annotation

The assemblies were aligned against P125109 using ABACAS (Assefa et al. 2009). Annotations were transferred to the output using “annotations_update.pl”. The resulting embl file was blasted against the reference using “bigger_blast.pl” and manually curated against the reference using the Artemis Comparison Tool (ACT), see chapter 2 for full description of methods. Sections of contigs which were incorporated into the alignment, but which did not align with P125109 were manually inspected and compared to the public databases using BLASTn (http://blast.ncbi.nlm.nih.gov). When these regions appeared to be novel prophage regions, they were annotated using the phage search tool PHAST and manually curated (Zhou et al. 2011).

8.3.3 Plasmid Biology

Plasmid DNA was extracted from isolates using the Kado & Lui method (chapter 2) and separated by gel-electrophoresis (see chapter 2) to estimate the number and size of plasmids present. DNA not incorporated into the chromosomal ABACAS alignment was then aligned against a reference *S*. Enteritidis plasmid, pSENV (from Nick Thomson, unpublished). Annotations were transferred from pSENV to this file as described in chapter 2. For novel regions genes were predicted using GLIMMER and manual annotations applied based on homology searches against the public databases, using both BLASTn and FASTA (see chapter 2 and 6).

8.4 Results

8.4.1 Strains

Malawian invasive strains A1636 (cluster 1) and D7795 (cluster 2c) were initially selected from the pilot study for detailed analysis. They were selected on the basis of their assembly statistics, which were the best on either of the two main branches of the tree. A1636 was a pre-epidemic strain, isolated from the blood of a Malawian adult in 1998 and was fully susceptible to antimicrobials. D7795 was isolated from the blood of a Malawian child in 2000, and was MDR (resistant to amoxicillin, chloramphenicol, cotrimoxazole and tetracycline).. and Both of these isolates were sequenced in 2009 and the reads generated were short (mean read length 54) and assembly of D7795 generated 59 contigs (n50=152,813) and when aligned against the reference P125109 revealed a high number of frameshift-pseudogenes at contigs breaks attributed to lower sequence quality rather than of biological relevance. D7795 was therefore replaced in the analysis by A7830, an adult MDR strain which had been more recently sequenced on the Illumina 2500, and which was phylogenetically closely related to D7795. The sequencing and assembly statistics for this isolate included a mean read length of 100, with only 42 contigs generated and the median contigs length (n50) was 255,784 bases. The short read length and contig length of A1636 did not complicate its alignment as it was so similar to reference P125109, it was straightforward to manually curate the alignment errors using the BAM file.

8.4.2 General features of the genomes of *S.* Enteritidis strains A1636 & A7830

Unsurprisingly, the draft genome sequences of iNTS Enteritidis strains A1636 and A7830 closely resemble the conserved genome of reference P125109. In comparison to P125109, however A7830 harbours a number of predicted pseudogenes, a different prophage repertoire and a substantially larger plasmid with an MDR region.

8.4.3 Whole genome comparisons of A1636, a fully susceptible blood-stream invasive Malawian isolate with reference *S.* Enteritidis P125109

Isolate A1636 was originally sequenced at WTSI in 2009. Depth of coverage was 20 and the Illumina sequence reads were on average of 50 bp long, which was standard at that time. When assembled, there were 850 contigs of a median length (n50) of 226387 kbp. These were aligned against P125109 and annotations were transferred from P125109. The draft genome of A1636 was then compared to P125109 in ACT (Figure 8.2). To view the sequence coverage independently of the aligned *de novo* assembly, WGS reads were also directly mapped to the P125019 reference genome sequence using SMALT and the resulting BAM (Binary sequence Alignment Mapping format) was simultaneously opened in ACT. In this way the draft genome was simultaneously manually curated using two models and the *de novo* assembly data could be directly compared to the mapping data where there were suspicions of mis-assemblies.

Comparison of the two genomes revealed high co-linearity of the core genome. On manual inspection for genomic insertions, deletions and rearrangements with respect to P125109, there were 13 areas of interest. All 13, however were related to regions

repeated thorough the genome (e.g. tRNA which is present 6 times) and could be discounted. Review of variable regions, as defined previously (Thomson et al. 2008), revealed no differences between A1636 and the reference P125109. All Salmonella Pathogenicity Islands (SPIs), fimbrial operons and prophage regions were present and fully represented in A1636. Similarly, the virulence plasmid was present and grossly intact.

Evaluation of the annotated genome of isolate A1636 on a gene by gene basis for smaller insertions and deletions (indels) and frameshift mutations that were predicted to ablate function identified 109 potential pseudogenes. 94 of these were discounted on the basis that they were frameshift mutations around repeat regions or contigs boundaries and not supported by the independently mapped reads in the BAM file which mapped across these regions, confirming they were assembly errors and not real mutations. This left five possible, eight likely and two definite high-quality pseudogenes (Table 8.1).

Amongst these genes, it is most interesting to that one of the propanediol utilisation enzymes, *pduD*, has been affected by a NS-SNP creating a stop codon. The gene may have retained its most important function as a subsequent start codon permits transcription of the its N-terminal subunit, which is the part necessary for packaging coenzyme B_{12}-dependent diol dehydratase (*Pdu*CDE) into the lumen of the Pdu microcompartment (Fan et al. 2011).

Table 8-1: Potential pseudogenes in A1636

Locus Tag	Name (if named)	Class	Mutation	Classification
SEN0194	*hrpB*	DNA-dependant helicase	Deletion	Likely pseudogene
SEN0383	*proY*	Proline specific permease	Deletion	Likely pseudogene
SEN0607	*rlpA*	Rare lipoprotein A precursor	Deletion	Likely pseudogene
SEN1363		Invasin like protein	SNP creates stop codon	Pseudogene
SEN1411	*hrpA*	Helicase	Insertion in repeat region	Uncertain
SEN1665		Putative hydrolase	Deletion at frameshift	Likely pseudogene
SEN1763		Hypothetical protein	Deletion at frameshift	Likely pseudogene
SEN1714	*yeaD*	Hypothetical protein	Deletion at frameshift	Likely pseudogene
SEN2039	*pduD*	Diol-dehydratase	SNP creates stop codon	Pseudogene
SEN2101	*manC*	Mannose-1-phosphate guanylytransferase	Insertion in repeat region	Uncertain
SEN3635	*ccmA*	Haem-exporter protein	Significant frameshift	Likely pseudogene
SEN2562	*lepB*	Signal peptidase	Insertion in repeat region	Uncertain
SEN3344	*yfiC*	Hypothetical protein	Deletion at frameshift	Uncertain
SEN2570	*rtcA*	RNA cyclase	Deletion at frameshift	Likely pseudogene
SEN3355		Putative dehydratase	Deletion at frameshift	Uncertain

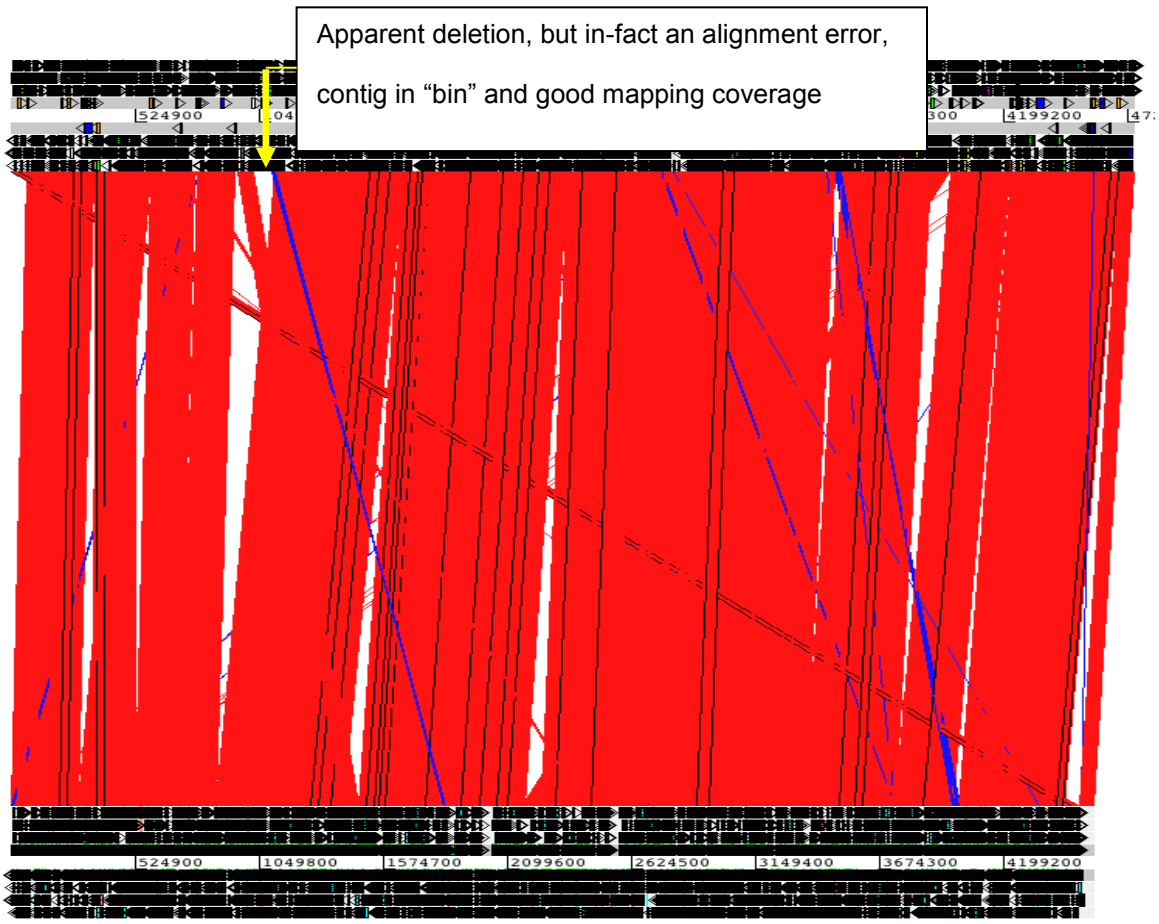


Figure 8-2: ACT comparison of matches between the whole genome sequences of P125109 (top) and A1636 (bottom). Forward and reverse strands of DNA are shown for each genome (dark grey lines). The red bars between the DNA lines represent matches, with inverted matches coloured blue. There is extensive synteny between the two genomes.

In conclusion, A1636 has a highly similar genome to that of the reference European isolate. This finding was predicted for the core genome by the pilot study (Figure 8.1), but the variable regions, including SPIs, prophage regions and plasmid had been excluded. The detailed analysis confirms that the accessory genome was also highly similar to the reference strain.

8.4.4 Whole genome comparisons of A7830, an MDR blood-stream invasive Malawian isolate with reference *S.* Enteritidis P125109

8.4.4.1 Overview

Alignment of the assembly of A7830, from the novel clade against P125109 suggests colinearity of the core genome of A7830 with P125109 and Figure 8.3 reveals the extensive synteny between the two chromosomes. Unlike A1636, its variable genome differs considerably from reference P125109, and additionally has an accumulation of definite pseudogenes and genes with nonsynonymous (NS) SNPs throughout the genome (Table 8.2). In total A7830 shares 95.2% of its coding sequences with P125109 and P125109 shares 97.0% of its CDS with A7830. There are a further 363 genes in A7830 which have NS-SNPs with respect to P125109.

Table 8-3: Summary of differences between P12509 and A7830

Differences with respect to P125109	Variable regions with differences	Genes gained in A7830	Genes lost in A7830
Additional prophage regions	3	150	0
"Missing" prophage regions	2	0	79
Other insertions	3	25	
Other deletions	3	0	3
Likely pseudogenes	43		45
Plasmid		56	4
Total		+221	-131

8.4.4.2 Evidence of ancestry from recent evolutionary events in P125109 and A7830

Evidence of recent gene acquisition in P125109 has previously been highlighted (Thomson et al. 2008); of the 130 CDSs specific to P125109, compared to *S.* Gallinarum strain 287/91, those associated with the novel genomic region denoted region of difference (ROD) 4 and prophages ΦSE10, ΦSE14 and ΦSE20 appear to be recent acquisitions with no evidence of them ever being present in *S.* Gallinarum strain 287/91. The same is partially true of A7830, which contains no remnant or genomic 'scar' to suggest the two neighbouring variable regions from P125109, ROD21 or ΦSE20 were ever present. It does, however contain the degraded remnant of a prophage Gifsy 2-like region, ΦSE12, although this region is even more degraded than in P125109. In contrast, P125109 contains the scar or remnant of a different Gifsy 2-like prophage

region, which appears complete in A7830. These signature events are consistent with A7830 and A1636/P125109 having a common ancestor but them not being directly evolved from one another.

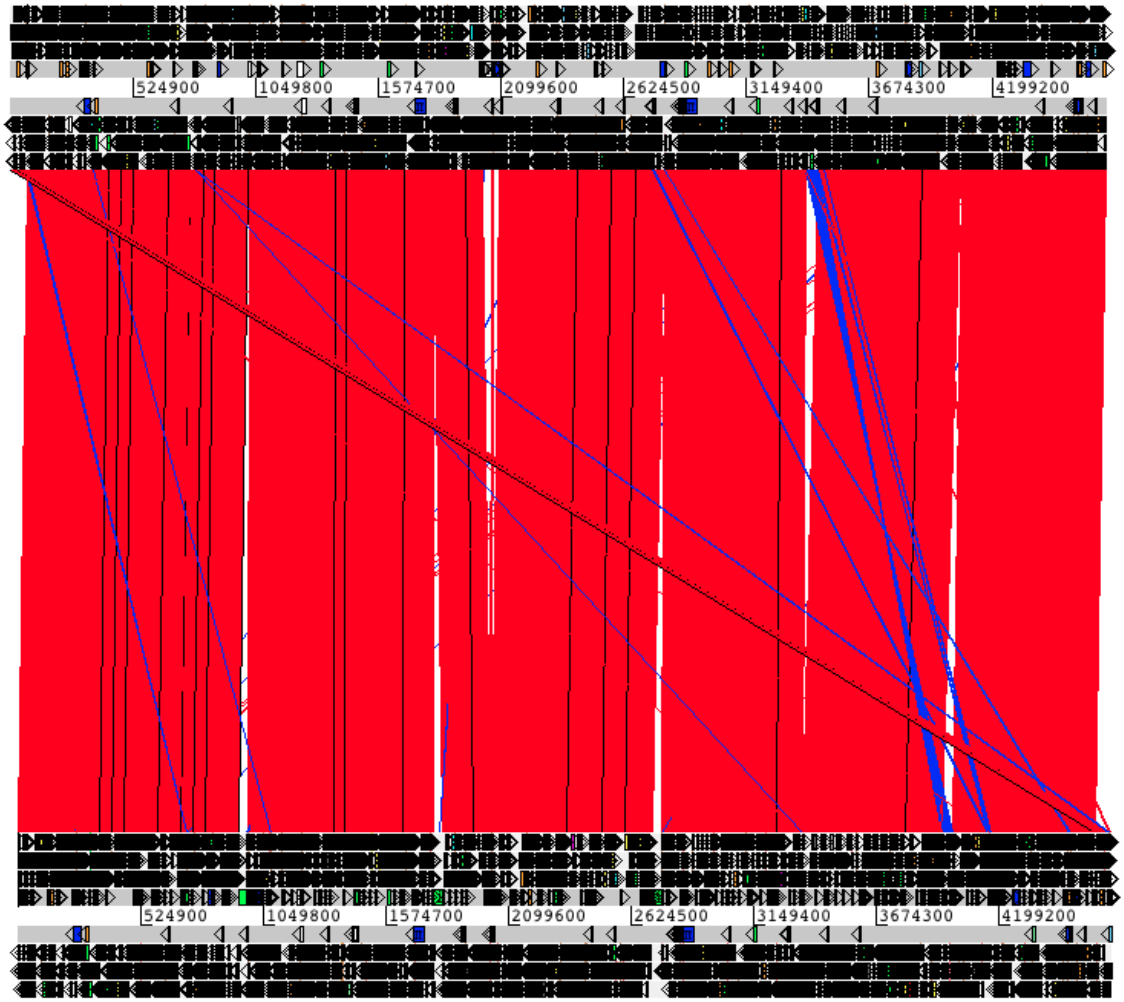


Figure 8-3 ACT comparison of P125109 (top) and A7830 (bottom) confirming colinearity of genomes. Forward and reverse strands of DNA are shown for each genome (dark grey lines). The red bars between the DNA lines represent matches, with inverted matches coloured blue. There is extensive synteny between the two genomes.

8.4.4.3 Gene-sets in present in P125109, but not A7830

The core genome of P125109 is largely conserved in A7830. All 12 SPIs and all 13 fimbrial operons associated with P125109 are present. The genes within the SPIs are also intact, however fimbrial protein Y in the *fim* operon is a pseudogene, and 7 other fimbrial operon genes contain non-synonymous SNPs. The fimbrial operons are otherwise intact with respect to P125109 and A7830 has maintained the multiple functional fimbrial operons common to host promiscuous Salmonellae (Townsend et al. 2001). All of these differences are put in detailed context in Appendix 5, Table A5 & A6.

The principle abnormality between the two chromosomes is in the prophage regions and genomic islands (RODs). P125019 contains prophage ΦSE20 and near to it, genomic island ROD 21. Prophage region ΦSE20 encodes 51 CDS in ~ 41 kbp. Potential cargo genes including the type three secretion system (TTSS) secreted effector protein *sseK3* and fragments of other TTSS effector genes including *sopE*, *sspH2* and *gogA* (Thomson et al. 2008). It has recently been demonstrated in both a mouse model (Silva et al. 2012) and cell-line model (Shah et al. 2012) that genes associated with region ΦSE20 are likely to be necessary for invasion in mice and chickens. The function of ROD21 is less clear. It is the largest genomic island in P125109 (~ 26 kb) and its structure has previously been described in detail (Thomson et al. 2008). Of note, it has a low G+C content and most of this island encodes paralogues of H-NS (*hnsB* – a down regulator of transcription) and/or an H-NS antagonist, *hnsT* (Williamson et al. 2005, Navarre et al. 2006, Doyle et al. 2007).

In addition to these large differences, some of the prophage regions and RODs have under gone further degradation involving specific important genes. ΦSE12 and ΦSE12A are prophage remnants lying adjacent to each other and carry remnants of the PhoPQ-activated genes *pagK* and *pagM*, an intact copy of *sopE* known to stimulate cytoskeletal re-organisation. This region has undergone further degradation in A7830, which does not carry gene *sodCI*, encoding a Cu/Zn superoxide dismutase known to be an important colonisation factor for *S.* Typhimurium (Figueroa-Bossi et al. 2001). Genomic island ROD 30 has lost the nickel/cobalt transporter *rcnA*. Both of these regions are likely to be ancient acquisitions as they are present in *S*. Typhimurium LT2.

8.4.4.4 Gene-sets present in A7830, but not P125109

A7830 contains three complete prophage regions that are absent from P12509, containing a total of 150 predicted CDS regions and including one Gifsy 2-like region, highly degraded in P125109, but retaining a further 10 genes in A7830. The three complete regions were annotated using Phast (see Table 8.3 for summary) and are closely related to phages Entero P2 (33.1 kb, 47 predicted CDS, including 3 cargo genes), *Yersinia* 413 (38.1 kb, 52 CDS, including 13 cargo genes) and a Gifsy 2-like prophage (33.1 kb, 52 CDS, including 5 cargo genes). The regions largely contain hypothetical genes or essential phage machinery, however the *Yersinia* 413 prophage carries a gene predicted to encode a leucine rich repeat (LRP) protein. These LRP-domains are protein interaction regions and have been implicated in suppression of gut-inflammation through inhibition of NF-κB-dependent gene expression by both *S*. Typhimurium and Shigellae (Haraga et al. 2003).

Table 8-3: Summary of contents of prophages of A7830

	Prophage 1	Prophage2	Prophage3
Length	33.1	38.1	30.1
Closest relative	Entero 2 (83%)	Yersinia 413C	Gifsy1
GC	53%	52	51%
Related Salmonellae Prophage	RE_2010	RE_2010	Gifsy1
Total CDS	47	51	52
Phage proteins	44	34	46
Hypothetical proteins	3	13	5
Phage +hypothetical protein %	100	92	98

8.4.4.5 Pseudogene formation

Having investigated the chromosome for large inversions, deletions and rearrangements, the next step was to go through the chromosome on a gene by gene basis in-order to discover if individual genes had been degraded. This is important as a single NS-SNP can generate a stop codon, calling an early halt to mRNA synthesis and leading to loss of protein function. Loss of a single critical protein may in turn disable an entire metabolic pathway.

Inspection of the draft assembly of A7830 reveals 43 putative pseudogenes (see Table 8.4 [summary] and Appendix 5, Table A7 [full list]). Whilst this only represents 0.1% of

the genome, it is striking feature is how many are involved in core metabolic pathways, specifically in genes critical for an enteric lifestyle, and how many genes from the same pathway contain NS-SNPs (Full list Appendix 5, Table A8).

Table 8-4: Function categories of pseudogenes in A7830

Functional category	Number of pseudogenes
Core metabolism	17
Virulence, host adaptation	3
Membrane	7
Transporter	7
DNA reproduction/repair	3
Phage protein	1
Hypothetical genes	5

8.4.4.6 Functional gene loss in A7830 associated with metabolism

The greatest concentration of pseudogenes in A7830 is within genes associated with metabolism. Some of them have catabolic roles in colonic mucus degradation, including SEN1434 (hexonate metabolism) and the sulphatase SEN 0035. Colonic mucus contains sugar acids, a source of nutrients which gut dwelling organisms must be able to utilize (Sweeney et al. 1996).

Several of the apparent pseudogenes are involved in anaerobic metabolism, including SEN 3902, a glycerol dehydrogenase. Glycerol metabolism is necessary for anaerobic

growth of enterobacteria on non-fermentable sources. Dimethyl sulphoxide reductase, a gene necessary for the use of terminal electron acceptors in anaerobic metabolism, has also been lost, together with two other sulphur metabolism genes. Two genes involved in formate metabolism have become degenerate. There are a further four metabolic pseudogenes, including mono-pentose pathway (L-fuculose kinase [*fucK*]*)*.

8.4.4.7 Cobalamine biosynthesis and 1, 2-propanediol utilization

Gene *pocR* is a pseudogene in A7830. This is one of the most important regulatory genes for the B12 synthesis and propanediol utilisation pathway (Bobik et al. 1992), a key enteric-adaptation pathway. Many *Enterobacteriaceae* have lost the capacity to synthesise cobalamine and therefore to degrade 1, 2-propanediol. However, Salmonella re-acquired a 40 kb metabolic island encoding both the *cbi* and *pdu* loci (Lawrence et al. 1996, Porwollik et al. 2002, Prentice et al. 2003). 1, 2-propanediol is an important source of energy for *S.* Typhimurium, especially within the intracellular compartment (Klumpp et al. 2007). Of note, the B12 transport gene *yncD* is also a pseudogene and a number of downstream genes in this pathway have acquired NS-SNPs including two tetrathionate and three propanediol utilisation genes, which suggests the possibility of degradation of this pathway. Degradation in this pathway is also seen in invasive, host-adapted serotype *S*. Gallinarum (Thomson et al. 2008).

8.4.4.8 Pseudogenes potentially involved in virulence and host adaptation

Like *S.* Typhi and *S.* Gallinarum 287/91, *S*. Enteritidis A7830 carries a number of mutations in a gene which is centrally involved with shedding - *shdA*. This gene is a surface-localized, fibronectin-binding protein whose expression is induced in vivo in the murine caecum (Kingsley et al. 2002, Kingsley et al. 2003) and is carried on a 25-kb genetic island named centisome 54 (CS54 island) in *S.* Typhimurium. Absence of this gene is associated with reduced faecal carriage and shedding of *S*. Typhimurium in mice, but not pigs (Kingsley et al. 2002) (Boyen et al. 2006). This gene is also a pseudogene in *S.* Typhi and *S.* Gallinarum and whilst it is intact in the African invasive *S*. Typhimurium ST313, the related gene *ratB* from CS54 has become a pseudogene (Thomson et al. 2008, Kingsley et al. 2009). As discussed above, there is one fimbrial gene (*fimY*) which is a pseudogene and one type III secretion system protein.

8.4.4.9 Other Pseudogenes

In addition to the metabolic signal suggestive of host adaptation, there are seven pseudogenes amongst membrane transport genes, including the two further genes involved in cobalamine metabolism. Cobalamine synthase incorporates Ni-Fe and Cobalt respectively, and it is interesting to note that A7830 has lost *rncA*, a high affinity Ni/Co transporter, through a deletion in ROD30 (see above)(Rodrigue et al. 2005) and the gene encoding the cobalamine transporter *yncB*. Five other putative, uncharacterised transport genes are pseudogenes. A further seven membrane-protein associated genes have significant mutations.

Three DNA replication and repair genes carry mutations, including a *mutT*-family gene. Only one pseudogene lies in an amino acid catabolic or biosynthetic pathway, L-serine (*L*-serine and *L*-threonine catabolism), for which there is redundancy (Sawers 1998). A further five hypothetical genes and one phage-protein gene have become pseudogenes. Table A6 in Appendix 5 provides a list of some of the common traits identified amongst the functions of genes lost independently by A7830, *S.* Typhi and *S.* Gallinarum. Some of the overlapping traits are striking; including the loss of genes involved in common metabolic processes such as cobalamine and propanediol utilisation and electron transport acceptor function. Figure 8.4 illustrates the positions of these mutations on a map of enzymatic pathways present in P125109.

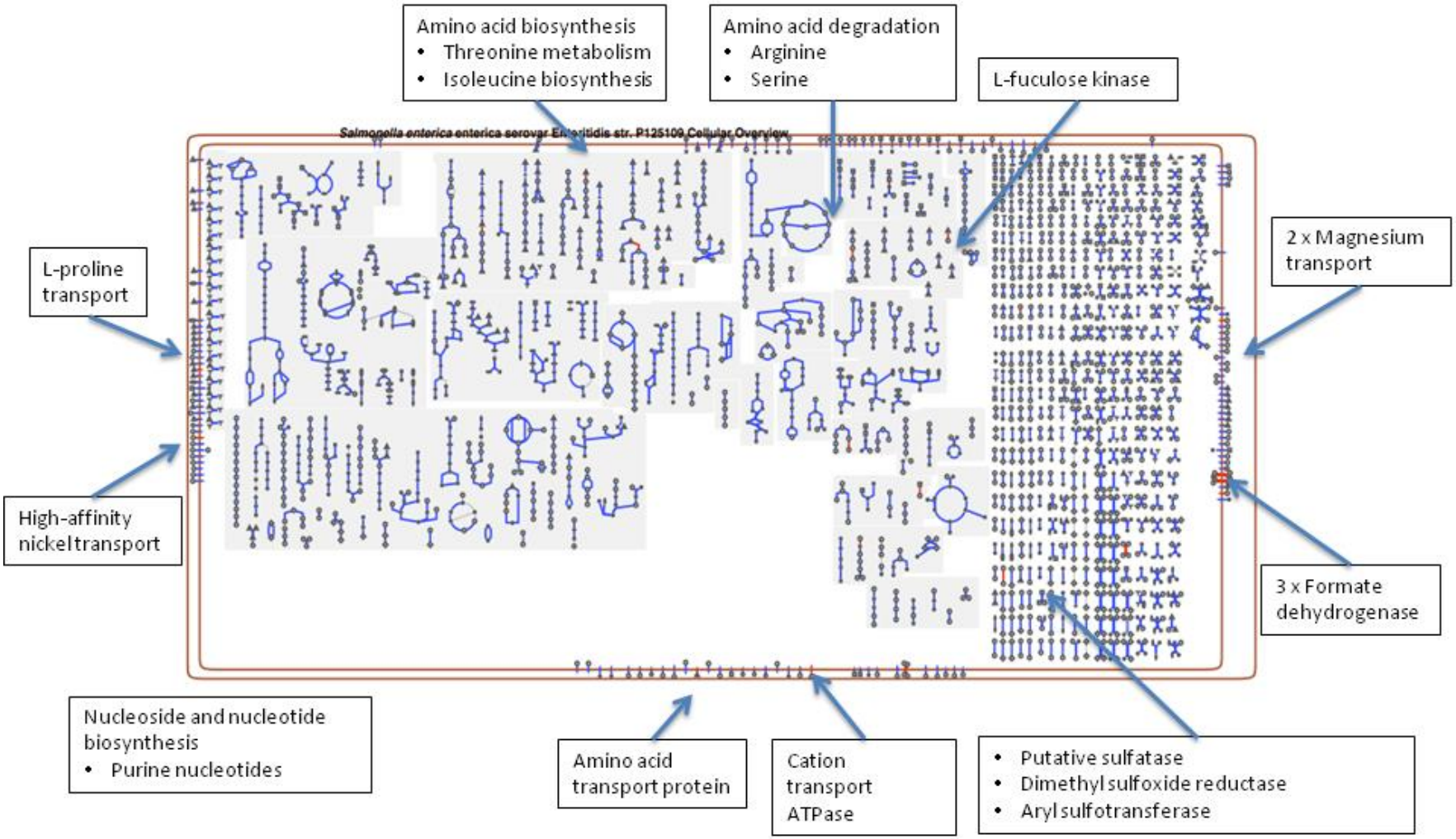


Figure 8-4: Graphical representation of biochemical pathways in P12509. The figure represents all the metabolic pathways present in *S.* Enteritidis. In this figure red indicates pathways which contain enzymes which have become pseudogenes in A7830.

8.4.4.10 NS-SNPs in coding regions

In addition to the large differences in terms of the variable region of the genome, there is further evidence of reductive evolution in A7830 in the form of 363 genes containing NS-SNPS (Table 8.5). The disproportionate clustering around membrane structures is yet another signal seen in host adaptation with both *S.* Typhi (Parkhill et al. 2001) and *S.* Gallinarum (Thomson et al. 2008).

Table 8-5 Functional breakdown of genes containing NS-SNPs

Functional class	Number of genes
Replication and repair	16
Metabolism	41
Membrane/surface structures	122
Degradation of macromolecules	12
Degradation of small molecules	18
Regulators	67
Conserved hypothetical	83
Phage	4
Total	363

Many of these genes will be functional, but it is likely that some have become pseudogenes and that others have altered functions. Certainly there is evidence of clustering around pathways (Figure 8.5), for example the cobalamine biosynthetic pathway.

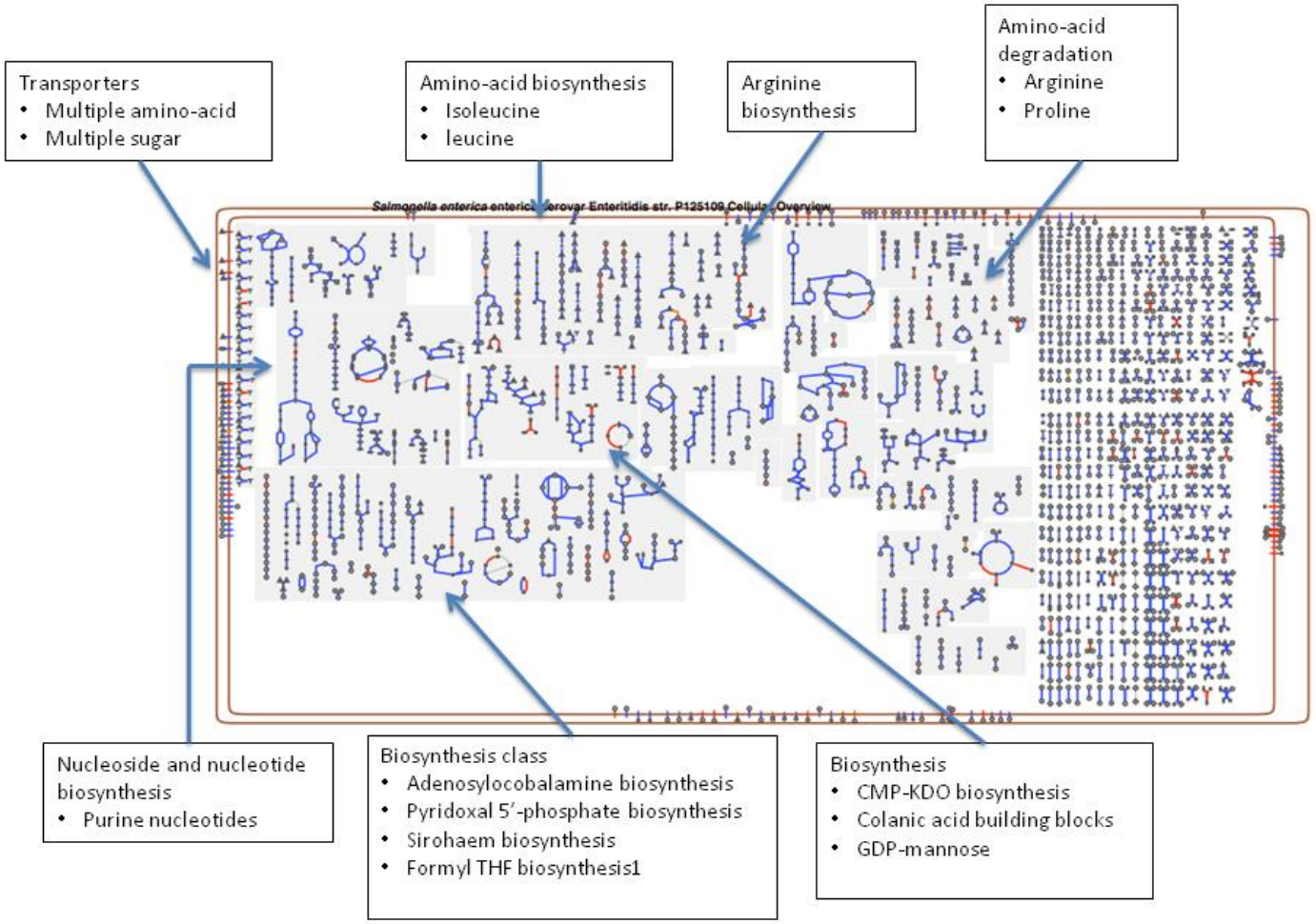


Figure 8-5: Graphical representation of biochemical pathways in P12509. Red indicates pathways which contain enzymes which contain NS-SNPs A7830. Pathways in which greater than 2 steps display evidence of degradation have been labeled

8.4.5 The MDR virulence plasmid of the novel *S.* Enteritidis clade

The 15 contigs which failed to align with P125109 chromosome were aligned against pSENV, a *S.* Enteritidis virulence plasmid from P125109 which has been sequenced, annotated and finished (unpublished NR Thomson). Four contigs aligned against pSENV with evidence of two deletions; the first contained genes *srgA, srgB and luxR*, part of the *lux* operon and the second was within gene pSENV0033, a putative integrase. These represented potential insertion sites for resistance cassettes. All the other genes on pSENV including the virulence operon, were intact (Figure 8.6).

There were 11 remaining contigs. BLASTn revealed that six, which contained all the predicted antimicrobial resistance genes, aligned against two sequenced fragments of an *S.* Enteritidis virulence plasmid (pUO-SeVR). This plasmid had been isolated from an African patient presenting with MDR iNTS Enteritidis in Spain (Rodriguez et al. 2012). A further five contigs contained segments of DNA which aligned with previously sequenced plasmids and one, 10Kbp contig contained predicted phage genes and has been described above.

In total, the 14 contigs contained 92 kbp of DNA likely to represent extra chromosomal DNA. In order to confirm that this represented just one, and not multiple plasmids, plasmid extraction by Kado and Lui alkaline lysis, followed by gel-electrophoresis was performed on MDR isolate D7795 as A7830 was not available in the UK (Figure 8.7).This revealed the presence of just one large plasmid.

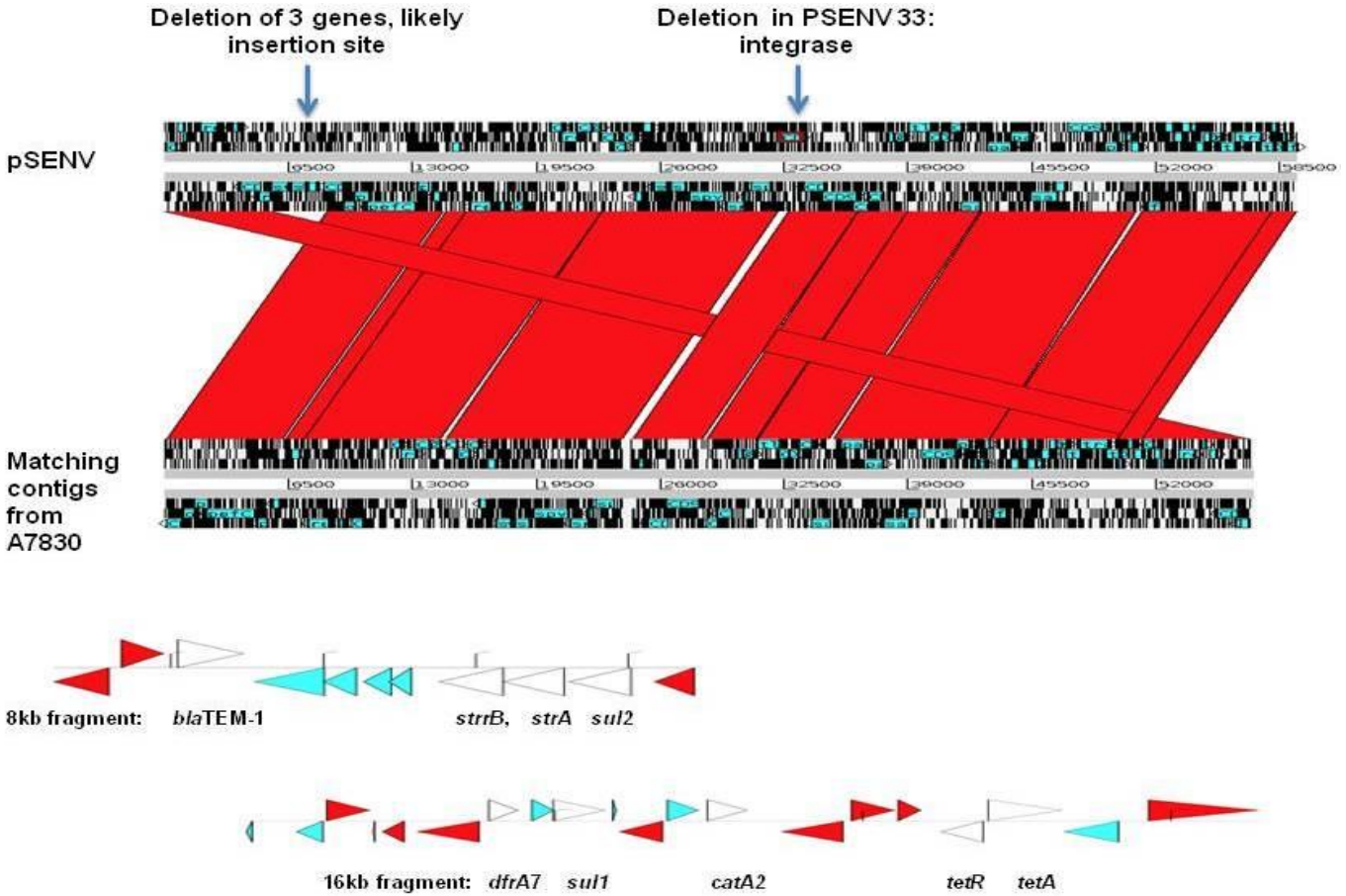


Figure 8-6: ACT comparison of contigs from A7830 matching pSENV reveals 2 deletions, which are likely to be MGE insertion points. The two fragments represent the alignment of the remaining contigs against sequenced fragments from pUO-Srv, a plasmid isolated from an MDR *S.* Enteritidis cultured from the blood of an African patient in Spain (Rodriguez et al. 2012)

A draft assembly including 14 contigs was assembled in 3 major (Figure 8.6) and 4 minor (all <5 kb) fragments. In total, nine antimicrobial resistance genes were identified, encoding resistance to amoxicillin (*bla*TEM-1), tetracycline (*tetR* and *tetA*), chloramphenicol (*cat*A2) the components of cotrimoxazole (*dfr*A7, *sul*1 and *sul*2) and aminoglycosides (*str* A and *str*B).

The plasmid contained a number of additional genes which might be associated with virulence and a toxin/antitoxin plasmid addiction system (see Appendix 5, Table A9 for full list of differences). These included *tir*, associated with gut wall attachment in enteropathogenic *E. Coli*, and associated with virulence in O157:H7 strain (Bono et al. 2007); a *pecM* gene which is a membrane spanning efflux protein which may have drug efflux properties (Rouanet et al. 2001); and *pncA* which is a nicotinamidase whose function unclear, however it is essential to the pathogenesis of *Borrelia burgdorferi* (Jewett et al. 2011). Lastly genes *mucA* and *mucB* are present. The latter has been shown to be a lesion bypass polymerase and although their precise role is unclear, together they may repair damaged plasmid DNA, or facilitate mutagenesis. This would be beneficial for an MGE transmitted through a number of bacterial hosts, by allowing faster adaptation to foreign intracellular environments (Goldsmith et al. 2000). A full list of additional genes in the MDR plasmid is provided in the appendices.

Like pSENV, the virulence plasmid contained an incomplete set of conjugal transfer genes (*tra* genes) and it was therefore predicted that this plasmid would not be capable of conjugal transfer. This was tested by conjugation experiments (see chapter 2) at 26^{o}C and 37^{o}C using the plasmid from A54560 (see chapter 6) and IncHI1 plasmid PHCM1 as positive controls and A1636 as a negative control. The plasmids of A1636 and D7795 failed to transfer, whilst the positive control successfully transferred.

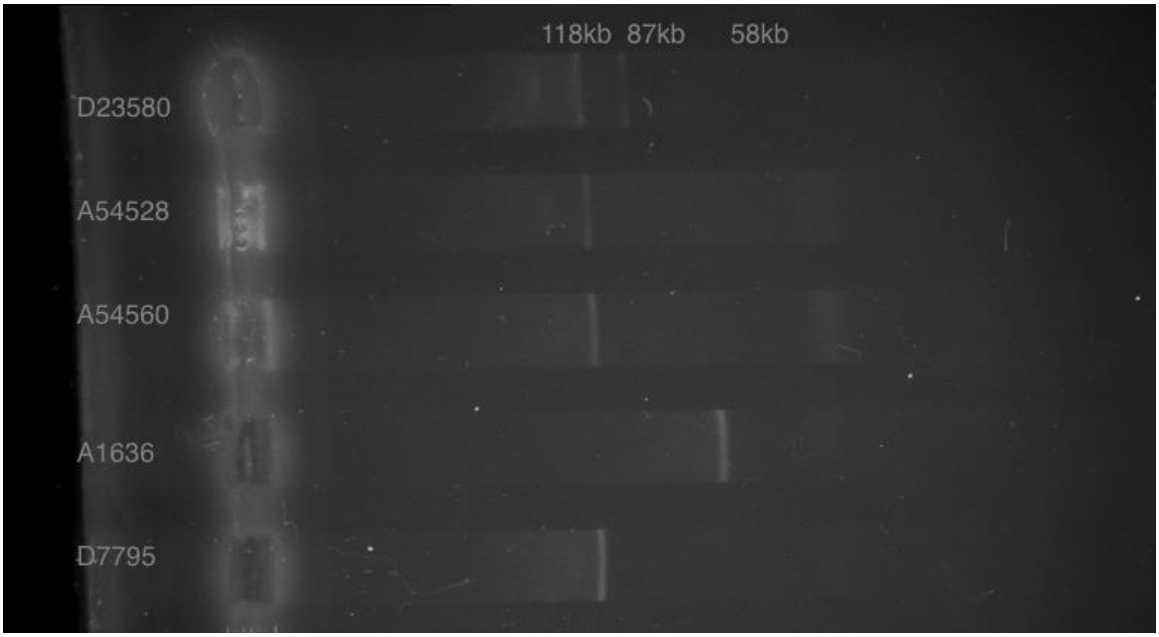


Figure 8-7: Plasmid Profile of MDR plasmid from isolate D7795, compared to plasmids pLST-BT (118kb, present in D23580, A54528, A54560) and pSENV (58kb, present in A1636) suggests the presence of 1 plasmid in D7795, which may be double the size of pSENV.

8.5 Conclusions

Comparative genomic analysis of two representative isolates of the 2 clades of *S*. Enteritidis proposed by the pilot study reveals them to be highly related to reference strain P12509. A1636 is extremely similar in all respects to P125109, however A7830 has a notably different prophage repertoire and evidence of genomic degradation. These include many signatures of host adaptation which has been seen before with multiple *Salmonella enterica* subsp*. enterica* serotypes, including *S*. Typhi (Parkhill et al. 2001), *S*. Gallinarum (Thomson et al. 2008) and *S*. Typhimurium ST313 (Kingsley et al. 2009).

S. Enteritidis is generally considered to be host promiscuous, being able to colonise and infect multiple hosts including chickens, cattle, mice and humans, in addition to producing

murine typhoid. A7830, however has lost virulence determinants associated with an ability to invade mice and chickens (ΦSE20) and has numerous pseudogenes in place of functional genes required for an enteric lifestyle. Some of these suggest convergent evolution with host-restricted serotype *S*. Gallinarum, which causes a fowl-typhoid in galliforme birds, but is relatively non-infectious in other hosts, including mice and does not colonise the gut of animals. The similarities between A7830 and *S.* Gallinarum are more striking than between A7830 and ST313. This is perhaps surprising as ST313 is a human bloodstream invasive pathogen, however *S.* Gallinarum is part of the same lineage of Salmonella as A7830.

In addition to the chromosomal changes, A7830 has a much larger plasmid than the reference. The evolution of the *S*. Enteritidis virulence plasmid is intriguing; it is the smallest of the *Salmonella* virulence plasmids, half the size of that of *S*. Typhimurium and much smaller than that of the closely related Gallinarum. In A7830 it has nearly doubled in size, but like pSENV lacks the *tra* genes necessary for conjugal transfer, which either indicates that MDR status has evolved through acquisition of MGEs multiple times or clonal expansion and vertical transmission of the plasmid to progeny. The former scenario seems unlikely as *S*. Enteritidis is not a commonly resistant pathogen in industrial settings.

8.6 Limitations

The findings of this study were based on improved draft assemblies. Illumina sequence reads were assembled then aligned against a reference and the chromosomal architecture was inferred from this. Finishing PCRs overlapping the contigs boundaries to confirm the hypothetical arrangement of the contigs were not performed.

Chapter 8B: High throughput phenotyping of a D7795, an "African" *Salmonella* Enteritidis

8.7 Introduction: Biolog™ microarray phenotyping

Phenotype microarrays enable the simultaneous quantitative measurement of a number of cellular phenotypes, and therefore the creation of a phenotypic profile under a variety of growth conditions. The Biolog™ platform provides a wide variety of tests as a set of 20 plate arrays of 96 wells (Tracy et al. 2002). It is based on redox chemistry, employing cell respiration as a universal reporter (http://www.biolog.com). If the phenotype is strongly "positive" in a well, the cells respire actively, reducing a tetrazolium dye and leading to a colour change. If it is weakly positive or negative, respiration is slowed or stopped, and minimal colour change occurs. Incubation and recording of phenotypic data is performed by an OmniLog plate reader which captures a digital image of the array four times/hour and stores the quantitative colour change values. These can be depicted as graphs, or the data analysed statistically, using software developed at WTSI (Dr L. Barquist).

8.8 Methods

8.8.1 Strains used: A1636 a European cluster isolate & D7795 an African cluster isolate

In these experiments, two replicates of two Malawian *S.* Enteritidis isolates A1636 (P125109-like) and D7795 (putative "African" type) were incubated at 28°C and 37°C to represent environmental and human temperatures. Biolog™ plates PM1-4 and 9 (Carbon

source [PM1,PM2], nitrogen source [PM3] and phosphor and sulphur source [PM4] metabolism and osmotic pressure [PM9]) were used. Each well was inoculated as described below, thereby testing 475 conditions at once (each plate has one negative control well).

8.8.2 Biolog protocol

The isolates were cultured overnight on LB-agar at 37^{o}C in air to exclude contamination. The following method is the Biolog protocol followed at WTSI and has been published elsewhere (Croucher et al. 2013). Colonies were scraped off plates and dispensed into IF-0a solution (Biolog) to a cell density corresponding to 81% transmittance. For each plate used, 880 µL of this cell suspension was added to 10 mL IF-10b GP/GP solution (Biolog) and 120 µL dye mix G (Biolog). This was then supplemented with a 1 mL solution of 7.5 mM D-ribose (Sigma), 2 mM magnesium chloride, 1 mM calcium chloride, 2 mM sodium pyrophosphate (Sigma), 25 µM L-arginine (Sigma), 25 µM L-methionine (Sigma), 25 µM hypoxanthine (Sigma), 10 µM lipoamide (Sigma), 5 µM nicotine adenine dinucleotide (Sigma), 0.25 µM riboflavin (Sigma), 0.005% by mass yeast extract (Fluka) and 0.005% by mass Tween 80 (Sigma). 100µl of this mixture was dispensed into each well on the assay plate. Plates were then allowed to equilibrate in air for 5 min prior to being sealed in airtight bags and loaded into the Omnilog machine (Biolog). Plates were scanned every 15 min for 48 hours while incubated at 28°C and 37°C in air. Culture under anaerobic conditions was unavailable. Two paired replicates were performed for each of the two isolates.

8.8.3 Analysis and interpretation of Biolog data

After completion of the run, the signal data were compiled and analysed. The data were first evaluated by examination of redox curves and discrepancies between replicates of the same isolate at the same temperature noted and repeated. Then signal data were exported from

the Biolog software and compiled using Microsoft Excel. The subsequent analysis was conducted in the statistical programming language 'R' by Dr Lars Barquist who has developed software for this purpose (Croucher et al. 2013). The data was transformed into signal values (SVs): the maximum SV for each well was used, but adjusted to control for any baseline variability by determining a "baseline" signal level for each well by calculating the average signal level for the first 2 h of the run. This value was then subtracted from the previously calculated signal value, yielding a final signal value (Homann et al. 2005). Dr Barquist summarised the data firstly as average log-fold difference between the 2 isolates under the two conditions and reported Benjamini-Hochberg corrected *p* values to determine statistical significance of differences controlling for a false discovery rate of 5% and secondly as a heat-map.

Significant differences in the data were subsequently evaluated and a log fold change of 0.5 was used as a cut-off for investigating a specific metabolite further. This was done by reviewing the graph of the tetrazolium reduction to ensure there was no obvious artefact i.e. two flat lines with one slight upward deviation at the end of the incubation period representing spontaneous reduction of the dye. The remaining differences were then visualised using Pathway Tools (Karp et al. 2010) and the metabolic change was related to pseudogenes and NS-SNPs in genes the genomes.

8.9 Results

Isolates A1636 and D7795 were incubated as described for 48 hours and the results exported from the Omnilog scanner to Excel. The findings from the pairs of replicates were compared using principal component analysis (PCA - Figure 8.8) and found to be highly consistent. PCA is a dimension reduction technique, which takes high-dimensional data and finds linear combinations of the variables (the substrates tested in this case) that have the

maximal variation across samples. These are used to summarise the variability of samples in a 2- dimensional space. The two components are the linear combinations of variables that 'explain' most of the variability in the data.

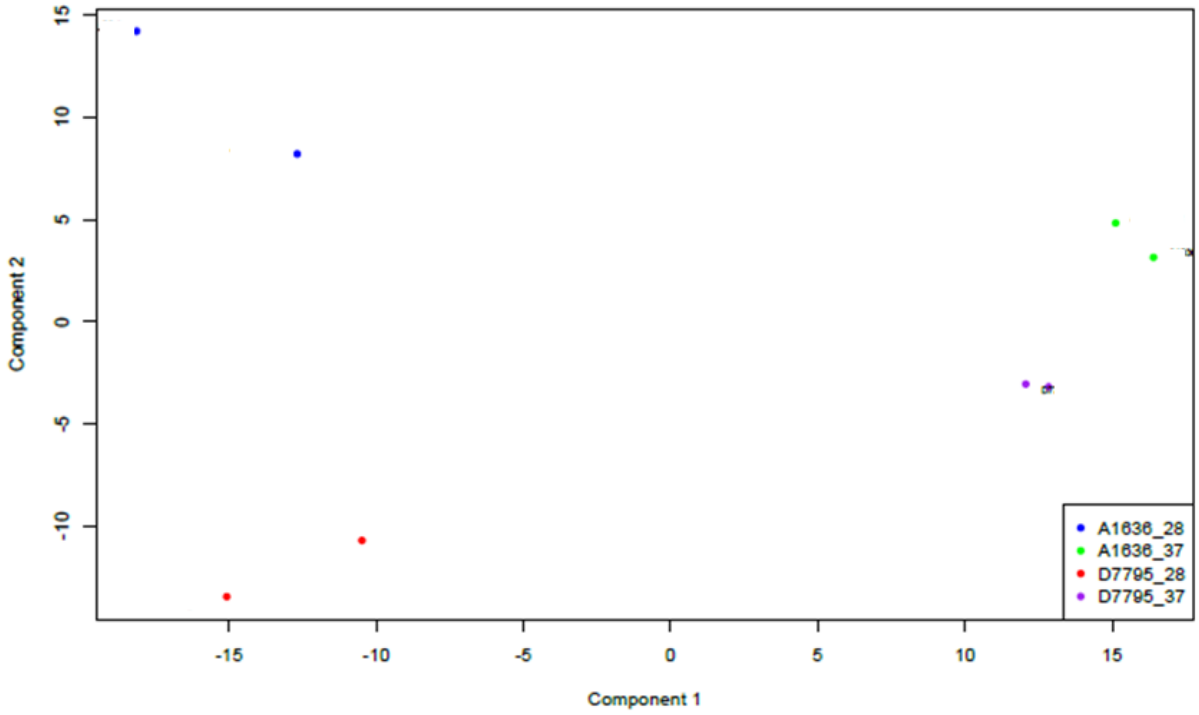


Figure 8-8: Principal component analysis summarising the Biolog output variables of the pairs of replicates

In total, there were 200 statistically significant differences between log-fold change in adjusted SVs between the 2 isolates; 97 at 28^{o}C and 103 at 37^{o}C. Following manual review of the signal from each well, 80 of the results were considered genuinely significant and pursued further (Table 8.6). The entire output is graphically displayed in Figures 8.9 (PM1 and 2 [carbon sources]) and 8.10 (both PM 3 [nitrogen] and 4 [phosphorous and sulphur]).

Table 8-6: Summary of phenotypic differences between A1636 and D7795. "Increased" and "decreased" refer to tetrazolium dye reduction by D7795 when compared to A1636

Condition (temp ^{o}C)		Number of conditions	
		28	**37**
Carbon sources (190)	Activity increased	7	8
	Activity decreased	13	20
Nitrogen sources (95)	Activity increased	0	0
	Activity decreased	13	10
Phosphorous & sulphur (95)	Activity increased	1	2
	Activity decreased	0	0
Osmotic stress (95)	Activity increased	3	2
	Activity decreased	1	0
Total differences		38	42

These pathways were then evaluated further using Pathway Tools software and the findings are graphically represented in Figures 8.11 (at 28^{o}C) and 8.12(at 37^{o}C). Pathway Tools was used to identify genes involved in each specific pathway with evidence of increased or decreased metabolic activity, and these were cross referenced against the list of pseudogenes and degraded genes in the genomic analysis in the previous chapter. In total 14/27 (52%) of pathways with evidence of decreased metabolic activity at 28^{o}C in D7795 displayed evidence of genomic degradation, and 12/30 (40%) of pathways with evidence of decreased metabolic activity at 37^{o}C in D7795 displayed evidence of genomic degradation.

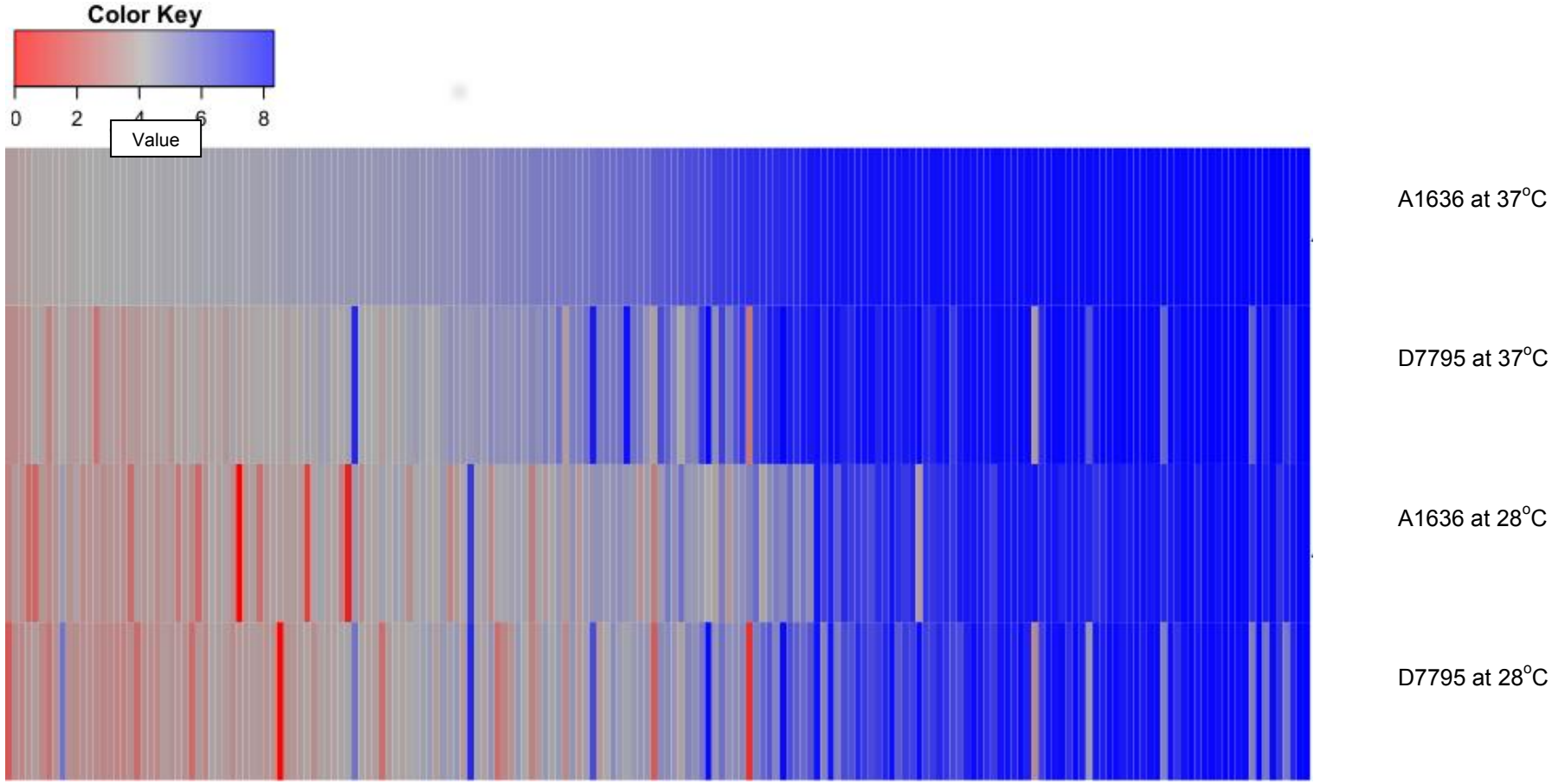


Figure 8-9 Heat map revealing metabolic activity of isolates A1636 and D7795 in response to 190 carbon sources. This is a graphical representation of the level of reduction of tetrazolium blue in the presence of each substrate in each well. Reduction is represented a coloured bar (see key above) and has been averaged across pairs of replicates.

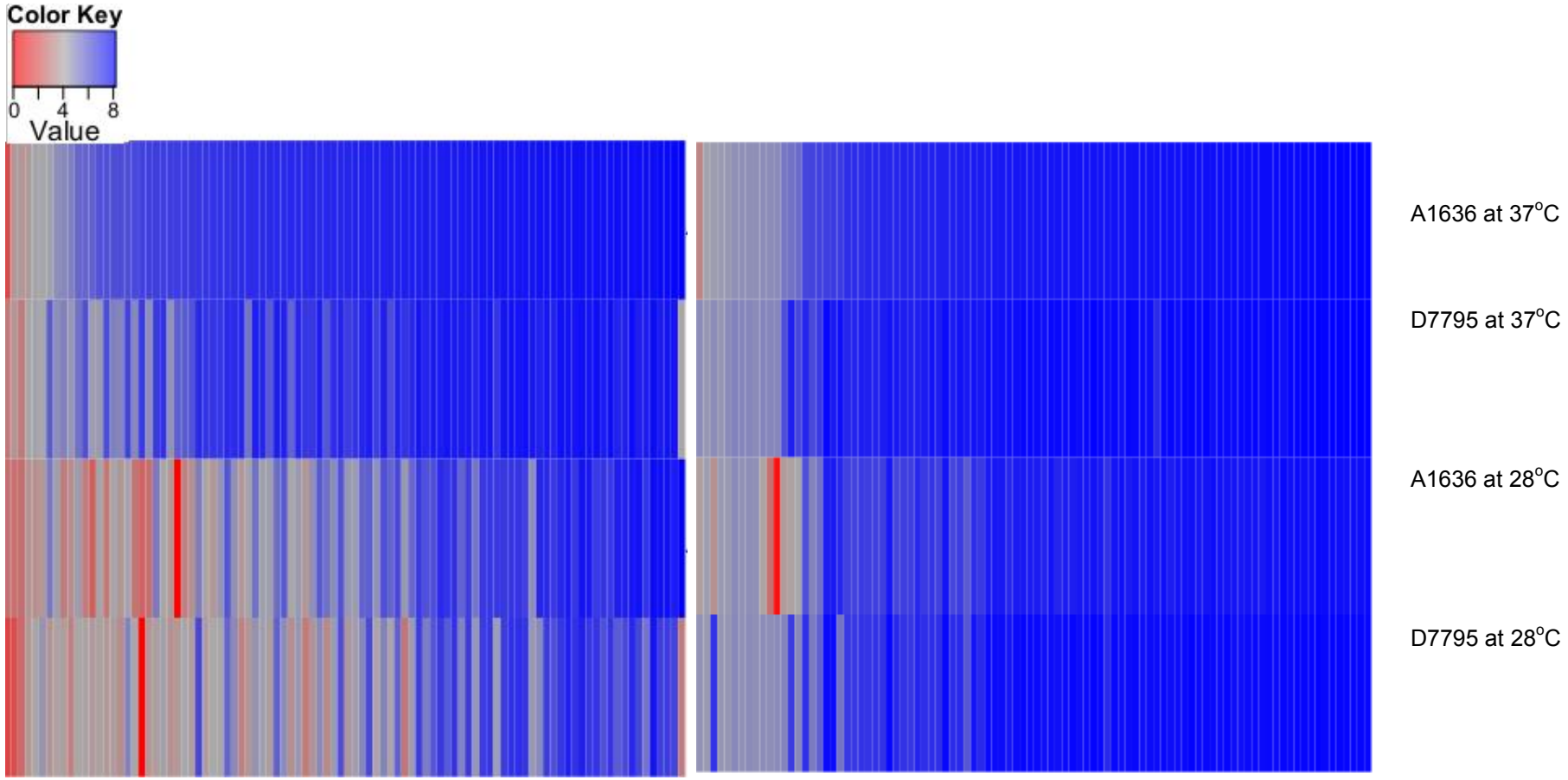


Figure 8-10 Heat map revealing metabolic activity of isolates A1636 and D7795 in response to 95 nitrogen (left) and 95 sulphur & phosphorous sources (right)

8.9.1 Differences in Carbon Utilization

The two replicates of D7795 showed enhanced metabolism of three simple carbohydrates, including glucose, sucrose and lactulose, also of D-saccharic acid, D-alanine, mucic (galactaric) acid and formic acid.

There were far more instances of reduced activity, many of which have a corresponding signature of genomic degradation, such as pseudogene formation or the presence of NS-SNPs in genes corresponding to the pathway. These included dulcitol and glycolic acid in the glycerol degradation pathway, propionic acid in the propanediol pathway and ethylamine and ethynolamine. Also there was reduced activity in response to three forms of butyric acid, a short chain fatty acid. This is another signature of loss of enteric adaptation, as the ability to metabolize short chain fatty acids is an extremely useful trait for enteric adaptation (Keeney et al. 2011). The full list of differences is detailed in Tables A10 & 11 in Appendix 5.

8.9.2 Differences in Nitrogen Utilization

There was no increased metabolism of any nitrogen sources in D7795 compared to A1636, however metabolism was reduced in several nitrogen sources (for full list see Table A10 & A11 in Appendix 5). The most interesting sources to be down regulated were alloxan and allantoic acid. Allantoin can be found in the serum of birds, and is utilised as a carbon source during *S.* Enteritidis infection of chickens (Dhawi et al. 2011) and pseudogenes relating to allantoin have been noted in *S.* Typhimurium ST313 (Kingsley et al. 2009).

8.9.3 Different metabolism under osmotic stress

D7795 showed increased metabolism in the presence of 6% sodium chloride solution at both temperatures, but decreased metabolism in the presence of 4% sodium formate, which is consistent with the numerous mutations in formate catabolizing genes in D7795.

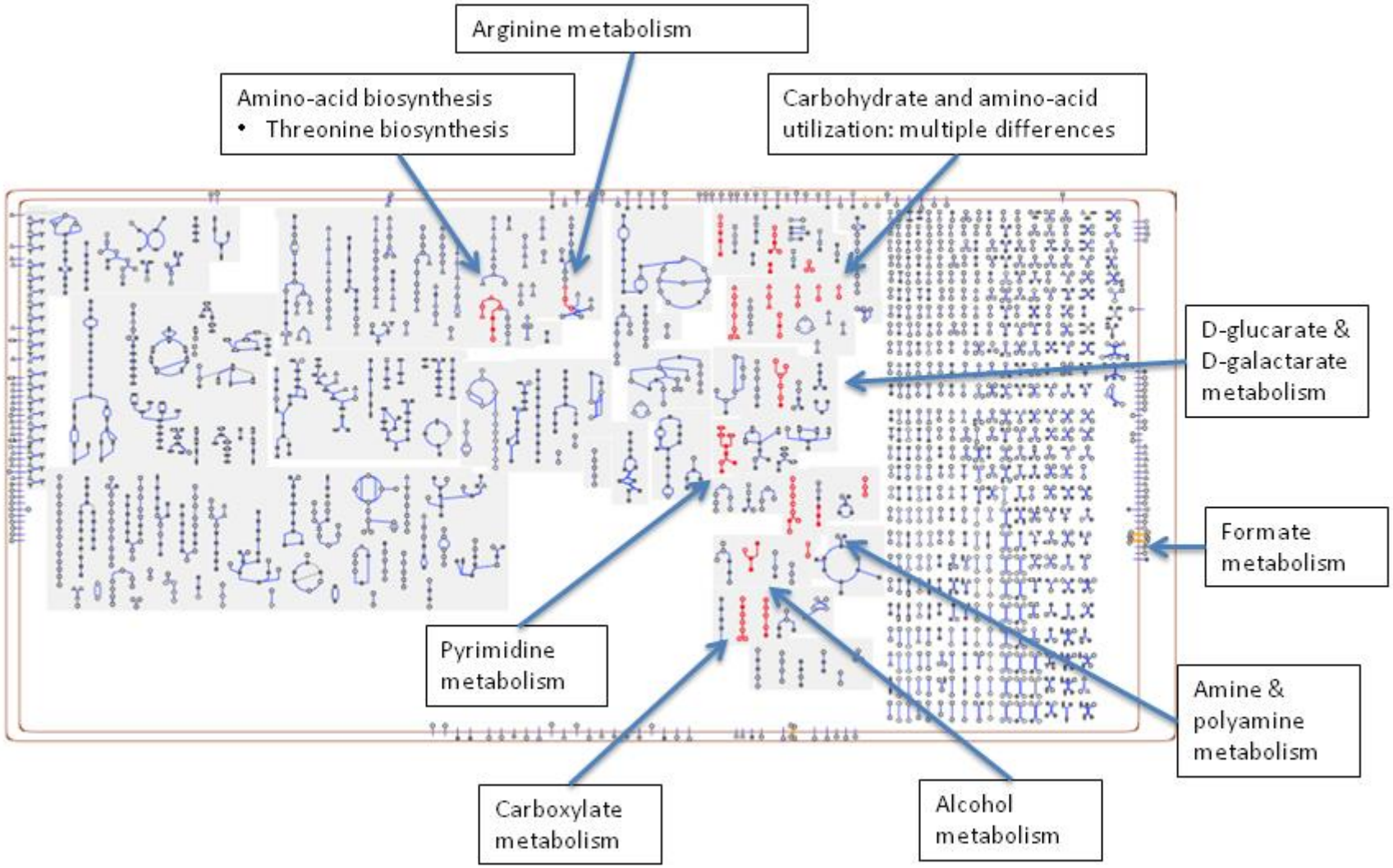


Figure 8-11: Metabolic pathways with different activity in D7795 compared to A1636 at 28°C

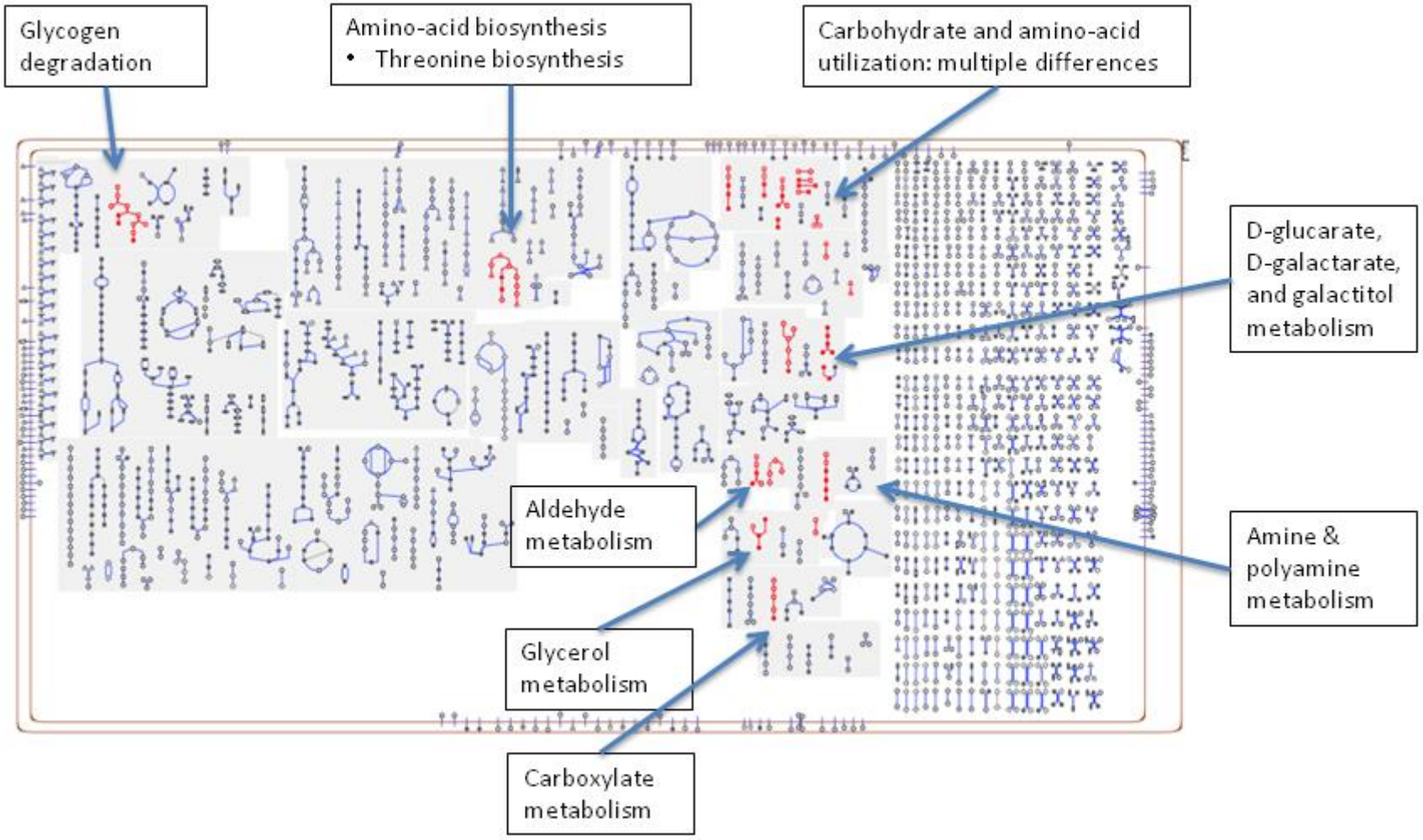


Figure 8-12: Metabolic pathways with different activity in D7795 compared to A1636 at 37°C

8.10 Discussion

Evaluation of the two strains of *S.* Enteritidis, one with a “classical” genome-type and one with an “African” type has added phenotypic evidence to the host-adaptation signature predicted by the pseudogenes in the genome of the “African” *S*. Enteritidis in chapter 8A. Furthermore, these experiments have provided phenotypic evidence to support the suggestion of genomic degradation hinted at by the concentration of genes with NS-SNPs, which are associated with a change in amino acid and therefore a possible impact on function, but no actual pseudogenes in certain metabolic pathways, including butyric acid pathways. There is also intriguing evidence of pathways with reduced metabolism that would suggest a change in host adaptation for which there is no obvious signature in the genome, including the allantoin pathway. It is possible that these pathways have been affected by more subtle changes in the genome, for example changes in transcriptional start sites, the positions of which are extremely hard to predict from primary sequence alone.

8.11 Limitations

In total there are 20 sets of 96 Biolog conditions. It would have been ideal to run several replicates of several strains of *S*. Enteritidis on all plates, at a greater variety of temperatures, including 42^{o}C (core chicken temperature) and 15^{o}C (a lower environmental temperature). It would also have been interesting to perform the assays under anaerobic conditions, especially given the genomic signature of reduced anaerobic metabolism in A7830. Further experiments targeting some of these additional conditions, while outside the scope of this study, are planned for the future.

8.12 Conclusions

The work undertaken in chapters 8A and 8B provides compelling evidence of a *Salmonella* Enteritidis clade with a distinct genotype and phenotype circulating in Malawi. This strains displays evidence of changing host adaptation, different virulence determinants and multi-drug resistance. a parallel situation to the evolutional history of *S*. Typhimurium ST313.

8.13 Future work

In order to confirm that these findings represent the situation across the continent, it is necessary to broaden the phylogeny described in the pilot study, using collections of Salmonellae from across Africa and placing them in the context of global strains to investigate:

a. Whether there is an pan-African epidemic clade of *S*. Enteritidis as is the case with *S.* Typhimurium ST313
b. Whether this clade has been seen anywhere else in the world

Chapter 9: A "first look" at the African and global phylogeny of *Salmonella* Enteritidis

9.1 Summary

A pilot study of the phylogeny of *Salmonella* Enteritidis has suggested that two clades are responsible for BSI in Malawi, and that their genomes display distinct virulence-repertoires and suggest differential host-adaptation. The strain collection was expanded to investigate whether this true throughout Africa, by inviting reference laboratories from across SSA and the world to contribute isolates representing the diversity of their collections. Strain selection was decided upon on the basis of geographical diversity, host diversity (whether human, animal or environmental), temporal range (1955-2013) and drug susceptibility profile. The final collection also included examples of *S*. Enteritidis used as rodenticides. They were Illumina sequenced and the reads were mapped to the genome of reference *S*. Enteritidis strain P125109 (referred to as P125109 from now on). The whole genome phylogenies of all *S*. Enteritidis included in this study were constructed using ML-modelling by RAxML.

The expanded phylogeny confirms the existence of two novel clades of *S*. Enteritidis which are so far restricted in their global distribution to Africa, one of which is predominantly restricted to western Africa and the second, to southern Africa. Both are associated with both bloodstream infection and drug-resistance. In Malawi, however it is

striking that the southern African, MDR iNTS clade co-exists as a cause of BSI with drug susceptible isolates from the wider global clade of *S*. Enteritidis.

This phylogeny confirms the existence of a second Salmonella serotype which has become a prominent cause of BSI in SSA and which displays differential host-adaption and. This work suggests that *S*. Enteritidis has exploited two different environmental niches to cause disease in immune-suppressed patients, the first is likely to be represented by industrial production of eggs and the second of which although likely to be different remains to be determined.

9.2 Introduction

S. Enteritidis is a common cause of BSI in SSA (Reddy et al. 2010) and a few invasive strains of *S.* Enteritidis isolated from African patients in Europe have previously been characterized by sub-genomic methods. These isolates had a distinct Phage Type (PT 42), and were associated with ~100kbp MDR-virulence plasmids (Rodriguez et al. 2011 & 2012). In Chapter 8, a pilot study was described, which suggested that iNTS in Malawi is caused by two clades of *S*. Enteritidis. Whilst these two clades have similar conserved regions, there has been degradation in genes associated with host adaptation and their accessory genomes are markedly different. The novel clade also had an enlarged, complex virulence plasmid when compared to pSENV.

In order to confirm that this novel clade is in circulation across SSA, the phylogeny needed to be broadened to include strains from across the continent. Additionally, the literature was reviewed to assess whether there are other examples of *S*. Enteritidis

displaying different invasiveness, host range or antimicrobial susceptibility to that which is typically associated with *S*. Enteritidis PT4, the classical European gastroenteritis strain. In addition to classical/enterocolitis and African/invasive strains, a third type of *S.* Enteritidis has been described, a which has been used as a rodenticidal agent since the mid-nineteenth century and variously called; “Ratin®”, the “Danysz virus” or “Liverpool-Rat-Virus” (Figure 9.1). This agent has been used throughout the world and is still commercially produced in Cuba and Vietnam under the name “Biorat®” (http://www.labiofam.cu/en/productos/biorat.html). It has also previously been implicated in disease outbreaks (Spray 1926, Williams 1908) and in 1973, WHO recommended that the use of bacterial rodenticides be discontinued on the grounds that they are potentially hazardous to humans and ineffective as a rodenticide (Wodzicki 1973). These agents were included to investigate the possibility that they might have been the causative agent of epidemic iNTS in Africa.

Figure 9-1: Advert for the Liverpool Virus, an *S*. Enteritidis based rodenticide

9.2.1 Research Question

Is there an “African” clade of *S*. Enteritidis, which has emerged in a similar way, with similar phenotypic characteristics to the “African” epidemic *S*. Typhimurium ST313?

9.3 Methods

9.3.1 Strain Selection

Collaborators (Table 9.1) were asked to select strains on the basis of diversity. Factors taken into account in order to distinguish isolates were:

- Time – date of original isolation
- Antibiogram – antimicrobial resistance spectrum
- Geography – site of original location
- Source – whether iNTS or from faeces, or animal or environmental sources. This enabled further categorization into human invasive (from a normally sterile site [blood, CSF, bone, pus]), non-invasive (from an “external” mucosal site [faeces, urine, sputum]) or non-human.
- Phage type – where available
- MLVA type – where available

Table 9-1: A list of institutes or people who donated strains, extracted DNA or contributed clinical data matching the strains

Institute	Country	Collaborators
Centre for Disease Control	USA	Xiangyu Deng
Centre for Vaccine Development	USA	Kristine Borgstein, Sharon Tennant, Myron Levine
Institute for Infection and Global Health	UK	Melita Gordon, Fred Dove, Neil French, Tom Humphrey
Institute Pasteur	France	Francois Weill
Instituto de Higiene, Facultad de Medicina	Uruguay	Jose A. Chabalgoity
Instituut voor Tropische Geneeskunde	Belgium	Jan Jacobs
Kenya Medical Research Institute	Kenya	Robert Onsare, Sam Kariyuki
Lancashire Teaching Hospitals	UK	John Cheesbrough
Liverpool School of Tropical Medicine	UK	Chris Parry
MLW	Malawi	Chisomo Msefula, Robert Heyderman
National Institute for Communicable Diseases	South Africa	Karen Keddy, Anthony Smith
Novartis Institute for Vaccines and Global Health	Italy	Cal MacLennan
University of Bristol	UK	Tristan Cogan
WTSI	UK	Robert Kingsley, Gordon Dougan, Nicholas Thomson

9.3.2 DNA Extraction and Whole Genome Sequencing

In Malawi and at WTSI, DNA was extracted using an automated system as described in chapter 2. Collaborators from University of Maryland, CDC (Atlanta), Institute Pasteur (Paris) and NICD (Johannesburg) provided genomic DNA. DNA was submitted to the sequencing pipeline at WTSI for quality control and WGS by Illumina HiSeq 2500 (Chapter 2). All *S*. Enteritidis isolates (n=94) previously sequenced at Sanger were also incorporated into the study (see appendices for full metadata).

9.3.3 Construction of Phylogeny

Sequences were mapped to the reference using SMALT. The output was curated using Seaview. Extreme outliers were checked by *in-silico* MLST and serotyping and discarded if not confirmed as being *S*. Enteritidis. The phylogeny was constructed from the final alignment file using the maximum-likelihood programme RAxML. The tree was annotated with the metadata using "report_lab_tests.py" (SR Harris), see chapter 2 for full description.

9.3.4 Statistical comparison

The phylogeny was used to assign strains to phylogenetic clusters. This permitted statistical comparison between groups using Pearson's $\chi 2$ test at the 95% level of significance and further comparisons were made using odds ratios. Stata v12 (statacorp Texas) was used for this analysis.

9.4 Results

9.4.1 Strains

Strains were supplied from a considerable variety of sources, enabling evaluation not just of the phylogeny of *S*. Enteritidis in Africa, but also allowing it to be placed in the context of a diverse selection of global strains. In total, DNA was successfully extracted from 608 isolates of *S*. Enteritidis from 40 countries and 6 continents (Table 9.2 and Figure 9.2). Although a smaller proportion came from industrialised settings, these isolates had been typed extensively, including 47 isolates from the University of Bristol which had been Phage Typed and 40 isolates from CDC which had been PFGE and MLVA typed. The isolates were collected over a 65 year time period from 1948 to 2013.

Table 9-2: Isolates included, broken down by region

Region	Number of Isolates	%
Industrial World	114	19
North Africa (Maghreb & Egypt)	12	2
Sub-Saharan Africa excl RSA	347	57
South Africa (RSA)	131	22
Rodenticide	3	<1

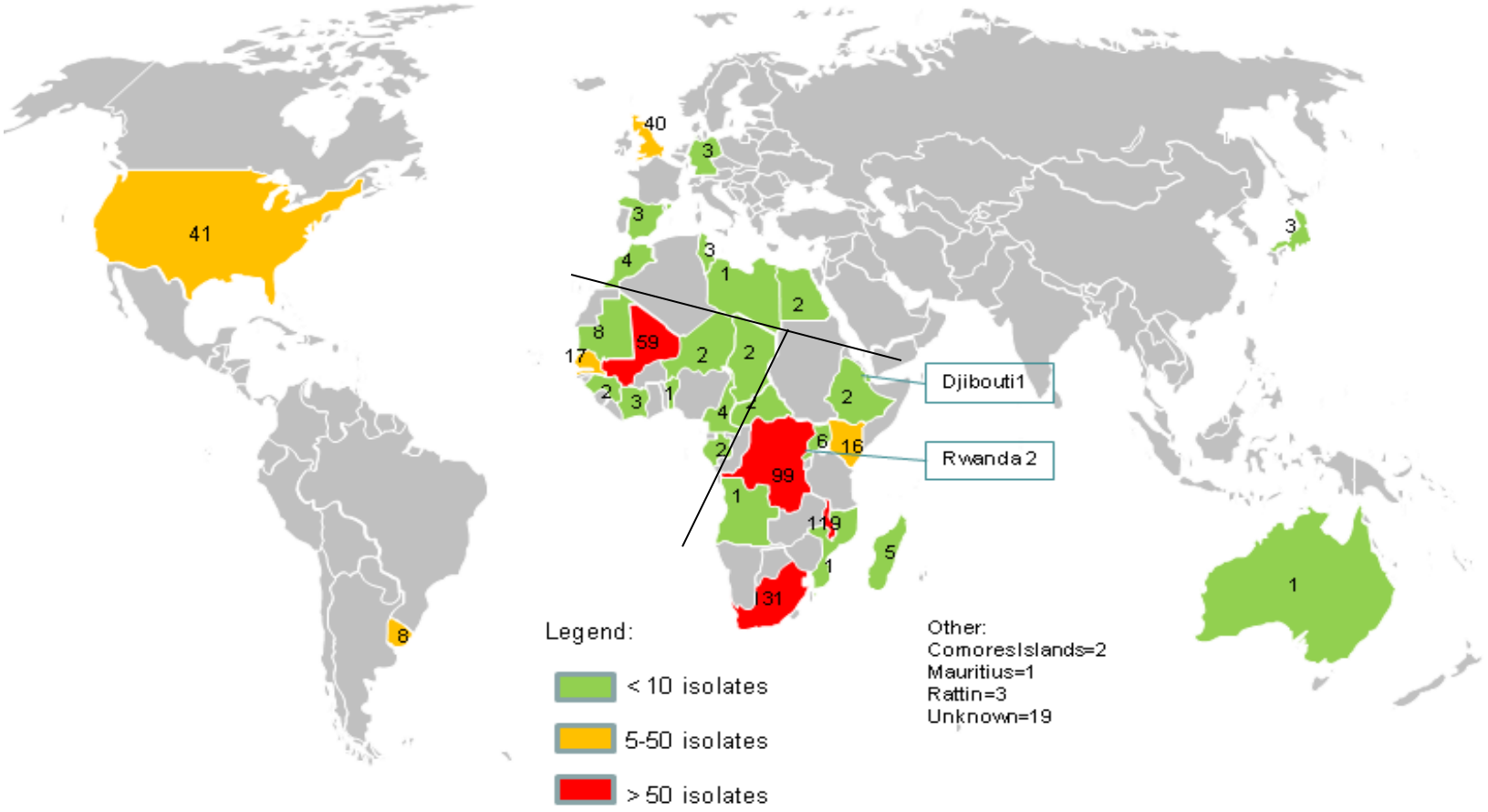


Figure 9-2: Map of the world showing the source and number of isolates in the study. Isolates of unknown source were either from collections donated to Bristol (UK) or CDC (USA). The lines across Africa divide the Maghreb & Egypt from SSA and arbitrarily divide SSA into western Africa and central/southern Africa.

9.4.2 Antimicrobial susceptibility

Many of the isolates were submitted as DNA and had not previously been tested for antimicrobial susceptibility (137/608 [23%]). Moreover where susceptibility testing had been performed, the panel of agents used varied considerably. The data were therefore classed into 9 categories of antimicrobial susceptibility (Table 9.3). 204/471 (43%) isolates were fully susceptible, and 267/471 (57%) had phenotypic evidence of resistance to one or more classes of drug antimicrobial and 155/471 (33%) isolates were MDR. Just 3 were ESBL-resistant and 2 were nalidixic acid (NA) resistant. Most of the resistant isolates come from sub-Saharan Africa (excluding South Africa) with 217/267 (81%) isolates with any resistance coming from this region, although 109/261 (80%) of isolates from outside of this region were of unknown drug susceptibility.

Table 9-3: Antimicrobial resistance types

Resistance type	Number	%
Fully susceptible	204	34
Amoxicillin resistant	22	4
Choramphenicol resistant	24	4
Cotrimoxazole resistant	14	2
Both amoxicillin and chloramphenicol	5	1
Both amoxicillin and cotrimoxazole	42	7
MDR	155	25
ESBL-producing	3	0
Nalidixic acid	2	0
Unknown	137	23

The source was known for 561 isolates. The majority of isolates were human invasive coming from blood (355 [63%]) or another normally sterile site such as bone or CSF (31 [6%]). 105 isolates (19%) were from human stool and a further 27 (5%) were potentially non-invasive (urine and sputum). The remainder were isolated from animal sources (11 [2%]) or food (29 [5%]). Three strains were known to come from the rodenticidal agents.

Table 9-4: Source from which isolates originally cultured

Source	Number	%
Human blood	355	58.39
Other human-invasive (CSF, bone, pus)	31	5.1
Human faeces	105	17.27
Other human (urine, sputum)	27	4.44
Animal	11	1.81
Food	29	4.77
Unknown	47	7.73
Rodenticide	3	0.49

9.4.3 Diversity of MLST

The collection was typed by *in-silico* MLST typing ("get_sequence_alignment.pl [A. Page WTSI]). The majority (573 [94%]) were of ST 11, however, 8 were of MLST type ST 136, the type associated with rodenticides (Table 9.5). This result suggests two possibilities which could not be resolved by MLST; either that one clade has been responsible for the majority of cases of *S.* Enteritidis or that MLST cannot adequately discriminate between pathotypes of *S.* Enteritidis. This is in contrast to the situation with "African" *S.* Typhimurium which is distinguishable from "industrial world" strains by its ST, which is 313 (Kingsley et al. 2009). This is not completely surprising as MLST is based on just seven house-keeping genes (Achtman et al. 2012).

Table 9-5 MLST typing of collection

MLST Type	Number	%
11	573	94
50	1	<1
136	8	1
168	1	<1
183	7	1
366	3	1
745	3	1
814	1	<1
892	1	<1
1479	2	<1
1632	8	1

In addition to the MLST typing, the 47 strains provided by the University of Bristol had been Phage Typed and 16 PTs were represented.

A full list of all isolates and their associated metadata is available in the appendices (Appendix 5,Table 12).

9.4.4 Phylogeny

In total, 608 *Salmonella* Enteritidis genomes were mapped to P125109, the variable regions and plasmid were then excluded and the remaining sites screened for SNPs (snp_sites.pl [SR Harris]). This left an alignment file containing a total of 28,858 variable sites at which one or more of the genomes in the collection had SNPs with respect to the reference genome P125109. ML-phylogeny was then constructed using RAxML (Figure 9.3 and Figure 9.4).

Figure 9-3: Midpoint rooted phylogeny of *S.* Enteritidis reveals 3 epidemic clades (clusters 3, 4 and 6), with 3 clusters of evolutionary outliers. There is clear evidence of 2 African clades, cluster 3 which is predominantly formed of Malian isolates and cluster 4 with includes Central/Southern African, but very few South African (RSA) clades. As the decades

progress, so too do the number of antimicrobial resistance categories in each of these two clades. Cluster 6 includes a mixture of isolates from around the world. Key to antimicrobial resistance (AMR) A=amoxicillin, C=chloramphenicol, SXT=cotrimoxazole, NA=nalidixic acid.

The structure seen in the phylogeny of *S.* Enteritidis is striking for many reasons. In addition to the differences in the phylogeny, there are significant differences between the geographical distribution of clusters ($p < 0.01$), antimicrobial resistance ($p < 0.01$) and invasiveness ($p < 0.01$), although there is selection bias towards the latter result as neither MLW nor ITM Antwerp regularly cultured faeces. There is clear evidence of not one, but two African clades, the first (cluster 3) predominantly composed of Malian isolates and the second (cluster 4) of Central/Southern African ones. As P125109 forms part of cluster 6, they have been described in reverse order.

9.4.5 Cluster 6: The global epidemic cluster

This was the cluster most closely associated with both P125109 and A1636. It includes 224 isolates from 23 countries, including all parts of Africa and from a 63 year period. It is noteworthy that this cluster included 43 isolates from Malawi and 82 from South Africa (RSA). 108/150 were fully susceptible, including 98% of the Malawian iNTS isolates. Only five were MDR and just one was nalidixic acid resistant. None were ESBL-producing isolates. The majority with drug resistance were cotrimoxazole mono-resistant isolates from South Africa (30). There were 93 invasive isolates and 68 from stool culture in this cluster.

9.4.6 Cluster 5: Outliers to the global epidemic

This cluster was closely related to P125109, sitting ~250 SNPs away. It is both geographically and temporally diverse in that there are 99 isolates present from five continents and a 64 year time period. 59/71 isolates were drug susceptible and only 1 was MDR. 49/79 of the isolates from humans were invasive and 27 were not. Perhaps most interesting is that there were 40 isolates from South Africa in this clade, which might have been associated with an epidemic there. This is of great interest as South Africa is a middle income country with high HIV rates, but very little malaria which is restricted to a few areas in the northeast. Nutrition, access to potable water and sanitation standards are generally higher than across the rest of SSA, but the borders are porous and legal and illegal immigrants come from across the continent, therefore frequent occurrence of *S.* Enteritidis disease caused by 'classical' rather than 'African' strains is striking, as one might predict a dominance of the African clade.

9.4.7 Cluster 4: Southern African BSI-epidemic

Global *S.* Enteritidis cluster 4 (Figure 9.3) included isolates D7795 and A7830. There were 165 isolates and all but one from the USA came from central and southern Africa (Central African Republic, Congo, Kenya, Malawi, Rwanda, Uganda, South Africa). It is striking that only 2 isolates from this cluster came from South Africa and none came from West or North Africa. This clade diverges from the global epidemic clade by ~1,000 SNPs. 126/153 (82%) of known susceptibility were MDR and 148/153 (97%) had some form of resistance and acquisition of resistance has been progressive over time; none were MDR in the 1980s, 50% were in the 90s and 100% since 2010. This clade was significantly more likely to be resistant than cluster 6 (OR 76 [95%CI 16-298]). 91% of Malawian isolates were MDR, but although the MDR clade was involved in an epidemic (chapter 3) it did not replace the fully

susceptible strains from cluster 1 as a cause of iNTS in Malawi. The oldest example of this clade was from 1985. 156/165 (95%) of these isolates were invasive, perhaps implicating HIV in its emergence.

9.4.8 Cluster 3: Western African BSI epidemic

Global *S.* Enteritidis cluster 3 (Figure 9.3) was significantly associated with West Africa with 70/76 isolates coming from one of Guinea, Gabon, Ivory Coast, Mali, Niger or Senegal. Three isolates came from the USA, two from Malawi and one from Madagascar. Interestingly, three isolates (Senegal and Guinea) were originally cultured in the 1950s, before the HIV pandemic. These isolates were drug susceptible. This clade diverges from the global epidemic clade by ~1,000 SNPs and the branch length to the Southern African clade is 300 SNPs. This clade was also significantly associated with drug resistance. Only 9/76 strains were fully susceptible and 25 were MDR including 3 ESBL strains. In this clade acquisition of resistance was also been stepwise over time, but more recent than in Southern Africa; none were MDR until the 1990s, and only 50% recent isolates are MDR. Compared to classical *S.* Enteritidis from cluster 6, the OR of drug resistance was 18 (95% CI 7-46). 99% of these isolates were human-invasive.

9.4.9 Cluster 2: Evolutionary Outliers

There were 34 isolates associated with Global *S.* Enteritidis cluster 2 (Figure 9.3), a highly diverse group which incldes 20 strains from SSA, 8 from industrial settings and 6 from S. Africa. These isolates have been cultured throughout the course of the study (1959-2012), with representatives from each decade. There was considerable diversity within this cluster (Figure 9.4); 7/34 were of ST 183 and they diverged from the global epidemic clade by 1,200

– 3,500 SNPs. Only 1/23 was MDR and 2/23 display any form of resistance. 20 were human-invasive, and 6 were from stool. These isolates appear to be sufficiently well adapted to and pathogenic in humans that they continue to cause disease, yet there is no evidence from this study that this clade has caused epidemics.

9.4.10 Cluster 1: Rodenticide strains

Isolates within Global *S.* Enteritidis cluster 1 were extreme outliers and are fascinating as they are more closely related to *S*. Gallinarum and *S*. Pullorum than *S*. Enteritidis (unpublished work G Langridge). Three of these isolates were known to have been sequenced from stocks of rodenticide and this clade is separated from the global epidemic by approximately 1,900 SNPs. This is known to be of MLST type 136 (unpublished work F. Weill), but it was in keeping with this that all 8 of the ST 136 isolates cluster around it. There is one isolate of ST50 which is associated with this cluster, but on a long branch of its own. The fact rodenticides have been industrially produced for over a century means it is possible that the branch length of these strains may result from multiple passages in industrial production, but they are basal to the rest of the tree suggesting that the original source predates all of the other *S.* Enteritidis isolates in this tree. They are phyletically remote from both global-epidemic and Africa-epidemic clusters.

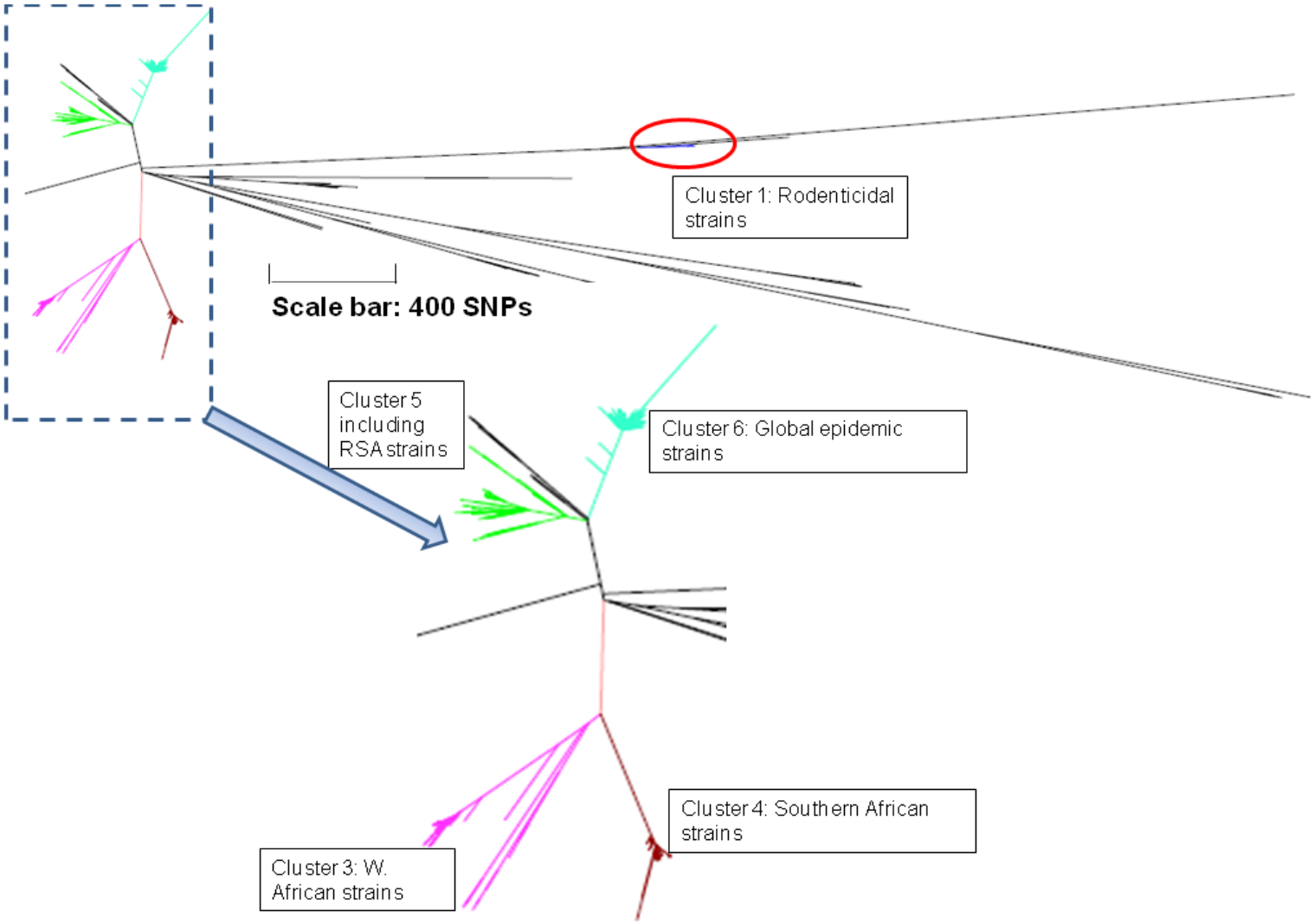


Figure 9.4: Midpoint rooted radial phylogeny of the *S.* Enteritidis collection

9.4.11 Plasmid phylogeny

The genomes were next mapped to the 58kb plasmid from P125108, pSENV. The same procedure was followed as described above (Chapter 2.9), yielding an alignment with 3,108 variable sites. The phylogeny was constructed using RAxML is displayed in Figure 9.4. 120 Genomes did not yield any matches to pSENV.

The plasmid phylogeny is strikingly similar to that of the chromosome suggesting that the plasmid has not been transmitted by conjugal transfer between lineages, but is highly conserved and has evolved alongside the chromosome extension of the chromosome. The absence of the conjugal *tra* genes and failure of conjugal transfer described in chapter 8A provide a genetic mechanism and phenotypic evidence to support and explain this. Chromosomal clusters 1-2 (plasmid cluster 1) and 5-6 (plasmid cluster 1) merge in this phylogeny, whereas the difference between East and West African clusters is maintained.

Of the five Cluster 6 isolates which were MDR, only one plasmid was found within the global collection, but intriguingly, the other three did not map to pSENV at all and one matched only 15% of the plasmid and these isolates should be investigated further in the future.

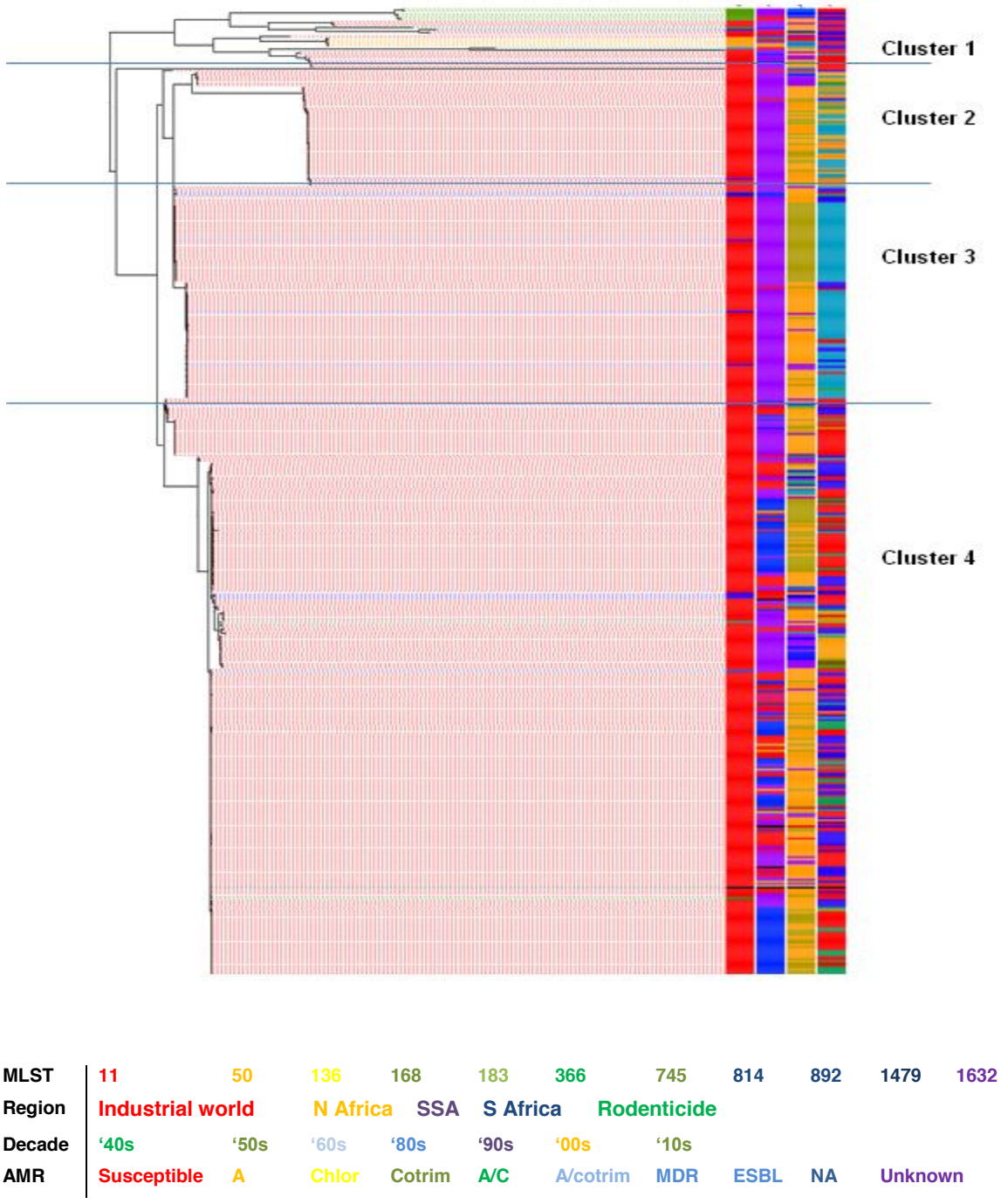


Figure 9-5 Phylogeny of plasmids associated with *S.* Enteritidis isolates mapped to 58kb plasmid pSENV. Key to antimicrobial resistance (AMR) A=amoxicillin, C=chloramphenicol, SXT=cotrimoxazole, NA=nalidixic acid.

9.5 Discussion

These detailed phylogenies provide new evidence of not one, but two clades of *Salmonella* Enteritidis causing epidemics in sub-Saharan Africa. They are both significantly associated with drug resistance, and the Southern African clade (cluster 4) has a distinct genotype with a novel prophage repertoire and plasmid, and evidence of the genomic degradation that is the signature of host-restriction in multiple Salmonella serotypes (Parkhill et al. 2001, Thomson et al. 2008, Kingsley et al. 2009). This evidence of host adaptation from the genome is both corroborated and enhanced by the results of high-throughput phenotyping, which suggests degradation of metabolic pathways involved in host adaptation not obvious from comparative genomics. The hypothesis following the pilot study, of an African *S.* Enteritidis is confirmed by the more extensive phylogeny.

The most important question posed by this study is why have two clades of Salmonella from serotypes normally considered to be weakly invasive emerged in Africa? HIV is highly likely to have been an important factor, but in contrast to the picture seen with *S*. Typhimurium, fully susceptible *S.* Enteritidis isolates from the global epidemic clade (cluster 6) have continued to cause disease alongside the novel clade. In Malawi, where there has been robust, long-term BSI surveillance, serotype replacement of classical/susceptible *S.* Enteritidis by the African clade did not occur. This strongly suggests that the two clades are not in competition outside of the human host, instead there are three alternative possibilities:

1. That they occupy different ecological niches and are indeed different “ecotypes”
2. They share the same niche without detriment to transmission
3. They demonstrate symbiosis within the same niche

This study provides compelling evidence of a need to understand what these ecological niches might be, and what the transmission pathways of African/invasive Salmonellae are.

Just as the presence of these strains in Africa is striking, so too is their absence from industrial settings; the phylogeny of these strains suggests the possibility that they occupy not just a different ecological niche, but a different socio-economic one too. It is striking, however, that there were only 2 "African" *S.* Enteritidis isolates amongst the strains submitted from NICD, South Africa. This is surprising since South Africa has large numbers of people living in extreme poverty, high HIV rates and, perhaps most relevant, has a large migrant population and is part of the same SADC trading group of nations as Malawi, however it might be of note that South Africa is considerably developed relative to the rest of SSA, and the poor in South Africa might have better access to sanitation and hygiene.

The difference in drug susceptibility is also interesting. Drug resistance was not a common feature amongst the *S*. Enteritidis from the global epidemic clade, which appears to have thrived without acquisition of drug resistance. In contrast, the sequential acquisition of drug resistance in SSA, prior to epidemics in each of Mali, Congo and Malawi, suggests that this it is strongly linked to the success of these lineages/ecotypes and is perhaps crucial to its success, as appears to have been the case with ST313 strains of *S*. Typhimurium (Gordon et al. 2012, Okoro et al. 2012(1)).

9.6 Limitations

The principal limitation of this study is that it was restricted to the few laboratories which both culture and store bacterial isolates and also to collaborators who were prepared to donate their isolates. This study suggests that the two clades of "African" *S*. Enteritidis are highly invasive, however neither the Congolese nor the Malawian laboratories routinely performed

stool culture at the time isolates were requested, therefore there was gross strain-selection bias towards invasive isolates. Of note, however the Global Enteric Multicentre Study (GEMS) has recently reported that Salmonella serotypes are not a common cause of diarrhoeal illness in African children (Kotloff et al. 2013), which supports the hypothesis that these strains are intrinsically more invasive and may have lost their ability to cause diarrhea. It is not, however, possible to estimate an invasiveness index for the African strains from these data in the absence of stool culture surveillance data contemporaneous to the BSI data.

It is also the case that the number of isolates from Africa was much greater than that from the rest of the world. Although industrial-world isolates were selected on the basis of PFGE and/or MLVA diversity, a truly representative survey of the global phylogeny must include a greater number of isolates from these settings and from Asia which was not well represented in this analysis. Meaningful surveillance of the genomic-epidemiology, or phylogeography of *S.* Enteritidis would require a prospective study with consistent inclusion criteria in well phenotyped populations, however decreasing sequencing costs and existing structures for the global surveillance of Salmonella (i.e. Pulsenet: http://www.cdc.gov/pulsenet) should make this feasible in the future.

9.7 Conclusions

Two clades of *Salmonella* Enteritidis, which are rarely seen outside of Africa, have emerged and have different phenotypes from the strains of *S.* Enteritidis circulating in the industrial world. They are likely to have different ecologies and/or host ranges, and are strongly associated with drug resistance have caused epidemics of BSI in at least three settings in SSA, yet are rarely responsible for disease in South Africa. An investigation into the environmental reservoirs and transmission of these pathogens is urgently required.

9.8 Future work

1. Phylogeny:
    a. Expand the strain collection:
        i. To be fully representative of the global epidemiology including confirmation of our findings in the Asian context
        ii. Using a consistent methodology for selecting truly representative or well phenotypes strains
    b. Investigate the diversity of the accessory genome of *S.* Enteritidis using software under development at Sanger which will map the variable regions of all 608 genomes. A detailed heat-map of these accessory regions will enable targeted investigation of variable regions and specific genes to further investigate host adaptation, virulence and antimicrobial resistance determinants.
2. Use Bayesian methods to estimate the dates of the branches of the clades, in order to investigate the role of HIV, antimicrobial resistance, or other temporal, or geographical factors involved in promoting the spread of the African clades.
3. Plasmids: undertake multiple plasmid extractions and sequence on the Pacific Biology sequencer to establish the structure of the African plasmids.
4. Investigate the epidemiology of invasive Nontyphoidal Salmonellae in sub-Saharan Africa, with a particular focus on exploring environmental reservoirs.

Chapter 10: Concluding remarks and future research priorities

10.1 Summary of findings

The Malawi Liverpool Wellcome Trust Clinical Research Programme has provided diagnostic microbiology service for routine medical admissions to QECH hospital, Blantyre since 1998. A review of ~150,000 blood cultures taken between 1998 and 2013 has revealed 3 epidemics of invasive Salmonella disease. The first caused by *S*. Enteritidis, the second by *S*. Typhimurium, and the most recent caused by *S*. Typhi. All were associated with the acquisition multidrug resistance prior to the epidemic. Whilst iNTS has declined, especially in children ≤ 1year of age, it remains a prominent pathogen (Figure10.1).

Understanding the epidemiology of paediatric iNTS is complicated by the considerable interactions between the principal risk factors, malaria, HIV and malnutrition. There is strong epidemiological data from Kilifi, Kenya, a low HIV prevalence area to support the hypothesis that malaria is the major risk factor for iNTS there (Scott et al. 2011). Like Kilifi, Blantyre has seen considerable falls in iNTS, in contrast, however has had great success in ART roll-out, but until 2010, only modest success in malaria control. Analysis of the temporal trend (2001-10) in paediatric iNTS in light of trends in malaria, rainfall and malnutrition data was undertaken using structural equation modelling, which unlike Poisson regression, permits interaction between the predictor variables. SEM confirmed that reduction in malaria likely played a significant role, but also revealed complex independent interactions between there factors, and additionally demonstrated that much of the highly significant fall in paediatric iNTS cannot be explained by the available data, suggesting a highly complex epidemiology, which remains incompletely understood.

The principal risk factor for iNTS in adults is HIV infection. As HIV is a risk factor for all causes of BSI, except that caused by *S*. Typhi (Reddy et al. 2010, Crump et al. 2011), it was predicted that roll-out of ART in Blantyre would lead to a reduction in incidence of all adult BSI. Recruitment of a cohort of 2,007 adult patients presenting to QECH with fever ≥37.5^{o}C revealed very significant declines in both incidence of and mortality from BSI when compared to a similar cohort recruited in the pre-ART era. Comparison of two cohorts from arbitrary time points invites a criticism of selection bias. The 2009/10 cohort was therefore stratified by HIV status and estimated incidence rate ratios were calculated for HIV negative subjects and those at different stages of ART-treatment. Patients that had been taking ART for ≥ 3 months were significantly less likely to develop BSI than HIV infected patients not on treatment, or in the first 3 months of ART, suggesting that ART does protect against all cause BSI. Despite the success of the ART programme, there remains a large group of highly immune-suppressed patients at high risk of BSI in Blantyre who remain highly vulnerable to BSI early in immune-reconstitution.

Three specific questions outstanding regarding recurrent iNTS disease in adults include:

1. What is the impact of ART on this recurring, AIDS defining illness?
2. Does recurrence lead to evolution of further antimicrobial resistance?
3. What is the site of persistence?

Recruitment of a cohort of adult iNTS patients confirmed the ongoing improvement in clinical outcomes from this condition at presentation. The gradual nature of this improvement over a number of years suggested that multiple factors were involved in this improvement on admission. The significant decline in both recurrence and death in the period following discharge from QECH suggested a specific role for ART in this improvement. It also suggested that ART gives rapid protection against recurrent disease. This drop in iNTS recurrence has also reduced the need for multiple courses of antimicrobials in patients with

persistent NTS, resulting in a decline in evolutionary selection pressure to acquire further antimicrobial resistance. Only one patient developed ESBL-producing, fluoroquinolone resistant iNTS disease and this case preceded commencement of the cohort study. The site of NTS persistence was not discovered. In future the gastrointestinal tract should be surveyed more intensively, using both molecular detection and gut-mucosal biopsy.

WGS of invasive strains of *S*. Typhimurium previously revealed the existence of a novel pathotype, which is highly adapted to cause invasive disease in the huge pool of immune-suppressed subjects in SSA (Kingsley et al. 2009). Phylogeny of African invasive strains of *S*. Enteritidis placed in the context of a diverse array isolates from several global collections by maximum-likelihood modelling has revealed the existence of not one but two African epidemic clades, which are distinct from the global epidemic clade. In-depth comparison was made between an example of the Southern-African clade and the reference sequenced isolate from the global epidemic clade (P125109). This revealed:

1. Genomic degradation in genes responsible for an enteric life-style, the signature of differential host adaptation in Salmonellae
2. A novel prophage repertoire
3. A complex, novel virulence plasmid with multidrug resistance encoding genes

Predictions from the genome were supported by high throughput phenotyping work. This is the second confirmed example of a clade emerging from a host-promiscuous serotype commonly associated with enterocolitis to cause invasive disease in susceptible patients and fundamentally challenges the simplistic clinical division of Salmonellae as being Typhoidal/invasive or non-typhoidal.

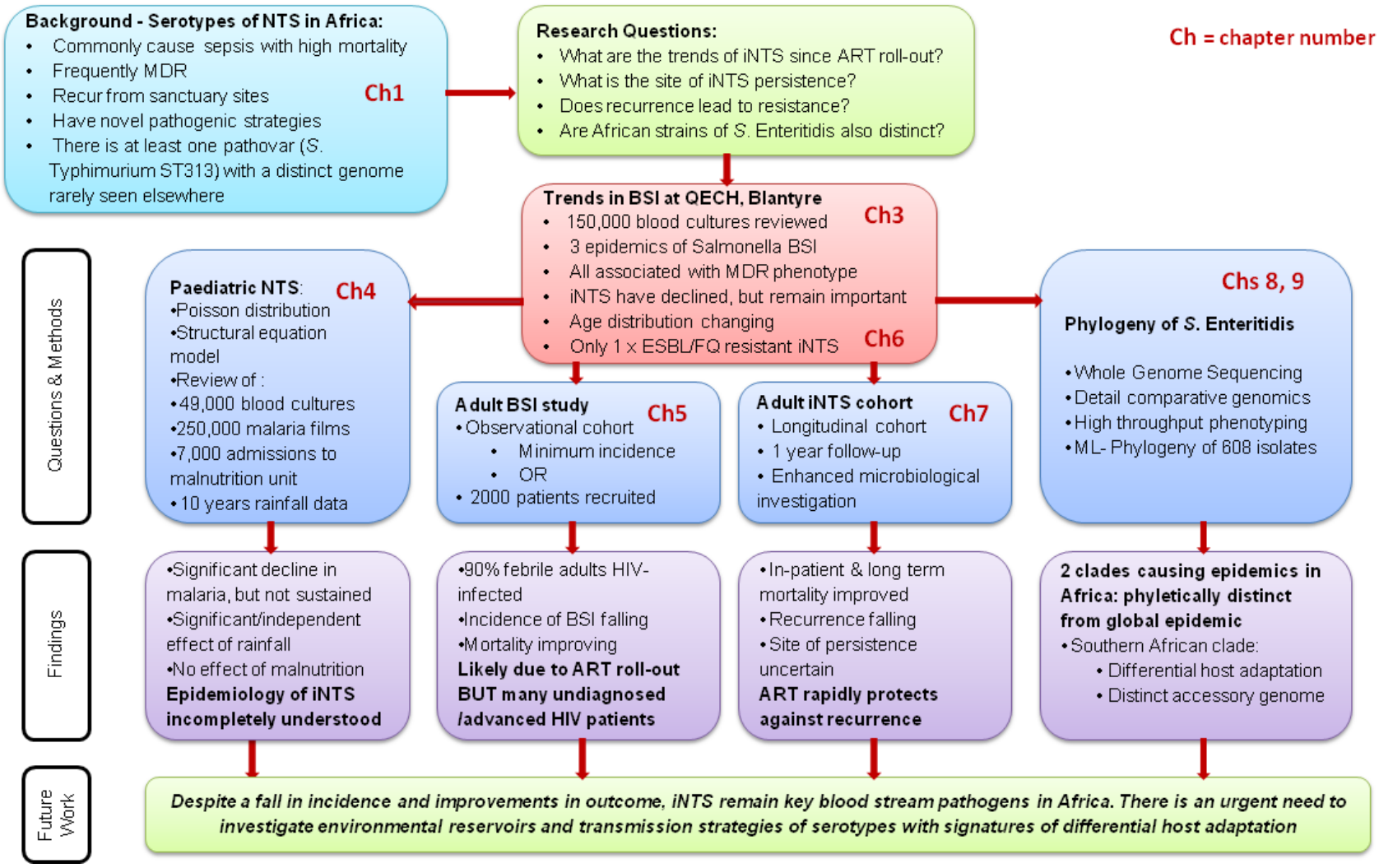
10.2 Future Research Priorities

10.2.1 Epidemiology

The true burden of Salmonella disease in Africa is unclear. A comprehensive epidemiological study of iNTS, diarrhoeal NTS and *S*. Typhi is urgently needed, addressing the possible modes of transmission. In particular, there is an urgent need to investigate the current epidemic of Typhoid in Blantyre, through an outbreak investigation, using a case-control study. Such a study might be enhanced by combining global positioning system (GPS) coordinates of patients' homes with WGS data from strains causing Typhoid to model genomic epidemiology of the outbreak in fine detail. The value of WGS as a tool in epidemiological investigations has already been proven in the case of *S*. Typhi in Asia (Baker et al. 2008). Both *S*. Typhimurium ST313 and "African" clades of *S*. Enteritidis display evidence of differential host-adaptation, and investigation of the reservoirs, and risk-factors for exposure and transmission, will permit hypothesis-driven approaches to preventative measures including vaccines, improved hygiene and sanitation, improved nutrition, malaria control and ART roll-out programs.

10.2.2 Rapid Diagnostics

Clinical algorithms fail to reliably identify iNTS in adults or children in Africa. Blood culture and microbiological diagnosis of Salmonella requires technical expertise, and investment in infrastructure, consumables and quality-control, all of which must be strengthened in the region. There is an urgent need for the development of rapid, accurate point of care diagnostics, to supplement conventional diagnostic microbiology. Targets for a NTS rapid multiplexed PCR diagnostic have been identified (Tennant et al. 2010), but low numbers of NTS bacilli in blood make this technically challenging (Gordon et al. 2010). Similar limitations



10-1: Summary of research questions, methodologies and outcomes

have been observed in typhoid fever (Nga et al. 2010). A novel ultra-fast methodology for detecting the Salmonella *ori C* chromosomal locus shows promise but requires validation and translation to the field (Tennant et al. 2011). Such molecular diagnostic tools will be invaluable both for clinical diagnosis and for epidemiological investigations.

10.2.3 Prevention

Vaccine development is a potential prospect for NTS control. The few *S. enterica* vaccines to date have targeted *S*. Typhi in humans, but several approaches could target a vaccine against iNTS. Inactivated whole cells have historically provided the basis for oral vaccines such as those for cholera (Lebens et al. 2011). This approach would be relatively cheap, but it is uncertain whether it would be effective against invasive disease. Live oral African NTS vaccine strains, attenuated by mutations in guanine synthesis and flagellum regulatory genes, protected mice against lethal infection with both *S*. Typhimurium and *S*. Enteritidis, and such strains could be used as part of a vaccination strategy A second approach, as pioneered using the Vi antigen of *S*. Typhi (Lin et al. 2001 N Engl J Med 2001), could target surface polysaccharides, particularly O side-chains, in the form of a conjugate vaccine, and some programmes are pursuing this line. Different NTS serotypes, however, have immunologically distinct O side-chains, and consequently a multiple-antigen vaccine may be required. A further approach could target surface protein antigens such as the porins or flagella (Gil-Cruz et al. 2009, Bobat et al. 2011). Protein antigens can be protective in mice, but this remains to be translated into human vaccines. NTS vaccine development is also complicated by the compromised nature of susceptible patients; HIV-infected individuals show dysregulated responses to Salmonella infection (MacLennan et al. 2010, Dougan et al. 2011) which must be addressed early in clinical development.

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Appendices

1 Case Report Forms: Adult BSI study

1.1 Blood culture nurse screening CRF

Date……/……. /……

Name…….

Date of birth/age ……...

Sex M F

Personal Identification Number

1. QUESTIONS

HIV Status?	Pos **+**	Neg **-**	DK	Date ……/……./,……..
On ARV?	Y	N	DK	First line Second line Start date ……/……./,……..
On Cotrimoxazole?	Y	N	DK	Start date ……/……./,……..
Antibiotics in last week?	Y	N	DK	

Hospital admission in last month?	Y	N	DK	Diagnosis if known?
Antimalarials in last week?	Y	N	DK	
Length of current illness				Start date ……/……./,……..

Blood culture	Result: S. pneuemoniae Non-typhi salmonellae E. coli Klebsiella spp. N. meningitidis Other streptococci Salmonella typhi S. aureus Cryptococcus Other Sensitivities if known?

Outcome at discharge	Alive	Dead	DK

1.2 Fieldworker CRF

Date ……/…… /……

Name…….

Date of birth/age ……...

Sex M F

Personal Identification Number

2. QUESTIONS

HIV Status?	Pos +	Neg -	DK	Date ……/……./,……..
On ARV?	Y	N	DK	First line Second line Start date ……/……./,……..
On Cotrimoxazole?	Y	N	DK	Start date ……/……./,……..
Antibiotics in last week?	Y	N	DK	
Hospital admission in last month?	Y	N	DK	Diagnosis if known?
Antimalarials in last week?	Y	N	DK	
Length of current illness				Start date ……/……./,……..

3. SYMPTOMS

Symptom				**Time in days**
Chronically ill (> 1 month)	Y	N	DK	
Fever > 1 month	Y	N	DK	
Fever > 39°C	Y	N	DK	
Reported weight loss	Y	N	DK	
Cough	Y	N	DK	
Cough < 3 weeks	Y	N	DK	
Pleuritic chest pain	Y	N	DK	
Shortness of breath	Y	N	DK	
Sputum production	Y	N	DK	
Vomiting	Y	N	DK	
Diarrhoea	Y	N	DK	
Abdominal pain	Y	N	DK	
Urinary symptoms	Y	N	DK	
Headache	Y	N	DK	
Confusion, ↓ LOC	Y	N	DK	
Fits	Y	N	DK	

4. EXAMINATION

Axillary Temp			
BP			
Pulse			
Resp rate			

Wasting	Y	N	DK
Pallor	Y	N	DK
Jaundice	Y	N	DK
Oral thrush	Y	N	DK
KS palate	Y	N	DK
KS skin	Y	N	DK
LNs	Y	N	DK
Shingles scar	Y	N	DK

5. <u>MANAGEMENT and RESULTS</u>
please fill in any results known during admission

			Results
Antibiotics given	Y	N	Ceftriaxone Cefotaxime Chloramphenicol Gentamycin Benzyl penicillin Ciprofloxacin Erythromycin Co-trimoxazole other
HIV test	**+**	**-**	
CD4 count			
MPs	**+**	**-**	
Hb			
WCC			

Platelets			
CXR	**+**	**-**	+ is abnormal. Please describe:
Blood culture	**+**	**-**	*S. pneumoniae* Nontyphoidal Salmonella *E. coli* *Klebsiella spp.* *N. meningitidis* Other streptococci *Salmonella* Typhi *S. aureus* *Cryptococcus* Other

6. PATIENT OUTCOME

Outcome at discharge	Alive	Dead	DK

2 Adult NTS Cohort CRF Set:

2.1 Recruitment

Consent to study	Y	N	NA

Date recruited	
Study Number	

SPINE number

Date of Birth

Occupation:

Residential address/location

Contact details:

Phone number:

Owner of phone if not patient:

Relationship to patient:

Aware of all diagnoses? **Yes** **No**

History

Been sick for …… days, with current / presenting illness

Symptom			
Fever	**Y**	**N**	**DK**
Sweats	**Y**	**N**	**DK**
Cough	**Y**	**N**	**DK**
Sputum produced	**Y**	**N**	**DK**
Chest pain	**Y**	**N**	**DK**
SOB	**Y**	**N**	**DK**
Acute diarrhoea	**Y**	**N**	**DK**
Blood in diarrhoea	**Y**	**N**	**DK**
Vomiting	**Y**	**N**	**DK**
Abdo Pain	**Y**	**N**	**DK**
Dysuria	**Y**	**N**	**DK**
Urinary frequency	**Y**	**N**	**DK**
Headache	**Y**	**N**	**DK**
Confusion, ↓LOC	**Y**	**N**	**DK**
Fits	**Y**	**N**	**DK**

Past History:			
Weight loss >3/12	**Y**	**N**	**DK**
Diarrhoea >3/12	**Y**	**N**	**DK**
Fever > 3/12	**Y**	**N**	**DK**
Cough > 3/12	**Y**	**N**	**DK**
Shingles	**Y**	**N**	**DK**
Generalised pruritis	**Y**	**N**	**DK**
Thrush	**Y**	**N**	**DK**
TB smear +	**Y**	**N**	**DK**
TB smear -	**Y**	**N**	**DK**
Crypto meningitis	**Y**	**N**	**DK**
Recurrent pneumonia	**Y**	**N**	**DK**
Dysphagia	**Y**	**N**	**DK**
Kaposi's sarcoma	**Y**	**N**	**DK**
Neurol. disability	**Y**	**N**	**DK**

Already taken antibiotics for this illness?

Yes No DK

If yes:

Antibiotic	Amoxil	SP	Cipro	MTZ	Ery
Dose					
Frequency					
Duration (days)					

Taken prophylactic cotrimoxazole before admission? Y**es** **No** **DK**

How many days ago _____

On ARVs? Yes No

Regimen?

Duration? Yes No

Examination

General

Pallor	**Y**	**N**	**DK**
Wasting	**Y**	**N**	**DK**
Jaundice	**Y**	**N**	**DK**
Oral thrush	**Y**	**N**	**DK**
KS palate	**Y**	**N**	**DK**
KS skin	**Y**	**N**	**DK**
Lymphadenopathy	**Y**	**N**	**DK**

Observations at presentation

Axillary Temp	**°C**
Pulse	**bpm**
SBP	**mmHg**
DBP	**mmHg**
Dehydrated?	**Y / N**

Abdomen

Abdo tender	**Y**	**N**	**DK**
Hepatomegaly	**Y**	**N**	**DK**
Splenomegaly	**Y**	**N**	**DK**
Ascites	**Y**	**N**	**DK**

Chest

Heart murmur/s	**Y**	**N**	**DK**
Crackles unilat	**Y**	**N**	**DK**
Crackles bilat	**Y**	**N**	**DK**
Bronchial breathing	**Y**	**N**	**DK**
Effusion	**Y**	**N**	**DK**

Admission management:

Chest X-Ray		Yes	No
Result:	evidence of TB	Yes	No
Abdominal USS		Yes	No
Result:	evidence of TB	Yes	No
	Splenomegaly	Yes	No
HIV test		Yes	No
Result:		positive	negative

Antibiotics given:

Name

Dose

Duration

OUTCOMES **Choose one only**	**On Date**	**On Day** **Recruitment =** **day 0**
Died in hospital		
Discharged with appt		
Absconded without appt		

Specimens taken for study at presentation:

Consented: **Yes** **No**

Sample	**Taken**	**Lab number**	**Result**
Blood:			
FBC			
CD4			
Malaria film			
Plasma save			
Serum save			
Bone Marrow Aspirate			
Pax gene			
Mycobatcerial culture			

Bacterial Culture			
Stool Culture			
Hairy String			

Discharge Plan:

Antimicrobial therapy:

Drug 1:	**Drug 2:**
Dose:	**Dose:**
Duration:	**Duration:**

Transport offered:

Follow up date:

Understands where follow up will be:

2.2 1st Review (4-6 weeks after first presentation)

Date:

Antiviral history:

Attended group counselling? Yes No

On ARVs? Yes No

Regimen?

Date Started Yes No

Duration? (Days)

Clinically well? **Yes** **No**

If "no" proceed to document clinical features again

Been sick for …… days, with current / presenting illness

Symptom				**Time days**
Fever	**Y**	**N**	**DK**	
Sweats	**Y**	**N**	**DK**	
Cough	**Y**	**N**	**DK**	
Sputum produced	**Y**	**N**	**DK**	
Chest pain	**Y**	**N**	**DK**	
SOB	**Y**	**N**	**DK**	
Acute diarrhoea	**Y**	**N**	**DK**	
Blood in diarrhoea	**Y**	**N**	**DK**	
Vomiting	**Y**	**N**	**DK**	
Abdo Pain	**Y**	**N**	**DK**	
Dysuria	**Y**	**N**	**DK**	
Urinary frequency	**Y**	**N**	**DK**	
Headache	**Y**	**N**	**DK**	
Confusion, ↓LOC	**Y**	**N**	**DK**	
Fits	**Y**	**N**	**DK**	

Past History:			
Weight loss >3/12	**Y**	**N**	**DK**
Diarrhoea >3/12	**Y**	**N**	**DK**
Fever > 3/12	**Y**	**N**	**DK**
Cough > 3/12	**Y**	**N**	**DK**
Shingles	**Y**	**N**	**DK**
Generalised pruritis	**Y**	**N**	**DK**
Thrush	**Y**	**N**	**DK**
TB smear +	**Y**	**N**	**DK**
TB smear -	**Y**	**N**	**DK**
Crypto meningitis	**Y**	**N**	**DK**
Recurrent pneumonia	**Y**	**N**	**DK**
Dysphagia	**Y**	**N**	**DK**
Kaposi's sarcoma	**Y**	**N**	**DK**
Neurol. disability	**Y**	**N**	**DK**

Further antibiotics taken? **Yes** **No** **DK**

If yes:

Antibiotic	Amoxil	SP	Cipro	MTZ	Ery
Dose					
Duration (days)					

Examination

General

Pallor	**Y**	**N**	**DK**
Wasting	**Y**	**N**	**DK**
Jaundice	**Y**	**N**	**DK**
Oral thrush	**Y**	**N**	**DK**
KS palate	**Y**	**N**	**DK**
KS skin	**Y**	**N**	**DK**
Lymphadenopathy	**Y**	**N**	**DK**

Observations at presentation

Axillary Temp	**°C**
Pulse	**bpm**
SBP	**mmHg**
DBP	**mmHg**
Dehydrated?	**Y / N**

Abdomen

Abdo tender	**Y**	**N**	**DK**
Hepatomegaly	**Y**	**N**	**DK**
Splenomegaly	**Y**	**N**	**DK**
Ascites	**Y**	**N**	**DK**

Chest

Heart murmur/s	**Y**	**N**	**DK**
Crackles unilat	**Y**	**N**	**DK**
Crackles bilat	**Y**	**N**	**DK**
Bronchial breathing	**Y**	**N**	**DK**
Effusion	**Y**	**N**	**DK**

1st Review: Samples taken

Sample	Taken	Lab no	Result
Blood:			
Culture (IF UNWELL)			
CD4			
Plasma save			
Serum save			
Bone Marrow Aspirate			
Pax gene			
Mycobatcerial culture			
Bacterial Culture			
Stool Culture			
Hairy String			

2.3 2nd Review (2-3 months after presentation):

Date:

Antiviral history:

Attended group counselling? Yes No

On ARVs? Yes No

Regimen?

Date Started Yes No

Duration? (Days)

History:

Temperature:

Sample	Taken	Lab no	Result
Blood:			
Culture (IF UNWELL)			
CD4			
Plasma save			
Serum save			
Stool Culture			

2.4 3rd Review (3-4 months after presentation):

Date:

Antiviral history:

Attended group counselling? Yes No

On ARVs? Yes No

Regimen?

Date Started Yes No

Duration? (Days)

History:

Temperature:

Sample	Taken	Lab no	Result
Blood:			

Culture (IF UNWELL)			
CD4			
Plasma save			
Serum save			
Stool Culture			

2.5 4th Review (1 year after presentation)

Date:

Antiviral history:

Attended group counselling? Yes No

On ARVs? Yes No

Regimen?

Date Started Yes No

Duration? (Days)

History:

Temperature:

Sample	**Taken**	**Lab no**	**Result**
Blood:			
CD4			
Plasma save			
Serum save			

3. Ethical Approvals

3.1 Approval for adult BSI study

UNIVERSITY OF MALAWI

Principal

Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.:

Your Ref.: P.05/08/672

College of Medicine
Private Bag 360
Chichiri
Blantyre 3
Malawi
Telephone: 677 245
677 291
Fax: 674 700
Telex: 43744

28 May 2009

Prof. R.S Heyderman
Wellcome Trust
Blantyre 3

Dear Prof Heyderman,

RE: P.05/08/672 – Surveillance for invasive bacterial infections in febrile adults in Malawi

I write to inform you that COMREC reviewed and **approved** the amendments of the above mentioned proposal. The amendment was approved at COMREC's meeting of 27th May 2009.

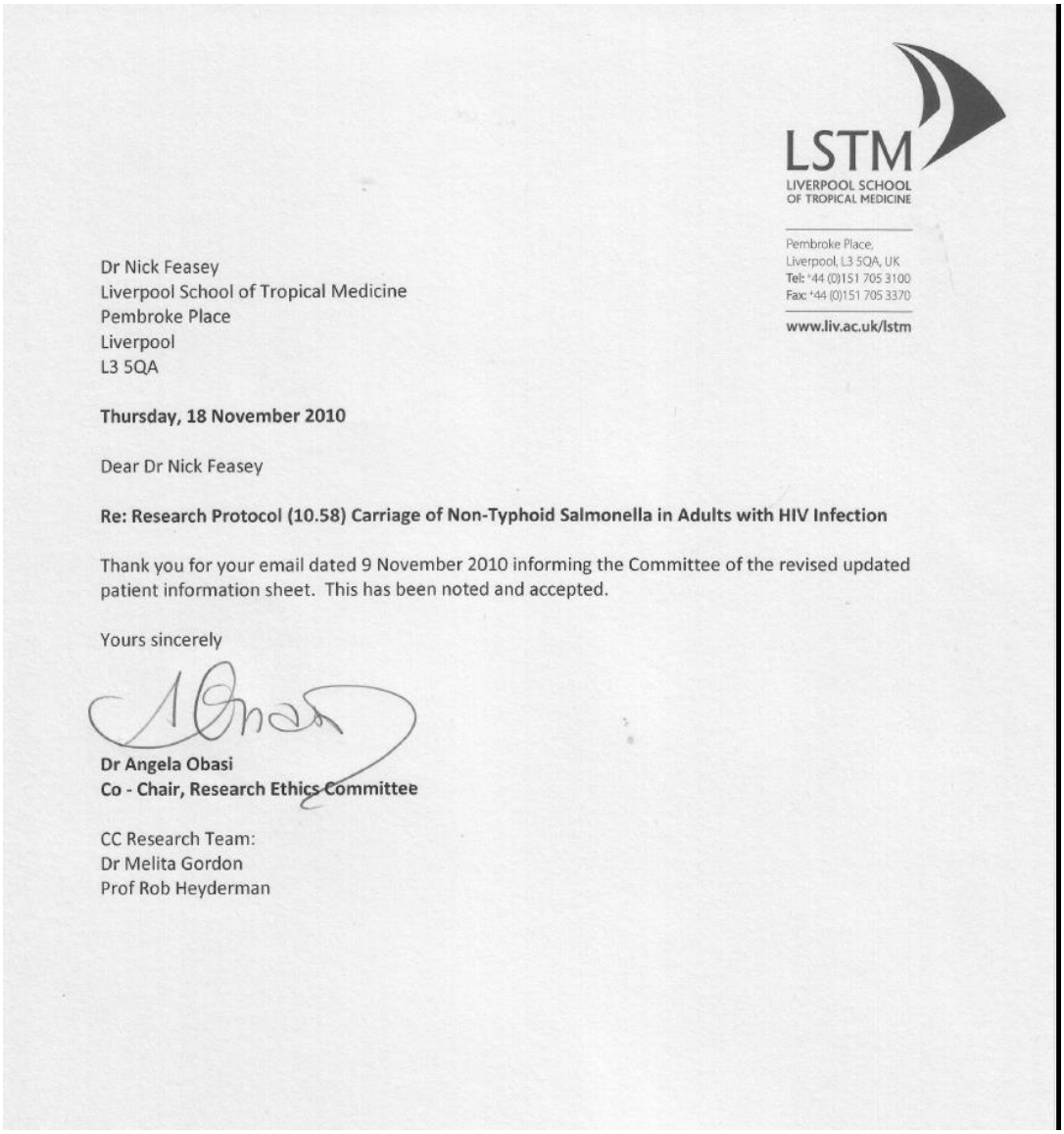
As you proceed with the implementation of your study we would like you to take note that all requirements by the college are followed as indicated on the attached page.

Sincerely,

Prof. J.M.Mfutso-Bengo
CHAIRMAN - COMREC

JMMB/ck

3.2 Ethical Approval for adult iNTS cohort from LSTM and University of Malawi COMREC



LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE

Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44 (0)151 705 3100
Fax: +44 (0)151 705 3370
www.liv.ac.uk/lstm

Dr Nick Feasey
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Thursday, 18 November 2010

Dear Dr Nick Feasey

Re: Research Protocol (10.58) Carriage of Non-Typhoid Salmonella in Adults with HIV Infection

Thank you for your email dated 9 November 2010 informing the Committee of the revised updated patient information sheet. This has been noted and accepted.

Yours sincerely

Dr Angela Obasi
Co - Chair, Research Ethics Committee

CC Research Team:
Dr Melita Gordon
Prof Rob Heyderman

UNIVERSITY OF MALAWI

Principal

Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.:

Your Ref.: P.07/09/808

College of Medicine
Private Bag 360
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Blantyre 3
Malawi
Telephone: 877 245
877 291
Fax: 874 700
Telex: 43744

30th September 2009

Prof. R.S Heyderman
Wellcome Trust
P/O Box 30096,
Blantyre

Dear Prof. Heyderman,

RE: P.07/09/808 – Carriage of non-Typhoid Salmonella in Adults with HIV Infection

I write to inform you that COMREC reviewed the above mentioned proposal which you submitted at its meeting of 30th September 2008. I am pleased to inform you that your proposal was approved.

As you proceed with the implementation of your study we would like you to take note that all requirements by the College are followed as indicated on the attached page.

Yours Sincerely,

UNIVERSITY OF MALAWI
COLLEGE OF MEDICINE
30 SEP 2009
COMREC
P/BAG 360, CHICHIRI, BLANTYRE 3

Prof. J.M.Mfutso-Bengo
CHAIRMAN - COMREC

JMMB/ck

4 Appendices to chapter 3

In view of the amount of data on antimicrobial resistance for the 4 types of *Salmonella*, these tables have been presented separately, for each of *S*. Typhimurium, *S*. Enteritidis, *Salmonella sp.* and *S*. Typhi. The data are presented in tables for each serotype: the top half of the table presents numbers (count) of each drug susceptibility phenotype, and the lower half presents proportion.

Table A1: Trends in drug susceptibility of *S.* Typhimurium (1998-2013)

Count	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Fully susceptible	5	5	6	2	9	19	8	15	13	5	12	10	8	4	7	2
Cotrim	0	0	1	1	4	8	2	0	7	1	1	0	1	1	2	1
Chlor	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Chlor/cotrim	0	0	0	0	1	1	2	3	0	0	1	0	1	2	0	1
Amoxil	25	22	21	13	18	15	17	9	6	0	3	3	2	2	3	3
Amox/Cotrim	220	257	270	191	104	82	51	34	31	3	10	22	29	33	9	13
Amox/Chlor	0	0	1	0	3	9	2	0	9	0	4	0	1	0	0	0
MDR	1	7	66	231	654	813	586	567	495	497	438	238	153	141	97	78
Total	253	298	382	454	847	1031	704	718	562	508	472	279	200	183	118	98
Proportion																
Fully susceptible	2	2	2	0	1	2	1	2	2	1	3	4	4	2	6	2
Cotrim	0	0	0	0	0	1	0	0	1	0	0	0	1	1	2	1
Chlor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlor/cotrim	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
Amoxil	10	7	5	3	2	1	2	1	1	0	1	1	1	1	3	3
Amox/Cotrim	87	86	71	42	12	8	7	5	6	1	2	8	15	18	8	13
Amox/Chlor	0	0	0	0	0	1	0	0	2	0	1	0	1	0	0	0
MDR	0	2	17	51	77	79	83	79	88	98	93	85	77	77	82	80
Missing data	1	2	4	4	6	8	5	13	0	0	1	2	3	0	0	0

Table A2: Trends in drug susceptibility of *S.* Enteritidis (1998-2013)

Count	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Fully susceptible	44	41	38	38	27	30	41	36	26	11	20	19	13	14	10	6
Cotrim	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Chlor	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	2
Chlor/cotrim	0	0	1	2	1	0	0	1	0	0	0	0	1	1	0	0
Amoxil	0	0	1	0	1	0	1	2	0	1	1	0	0	0	3	0
Amox/Cotrim	1	0	0	2	1	0	0	2	1	0	0	0	1	0	0	1
Amox/Chlor	1	1	9	23	3	1	1	1	2	1	0	0	0	0	0	0
MDR	1	141	142	170	87	53	24	26	18	34	20	17	16	7	5	3
Total	48	186	199	240	137	95	77	76	49	47	44	36	33	22	19	12
Proportion																
Fully susceptible	92	22	19	16	20	32	53	47	53	23	45	53	39	64	53	50
Cotrim	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Chlor	2	0	0	0	0	1	0	0	2	0	0	0	0	0	5	17
Chlor/cotrim	0	0	1	1	1	0	0	1	0	0	0	0	3	5	0	0
Amoxil	0	0	1	0	1	0	1	3	0	2	2	0	0	0	16	0
Amox/Cotrim	2	0	0	1	1	0	0	3	2	0	0	0	3	0	0	8
Amox/Chlor	2	1	5	10	2	1	1	1	4	2	0	0	0	0	0	0
MDR	2	76	71	71	64	56	31	34	37	72	45	47	48	32	26	25
Missing data	0	2	4	2	12	9	13	11	2	0	7	0	6	0	0	0

Table A3: Trends in drug susceptibility of *Salmonella sp.* (1998-2013)

Count	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Fully susceptible	9	19	25	14	14	14	14	13	11	1	2	7	4	4	2	5
Cotrim	0	0	2	1	0	2	0	0	0	0	0	0	1	1	0	0
Chlor	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Chlor/cotrim	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amoxil	0	0	3	0	2	1	1	0	0	0	1	0	0	0	0	0
Amox/Cotrim	0	3	7	5	15	5	3	1					2	1	1	1
Amox/Chlor	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MDR	0	5	13	14	35	35	38	16	5	8	8	11	13	2	1	1
Total	10	34	51	35	72	62	60	34	16	9	12	18	20	8	4	7
Proportion																
Fully susceptible	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Cotrim	90	56	49	40	19	23	23	38	69	11	17	39	20	50	50	71
Chlor	0	0	4	3	0	3	0	0	0	0	0	0	5	13	0	0
Chlor/cotrim	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Amoxil	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amox/Cotrim	0	0	6	0	3	2	2	0	0	0	8	0	0	0	0	0
Amox/Chlor	0	9	14	14	21	8	5	3	0	0	0	0	10	13	25	14
MDR	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Missing data	0	15	25	40	49	56	63	47	31	89	67	61	65	25	25	14
Unknown	10	6	2	0	8	8	7	12	0	0	8	0	0	0	0	0

Table A4: Trends in drug susceptibility of *S.* Typhi (1998-2013)

Count	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Fully susceptible	23	11	5	2	4	6	16	16	18	6	16	16	8	18	11	11
Cotrim	1	0	0	0	0	0	0	0	0	0	0	2	2	1	1	1
Chlor	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Chlor/cotrim	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Amoxil	0	0	0	0	0	0	0	1	0	0	0	0	0	2	1	1
Amox/Cotrim	0	2	0	0	0	1	0	0	0	0	0	0	1	0	3	5
Amox/Chlor	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
MDR	0	0	0	2	0	0	0	0	0	4	0	0	6	42	168	731
Total	24	13	5	4	4	9	17	19	18	10	16	19	18	67	186	750
Proportion																
Fully susceptible	96	85	100	50	100	67	94	84	100	60	100	84	44	27	6	1
Cotrim	4	0	0	0	0	0	0	0	0	0	0	11	11	1	1	0
Chlor	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Chlor/cotrim	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Amoxil	0	0	0	0	0	0	0	5	0	0	0	0	0	3	1	0
Amox/Cotrim	0	15	0	0	0	11	0	0	0	0	0	0	6	0	2	1
Amox/Chlor	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
MDR	0	0	0	50	0	0	0	0	0	40	0	0	33	63	90	97
Unknown	0	0	0	0	0	22	6	11	0	0	0	5	6	1	1	0

5 Appendices to Chapter 8

Table A5: Comparison of the variable regions of P125019, A1636, A7830 *S.* Gallinarum and *S.* Typhimurium LT2 adapted from Thomson et al 2008

Label$	PT4 CDS range	GAL CDS range	Locus tag	Size in PT4	General description of locus	Where present
bcf	SEN0020-SEN0027	SG0023-SG0030	bcf	7.6 kb	Fimbrial operon (bovine colonisation factor)	GAL PT4 LT2 A7830
sti	SEN0179-SEN0182	SG0177-SG0180	sti	4.9 kb	Fimbrial operon (*S.* Typhi I)	GAL PT4 LT2 A7830
stf	SEN0200-SEN0205	SG0199-SG0204	stf	6.4 kb	Fimbrial operon (*S.* Typhi F)	GAL PT4 LT2 A7830
4	SEN0216	NP	ROD 4	3 kb	Viral enhancing factor (metalloprotease)	PT4 A7830 Not: LT2 or GAL
SPI-6	SEN0267-SEN0290	SG0263-SG0318	SPI-6	17.6 kb (44 kb)	Salmonella Pathogenicity Island (tRNA-asp)	GAL PT4 LT2 A7830 (variable in all)
saf	SEN0281-SEN0284	SG0308-SG0312	saf	4.3 kb	Part of SPI-6. Fimbriae: Salmonella atypical fimbriae	GAL PT4 LT2 A7830
stb	SEN0319-SEN0323	SG0346-SG0350	stb	5.9 kb	Fimbrial operon (*S.* Typhi B)	GAL PT4 LT2 A7830
fim	SEN0524-	SG0555-	fim	9 kb	Fimbrial operon	GAL PT4 LT2

	SEN0533	SG0564			(tRNA-arg)	Pseudogene in A7830
SPI-16	SEN0535-SEN0537	SG0567-SG0569	SPI-16	3.3 kb	phage remnant. Cargo: LPS modification genes (tRNA-arg)	GAL PT4 LT2 A7830
SPI--14	SEN0800-SEN0805	SG0835-SG0840	SPI-14	6.8 kb	Electron transfer & regulatory CDS	GAL PT4 LT2 A7830
ΦSE10	SEN0908A-SEN0921	NP	SE10	8.2 kb	Prophage (remnant). Related to Gifsy-2. Cargo: sseI, gtgE, gtgF	PT4 LT2, NI: GAL Further degradation to A7830
SPI-5	SEN0951-SEN0958	SG0976-SG0985	SPI-5	6.6 kb	Salmonella Pathogenicity Island (tRNA-ser)	GAL PT4 LT2 A7830
9	SEN0995-SEN1013B	SG1023-SG1058	ROD 9	13.7 kb (42 kb)	RHS element, exported proteins & an IcmF-like CDS	GAL. NI: LT2, degenerate in PT4 & A7830
ΦSE12	SEN1131-SEN1156	SG1180-SG1234	fSE12[fSG12]	18 kb(45 kb)	Prophage (remnant). Related to Gifsy-2 Cargo: sodCI, ompX*, sopE, gogA*, hokW	GAL. NI: LT2, degenerate in PT4 & A7830 (loss of sodCI)
ΦSE12A	SEN1158-SEN1171B	SG1236-SG1249	fSE12A [fSG12A]	8 kb	Prophage (remnant). Related to Gifsy-2 Cargo: pagK, pagM*	GAL PT4 LT2 A7830
ΦSE14	SEN1378-	NP	fSE1	12.6 kb	Prophage (remnant)	PT4, NI: LT2 GAL

	SEN1398		4			A7830
13	SEN1432-SEN1436	SG1499-SG1503	ROD 13	6 kb	possible hexonate uptake and catabolism operon	GAL PT4, NI LT2, degraded with pseudogene in A7830
14	SEN1499-SEN1509	SG1573*-SG1576*	ROD 14	11.7kb (1.8 kb)	Drug efflux system, pqaA	GAL PT4 LT2 A7830: variable in all
15	SEN1565-SEN1567	SG1636*	ROD 15	2.2 kb (0.6 kb)	exported protease	PT4 LT2 A7830, remnant in GAL
SPI-2	SEN1623-SEN1666	SG1694-SG1738	SPI-2	39.8 kb	Salmonella Pathogenicity Island (tRNA-val)	PT4 GAL LT2 A7830
17	SEN1751-SEN1758	SG1824-SG1832	ROD 17	9.3kb	Major facilitator-family transport system	PT4 A7830 GAL, remnant in LT2 (1370980-1371012)
18	SEN1766-SEN1769	SG1841*	ROD 18	6.9 kb (0.3 kb)	MipA – outer-membrane scaffold	PT4 A7830 LT2; remnant in GAL
csg	SEN1903-SEN1909	SG1977-SG1983	csg	4.4 kb	curli fimbrial operon	PT4 GAL LT2 A7830
ΦSE20	SEN1919A-SEN1966	NP	fSE20	40.6 kb	Prophage. Related to ST64B. Cargo: sopE*, sspH1*, orgA (tRNA-ser)	PT4 only NOT: GAL LT2 A7830
21	SEN1970-SEN1999	SG1996-SG2025	ROD 21	26.5 kb	Genomic Island (24 bp DR; tRNA-asn)	PT4 GAL, NOT: LT2 A7830
22	SEN2085A-SEN2085D	SG2117-SG2120	ROD 22	4.9 kb	Group D LPS O-chain genes rfbE and rfbS	PT4 A7830 GAL, different genes in LT2 (group B O-chain)

peg	SEN2144A-SEN2145B	SG2182-SG2186	peg	4.8 kb	Fimbrial operon	PT4 A7830 GAL, NI: LT2
SPI-17	SEN2375A-SEN2378	SG2425-SG2430A	SPI-17	3.6 kb	Prophage remnant. Cargo: gtrA, gtrB* gtrC (tRNA-arg)	PT4 A7830 GAL, NI: LT2
25	SEN2471-SEN2473	SG2522-SG2524	ROD 25	4kb	membrane transport system	PT4 A7830 GAL, remnant in LT2 (STM2492*)
SPI-9	SEN2609-SEN2612	SG2666-SG2671	SPI-9	16.3 kb	Salmonella Pathogenicity Island (10Sa_RNA)	PT4 A7830 GAL LT2
SPI-1	SEN2703-SEN2744	SG2764-SG2806	SPI-1	40.2 kb	Salmonella Pathogenicity Island	PT4 A7830 GAL LT2
28	SEN2746-SEN2746A	SG2808-SG2811	ROD 28	2 kb	Membrane proteins	PT4 A7830 GAL, NI: LT2
ste	SEN2794-SEN2799	SG2859-SG2864	ste	5.6 kb	Fimbrial operon (*S.*Typhi E)	PT4 A7830 GAL, Remnant in LT2 (major pilin remnant: 3102016-3102150)
30	SEN2864-SEN2878	SG2930*	ROD 30	13 kb (156 bp)	rcnA, the std fimbrial, operon	PT4 LT2. Remnant in GAL A7830, rcnA is pseudogene
std	SEN2871-SEN2873	NP	std	5.0 kb	Within ROD30. Fimbrial operon S. Typhi D)	PT4 A7830 LT2, NI: GAL
SPI-13	SEN2960-SEN2966	SG3011-SG3017	SPI-13	7.4 kb	Salmonella Pathogenicity Island	PT4 A7830 GAL LT2

					(tRNA-phe)	
lpf	SEN3459-SEN3463	SG3793-SG3798	lpf	5.5 kb	Fimbrial operon (long polar fimbriae)	PT4 A7830 GAL LT2
SPI-3	SEN3572-SEN3586	SG3665-SG3680	SPI-3	16.6 kb	Salmonella Pathogenicity Island (tRNA-selC)	PT4 A7830 GAL LT2
34	SEN3896-SEN3898	SG3312-SG3314	ROD 34	4.2 kb	Amino acid metabolic CDS	PT4 A7830 GAL, NI: LT2
35	SEN3977-SEN3981	SG4054-SG4058	ROD 35	4.5 kb	Membrane transport system	PT4 A7830 GAL, NI: LT2
SPI-4	SEN4026-SEN4032	SG4100-SG4105	SPI-4	25 kb	Salmonella Pathogenicity Island	PT4 A7830 GAL LT2
37	SEN4165-SEN4166	SG4241-SG4242	ROD 37	3 kb	exported proteins	PT4 A7830 GAL, NI: LT2
SPI-10	SEN4244-SEN4254	SG4311-SG4325	SPI-10	10 kb	Salmonella Pathogenicity Island (tRNA-leu)	GAL PT4 A7830
sef	SEN4247-SEN4251	SG4318-SG4322	sef	5.1 kb	Fimbrial operon (Salmonella Enteritidis fimbriae)	PT4 A7830 GAL, NI: LT2
40	SEN4283-SEN4292	SG4349-SG4357	ROD 40	13.5 kb (10 kb)	Immigration control region	PT4 A7830 , degenerate in GAL, NI: LT2
sth	SEN4347-SEN4351	SG4413-SG4417	sth	5.5 kb	Fimbrial operon (*S.* Typhi H)	PT4 GAL LT2 A7830
42	SEN3843*-SEN3844*	SG3367-SC3368	ROD 42	1 kb	C4-dicarboxylate transporters	GAL LT2, Deleted from PT4 & A7830

[$] Labels used to mark these regions on the outer ring of Figure 2.

*gene remnant

[@] numbers in parentheses indicate that the size of the analogous region in *S.* Gallinarum. Only shown where significantly different from that found in *S.* Enteritidis PT4.

[%] square brackets indicate the name in *S.* Gallinarum 287/91

NI: – not detected in:

LPS – lipopolysaccharide locus

LT2 – *S.* Typhimurium LT2

GAL – *S.* Gallinarum 287/91

PT4 – *S.* Enteritidis PT4

Table A6: Summary of the common traits identified amongst the functions of genes lost independently by *S.* Typhi CT18, *S.* Gallinarum 287/91 and A7830

Process/Pathway	*S.* Gallinarum 278/91$	*S.* Typhi CT18£	A7830
Cell interactions			
	slrP, sopA, sifB, sspH2, sinH, pipB2, pagK, bigA	sopD2(STY0971)	sodCI
		sopA(STY2275) sopE2(STY1987) sseJ(STY1439a) cigR(STY4024) misL(STY4030) marT(STY4027) sivH(STY2767)	
		slrP(STY0833) bigA(STY4318)	
Feacal Shedding			
	shdA, ratB	shdA(STY2755), ratB(STY2758), sivH(STY2767)	shdA
Fimbriae			
	stdD, stiC, stfF, safC, stbC, pegC, lpfC, sefD & C, sthB C & E	bcfC(STY0026) fimI(STY0590) steA(STY3084)	fimY
		safE(STY0333) stgC(STY3920) ushA(STY0539) sefA(STY4836a) sefD(STY4839) sefR(STY4841)	
		sthC(STY4938) sthE(STY4938)	
Flagella/motility			
	cheM flhB flhA flgK flgI	fliB(STY2166)	
Type I restriction modification			
	hsdR hsdM	hsdM(STY4833)	
Type III restriction modification			

restriction enzyme StyLT1	mod	res(STY0389)	
Cobalamine biosynthesis			
	pocR, cobD, cbiD, cbiC	cbiM(STY2226) cbiK(STY2229) cbiJ(STY2231)	pocR
Propanediol utilisation			
	pduG pduO	pduN(STY2254)	
Metal/drug resistance and transport			
Copper	cusA cusS	cusA (STY0610)	
		cusS(STY0609a)	
Nickel /Cobalt	rcnA	rcnA (STY3169)	rcnA
Nickel	nxiA	nxiA (STY2901)	
Acriflavin	acrF	acrE(STY3569) acrF(STY3570)	
Tetrathioante respiration			
	ttrB ttrC	ttrS(STY1735)	
Trehalose degradation/synthesis			
	treC	treA(STY1924)	
Hydrogenase I			
	hyaF	hyaB2(STY1525) hyaA(STY1319)	
Ornithine catabolism			
	speC	speC(STY3270)	SEN1740
		speF (STY0739)	
Amino acid catabolism			
L-serine/L-threonine	tdcG	tdcC(STY3426)	L-serine dehydratase
Cellulose biosynthesis			
	bcsG	bcsC (STY4184)	
Surface polysaccharide			
LPS O-chain	gtrB	gtrB(STY2627b)	

LPS core	rfaZ (waaZ)	wcaK(STY2311) wcaD(STY2324) wcaA(STY2328)	SEN2279
Alternative terminal electron acceptors			
dimethyl sulfoxide reductase	dmsA2	dmsA2(STY4503) dmsB2(STY4506)	SEN2510
	dmsA1		
Trimethylamine N-oxide (TMAO)	torS	torR(STY3954) torC(STY3955)	
Carbon source			
D-Glucarate uptake & degradation	gudD	gudP(STY4097)	
Rhamnose degradation	rhaR	rhaD(STY3828)	
Maltose/Maltodextrins uptake & degradation	malY	malY(STY1657a) malX(STY1657)	
Fuculose			L fuculose kinase
Glycerol			Glycerol dehydrogenase
Formate metabolism			Formate dehydrogenase

[£]Parkhill et al., 2001 (Parkhill et al. 2001)

Table A7: Pseudogenes of A7830

Locus Tag	Nature of change	Gene function
SEN0005	Frameshift	Putative amino-acid transport protein
SEN0008	Frameshift	Conserved hypothetical protein
SEN0035	Frameshift	Putative secreted sulfatase
SEN0110B	SNP generating early stop codon	Hypothetical gene
SEN0336	Frameshift	Putative cation transport ATPase
SEN0354	Insertion mid gene	Propionyl-coA synthetase
SEN0531	Frameshift	Fimbriae Y protein
SEN0546	Frameshift	Outer membrane esterase
SEN1006	Frameshift	Hypothetical protein
SEN1101	SNP generating early stop codon	Putative hydrolase
SEN1393	Insertion mid gene	Phage protein
SEN1396	SNP generating early stop codon	Putative phage encoded exported protein
SEN1434	Frameshift	Putative hexonate sugar transport protein
SEN1468	Insertion mid gene	Probable tonb-dependent receptor yncd precursor
SEN1504	Partial deletion	Putative isomerase
SEN1624	Frameshift	Putative type III secretion protein
SEN1694	Frameshift	Putative ligase/synthetase
SEN1740	SNP generating early	Succinylornithine transaminase

	stop codon	
SEN1742	SNP generating early stop codon	Putative mutT-family protein
SEN1914	Frameshift	Hypothetical protein
SEN2034	Frameshift	Pdu/cob regulatory protein pocR
SEN2122	SNP generating early stop codon	Putative efflux system protein
SEN2297	SNP generating early stop codon	Lipoprotein
SEN2493	Frameshift	Shda host colonisation factor
SEN2510	Partial deletion	Dimethyl sulfoxide reductase
SEN2701	Insertion mid gene	Transcriptional activator of the formate hydrogenlyase system
SEN2702	Frameshift	Conserved hypothetical protein
SEN2821	Frameshift	L-fuculose kinase
SEN2867	Partial deletion	Rcna (nickel & cobolat res)
SEN2876	Frameshift	Putative membrane protein
SEN2942	Partial deletion	Type II secretion, ATP-binding, protein
SEN2985	SNP generating early stop codon	Possible ABC-transport protein, periplasmic-binding component
SEN3034	Frameshift	Probable arylsulfate sulfotransferase
SEN3080	Partial deletion	Putative membrane transport protein
SEN3081	Frameshift	L-serine dehydratase
SEN3200	Frameshift	Probable lysr-family transcriptional regulator

SEN3409	Partial deletion	Hypothetical ABC transporter ATP-binding protein
SEN3432	Insertion mid gene	Hypothetical metabolite transport protein
SEN3902	Frameshift	Glycerol dehydrogenase
SEN3930	Expanding repeat	Elongation factor Tu
SEN4056	Frameshift	Formate dehydrogenase H
SEN4208	SNP generating early stop codon	Putative membrane protein
SEN4277	SNP generating early stop codon	Putative membrane protein

Table A8: Genes of A7830 with NS-SNPs

Locus Tag	Position (A7830)	Product	Locus Tag	Position (A7830)	Product
SEN0020	24235..24777	fimbrial subunit	**SEN2119**	2151125..2154115	putative membrane protein
SEN0046	4664311..4667145	isoleucyl-tRNA synthetase	**SEN2122**	2156879..2158120	putative efflux system protein
SEN0054	complement(4672250..4673869)	sensor kinase	**SEN2126**	2165732..2167135	putative two-component system sensor kinase
SEN0061	3389..4258	citrate lyase beta chain	**SEN2133**	complement(2174279..2174695)	conserved hypothetical protein
SEN0065	7406..8227	dihydrodipicolinate reductase	**SEN2140**	2180347..2181207	putative sugar kinase
SEN0068	13757..14152	transcriptional activator CaiF	**SEN2154**	2197884..2198615	putative transcriptional regulator
SEN0080	25389..26729	putative metabolite transport protein	**SEN2156**	complement(2198763..2199494)	putative permease transmembrane component
SEN0104	complement(52742..54451)	L-ribulokinase	**SEN2158**	complement(2200418..2201587)	putative permease transmembrane component
SEN0110B	complement(62033..62359)	hypothetical gene	**SEN2160**	complement(2202689..2204986)	periplasmic beta-glucosidase precursor
SEN0115	68039..68983	probable activator protein in leuABCD operon	**SEN2165**	complement(2209519..2210280)	putative oxidoreductase
SEN0129	83427..84494	acetylglucosamine transferase	**SEN2180**	2224145..2225380	putative oxidoreductase
SEN0134	89153..90304	cell division protein FtsZ	**SEN2181**	complement(2225455..2226465)	galactoside transport system permease protein MglC
SEN0136	92160..94865	preprotein translocase SecA subunit	**SEN2183**	complement(2228135..2229133)	D-galactose-binding periplasmic protein precursor
SEN0152	complement(106056..107006	putatuve glycosysl hydrolase	**SEN2208**	2256074..2257630	rtn protein
SEN0164	complement(124568..124891)	conserved hypothetical protein	**SEN2012**	2261673..2263262	putative ABC-transporter ATP-binding protein
SEN0168	127970..128953	4- phosphohydroxy-L-threonine dehydrogenase	**SEN2273**	complement(2320258..2321475)	putative MR-MLE-family protein

SEN0175	complement(140641..143187)	Putativie fimbrial usher	**SEN2276**	complement(2323820..2324467)	conserved hypothetical protein
SEN0184	145356..146585	conserved hypothetical protein	**SEN2281**	2328137..2330119	putative lipopolysaccharide modification protein
SEN0207	complement(172823..174103)	Glutamate-1-semialdehyde 2,1-aminomutase	**SEN2297**	complement(2343729..2345519)	lipoprotein
SEN0210	complement(176221..176844	putative membrane protein	**SEN2311**	complement(2361946..2362098)	hypothetical protein
SEN0215	181684..182841	carbohydrate diacid regulator	**SEN2332**	2383253..2384146	conserved hypothetical protein
SEN0216	complement(182898..185929	putative viral enhancing factor	**SEN2341**	complement(2391820..2393241)	putative amino acid transporter
SEN0217	complement(186115..186501	conserved hypothetical protein	**SEN2375**	2426874..2427796	putative membrane protein
SEN0242	217016..217405	conserved hypothetical protein	**SEN2383**	complement(2435538..2437544)	phosphoglycerate transport system sensor protein PgtB
SEN0243	217549..218760	cell cycle protein MesJ	**SEN2387**	2441600..2442520	putative acyltransferase
SEN0262	complement(235168..236535	membrane-bound lytic murein transglycosylase d precursor	**SEN2395**	2452344..2453546	nucleoside permease NupC
SEN0281	252024..252533	lipoprotein	**SEN2404**	2462987..2463748	conserved hypothetical protein
SEN0284	325633..326103	fimbrial structural subunit	**SEN2408**	complement(2466041..2468056)	DNA ligase
SEN0287	258424..259371	LysR-family transcriptional regulator	**SEN2424**	complement(2481927..2482760)	sulphate transport system permease protein CysT
SEN0312	282341..283762	3-isopropylmalate dehydratase large subunit 2	**SEN2432**	2488540..2489439	Coproporphyrinogen III oxidase
SEN0319	complement(288550..289308)	fimbrial chaperone protein	**SEN2441**	complement(2497548..2498735)	putative alchohol dehydrogenase
SEN0371	complement(290604..293165)	outer membrane fimbrial usher protein	**SEN2443**	complement(2499575..2500978)	putative aldehyde dehydrogenase
SEN0324	295141..295905	possible transmembrane regulator	**SEN2467**	complement(2528459..2529493)	putative lipoprotein

SEN0334	complement(303464..306631)	putative cation efflux protein	**SEN2471**	complement(2531623..2532762)	probable glycerate kinase
SEN0335	complement(306628..307854)	putative cation efflux pump	**SEN2472**	complement(2532768..2534057)	putative permease
SEN0340	312900..314858	type III restriction-modification system enzyme (StyLTI) modification methylase	**SEN2475**	2536843..2538306	putative exported protein
SEN0360	341408..342502	putative lipoprotein	**SEN2480**	2541929..2542981	Phosphoribosylformylglycinamidine cyclo-ligase
SEN0370	350016..350471	conserved hypothetical protein	**SEN2496**	complement(2576181..2577140)	putative exported protein
SEN0378	complement(356052..359192)	exonuclease SbcC	**SEN2500**	complement(2581489..2582667)	putative lipoprotein
SEN0403	complement(383410..384384)	putative oxidoreductase	**SEN2502**	complement(2583312..2584586)	Histidyl-tRNA synthetase
SEN0419	400055..401566	Putative periplasmic protein	**SEN2508**	complement(2589926..2590735)	putative anaerobic reductase component
SEN0431	413879..415150	ATP-dependent clp protease ATP-binding subunit ClpX	**SEN2509**	complement(2590728..2591357)	putative anaerobic reductase component
SEN0440	complement(424894..425949)	putative lyase	**SEN2510**	complement(join(2591354..2593120,2593117..2593686))	dimethyl sulfoxide reductase
SEN0454	complement(437928..438146)	haemolysin expression modulating protein	**SEN2511**	complement(2593815..2596130)	penicillin-binding protein 1C
SEN0488	476353..477039	hypothetical ABC transporter ATP-binding protein	**SEN2515**	complement(2603599..2604384)	sseB protein
SEN0501	490996..492243	probable metabolite transport protein	**SEN2516**	complement(2604485..2605768)	Peptidase B
SEN0514	complement(505951..507018)	phosphoribosylaminoimidazole carboxylase ATPase subunit	**SEN2562**	complement(2654080..2655054)	signal peptidase I
SEN0520	complement(511284..511721)	putative exported protein	**SEN2569**	complement(2661843..2662580)	conserved hypothetical protein
SEN0523	complement(512600..513466)	FolD bifunctional protein [includes:	**SEN2572**	2665064..2665651	putative membrane protein

		methylenetetrahydrofolate dehydrogenase; methenyltetrahydrofolate cyclohydrolase			
SEN0524	514028..514570	type-1 fimbrial protein, a chain precursor	**SEN2581**	complement(2674182..2675483)	alpha-ketoglutarate permease
SEN0531	complement(join(521388..522038,522038..522109))	fimbriae Y protein	**SEN2582**	complement(2675587..2676042)	conserved hypothetical protein
SEN0539	complement(529130..529984)	hypothetical araC-family transcriptional regulator	**SEN2589**	complement(2683890..2684798)	conserved hypothetical protein
SEN0542	531931..532491	putative membrane protein	**SEN2609**	2702033..2713507	large repetitive protein
SEN0546	join(537073..537390,537390..539042)	outer membrane esterase	**SEN2634**	2780265..2781533	putative GAB DTP gene cluster repressor
SEN0557	617330..621214	enterobactin synthetase component F	**SEN2650**	2793277..2793522	putative glutaredoxin
SEN0572	567172..568332	putative aminotransferase	**SEN2672**	complement(2818261..2819340)	membrane-bound lytic transglycosylase B precursor
SEN0574	complement(568923..570158)	conserved hypothetical protein	**SEN2680**	complement(2824652..2826172)	putative sigma-54-dependent transriptional regulator
SEN0594	590309..591897	sensor kinase DpiB	**SEN2683**	complement(2829024..2831264)	hydrogenase maturation protein
SEN0595	592104..592784	transcriptional regulatory protein	**SEN2689**	complement(2833863..2834630)	formate hydrogenlyase subunit 7
SEN0600	596103..596891	possible hydrolase	**SEN2692**	complement(2836909..2837832)	formate hydrogenlyase subunit 4
SEN0603	complement(598480..599433)	lysR-family transcriptional regulator	**SEN2720**	complement(2863528..2865159)	tyrosine phosphatase

SEN0617	complement(611560..614142)	leucyl-tRNA synthetase	**SEN2741**	2885845..2886288	cell adherance/invasion protein
SEN0620	615959..616912	probable permease	**SEN2750**	complement(2894835..2896043)	possible membrane transport protein
SEN0635	complement(632607..634145	apolipoprotein N-acyltransferase	**SEN2781**	complement(2922390..2922944)	hypothetical protein
SEN0640	638490..639665	putative monooxygenase	**SEN2784**	2927617..2928570	possible secreted protein
SEN0644	complement(641373..643037)	asparagine synthetase B	**SEN2786**	complement(2929494..2931206)	sulfite reductase (NADPH) hemoprotein alpha subunit
SEN0670	complement(675005..676684)	potassium-transporting ATPase A chain	**SEN2800**	2946800..2947831	hypothetical protein
SEN0687	694521..695729	dihydrolipoamide succinyltransferase component (E2)	**SEN2803**	2951820..2954576	sensor protein
SEN0756	763484..764044	putative inner membrane protein	**SEN2806**	complement(2957201..2958541)	glucarate dehydratase-related protein
SEN0781	790695..791747	putative membrane protein	**SEN2812**	2963182..2964030	conserved hypothetical protein
SEN0783	792930..794522	ABC transporter ATP-binding protein	**SEN2813**	3007529..3008893	conserved hypothetical protein
SEN0794	805786..807657	hypothetical ABC transporter ATP-binding protein	**SEN2814**	2966072..2967361	putative serine transporter
SEN0835	complement(847989..848720)	arginine-binding periplasmic protein 1 precursor	**SEN2852**	complement(3011964..3013166)	putative membrane protein
SEN0890	916920..918152	conserved hypothetical protein	**SEN2853**	complement(3013159..3015318)	2-acylglycerophosphoethanolamine acyl transferase/acyl carrier protein synthetase
SEN0892	918383..919129	3-deoxy-manno-octulosonate cytidylyltransferase	**SEN2857**	3019362..3020297	transcriptional activator protein LysR
SEN0977	1044709..1045071	membrane protein, suppressor for copper-sensitivity A	**SEN2893**	complement(3053400..3053711)	conserved hypothetical protein

SEN0986A	complement(1052152..1056132)	proline dehydrogenase (proline oxidase)	**SEN2895**	complement(3055350..3056243)	hypothetical protein
SEN0992	1065235..1065927	putative secreted protein	**SEN2909**	complement(3072152..3072787)	putative membrane protein
SEN0998	complement(1069918..1070376)	putative exported protein	**SEN2913**	complement(3076447..3077493)	D-erythrose 4-phosphate dehydrogenase
SEN1010	1080668..1080976	putative transposase	**SEN2958**	complement(3116972..3119107)	ornithine decarboxylase, constitutive
SEN1017	1087111..1087806	conserved hypothetical protein	**SEN2985**	complement(3150270..3151322)	possible ABC-transport protein, periplasmic-binding component
SEN1040	complement(1105418..1107100)	flagellar basal-body M-ring protein	**SEN3009**	3170779..3172080	possible membrane transport protein
SEN1045	complement(1110476..1111960)	cytoplasmic alpha-amylase	**SEN3035**	3200515..3201186	disulfide isomerase
SEN1065	1128380..1129819	putative exported protein	**SEN3041**	3204616..3206295	putative exported protein
SEN1068	complement(1131337..1132548)	tyrosine-specific transport protein	**SEN3043**	complement(3208235..3211078)	adenyl-transferase
SEN1077	complement(1139666..1140094)	conserved hypothetical protein	**SEN3073**	3242147..3242632	putative membrane protein
SEN1090	1152658..1154736	flagellar biosynthesis protein FlhA	**SEN3080**	complement(join(3247553..3247954,3247951..3248805))	putative membrane transport protein
SEN1101	complement(1165174..1165740)	putative hydrolase	**SEN3092**	3265022..3266293	putative sugar kinase
SEN1117	1181124..1182599	glucose 6-phosphate dehydrogenase	**SEN3095**	3267091..3268464	PTS system, galactitol-specific IIC component
SEN1118	1182834..1184645	6-phosphogluconate dehydratase	**SEN3013**	3282389..3282919	conserved hypothetical protein
SEN1137	1231197..1231796	hypothetical phage protein	**SEN3153**	3322320..3323753	RNA polymerase sigma-54 factor (sigma-N)
SEN1193	1237748..1238629	heat shock protein	**SEN3197**	3991020..3992987	putative membrane protein
SEN1197	1241626..1241769	putative membrane protein	**SEN3209**	3976177..3978117	putative lipoprotein
SEN1209	1250629..1252188	putative membrane protein	**SEN3304**	3412442..3413815	siroheme synthase
SEN1213	complement(1256159..1257523)	para-aminobenzoate synthase	**SEN3319**	3430583..3433159	penicillin-binding protein 1A

		component I			
SEN1246	complement(1288374..1289222)	hydrogenase-1 operon protein HyaF	**SEN3325**	complement(3438430..3440139)	putative membrane protein
SEN1255	complement(1296649..1297740)	putative ATP/GTP-binding protein	**SEN3353**	complement(3474609..3476162)	putative membrane protein
SEN1265	1307057..1307446	putative regulator	**SEN3361**	complement(3487892..3489868)	glycogen operon protein
SEN1267	1308297..1309151	aldolase	**SEN3380**	complement(3510411..3511727)	glycerol-3-phosphate-binding periplasmic protein
SEN1302	1351234..1351872	putative outer membrane protein	**SEN3387**	complement(3516442..3517551)	leucine-specific binding protein
SEN1317	1365634..1366680	putative protease	**SEN3408**	complement(3535882..3537006)	putative ABC-2 type superfamily transport protein
SEN1320	1370384..1371358	cys regulon transcriptional activator	**SEN3417**	complement(3547212..3549254)	oligopeptidase A
SEN1328	complement(1380101..1380853)	putative regulatory protein	**SEN3424**	complement(3556648..3557625)	putative phosphosugar-binding protein
SEN1330	complement(1383485..1385419)	exoribonuclease II	**SEN3430**	3563973..3564872	hypothetical lysR-family transcriptional regulator
SEN1341	complement(1394438..1396087	peptide transport periplasmic protein SapA precursor	**SEN3439**	complement(3577331..3580762)	Cellulose biosynthesis protein subunit C
SEN1348	1399435..1400838	putative ATP-binding protein	**SEN3480**	3627287..3628282	putative membrane protein
SEN1356	1408913..1409818	putative transcriptional regulator	**SEN3496**	3644883..3646379	putative L-xylulose kinase
SEN1364	1418595..1419419	putative exported protein	**SEN3516**	3672218..3673408	putative L-lactate dehydrogenase
SEN1386	complement(1437432..1438364	predicted phage protein	**SEN3532**	3688998..3689930	ADP-L-Glycero-D-mannoheptose-6-epimerase
SEN1404	complement(1455069..1455479)	heat shock protein (hslJ)	**SEN3558**	complement(3712119..3712976)	putative regulatory protein
SEN1406	1456819..1459455	putative exported protein	**SEN3571**	complement(3729770..3731152)	sodium:galactoside family symporter
SEN1413	1466255..1466785	cytochrome b561	**SEN3585**	complement(3744340..3747066)	Magnesium transport ATPase, P-type 2
SEN1417	complement(1469869..1470549)	putative amino acid ABC transporter	**SEN3608**	complement(3769481..3770797)	probable permease
SEN1426	1479105..1480730	methyl-accepting chemotaxis protein	**SEN3610**	3772073..3772858	putative DeoR-family transcriptional regulator

		III			
SEN1432	complement(1487731..1488450)	putative GntR-family regulatory protein	**SEN3612**	complement(3773154..3774842)	acetohydroxy acid synthase I, small subunit
SEN1447	1501381..1502394	tellurite resistance protein TehA	**SEN3659**	3817705..3819351	putative membrane protein
SEN1450	complement(1503855..1505033)	putative benzoate membrane transport protein	**SEN3663**	3823313..3824068	conserved hypothetical protein
SEN1454	complement(1508210..1509160)	hypothetical protein	**SEN3675**	complement(3835997..3837826)	glucosamine--fructose-6-phosphate aminotransferase
SEN1477	complement(1535369..1536913)	respiratory nitrate reductase 2 beta chain	**SEN3689**	complement(3851128..3851571)	MioC protein
SEN1478	complement(1536910..1540638)	respiratory nitrate reductase 2 alpha chain	**SEN3693**	complement(3854712..3856208)	conserved hypothetical protein
SEN1479	complement(1540730..1542118)	nitrite extrusion protein	**SEN3732**	3897794..3898873	conserved hypothetical protein
SEN1492	1589124..1589555	osmotically inducible protein C	**SEN3750**	complement(3920261..3921148)	chloramphenicol-sensitive protein RarD
SEN1513	1581523..1582626	uptake hydrogenase small subunit	**SEN3761**	3932571..3933914	putative regulatory protein
SEN1518	1586035..1586445	hydrogenase-1 operon protein HyaE2	**SEN3771**	3942323..3943117	putative deoxyribonuclease
SEN1538	complement(1604169..1604351	conserved hypothetical protein	**SEN3777**	complement(3949511..3951700)	large (alpha) subunit of the fatty acid-oxidizing multienzyme complex
SEN1544	complement(1611933..1612952)	starvation sensing protein RspB	**SEN3780**	3953874..3955325	trk system potassium uptake protein
SEN1551	1617031..1619472	putative dimethyl sulphoxide reductase subunit A	**SEN3807**	complement(4029520..4031556)	putative glycosyl hydrolase
SEN1552	1619571..1622006	putative dimethyl sulphoxide	**SEN3809**	complement(4032461..4033702)	conserved hypothetical protein

		reductase subunit			
SEN1560	1628037..1629326	Putative chloride channel protein clcB-like	**SEN3811**	complement(4034619..4035515)	putative oxidoreductase
SEN1569	complement(1637801..1639189)	pyridine nucleotide transhydrogenase subunit-beta	**SEN3812**	4035674..4036576	putative sugar kinase
SEN1578	1650114..1651043	Ter protein	**SEN3826**	complement(join(4045613..4048075,4048079..4048663))	formate dehydrogenase-O, major subunit
SEN1582	1654457..1655632	mannose-6-phosphate isomerase	**SEN3830**	4051207..4052265	putative lipoprotein
SEN1596	1670006..1670611	glutathione S-transferase	**SEN3846**	4067147..4067836	conserved hypothetical protein
SEN1598	complement(1671590..1672864)	tyrosyl-tRNA synthetase	**SEN3875**	complement(4093464..4094474)	putative glycerol metabolic protein
SEN1606	1677765..1679804	putative membrane protein	**SEN3888**	complement(4107127..4108917)	putative arylsulfate sulfotransferase
SEN1607	complement(1679780..1680301)	copper-zinc superoxide dismutase	**SEN3913**	complement(4137667..4139400)	putative membrane protein
SEN1608	complement(1680381..1681277)	putative oxidoreductase	**SEN3915**	complement(4142631..4143782)	acetylornithine deacetylase
SEN1614	complement(1684727..1685074)	conserved hypothetical protein	**SEN3920**	complement(4148337..4149737)	possible pyridine nucleotide-disulphide oxidoreductase
SEN1616	1686358..1686939	superoxide dismutase	**SEN3948**	complement(4181248..4182006)	thiamine biosynthesis protein
SEN1629	complement(1698979..1699356)	putative type III secretion protein	**SEN3959**	4190578..4191975	two-component system sensor protein
SEN1653	1716692..1719454	putative two-component sensor kinase	**SEN3969**	complement(4203902..4204726)	acetate operon repressor
SEN1659	complement(1723188..1724966)	putative histidine kinase,	**SEN3994**	complement(4228850..4229740)	maltose transport inner membrane protein
SEN1662	1726898..1729960	tetrathionate reductase subunit A	**SEN4007**	4242138..4243463	putative DNA-damage-inducible membrane protein
SEN1669	1737019..1737261	major outer membrane lipoprotein	**SEN4030**	4264577..4281256	large repetitive protein

SEN1684	1753729..1754964	Putative MFS-family transport protein	**SEN4035**	complement(4286073..4286396)	regulatory protein SoxS
SEN1696	1769031..1769864	conserved hypothetical protein	**SEN4038**	4288250..4289599	putative xanthine/uracil permeases family protein
SEN1699	complement(1771449..1772891)	conserved hypothetical protein	**SEN4045**	complement(4295818..4297776)	acetyl-coenzyme A synthetase
SEN1711	complement(1820637..1822565)	threonyl-tRNA synthetase	**SEN4046**	4297963..4298109	hypothetical protein
SEN1713	complement(1825371..1826225)	Putative DNA/RNA non-specific endonuclease	**SEN4049**	4300319..4300990	cytochrome c-type biogenesis protein
SEN1724	complement(1834782..1835024)	cell division activator CedA	**SEN4053**	4304171..4304791	NrfG protein
SEN1726	complement(1837685..1838443)	conserved hypothetical protein	**SEN4057**	complement(4309971..4310879)	putative membrane-bound beta-hydroxylase
SEN1730	complement(1841187..1842545)	PTS system, cellobiose-specific IIC component	**SEN4068**	complement(4323316..4324248)	melibiose operon regulatory protein
SEN1733	1843809..1844636	NAD synthetase	**SEN4125**	4380539..4381858	N-acetylmuramoyl-L-alanine amidase
SEN1734	1844730..1845638	conserved hypothetical protein	**SEN4136**	4394632..4395036	conserved hypothetical protein
SEN1737	complement(1847495..1848838)	succinylarginine dihydrolase	**SEN4141**	4397901..4398539	conserved hypothetical protein
SEN1740	complement(1851335..1852561)	succinylornithine transaminase	**SEN4144**	complement(4401457..4401777)	conserved hypothetical protein
SEN1742	1853886..1854302	putative MutT-family protein	**SEN4145**	complement(4401863..4402192)	putative exported protein
SEN1765	complement(1877694..1878890)	putative regulatory protein	**SEN4155**	complement(4409944..4410219)	conserved hypothetical protein
SEN1769	1884336..1885829	membrane protein	**SEN4164**	4415023..4416426	D-serine/D-alanine/glycine transporter
SEN1773	1888028..1889212	putative membrane protein	**SEN4171**	complement(4422564..4424507)	2',3'-cyclic-nucleotide 2'-phosphodiesterase
SEN1786	complement(1895080..1895928)	putative transcriptional regulator	**SEN4178**	4429057..4430790	putative exported protein
SEN1792	complement(1900211..1900801	putative ABC transport ATP-binding subunit	**SEN4182**	complement(4436172..4437335)	hypothetical protein

SEN1822	complement(1929475..1930704)	peptidase	**SEN4204A**	complement(4456779..4458197)	PTS system, trehalose-specific IIBC component
SEN1837	complement(1947644..1948183)	putative secreted protein	**SEN4207**	4459666..4462374	Mg(2+) transport ATPase, P-type
SEN1845	1954059..1956233	outer-membrane receptor for Fe(iii)-coprogen, fe(iii)-ferrioxamine b and fe(iii)-rhodotrulic acid	**SEN4208**	complement(4462490..4462936)	putative membrane protein
SEN1846	complement(1956318..1957751)	PTS system, glucose-specific IIBC component	**SEN4211**	complement(4463948..4464883)	aspartate carbamoyltransferase catalytic subunit
SEN1863	1972559..1975765	ribonuclease E	**SEN4213**	complement(4465111..4465599)	putative transcriptional regulator
SEN1870	complement(1982314..1983069)	putative flagellar basal-body rod protein FlgF (proximal rod protein)	**SEN4216**	complement(4467245..4468249)	ornithine carbamoyltransferase
SEN1878	complement(1987572..1989146)	virulence factor MviN	**SEN4232**	4482341..4483441	putative innermembrane protein
SEN1879	complement(1989411..1990334)	putative virulence factor MviM	**SEN4247**	4495099..4495635	fimbrial protein precursor
SEN1901	complement(2010558..2011097)	conserved hypothetical protein	**SEN4249**	4496514..4498964	outer membrane fimbrial usher protein
SEN2003	2026804..2027502	Putative DNA-binding protein	**SEN4253**	4501622..4502275	Thiol:disulfide interchange protein
SEN2017	complement(2041078..2042598)	putative cobyric acid synthase	**SEN4254**	4502462..4503293	putative outer membrane protein
SEN2027	complement(2049156..2049929)	precorrin-4 C11-methyltransferase	**SEN4259**	4505177..4505887	hypothetical protein
SEN2041	2062249..2064081	propanediol utilization protein	**SEN4277**	complement(4523225..4524496)	putative membrane protein
SEN2051	2070132..2071487	propanediol utilization ferredoxin	**SEN4304**	4554655..4555713	putative sugar isomerase
SEN2054	2072399..2072851	putative propanediol utilization protein PduV	**SEN4317**	4567545..4567781	conserved hypothetical protein
SEN2083	complement(2105268..2106707)	mannose-1-phosphate guanylyltransferase	**SEN4325**	4575046..4575819	conserved hypothetical protein
SEN2085B	complement(2110292..2111590)	O-antigen transporter	**SEN4352**	complement(4606768..4607439)	putative transcriptional regulator

SEN2085D	complement(2112674..2113513)	paratose synthase	**SEN4354**	complement(4608405..4609121)	global response regulator
SEN2091	complement(2118295..2119173)	**TDP-glucose pyrophosphorylase**			
SEN2095	complement(2122653..2124056)	**putative colanic acid biosynthesis protein**			
SEN2100	complement(2129614..2130984)	**phosphomannomutase**			

Table A9: Differences between pSENV and African MDR plasmid

Difference	Name (if named)	Function where known
Deletions with respect to pSENV	srgB	sdiA-regulated putative putative bacterial regulatory proteins, luxR famil thiol-disulfide isomerase or thioredoxin gene; putative outer membrane protein
	srgA	putative putative bacterial regulatory proteins, luxR famil thiol-disulfide isomerase or thioredoxin
	orf&	putative bacterial regulatory proteins, luxR family
New pseudogenes with respect pSENV	Integrase (pSENV 33)	
Novel genes with respect to pSENV (46)	sulI	Dihydropteroate synthetase
	dhfrVI	Dihydrofolate reductase
	integrase	
	tnpM	Tn21 modulator protein
	hypothetical protein	
	hypothetical protein	
	hypothetical protein	
	hypothetical protein	
	mucB	
	transposase	Tn3 transposase
	tnpa	
	hypothetical protein	
	catA2	chloramphenicol acetyltransferase
	hypothetical protein	

	rop	
	hypothetical protein	
	hypothetical protein	
	hypothetical protein	
	hypothetical protein	
	hypothetical protein	
	istb-like atp-binding protein	
	integrase catalytoc subunit	
	tnpA	transposase
	tetr (frag)	tetr fragment
	tetr	tetracycline repressor protein
	tetE	tetracycline efflux protein
	pecM	drug transporter
	pncA	putative amidase
	tnpa	transposase
	hypothetical protein	
	restriction endonuclease	restriction endonuclease
	hypothetical protein	
	hypothetical protein	
	tnpA	Tn3 transposase
	resolvase	
	bla-TEM1	beta-lactamase
	mucB	mucin production
	mucA	mucin production
	pemK	Toxin/antitoxin addiction system
	PEMI	Toxin/antitoxin addiction system
	tir	facilitates gut wall attachement in EPEC

	strA	Streptomycin 3-kinase
	strB	aminoglycoside phosphotransferase
	sul2	Dihydropteroate synthetase
	rep	replication protein
		regulatory protein

Table A10 Full list of phenotypic differences between D7795 and A1636 at 28°C and corresponding genetic differences

Plate ID	log-fold change in signal	P.Value	Adj.P.Val	Compound	Pathway	Genes	Mutation in A7830	Difference
PM1-.A04	3.13	< 0.01	< 0.01	D-Saccharic Acid	D-glucarate degradation	2805-6	2806	NSSNP
PM1-.A06	-0.64	< 0.01	0.03	Galactose degradation	galactose degradation	718-721		
PM1-.A09	0.65	< 0.01	0.03	D-Alanine	alanine degradation 1	1234-6		
PM1-.A12	-1.14	< 0.01	0.04	Dulcitol	glycerol degradation	3093-3096	3095	NSSNP
PM1-.B04	-2.33	< 0.01	0.02	L-Fucose	fucose degradation	2818-2823	2821	pseudogene
PM1-.B10	1.43	< 0.01	0.01	Formic acid	formylTHF biosynthesis			3 pseudogenes and 4 NSSNPS
PM1-.C05	-2.29	< 0.01	0.01	Tween 20				
PM1-.C07	-0.58	< 0.01	0.03	D-fructose	fructose degradation	3675	3675	NSSNP
PM1-.C11	-1.19	< 0.01	0.01	D-Melibiose	melibiose degradation	4068-70	4068	NSSNP

PM1-.D05	-2.29	< 0.01	0.01	Tween, polysorbate detergent				
PM1-.D07	-0.52	0.02	0.08	α-keto-butyric acid	melibiose degradation	4068-70	4068	NSSNP
PM1-.D08	-3.01	< 0.01	0.04	α -methyl-D-galactoside	sugar degradation			4 genes with NSSNPs
PM1-.D09	1.19	< 0.01	0.02	α -D-Glucose	glucose degradation	718-21		
PM1-.D10	1.78	< 0.01	0.01	Lactulose				
PM1-.D11	1.42	< 0.01	0.01	Sucrose				
PM1-.E05	-2.06	< 0.01	0.01	Tween, polysorbate detergent				
PM1-.E07	-0.79	< 0.01	0.03	α -Hydroxy butyric acid	glycolysis	686 2636		
PM1-.E08	-1.63	< 0.01	0.01	ß-Methyl-D-glucoside	glycolysis	2160	2160	NSSNP
PM1-.F07	-0.73	< 0.01	0.04	propionic acid	propiaonate degradtion	731-3		
PM1-.F08	4.19	0.01	0.08	Mucic acid				
PM3-B.A11	-5.27	< 0.01	0.02	L-Cysteine	cysteine degradation	1320	1320	NSSNP
PM3-B.B09	-1.12	< 0.01	0.01	L-Proline	proline degradation		986A	NSSNP
PM3-B.B10	-1.13	< 0.01	0.02	L-Serine	serine degradation		2184,3081, 4164	2 pseudogenes (2184, 4164) Pseudogene 3081

PM3-B.B11	-0.61	< 0.01	0.03	L-Threonine	threonine degradation		2056, 3520	
PM3-B.C08	-1.06	< 0.01	0.02	D-Serine	serine degradation		2184,3081, 4164	2 pseudogenes (2184, 4164) Pseudogene 3081
PM3-B.C12	-1.77	< 0.01	0.01	L-Ornithine	arginine biynthesis	4214-18	4216	NSSNP
PM3-B.E08	-2.43	< 0.01	0.02	D-Glucosamine	N-acetyl glucosamine degradation	3169-72	1,293,675	
PM3-B.E11	-2.48	< 0.01	0.01	D-Glucosamine	N-acetyl glucosamine degradation			
PM3-B.G04	-1.21	0.01	0.07	Alloxan	allantoin degradation	508		
PM3-B.G05	-1.27	< 0.01	0.02	Allantoin	allantoin degradation	508		
PM3-B.G08	-0.56	0.02	0.09	γ-amino-N-Butyric acid	4-aminobutyrate degradation 1	2636		
PM3-B.G09	-1.07	< 0.01	0.03	ε-amino-caproic acid				
PM3-B.H09	-0.73	< 0.01	0.02	Gly-Gln				
PM4-A.E07	0.62	0.01	0.08	2-aminehtyl-phosphonic acid		2437/8		
PM9-.B08	0.61	< 0.01	0.03	NaCl 6% + choline				
PM9-.B10	0.89	0.01	0.06	NaCl 6% + creatine				

PM9-.C07	0.64	< 0.01	0.03	NaCl 6% + gamma-amino-butyric-acid
PM9-.E04	-1.23	0.01	0.06	Sodium formate 4%

Table A11 Full list of phenotypic differences between D7795 and A1636 at 37°C and corresponding genetic differences

Plate ID	log-fold change in signal	P.Value	Adj.P.Val	Compound	Pathway	Genes	Mutation in A7830	Difference
PM1-.A02	-1.06	< 0.01	0.01	L-Arabinose	L-arabinose degradation	104-106	104	NSSNP
PM1-.A03	-0.34	0.01	0.05	N-Acetyl-D- Glucosamine	N-acetyl glucosamine degradation	721		
PM1-.A04	0.79	< 0.01	0.03	D-Saccharic Acid	D-glucarate degradation	2805-6	2806	NSSNP
PM1-.A06	-0.32	0.01	0.04	D-Galactose	galactose degradation	718-721		
PM1-.A11	-0.45	< 0.01	0.03	D-Mannose	mannose degradation	2978-80	1582	NSSNP
PM1-.A12	-0.40	< 0.01	0.03	Dulcitol	glycerol degradation	3093-3096	3095	NSSNP
PM1-.B04	-0.89	< 0.01	0.03	L-Fucose	fucose degradation	2818-2823	2821	pseudogene
PM1-.B11	-0.56	< 0.01	0.03	D-Mannitol	mannitol degradation	3508-10		
PM1-.C06	-0.66	< 0.01	0.02	L-Rhamnose	rhamnose degradation	3836-8		
PM1-.C07	-0.76	< 0.01	0.01	D-fructose	fructose degradation	3675	3675	NSSNP
PM1-.C09	-0.51	< 0.01	0.03	α-D-Glucose	glucose	10 pathways		

PM1-.C11	-0.21	0.01	0.05	D-Melibiose	melibiose degradation	4068-70	4068	NSSNP
PM1-.D05	-2.30	< 0.01	< 0.01	Tween, polysorbate detergent				
PM1-.D07	-0.55	< 0.01	0.03	α -Methyl-D- Galactoside	melibiose degradation	4068-70	4068	NSSNP
PM1-.D10	2.20	< 0.01	0.01	Lactulose				
PM1-.D11	0.42	0.02	0.06	Sucrose				
PM1-.E03	0.29	< 0.01	0.04	D-Glucose-1-Phosphate		10 pathways		
PM1-.E05	-2.29	< 0.01	< 0.01	Tween, polysorbate detergent				
PM1-.E07	-0.34	< 0.01	0.03	α -Hydroxy butyric acid	glycolysis	686 2636		
PM1-.E08	-0.44	0.01	0.05	ß-Methyl-D- Glucoside	glycolysis	2160	2160	NSSNP
PM1-.F01	0.19	0.01	0.06	Glycyl-L-Aspartic Acid	aspartate degradation	4210-11	4211	NSSNP
PM1-.F09	-2.04	0.01	0.04	Glycolic Acid	glycolate and glyoxylate degreadtion	?		
PM1-.G04	0.32	0.02	0.06	L-Threonine	threonine degradation	168	168	NSSNP
PM1-.G09	-0.49	0.03	0.09	N-Acetyl-ß-D- Mannosamine	N-acetylneuraminate and N-acetlymannosamine degradation	3170		
PM1-.G11	2.81	< 0.01	< 0.01	Malic acid	gluconeogenesis	2451		

PM2-A.F03	-4.25	< 0.01	< 0.01	Melibionic acid	melibiose degradation	4067-9	4068	NSSNP
PM2-A.F12	2.17	< 0.01	< 0.01	L-Tartaric Acid	tartaric degradation	3186-7		
PM2-A.H09	-4.23	< 0.01	< 0.01	Dihydroxyacetone	threonine metabolism	3902	3902	pseudogene
PM3-B.A06	-0.56	< 0.01	0.03	Biuret	Enteric adaptation			
PM3-B.A11	-4.21	< 0.01	0.01	L-Cysteine	cysteine degradation	1320	1320	NSSNP
PM3-B.B06	-1.18	< 0.01	0.03	L-Lysine	lysine degradation	2537-40		
PM3-B.B12	-0.70	0.01	0.06	L-Tryptophan	typtophan degradation	4017		
PM3-B.C12	-1.10	0.01	0.06	L-Ornithine	arginine degradation	4214-18	4216	NSSNP
PM3-B.D02	-0.77	0.02	0.06	N-Phthaloyl-L- Glutamic Acid				
PM3-B.D08	-0.52	0.01	0.06	Ethylamine				
PM3-B.D09	-1.20	< 0.01	< 0.01	Ethanolamine		2437/8		
PM3-B.E11	-0.76	< 0.01	0.03	N-Acetyl-D- Glucosamine	as above			
PM4-A.B01	0.51	0.02	0.07	Thiophosphate				
PM4-A.E09	2.02	< 0.01	0.03	Thymidine- 3Í- monophosphate	purine and pyrimdine salvage	1847-53		
PM9-.B01	0.59	0.02	0.07	NaCl 6%				

PM9-.B05	1.43	< 0.01	0.03	NaCl 6% + Dimethyl sulphonyl propionate

Table A12: *S.* Enteritidis metadata and sequencing statistics

Lane_tag	Cluster	Name	Country	Year	Antibiogram	Compartment	Serotype	MLST	Total Length	No Contigs	Avg Contig Length	Largest Contig	N50	Contigs in N50	Average Read Length
10071_5#1	6	482916	SA	2010	Sensitive	PUS	Enteritidis	11	4641620	19	244295.79	1537004	489950	3	100
10071_5#10	6	332287	SA	2009	Sensitive	URINE	Enteritidis	11	4704309	16	294019.31	1545637	489943	3	100
10071_5#11	4	250994	SA	2006	Sensitive	Blood	Enteritidis	1479	4758256	29	164077.79	1481378	483484	3	100
10071_5#13	5	616854	SA	2012	Sensitive	PUS	Enteritidis	11	4706605	22	213936.59	1508517	490107	3	100
10071_5#14	6	584643	SA	2012	Sensitive	Blood	Enteritidis	11	4703100	15	313540	1268015	694545	3	100
10071_5#15	6	503944	SA	2011	Sensitive	Stool	Enteritidis	11	4703281	16	293955.06	1549410	489943	3	100
10071_5#16	5	454790	SA	2006	Sensitive	FLUID	Enteritidis	11	4699547	18	261085.94	1726720	729300	2	100
10071_5#17	6	285561	SA	2009	Sensitive	Stool	Enteritidis	11	4767070	20	238353.5	1549414	489934	3	100
10071_5#18	6	283959	SA	2009	Sensitive	Blood	Enteritidis	11	4702607	18	261255.94	1532543	509754	3	100
10071_5#19	6	238663	SA	2008	Sensitive	Stool	Enteritidis	11	4710545	20	235527.25	1549425	489946	3	100
10071_5#2	6	361592	SA	2010	Sensitive	Stool	Enteritidis	11	4704012	21	224000.57	1549442	490601	3	100
10071_5#20	6	120673	SA	2007	Sensitive	PUS	Enteritidis	11	4700392	15	313359.47	1544961	694432	3	100
10071_5#21	5	624887	SA	2012	Sensitive	PUS	Enteritidis	11	4694604	17	276153.18	1591549	490096	3	100
10071_5#22	6	584640	SA	2012	cotrimoxazole	URINE	Enteritidis	11	4702474	14	335891	1549451	489949	3	100
10071_5#23	5	508751	SA	2011	cotrimoxazole	URINE	Enteritidis	11	4699486	14	335677.57	1190569	708310	3	100
10071_5#24	6	1029414	SA	2010	cotrimoxazole	Blood	Enteritidis	11	4805937	18	266996.5	1959409	726457	2	100
10071_5#25	6	380410	SA	2010	Sensitive	URINE	Enteritidis	11	4700381	15	313358.73	1549422	523211	3	100
10071_5#26	4	258179	SA	2008	cotrimoxazole	Blood	Enteritidis	1479	4758241	31	153491.65	1337285	488225	3	100
10071_5#27	6	180211	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4699926	16	293745.38	1549420	490359	3	100
10071_5#28	6	247274	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4704191	21	224009.1	1374372	492827	3	100
10071_5#29	6	631484	SA	2012	cotrimoxazole	Blood	Enteritidis	11	4708343	16	294271.44	1498373	489996	3	100
10071_5#3	6	283954	SA	2009	Sensitive	Stool	Enteritidis	11	4704705	18	261372.5	1549412	489948	3	100
10071_5#30	5	570890	SA	2012	Sensitive	SPUTUM	Enteritidis	11	4696862	12	391405.17	1827376	728732	2	100
10071_5#31	5	684394	SA	2011	Sensitive	Blood	Enteritidis	11	4701548	20	235077.4	1508498	478705	3	100
10071_5#32	6	172848	SA	2007	MDR	Blood	Enteritidis	11	4697697	13	361361.31	1632523	489937	3	100
10071_5#33	6	481587	SA	2010	Sensitive	PUS	Enteritidis	11	4698938	15	313262.53	1549417	489948	3	100
10071_5#34	6	245880	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4703086	14	335934.71	1780118	694970	2	100
10071_5#35	5	468205	SA	2010	Sensitive	Stool	Enteritidis	11	4734731	19	249196.37	1494381	709328	3	100
10071_5#36	6	236817	SA	2008	cotrimoxazole	URINE	Enteritidis	11	4699669	14	335690.64	1632981	489942	3	100
10071_5#37	6	626279	SA	2012	Ampicillin+cotrimoxazole	Stool	Enteritidis	11	4701783	13	361675.62	1549642	489941	3	100
10071_5#38	5	492221	SA	2011	Sensitive	TISSUE	Enteritidis	11	4697866	18	260992.56	1508517	489396	3	100
10071_5#39	5	684397	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4702886	19	247520.32	1591620	490094	3	100
10071_5#4	2	603671	SA	2012	Sensitive	ABSCESS	Enteritidis	11	4769153	22	216779.68	1365245	488714	3	100
10071_5#40	5	468192	SA	2010	Sensitive	Blood	Enteritidis	11	4733877	22	215176.23	1592086	490085	3	100
10071_5#41	6	119691	SA	2007	Sensitive	PUS	Enteritidis	11	4793593	25	191743.72	1549431	490356	3	100
10071_5#42	6	283957	SA	2009	Sensitive	Stool	Enteritidis	11	4703749	16	293984.31	1632793	489835	3	100
10071_5#43	5	459755	SA	2010	Sensitive	URINE	Enteritidis	11	4699435	17	276437.35	1508497	696415	3	100
10071_5#44	6	485533	SA	2009	Sensitive	Blood	Enteritidis	11	4699295	15	313286.33	2037644	695035	2	100
10071_5#45	6	626285	SA	2012	cotrimoxazole	Stool	Enteritidis	11	4703207	14	335943.36	1780139	694967	2	100
10071_5#46	6	290914	SA	2006	Sensitive	Stool	Enteritidis	11	4702038	15	313469.2	1549653	694530	3	100
10071_5#48	6	238579	SA	2008	Sensitive	Stool	Enteritidis	11	4745318	18	263628.78	1813871	694970	2	100
10071_5#49	6	204225	SA	2008	cotrimoxazole	URINE	Enteritidis	11	4700534	19	247396.53	1374243	489945	3	100
10071_5#5	5	616851	SA	2012	Sensitive	ABSCESS	Enteritidis	11	4696676	15	313111.73	1739052	729229	2	100
10071_5#50	6	254602	SA	2008	Sensitive	Stool	Enteritidis	11	4699090	14	335649.29	1780083	694964	2	100
10071_5#51	6	358520	SA	2006	Sensitive	Stool	Enteritidis	11	4700734	16	293795.88	1268009	694241	3	100

10071_5#52	6	533511	SA	2011	cotrimoxazole	TISSUE	Enteritidis	11	4705431	17	276790.06	1549452	490050	3	100
10071_5#53	6	570523	SA	2012	Sensitive	SPUTUM	Enteritidis	11	4702566	12	391880.5	1632691	694989	3	100
10071_5#54	2	261627	SA	2008	Sensitive	Stool	Enteritidis	366	4801819	22	218264.5	1058490	489314	4	100
10071_5#55	5	492225	SA	2011	Sensitive	TISSUE	Enteritidis	11	4697594	17	276329.06	1591567	478694	3	100
10071_5#56	6	348823	SA	2010	cotrimoxazole	Stool	Enteritidis	11	4705448	19	247655.16	1549418	489953	3	100
10071_5#57	6	245891	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4702139	14	335867.07	1780120	694989	2	100
10071_5#58	6	319189	SA	2009	Sensitive	URINE	Enteritidis	11	4705415	20	235270.75	1549414	489953	3	100
10071_5#59	6	319184	SA	2009	cotrimoxazole	SPUTUM	Enteritidis	11	4742532	18	263474	1594513	964236	2	100
10071_5#6	5	590212	SA	2012	Sensitive	Stool	Enteritidis	11	4697916	18	260995.33	1517214	478986	3	100
10071_5#60	6	380425	SA	2010	Sensitive	URINE	Enteritidis	11	4698496	20	234924.8	1549152	492821	3	100
10071_5#61	5	569257	SA	2011	Sensitive	Stool	Enteritidis	11	4699019	19	247316.79	1508515	729166	3	100
10071_5#62	6	249309	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4700682	20	235034.1	1555464	489938	3	100
10071_5#63	5	496155	SA	2011	cotrimoxazole	URINE	Enteritidis	11	4697738	17	276337.53	1508519	729236	3	100
10071_5#64	5	317576	SA	2009	Sensitive	URINE	Enteritidis	11	4674417	20	233720.85	1600621	478692	3	100
10071_5#65	5	472274	SA	2010	cotrimoxazole	Stool	Enteritidis	11	4771126	20	238556.3	1508878	492861	3	100
10071_5#66	6	115452	SA	2006	Sensitive	Blood	Enteritidis	11	4700665	17	276509.71	1775788	694535	2	100
10071_5#67	6	250909	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4702402	14	335885.86	1549402	813196	2	100
10071_5#7	6	503933	SA	2011	cotrimoxazole	URINE	Enteritidis	11	4701705	15	313447	1549410	489943	3	100
10071_5#8	6	358519	SA	2010	Sensitive	URINE	Enteritidis	11	4702170	22	213735	1549435	489964	3	100
10071_5#9	6	332662	SA	2009	Sensitive	Stool	Enteritidis	11	4700391	20	235019.55	1548013	647767	3	100
10156_1#1	5	463134	SA	2010	Sensitive	Blood	Enteritidis	11	4732473	18	262915.17	1509070	490082	3	100
10156_1#10	2	603668	SA	2012	Sensitive	Stool	Enteritidis	11	4768007	22	216727.59	957783	488708	4	100
10156_1#11	5	512103	SA	2011	cotrimoxazole	URINE	Enteritidis	11	4698023	20	234901.15	1496118	490082	3	100
10156_1#12	5	511112	SA	2011	Sensitive	Stool	Enteritidis	11	4698922	15	313261.47	1508527	665421	3	100
10156_1#13	6	250545	SA	2006	Sensitive	Blood	Enteritidis	11	4701802	17	276576.59	1549436	695013	3	100
10156_1#14	6	115456	SA	2006	Sensitive	Blood	Enteritidis	11	4699579	15	313305.27	1544986	964243	2	100
10156_1#15	6	360688	SA	2010	Sensitive	Stool	Enteritidis	11	4701811	23	204426.57	1549452	489843	3	100
10156_1#16	6	177883	SA	2007	MDR	Blood	Enteritidis	11	4698685	14	335620.36	1549470	489949	3	100
10156_1#17	5	575404	SA	2006	Sensitive	FLUID	Enteritidis	11	4732369	19	249072.05	1508915	490085	3	100
10156_1#18	5	596719	SA	2012	Sensitive	Stool	Enteritidis	11	4732327	18	262907.06	937585	536267	4	100
10156_1#19	5	516364	SA	2011	Sensitive	Blood	Enteritidis	11	4731926	18	262884.78	1592097	490088	3	100
10156_1#2	6	547922	SA	2011	Sensitive	Stool	Enteritidis	11	4700579	14	335755.64	1549426	964545	2	100
10156_1#20	6	272011	SA	2009	Sensitive	Stool	Enteritidis	11	4702082	14	335863	1619593	492808	3	100
10156_1#21	6	249308	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4700728	16	293795.5	1537037	694579	3	100
10156_1#22	6	201566	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4699989	15	313332.6	1549451	489946	3	100
10156_1#23	6	469500	SA	2010	Sensitive	Stool	Enteritidis	11	4703591	20	235179.55	1549432	489955	3	100
10156_1#24	6	1030047	SA	2011	Sensitive	Blood	Enteritidis	11	4805343	13	369641.77	1553141	1036441	2	100
10156_1#25	5	624885	SA	2012	cotrimoxazole	Blood	Enteritidis	11	4695174	19	247114.42	1508411	490693	3	100
10156_1#26	6	586365	SA	2012	Sensitive	Blood	Enteritidis	11	4701853	14	335846.64	1956248	694534	2	100
10156_1#27	5	516360	SA	2011	Sensitive	Blood	Enteritidis	11	4731775	18	262876.39	970310	761392	3	100
10156_1#28	6	318064	SA	2009	cotrimoxazole	SPUTUM	Enteritidis	11	4702307	16	293894.19	1839595	694970	2	100
10156_1#29	6	262647	SA	2009	Sensitive	URINE	Enteritidis	11	4703002	19	247526.42	1547848	489966	3	100
10156_1#3	6	547857	SA	2011	cotrimoxazole	TISSUE	Enteritidis	11	4699691	17	276452.41	1549446	489962	3	100
10156_1#30	6	238577	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4701950	12	391829.17	1555506	694980	3	100
10156_1#31	6	317646	SA	2009	Sensitive	URINE	Enteritidis	11	4702868	13	361759.08	1272678	694529	3	100
10156_1#32	6	171403	SA	2007	cotrimoxazole	URINE	Enteritidis	11	4699536	14	335681.14	1555469	492808	3	100
10156_1#33	6	236888	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4703807	17	276694.53	1323700	490360	3	100
10156_1#34	6	249299	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4702593	14	335899.5	1632673	694520	3	100
10156_1#35	6	348816	SA	2010	cotrimoxazole	Stool	Enteritidis	11	4704912	16	294057	1549456	490161	3	100
10156_1#36	2	1025080	SA	2008	MDR	Blood	Enteritidis	11	4700951	21	223854.81	1326052	654438	3	100
10156_1#37	5	465481	SA	2010	cotrimoxazole	Stool	Enteritidis	11	4735433	19	249233.32	1020893	490091	4	100
10156_1#38	6	384986	SA	2010	Sensitive	URINE	Enteritidis	11	4702417	19	247495.63	1537030	489962	3	100

10156_1#39	2	1025081	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4700109	25	188004.36	920623	412281	4	100
10156_1#4	6	236885	SA	2008	cotrimoxazole	Stool	Enteritidis	11	4736580	16	296036.25	1549445	694958	3	100
10156_1#40	6	466244	SA	2010	Sensitive	Blood	Enteritidis	11	4702680	16	293917.5	1555465	489955	3	100
10156_1#41	6	426007	SA	2010	Sensitive	Stool	Enteritidis	11	4703837	17	276696.29	1549456	814120	2	100
10156_1#42	6	333519	SA	2009	Sensitive	Stool	Enteritidis	11	4705197	16	294074.81	1543046	489952	3	100
10156_1#43	6	308821	SA	2009	Sensitive	Stool	Enteritidis	11	4704278	18	261348.78	1549416	492808	3	100
10156_1#44	6	383167	SA	2010	Sensitive	URINE	Enteritidis	11	4703376	18	261298.67	1549435	489947	3	100
10156_1#45	5	464793	SA	2010	Sensitive	Blood	Enteritidis	11	4699642	20	234982.1	1514539	490092	3	100
10156_1#46	6	679982	SA	2010	cotrimoxazole	Blood	Enteritidis	11	4702280	16	293892.5	1632741	489962	3	100
10156_1#47	5	672637	SA	2010	Sensitive	Blood	Enteritidis	11	4699000	21	223761.9	1420117	490106	3	100
10156_1#48	6	682773	SA	2011	Sensitive	Blood	Enteritidis	11	4703451	17	276673.59	1549431	694582	3	100
10156_1#5	6	263174	SA	2009	Sensitive	Stool	Enteritidis	11	4704920	18	261384.44	1549439	489954	3	100
10156_1#50	2	261629	SA	2009	Sensitive	URINE	Enteritidis	366	4803381	18	266854.5	1288336	677681	3	100
10156_1#51	5	682965	SA	2011	cotrimoxazole	Blood	Enteritidis	11	4698308	16	293644.25	1508504	887640	2	100
10156_1#52	6	649763	SA	2012	Sensitive	Stool	Enteritidis	11	4740226	19	249485.58	1268055	490086	3	100
10156_1#53	5	655136	SA	2012	Sensitive	Stool	Enteritidis	11	4698266	19	247277.16	1514242	493104	3	100
10156_1#54	5	672246	SA	2004	Sensitive	Blood	Enteritidis	11	4769203	26	183430.88	1282778	492807	3	100
10156_1#55	5	656744	SA	2012	Sensitive	Stool	Enteritidis	11	4699301	16	293706.31	1508511	490111	3	100
10156_1#56	6	651579	SA	2012	cotrimoxazole	Stool	Enteritidis	11	4698613	14	335615.21	1549335	964246	2	100
10156_1#57	5	672325	SA	2004	Sensitive	Blood	Enteritidis	11	4699537	16	293721.06	1508541	729307	3	100
10156_1#58	5	672632	SA	2009	cotrimoxazole	Blood	Enteritidis	11	4698808	22	213582.18	1508514	664254	3	100
10156_1#59	5	658902	SA	2004	Sensitive	Blood	Enteritidis	11	4697936	19	247259.79	1508527	490394	3	100
10156_1#6	6	462913	SA	2010	Sensitive	URINE	Enteritidis	11	4699797	17	276458.65	1552133	489962	3	100
10156_1#60	5	658910	SA	2004	Sensitive	Blood	Enteritidis	11	4698822	15	313254.8	1508525	729236	3	100
10156_1#61	5	672461	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4812134	21	229149.24	1508499	490088	3	100
10156_1#62	6	672458	SA	2008	MDR	Blood	Enteritidis	11	4703863	21	223993.48	1549471	489966	3	100
10156_1#63	5	645899	SA	2012	Sensitive	Blood	Enteritidis	11	4698990	17	276411.18	1508504	490106	3	100
10156_1#64	5	645902	SA	2012	Sensitive	Blood	Enteritidis	11	4698382	20	234919.1	1739122	729182	2	100
10156_1#65	6	634948	SA	2012	Ampicillin+cotrimoxazole	Stool	Enteritidis	11	4709503	16	294343.94	2041885	694919	2	100
10156_1#66	6	649762	SA	2012	Sensitive	Stool	Enteritidis	11	4699146	15	313276.4	1549495	489948	3	100
10156_1#67	6	672456	SA	2008	cotrimoxazole	Blood	Enteritidis	11	4703193	15	313546.2	1267991	492810	3	100
10156_1#7	5	459756	SA	2010	Sensitive	URINE	Enteritidis	11	4699211	17	276424.18	1739068	729034	2	100
10156_1#8	6	238661	SA	2008	Sensitive	Stool	Enteritidis	11	4708050	18	261558.33	1763219	694532	2	100
10156_1#9	6	584634	SA	2012	cotrimoxazole	URINE	Enteritidis	11	4702913	16	293932.06	1549417	492808	3	100
10512_1#63	1	F8104	Ratin		Unknown	Ratin	Enteritidis	136	4763919	22	216541.77	1494263	615988	3	150
10512_1#64	1	F8022	ratin		Unknown	Ratin	Enteritidis	136	4783508	29	164948.55	1577855	489191	3	150
10512_1#65	1	F3124	Ratin		Unknown	Ratin	Enteritidis	136	4782414	25	191296.56	1494665	489174	3	150
10665_6#1	3	P149	Mali	2006	Ampicillin	Blood	Enteritidis	11	4713159	20	235657.95	657589	505671	4	100
10665_6#10	2	1_59	Senegal	1959	Sensitive	Blood	Enteritidis	183	4867673	25	194706.92	1030707	474700	4	100
10665_6#11	3	93_9986	Gabon	1993	Ampicillin+cotrimoxazole	Blood	Enteritidis	745	4832061	26	185848.5	892020	483691	4	100
10665_6#12	5	93_5337	Angola	1993	Sensitive	selles	Enteritidis	11	4745365	17	279139.12	1741868	664025	2	100
10665_6#13	5	17_60	Mozambique	1960	Sensitive	Guinea pig blood	Enteritidis	11	4651972	18	258442.89	1557144	478723	3	100
10665_6#14	4	94_4688	Rwanda	1993	MDR	Blood	Enteritidis	11	4784598	21	227838	1386849	608540	3	100
10665_6#15	6	93_2136	Djibouti	1992	Sensitive	selles	Enteritidis	11	4705360	15	313690.67	1550128	489834	3	100
10665_6#16	5	201101618	Tunisia	2011	Nal	Pericardic	Enteritidis	11	4698316	18	261017.56	1508499	664029	3	100
10665_6#18	3	S89_347	Niger	1983	Sensitive	Abscess	Enteritidis	745	4709679	20	235483.95	1299947	647671	3	100
10665_6#19	6	06_6063	Senegal	2006	K, Te, Nal	Blood	Enteritidis	11	4810569	15	320704.6	1552244	694855	3	100
10665_6#2	3	Q64	Mali	2007	Ampicillin		Enteritidis	11	4721270	365	12934.99	114175	22239	69	100
10665_6#20	6	06_2966	Cameroon	2006	Sensitive	Blood	Enteritidis	11	4699535	16	293720.94	1780079	694930	2	100
10665_6#21	2	6_66	Camero	1966	Sensitive	Blood	Enteritidis	183	4878338	25	195133.52	1039342	478191	4	100

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10665_6#22	3	94_8355	Guinea	1994	Sensitive	Blood	Enteritidis	11	4667879	18	259326.61	1324015	490360	3	100
10665_6#23	1	6_56	Ethiopia	1956	Sensitive	Stools	Enteritidis	136	4716135	18	262007.5	1496447	489173	3	100
10665_6#24	6	201009544	Madagascar	2010	Ampicillin	Blood	Enteritidis	11	4714342	21	224492.48	1270842	489943	3	100
10665_6#25	4	94_8098	Rwanda	1994	MDR	pr_lvement humain	Enteritidis	11	4755615	44	108082.16	748107	340144	5	100
10665_6#26	5	8_55	Senegal	1955	Sensitive	Mouse brain (Yellow fever culture process for vaccine)	Enteritidis	11	4636738	15	309115.87	1915015	682943	2	100
10665_6#27	5	34_58	Congo	1958	Sensitive	Osteomyelitis	Enteritidis	11	4713663	16	294603.94	1049492	695346	3	100
10665_6#28	3	201005749	Mali	2010	Ampicillin	Blood	Enteritidis	11	4714237	21	224487.48	738942	416254	5	100
10665_6#29	6	201000446	Comores	2010	Ampicillin	Blood	Enteritidis	11	4714306	16	294644.12	1549346	492808	3	100
10665_6#3	3	T27	Mali	2009	Ampicillin		Enteritidis	11	4714455	22	214293.41	657594	633570	4	100
10665_6#30	3	01_4533	Mali	2001	ESBL	CSF	Enteritidis	11	4772190	24	198841.25	1157541	405367	4	100
10665_6#31	5	00_7482	Cameroon	2000	Sensitive	Pastry (foodpoisoning 26 people)	Enteritidis	11	4697662	16	293603.88	1591888	478718	3	100
10665_6#32	2	09_4553	Comores	2009	S	Blood	Enteritidis	11	4851323	24	202138.46	955566	401122	4	100
10665_6#33	2	18_62	Senegal	1962	Sensitive	Blood	Enteritidis	183	4879777	28	174277.75	1278087	699851	3	100
10665_6#34	3	00_2993	Ivory Coast	2000	Ampicillin	Blood	Enteritidis	11	4706343	26	181013.19	650742	422096	5	100
10665_6#35	4	SE85_1233	CAR	1985	Ampicillin+cotrimoxazole	Blood	Enteritidis	11	4797748	32	149929.62	1079411	402139	4	100
10665_6#36	6	03_0448	Kenya	2003	Sensitive	Blood	Enteritidis	11	4643718	14	331694.14	1488617	852771	2	100
10665_6#37	3	16_57	Senegal	1957	Unknown	Unknown	Enteritidis	11	4705881	22	213903.68	1293004	489975	3	100
10665_6#38	6	201301913	Mauritania	2013	Sensitive	L1-L2 intervertebral disk	Enteritidis	11	4738267	21	225631.76	1549421	490006	3	100
10665_6#39	5	201003883	Mauritania	2010	Sensitive	Selles	Enteritidis	11	4697987	21	223713.67	1227086	490091	3	100
10665_6#4	3	R42	Mali	2007	Ampicillin		Enteritidis	11	4714147	25	188565.88	657589	490359	4	100
10665_6#40	1	10_63	Morocco	1963	Sensitive	CSF	Enteritidis	136	4720394	16	295024.62	1494619	1395533	2	100
10665_6#41	1	6_52	Congo	1952	Unknown	Unknown	Enteritidis	136	4808533	26	184943.58	780329	489129	4	100
10665_6#42	3	02_3084	Mali	2002	Ampicillin+cotrimoxazole	Blood	Enteritidis	11	4803783	24	200157.62	729204	490105	4	100
10665_6#43	5	7_55	Senegal	1955	Sensitive	Mouse brain (Yellow fever culture process for vaccine)	Enteritidis	11	4675608	19	246084.63	1571239	489954	3	100
10665_6#44	6	201008943	Egypt	2010	Sensitive	Blood	Enteritidis	11	4660923	19	245311.74	1472175	708232	3	100
10665_6#45	6	06_6191	Madagascar	2006	Ampicillin	CSF	Enteritidis	11	4713662	15	314244.13	1546836	492932	3	100
10665_6#46	5	06_0237	Ivory Coast	2006	Sensitive	Blood	Enteritidis	11	4706426	24	196101.08	1502082	489971	3	100
10665_6#47	6	04_5767	Maroc	2003	Sensitive	Blood	Enteritidis	11	4705545	21	224073.57	1268000	489733	3	100
10665_6#48	3	94_9599	Senegal	1994	Ampicillin	Blood	Enteritidis	11	4647753	25	185910.12	656243	403838	5	100
10665_6#49	3	03_2506	Mali	2003	ESBL	Stools (carrier)	Enteritidis	11	4757954	33	144180.42	646734	401126	6	100
10665_6#5	6	93_7053	Benin	1993	Sensitive	Blood	Enteritidis	11	4702720	13	361747.69	1863196	772080	2	100
10665_6#50	5	16_65	Senegal	1965	Sensitive	Abscess	Enteritidis	11	4696252	22	213466	1495186	478693	3	100
10665_6#51	5	11_66	Senegal	1966	Sensitive	Blood	Enteritidis	11	4640012	18	257778.44	1798909	729352	2	100
10665_6#52	2	9_63	Chad	1963	Sensitive	Stools	Enteritidis	11	4620959	16	288809.94	1523827	852895	2	100
10665_6#53	3	S89_348	Niger	1989	Sensitive	Blood	Enteritidis	745	4649741	20	232487.05	1180870	480478	4	100
10665_6#54	2	34_61	Madagascar	1961	Sensitive	Snake	Enteritidis	11	4774499	18	265249.94	1019054	489852	4	100
10665_6#56	5	08_7427	Tunisia	2008	Sensitive	Blood	Enteritidis	11	4638701	16	289918.81	1495774	480433	3	100
10665_6#58	5	3_66	Senegal	1966	Sensitive	CSF	Enteritidis	11	4697156	16	293572.25	1504294	710520	3	100

10665_6#59	5	201008533	Mauritius	2010	Sensitive	Blood	Enteritidis	11	4705015	17	276765.59	1579122	537007	3	100
10665_6#6	6	98_2244	Cameroon	1994	Sensitive	Blood	Enteritidis	11	4705339	16	294083.69	1386716	490417	3	100
10665_6#60	3	03_0779	Mali	2003	ESBL	Blood	Enteritidis	11	4776939	33	144755.73	490366	403844	6	100
10665_6#61	6	05_3223	Congo	2004	Sensitive	Blood	Enteritidis	366	4700502	19	247394.84	1536969	696770	3	100
10665_6#62	3	98_10272	Madagascar	1998	Ampicillin+cotrimoxazole	CSF	Enteritidis	11	4710474	24	196269.75	1152401	483498	4	100
10665_6#63	5	05_6321	Ivory Coast	2005	Sensitive	Blood	Enteritidis	11	4697449	18	260969.39	1365796	478691	3	100
10665_6#64	3	01_4553	Mali	2001	Ampicillin+cotrimoxazole	Abscess	Enteritidis	11	4851139	26	186582.27	1077953	483697	4	100
10665_6#65	6	201004918	Central African Republic	2010	Sensitive	Blood	Enteritidis	11	4702679	22	213758.14	1311240	489794	3	100
10665_6#66	6	04_6072	Madagascar	2004	Ampicillin	Blood	Enteritidis	11	4714978	17	277351.65	1632710	489941	3	100
10665_6#67	3	30_58	Guinea-Conackry	1958	Sensitive	Osteomyelitis	Enteritidis	11	4712854	20	235642.7	813963	490362	4	100
10665_6#68	3	02_3077	Mali	2002	Ampicillin	CSF	Enteritidis	11	4713818	23	204948.61	657903	474669	5	100
10665_6#69	3	02_3090	Mali	2002	Ampicillin	CSF	Enteritidis	11	4689637	22	213165.32	1028970	405364	4	100
10665_6#7	6	92_3273	Morocco	1992	Sensitive	Blood	Enteritidis	11	4704257	18	261347.61	1549399	489942	3	100
10665_6#70	6	3_58	Senegal	1958	Sensitive	Stools	Enteritidis	11	4705338	13	361949.08	1944382	698133	2	100
10665_6#71	2	15_63	Congo	1963	S	Osteomyelitis	Enteritidis	183	4877698	23	212073.83	1333276	513294	3	100
10665_6#72	6	00_5185	CAR	2000	Sensitive	Blood	Enteritidis	11	4703647	15	313576.47	1549317	490002	3	100
10665_6#73	3	04_7098	Senegal	2004	Ampicillin	Blood	Enteritidis	11	4713611	24	196400.46	1064613	416070	4	100
10665_6#74	6	201008619	Egypt	2010	Sensitive	Blood	Enteritidis	11	4696973	18	260942.94	2036057	695368	2	100
10665_6#75	1	5_51	Tunisia	1951	Unknown	Unknown	Enteritidis	136	4713594	20	235679.7	1183784	489347	3	100
10665_6#76	6	201007105	Algeria	2010	Sensitive	Blood	Enteritidis	11	4704480	15	313632	1549404	489956	3	100
10665_6#77	3	02_8821	Senegal	2002	Sensitive	Blood	Enteritidis	11	4654031	24	193917.96	663732	405359	5	100
10665_6#78	3	201007599	Mali	2010	MDR	Blood	Enteritidis	11	4706873	30	156895.77	694626	400979	5	100
10665_6#79	5	4_55	Senegal	1955	Sensitive	Blood	Enteritidis	11	4679028	20	233951.4	1660281	683876	2	100
10665_6#8	6	201300358	MAROC	2013	Nal	stools	Enteritidis	11	4709335	17	277019.71	1549336	489819	3	100
10665_6#80	2	SE89_327	Senegal	1980	Unknown	Unknown	Enteritidis	183	4872334	22	221469.73	1026680	679479	3	100
10665_6#81	5	17_65	Senegal	1965	Sensitive	Blood	Enteritidis	11	4698287	20	234914.35	1739356	729104	2	100
10665_6#9	3	20_55	Senegal	1955	Sensitive	Blood	Enteritidis	11	4648232	16	290514.5	1327659	489951	3	100
10702_4#46	3	Q48	Mali	2006	Ampicillin+cotrimoxazole		Enteritidis	11	4765910	24	198579.58	1064624	474684	4	100
10702_4#47	3	P83	Mali	2005	Ampicillin		Enteritidis	11	4720410	26	181554.23	1066121	416570	4	100
10702_4#48	3	P115	Mali	2005	Ampicillin+cotrimoxazole		Enteritidis	11	4759459	25	190378.36	828569	422573	4	100
10702_4#49	3	I60	Mali	2002	MDR		Enteritidis	11	4834707	27	179063.22	835648	525864	4	100
10702_4#50	3	T46	Mali	2009	MDR		Enteritidis	11	4732497	31	152661.19	1067892	405138	4	100
10702_4#51	3	S90	Mali	2009	Amp+chlor		Enteritidis	11	4727316	29	163010.9	1064895	402434	4	100
10702_4#52	3	P52	Mali	2005	Ampicillin		Enteritidis	11	4713279	21	224441.86	1064612	416556	4	100
10702_4#53	3	I74	Mali	2003	Ampicillin		Enteritidis	11	4743115	26	182427.5	830435	491217	4	100
10702_4#54	3	J35	Mali	2004	MDR		Enteritidis	11	4906493	33	148681.61	1303945	411510	4	100
10702_4#55	3	V39	Mali	2010	MDR		Enteritidis	11	4730019	36	131389.42	828585	270147	5	100
10702_4#56	3	Q76	Mali	2007	MDR		Enteritidis	11	4732177	29	163178.52	829982	400970	5	100
10702_4#57	3	J73	Mali	2004	Ampicillin		Enteritidis	11	4713597	21	224457	895355	416299	4	100
10702_4#58	3	T145	Mali	2009	Amp+chlor		Enteritidis	11	4733132	32	147910.38	657601	402433	5	100
10702_4#59	3	V55	Mali	2011	Ampicillin+cotrimoxazole		Enteritidis	11	4760584	29	164158.07	740979	416290	5	100
10702_4#60	3	J39	Mali	2004	MDR		Enteritidis	11	4907068	29	169209.24	1064925	416130	4	100
10702_4#61	3	T103	Mali	2009	MDR		Enteritidis	11	4734135	44	107593.98	489190	274824	7	100
10702_4#62	3	V7	Mali	2010	Amp+chlor		Enteritidis	11	4733085	32	147908.91	657901	404268	5	100

10702_4#63	3	D79	Mali	2002	MDR		Enteritidis	11	4813917	35	137540.49	646458	419163	5	100
10702_4#64	3	R53	Mali	2007	MDR		Enteritidis	11	4908978	27	181814	839752	648299	4	100
10702_4#65	3	T159	Mali	2010	Amp+chlor		Enteritidis	11	4729913	30	157663.77	741125	400963	5	100
10702_4#66	3	X36	Mali	2011	MDR		Enteritidis	11	4732988	34	139205.53	784133	474507	4	100
10702_4#67	3	I23	Mali	2002	Ampicillin+cotrimoxazole		Enteritidis	11	4818389	30	160612.97	812386	474666	4	100
10702_4#68	3	W47	Mali	2012	MDR		Enteritidis	11	4732146	29	163177.45	740982	274811	5	100
10702_4#69	3	V95	Mali	2011	MDR		Enteritidis	11	4818445	39	123549.87	1236738	402432	4	100
10702_4#70	3	X41	Mali	2011	MDR		Enteritidis	11	4733431	32	147919.72	657601	400976	5	100
10702_4#71	3	W42	Mali	2011	Amp+chlor		Enteritidis	11	4734386	35	135268.17	1065937	364782	4	100
10702_4#72	3	R74	Mali	2008	MDR		Enteritidis	11	4733361	27	175309.67	829982	405371	5	100
10702_4#73	3	S76	Mali	2008	MDR		Enteritidis	11	4731862	26	181994.69	694624	402745	5	100
10702_4#74	3	S4	Mali	2008	Amp+chlor		Enteritidis	11	4732039	27	175260.7	740981	453508	4	100
10702_4#75	3	V91	Mali	2011	MDR		Enteritidis	11	4732771	28	169027.54	1128272	597053	3	100
10702_4#76	3	X25	Mali	2010	Ampicillin		Enteritidis	11	4713869	21	224469.95	746576	422603	4	100
10702_4#77	3	S85	Mali	2009	MDR		Enteritidis	11	4734659	38	124596.29	520950	337225	6	100
10702_4#78	3	S56	Mali	2008	Ampicillin		Enteritidis	11	4713610	22	214255	729218	490355	4	100
10702_4#79	3	I15	Mali	2002	Sensitive		Enteritidis	11	4713319	20	235665.95	729221	494156	4	100
10702_4#80	3	T129	Mali	2009	MDR		Enteritidis	11	4731028	36	131417.44	663736	367212	5	100
10702_4#81	3	W19	Mali	2011	Ampicillin		Enteritidis	11	4680339	32	146260.59	657604	329328	5	100
10702_4#82	3	P177	Mali	2006	Ampicillin+cotrimoxazole		Enteritidis	11	4759999	24	198333.29	812254	633670	4	100
10702_4#83	3	R20	Mali	2007	Ampicillin+cotrimoxazole		Enteritidis	11	4761345	24	198389.38	1141102	404965	3	100
10702_4#84	3	W4	Mali	2011	Amp+chlor		Enteritidis	11	4731582	27	175243.78	680374	401048	5	100
10702_4#85	3	W24	Mali	2011	MDR		Enteritidis	11	4732337	29	163184.03	657597	367220	5	100
10702_4#86	3	T66	Mali	2009	Ampicillin		Enteritidis	1632	4713179	21	224437.1	740980	490249	4	100
10702_4#87	3	J55	Mali	2004	Ampicillin+cotrimoxazole		Enteritidis	11	4761642	27	176357.11	877632	416080	4	100
4407_7#1	6	D3063	Malawi	1997	Sensitive	Blood	Enteritidis	11	4698813	82	57302.6	410633	86142	15	54
4407_7#10	6	A3582	Malawi	1999	Sensitive	Blood	Enteritidis	11	4701167	35	134319.06	735431	284021	5	54
4407_7#11	4	D3314	Malawi	1999	MDR	Blood	Enteritidis	1632	4791685	85	56372.76	353811	120928	13	54
4407_7#12	6	D1374	Malawi	1999	Sensitive	Blood	Enteritidis	11	4700300	35	134294.29	703787	289816	6	54
4407_7#2	4	A9395	Malawi	2000	MDR	Blood	Enteritidis	11	4786245	82	58368.84	270650	119451	14	54
4407_7#3	4	D10599	Malawi	2000	MDR	Blood	Enteritidis	11	4791125	62	77276.21	799673	178958	8	54
4407_7#4	4	D7795	Malawi	2000	MDR	Blood	Enteritidis	11	4801421	59	81380.02	810044	152813	9	54
4407_7#5	5	D3098	Malawi	1997	Sensitive	Blood	Enteritidis	11	4688867	43	109043.42	1035547	188699	7	54
4407_7#6	2	D3493	Malawi	1997	Sensitive	Blood	Enteritidis	11	4801767	83	57852.61	371820	113118	13	54
4407_7#7	6	A1636	Malawi	1998	Sensitive	Blood	Enteritidis	11	4701599	40	117539.98	755756	226387	7	54
4407_7#8	6	A2331	Malawi	1998	Sensitive	Blood	Enteritidis	11	4742537	44	107784.93	650946	207979	7	54
4407_7#9	2	A850	Malawi	1998	Sensitive	Blood	Enteritidis	11	4692952	43	109138.42	651885	172058	7	54
4407_8#1	4	A13085	Malawi	2001	MDR	Blood	Enteritidis	11	4791932	264	18151.26	172642	42788	36	54
4407_8#10	6	D23847	Malawi	2004	Sensitive	Blood	Enteritidis	11	4705300	91	51706.59	356119	153238	11	54
4407_8#11	4	D21704	Malawi	2003	MDR	Blood	Enteritidis	11	4783746	115	41597.79	375327	140125	12	54
4407_8#12	4	A25257	Malawi	2003	MDR	Blood	Enteritidis	11	4758371	145	32816.35	246296	109454	16	54
4407_8#2	4	D28919	Malawi	2004	MDR	Blood	Enteritidis	11	4797484	160	29984.28	355013	106198	14	54
4407_8#3	4	A32803	Malawi	2004	MDR	Blood	Enteritidis	11	4837760	136	35571.76	243161	124252	14	54
4407_8#5	4	D11474	Malawi	2001	MDR	Blood	Enteritidis	1632	4834154	87	55564.99	602811	171113	10	54
4407_8#6	4	D11509	Malawi	2001	MDR	Blood	Enteritidis	11	4805207	155	31001.34	390985	103874	15	54
4407_8#7	6	D16885	Malawi	2002	Sensitive	Blood	Enteritidis	11	4714879	162	29104.19	228607	80085	20	54
4407_8#8	4	A17892	Malawi	2002	MDR	Blood	Enteritidis	11	4791730	102	46977.75	651463	130593	11	54
4407_8#9	4	A17731	Malawi	2002	MDR	Blood	Enteritidis	11	4841259	154	31436.75	323403	119152	14	54
4414_7#1	6	A38441	Malawi	2005	Sensitive	Blood	Enteritidis	11	4700866	101	46543.23	218811	91693	19	54
4414_7#10	4	D36956	Malawi	2006	Unknown	Blood	Enteritidis	11	4767267	51	93475.82	911626	195740	7	54

4414_7#11	6	D25809	Malawi	2004	Unknown	Blood	Enteritidis	11	4697703	41	114578.12	597176	199579	8	54
4414_7#12	2	D23652	Malawi	2004	Sensitive	Blood	Enteritidis	11	4691217	44	106618.57	602939	207965	7	54
4414_7#2	6	D23145	Malawi	2003	Sensitive	Blood	Enteritidis	11	4911712	137	35851.91	254617	80505	19	54
4414_7#3	4	D40290	Malawi	2006	MDR	Blood	Enteritidis	11	4828947	98	49274.97	246934	112009	17	54
4414_7#4	2	D23071	Malawi	2003	Sensitive	Blood	Enteritidis	11	4857584	40	121439.6	647118	228847	7	54
4414_7#5	2	D35807	Malawi	2005	Sensitive	Blood	Enteritidis	11	4758523	33	144197.67	894998	379058	5	54
4414_7#7	6	A39243	Malawi	2006	Sensitive	Blood	Enteritidis	11	4700208	63	74606.48	546641	148920	10	54
4414_7#8	6	A39959	Malawi	2006	Sensitive	Blood	Enteritidis	11	4701153	32	146911.03	987870	273804	5	54
4414_7#9	4	D36263	Malawi	2006	MDR	Blood	Enteritidis	11	4773001	44	108477.3	962233	232307	6	54
4414_8#1	2	D24359	Malawi	2004	Sensitive	Blood	Enteritidis	1632	4768069	557	8560.27	47398	14594	106	54
4414_8#5	2	D23974	Malawi	2004	Unknown	Blood	Enteritidis	11	4875726	43	113388.98	624654	318176	6	54
4414_8#6	4	D24443	Malawi	2004	MDR	Blood	Enteritidis	11	4828590	112	43112.41	255038	86821	17	54
4414_8#7	6	D21685	Malawi	2003	Sensitive	Blood	Enteritidis	892	6486258	7423	873.81	177449	62461	35	54
5187_2#11	3	MLP184	Mali	2006	Amp+Chlor	Blood	Enteritidis	11	4893686	51	95954.63	450944	256063	7	76
5187_2#12	3	MLI21	Mali	2002	MDR	Blood	Enteritidis	11	4902168	67	73166.69	644772	255760	6	76
5187_2#7	3	MLI99	Mali	2003	Amp+Chlor	Blood	Enteritidis	11	4755133	49	97043.53	492092	282141	7	76
5187_2#9	3	MLP43	Mali	2005	MDR	Blood	Enteritidis	11	4707970	45	104621.56	646123	284009	6	76
5886_5#1	2	01-00493-2	Germany	2001	Unknown	Reptile	Enteritidis	168	4584851	29	158098.31	646614	500484	4	76
5886_5#10	6	Salm0053		Unknown	Unknown		Enteritidis	11	4694700	28	167667.86	900393	305254	5	76
5886_5#11	2	99-02302	Germany	1999	Unknown	Hedgehog	Enteritidis	183	4898575	44	111331.25	768470	296751	5	76
5886_5#5	1	MZ0743	USA	1948	Unknown	Guinea pig	Enteritidis	50	5067376	219	23138.7	555590	254843	8	76
5886_6#2	2	00-03508	Germany	2000	Unknown	Hedgehog	Enteritidis	183	4894266	58	84383.9	741998	375582	5	76
5886_6#5	5	LH_M296	USA	2001	Unknown	Unknown	Enteritidis	11	4686968	28	167391.71	900099	305559	4	76
5886_6#8	2	SARB18_FB	USA	1988	Unknown	NK	Enteritidis	11	4857304	48	101193.83	777808	401830	5	76
5969_5#8	5	31/88	Uruguay	1988	Sensitive	Gastroenteritis	Enteritidis	1632	4707746	44	106994.23	790594	440717	4	76
5969_6#1	5	8*89	Uruguay	1989	Sensitive	Blood	Enteritidis	1632	4710701	49	96136.76	647020	249462	6	76
5969_6#2	6	53*94	Uruguay	1994	Sensitive	Food	Enteritidis	11	4702158	38	123741	925928	249522	5	76
5969_6#3	6	206*99	Uruguay	1999	Sensitive	Food	Enteritidis	11	4608090	35	131659.71	646938	249352	6	76
5969_6#4	6	251*01	Uruguay	2001	Sensitive	Food	Enteritidis	11	4702392	31	151690.06	729453	297685	5	76
5969_6#5	6	8*02	Uruguay	2002	Sensitive	Stool	Enteritidis	11	4706417	39	120677.36	647766	249461	6	76
5969_6#6	6	214*02	Uruguay	2002	Sensitive	Blood	Enteritidis	11	4640964	27	171887.56	696005	477409	4	76
5969_6#7	6	10*05	Uruguay	2005	Sensitive	Stool	Enteritidis	11	4705913	34	138409.21	647376	297453	5	76
6601_8#1	6	KUMAR1	UK	Unknown	Unknown		Enteritidis	11	4701873	18	261215.17	1544932	647872	3	75
6601_8#10	6	KUMAR10	UK	2009	Unknown	Stool	Enteritidis	11	4702757	13	361750.54	1549392	813310	2	75
6601_8#11	6	KUMAR11	UK	2009	Unknown	Stool	Enteritidis	11	4702527	22	213751.23	1223933	647865	3	75
6601_8#13	6	KUMAR13	UK	2009	Unknown	Stool	Enteritidis	11	4705989	20	235299.45	1323636	696207	3	75
6601_8#14	6	KUMAR14	UK	2009	Unknown	Stool	Enteritidis	11	4646238	14	331874.14	1632926	694909	2	75
6601_8#15	6	KUMAR15	UK	2009	Unknown	Stool	Enteritidis	11	4707763	20	235388.15	1549416	489907	3	75
6601_8#16	6	KUMAR16	UK	2009	Unknown	Stool	Enteritidis	11	4703790	19	247567.89	1549395	489905	3	75
6601_8#17	6	KUMAR17	UK	2009	Unknown	Stool	Enteritidis	11	4702173	20	235108.65	1223919	667957	3	75
6601_8#18	6	KUMAR18	UK	2009	Unknown	Stool	Enteritidis	11	4699354	22	213607	1223722	647758	3	75
6601_8#19	5	KUMAR19	UK	2010	Sensitive	Stool	Enteritidis	11	4702827	20	235141.35	1508466	478636	3	75
6601_8#2	5	KUMAR2	UK	Unknown	Unknown		Enteritidis	11	4697086	20	234854.3	1508867	483674	3	75
6601_8#20	6	KUMAR20	UK	2010	Sensitive	Stool	Enteritidis	11	4831375	17	284198.53	2042124	729677	2	75
6601_8#5	6	KUMAR5	UK	2008	Unknown	Stool	Enteritidis	11	4701157	13	361627.46	1630720	489913	3	75
6601_8#6	6	KUMAR6	UK	2008	Unknown	Stool	Enteritidis	11	4749803	17	279400.18	1549388	489902	3	75
6601_8#7	6	KUMAR7	UK	2008	Unknown	Stool	Enteritidis	11	4772237	25	190889.48	1224380	406127	4	75
6601_8#8	6	KUMAR8	UK	2008	Unknown	Stool	Enteritidis	11	4703661	12	391971.75	2125318	694502	2	75

6601_8#9	6	KUMAR9	UK	2008	Unknown	Stool	Enteritidis	11	4707319	14	336237.07	1549405	663818	3	75
6938_2#2	4	D24954	Malawi	2004	MDR	Blood	Enteritidis	11	4763382	33	144344.91	1308873	402043	4	75
6938_2#3	6	D24793	Malawi	2004	Sensitive	Blood	Enteritidis	11	4705036	17	276766.82	1544914	663711	3	75
6938_2#4	6	D23682	Malawi	2004	Sensitive	Blood	Enteritidis	11	4706087	13	362006.69	1555458	647880	3	75
6938_2#5	6	D24953	Malawi	2004	Sensitive	Blood	Enteritidis	11	4706189	19	247694.16	1544949	489903	3	75
8376_8#91	6							11	4706784	18	261488	1549481	489984	3	100
8525_2#14	6	Ke276	Kenya	2004	Unknown	Stool	Enteritidis	11	4700512	19	247395.37	1548285	694520	3	100
8525_2#21	6	Ke292	Kenya	2004	Unknown	CSF	Enteritidis	11	4701533	25	188061.32	855378	489767	4	100
8525_2#29	6	Ke304	Kenya	2001	Unknown	Stool	Enteritidis	11	4701806	22	213718.45	1470156	647639	3	100
8525_2#84	6	Ke409	Kenya	2000	Unknown	Stool	Enteritidis	11	4711116	13	362393.54	1549420	891959	2	100
8525_2#9	6	Ke267	Kenya	2003	Unknown	Stool	Enteritidis	11	4703339	12	391944.92	1719453	813371	2	100
8525_2#92	4	Ke417	Kenya	2003	Unknown	Stool	Enteritidis	11	4853458	32	151670.56	1074381	405356	4	100
8525_3#1	6	Ke422	Kenya	2004	Unknown	Stool	Enteritidis	11	4738325	16	296145.31	2038742	817321	2	100
8525_3#13	4	Ke434	Kenya	2003	Unknown	Stool	Enteritidis	11	6420437	4717	1361.13	1077253	229704	6	100
8525_3#24	6	Ke445	Kenya	2005	Unknown	Stool	Enteritidis	11	4700961	14	335782.93	1779844	813179	2	100
8525_3#27	4	Ke448	Kenya	2000	Unknown	Stool	Enteritidis	11	4878482	141	34599.16	1074500	486542	4	100
8525_3#63	4	Ke484	Kenya	2000	Unknown	Stool	Enteritidis	11	4858341	30	161944.7	1400740	559838	3	100
8525_3#64	4	Ke485	Kenya	2001	Unknown	Stool	Enteritidis	11	4793171	31	154618.42	1072017	515076	3	100
8525_3#65	6	Ke486	Kenya	2001	Unknown	Stool	Enteritidis	11	4708991	17	276999.47	1543775	489817	3	100
8525_3#67	4	Ke488	Kenya	2001	Unknown	Stool	Enteritidis	11	4802509	39	123141.26	1010465	395446	4	100
8525_3#70	4	Ke491	Kenya	2001	Unknown	Blood	Enteritidis	11	4795058	38	126185.74	1074350	327213	4	100
8987_8#1	5	101	Malawi	1996	Sensitive	Blood	Enteritidis	11	4699495	77	61032.4	681273	282166	5	100
8987_8#10	4	D7419	Malawi	2000	MDR	Blood	Enteritidis	11	4778658	44	108605.86	820330	255887	6	100
8987_8#11	3	253	Malawi	1996	Sensitive	Blood	Enteritidis	11	4753882	24	198078.42	812481	441551	4	100
8987_8#12	4	A17787	Malawi	2002	MDR	Blood	Enteritidis	11	4830986	43	112348.51	944449	284597	5	100
8987_8#13	4	D6007	Malawi	2000	MDR	Blood	Enteritidis	11	4841594	40	121039.85	813499	405044	5	100
8987_8#14	4	A54341	Malawi	2009	MDR	Blood	Enteritidis	11	4840134	41	118052.05	1101044	405181	4	100
8987_8#15	4	A55767	Malawi	2009	Sensitive	Blood	Enteritidis	11	4804026	36	133445.17	1326089	401394	4	100
8987_8#16	6	A32229	Malawi	2004	Sensitive	Blood	Enteritidis	11	6678622	1293	5165.21	281222	62433	29	100
8987_8#18	2	A40500	Malawi	2006	Sensitive	Blood	Enteritidis	11	4701278	39	120545.59	993236	405728	4	100
8987_8#19	4	D5293	Malawi	2000	MDR	Blood	Enteritidis	11	4783971	39	122665.92	912425	284502	5	100
8987_8#20	4	D6437	Malawi	2000	MDR	Blood	Enteritidis	11	4795330	50	95906.6	898002	284312	5	100
8987_8#23	4	A7830	Malawi	2000	Sensitive	Blood	Enteritidis	11	4778101	42	113764.31	814016	255784	6	100
8987_8#25	6	A51300	Malawi	2008	Sensitive	Blood	Enteritidis	11	4704232	20	235211.6	1000240	647825	3	100
8987_8#26	4	A58824	Malawi	2010	MDR	Blood	Enteritidis	11	4770384	28	170370.86	1648654	695432	3	100
8987_8#27	6	A28666	Malawi	2004	Sensitive	Blood	Enteritidis	11	4702380	22	213744.55	899861	439428	4	100
8987_8#28	6	A30023	Malawi	2004	Sensitive	Blood	Enteritidis	11	4705709	22	213895.86	900796	548344	4	100
8987_8#29	4	D3419	Malawi	1999	MDR	Blood	Enteritidis	11	4788367	36	133010.19	652839	405117	5	100
8987_8#30	2	A1284	Malawi	1998	Sensitive	Blood	Enteritidis	11	4694416	25	187776.64	1142913	439639	4	100
8987_8#31	6	548	Malawi	1996	Sensitive	Blood	Enteritidis	11	4756167	24	198173.62	950134	647835	3	100
8987_8#32	4	A6379	Malawi	1999	MDR	Blood	Enteritidis	11	4803513	37	129824.68	944384	511518	4	100
8987_8#33	4	D7378	Malawi	2000	MDR	Blood	Enteritidis	11	4797994	40	119949.85	819979	259002	6	100
8987_8#35	6	A49455	Malawi	2007	Sensitive	Blood	Enteritidis	11	4704842	19	247623.26	900461	489038	4	100
8987_8#38	6	A46872	Malawi	2007	Sensitive	Blood	Enteritidis	11	4703357	24	195973.21	900569	403336	4	100
8987_8#39	4	D3421	Malawi	1999	MDR	Blood	Enteritidis	11	4796551	41	116989.05	821405	258957	6	100
8987_8#4	4	A39959a	Malawi	2006	Unknown	Blood	Enteritidis	11	4855758	41	118433.12	511639	256210	7	100
8987_8#40	4	A13216	Malawi	2001	MDR	Blood	Enteritidis	11	4803532	41	117159.32	813820	265860	6	100
8987_8#41	5	2702	Malawi	1996	Sensitive	Blood	Enteritidis	11	4672050	20	233602.5	1279254	479646	3	100
8987_8#42	4	A7369	Malawi	2000	MDR	Blood	Enteritidis	11	4798294	38	126270.89	813778	405069	5	100
8987_8#45	6	A48460	Malawi	2007	Sensitive	Blood	Enteritidis	11	4708061	23	204698.3	966162	441530	4	100
8987_8#46	6	A59024	Malawi	2010	Sensitive	Blood	Enteritidis	11	4753424	25	190136.96	1132170	402805	4	100
8987_8#47	6	A34057	Malawi	2005	Sensitive	Blood	Enteritidis	11	4699707	20	234985.35	900331	489186	4	100

8987_8#48	6	A46756	Malawi	2007	Sensitive	Blood	Enteritidis	11	4703585	24	195982.71	900582	490554	4	100
8987_8#49	4	A17698	Malawi	2002	MDR	Blood	Enteritidis	11	4790511	43	111407.23	813530	255769	6	100
8987_8#5	4	A28721	Malawi	2004	MDR	Blood	Enteritidis	11	4798316	38	126271.47	819979	501642	4	100
8987_8#50	4	A12756	Malawi	2001	MDR	Blood	Enteritidis	11	4807544	48	100157.17	814490	255719	6	100
8987_8#51	6	3857	Malawi	1997	Sensitive	Blood	Enteritidis	11	4648406	19	244652.95	1821512	647696	2	100
8987_8#52	4	A9086	Malawi	2000	MDR	Blood	Enteritidis	11	4831916	38	127155.68	813416	405073	5	100
8987_8#54	4	A59040	Malawi	2010	MDR	Blood	Enteritidis	11	4827468	35	137927.66	511794	397222	6	100
8987_8#55	4	D43980	Malawi	2007	Chloramphenicol	Blood	Enteritidis	11	4772879	36	132579.97	813844	369337	5	100
8987_8#56	6	A57601	Malawi	2010	Sensitive	Blood	Enteritidis	11	4706400	19	247705.26	1341045	516236	3	100
8987_8#58	4	A30782	Malawi	2004	MDR	Blood	Enteritidis	11	4795517	40	119887.93	1073929	330858	5	100
8987_8#59	4	D3558	Malawi	1999	Unknown	Blood	Enteritidis	11	4778873	47	101678.15	820109	255789	6	100
8987_8#6	4	A53601	Malawi	2008	MDR	Blood	Enteritidis	11	4787312	37	129386.81	760532	294192	5	100
8987_8#60	6	A12683	Malawi	2001	Sensitive	Blood	Enteritidis	11	4704932	22	213860.55	1003615	480814	4	100
8987_8#61	2	6567	Malawi	1997	Sensitive	Blood	Enteritidis	11	4693937	33	142240.52	916040	405582	4	100
8987_8#63	3	A51583	Malawi	2008	Sensitive	Blood	Enteritidis	11	4765368	35	136153.37	646403	301945	6	100
8987_8#65	6	A54595	Malawi	2009	Sensitive	Blood	Enteritidis	11	4700060	22	213639.09	900624	476857	4	100
8987_8#66	6	A56756	Malawi	2009	Sensitive	Blood	Enteritidis	11	4700077	25	188003.08	900444	477369	4	100
8987_8#67	4	A53016	Malawi	2008	MDR	Blood	Enteritidis	11	4775480	40	119387	813715	281990	5	100
8987_8#68	6	A31243	Malawi	2004	Sensitive	Blood	Enteritidis	11	4702385	22	213744.77	1071273	440023	4	100
8987_8#69	4	A9215	Malawi	2000	MDR	Blood	Enteritidis	11	4802207	76	63186.93	692563	374930	5	100
8987_8#70	4	D11119	Malawi	2000	MDR	Blood	Enteritidis	11	4783232	45	106294.04	601354	259004	6	100
8987_8#71	6	7909	Malawi	1998	Sensitive	Blood	Enteritidis	11	4701777	29	162130.24	900551	439681	4	100
8987_8#73	6	A54059	Malawi	2009	Unknown	Blood	Enteritidis	11	4705043	24	196043.46	900431	439986	4	100
8987_8#74	6	D54684	Malawi	2009	Unknown	Blood	Enteritidis	11	4702713	26	180873.58	922151	439543	4	100
8987_8#75	6	A36950a	Malawi	2005	Sensitive	Blood	Enteritidis	11	4703807	21	223990.81	923137	649608	3	100
8987_8#76	4	A46277	Malawi	2007	MDR	Blood	Enteritidis	11	4865750	59	82470.34	1305439	410182	4	100
8987_8#77	4	A31878	Malawi	2004	MDR	Blood	Enteritidis	11	4793954	58	82654.38	816770	227679	7	100
8987_8#78	4	D16245	Malawi	2002	MDR	Blood	Enteritidis	11	4885786	81	60318.35	923383	480698	4	100
8987_8#8	6	A40882	Malawi	2006	Sensitive	Blood	Enteritidis	11	4701728	23	204422.96	900349	440882	4	100
8987_8#80	5	9486	Malawi	1998	Unknown	Blood	Enteritidis	11	4747421	115	41281.92	897359	405858	4	100
8987_8#81	6	A51073	Malawi	2008	MDR	Blood	Enteritidis	11	4647697	21	221318.9	920093	651867	3	100
8987_8#83	4	A53811	Malawi	2009	MDR	Blood	Enteritidis	11	7624358	2217	3439.04	217566	26739	63	100
8987_8#84	6	A36678	Malawi	2005	Sensitive	Blood	Enteritidis	11	4700708	24	195862.83	900652	305281	5	100
8987_8#86	4	A53742	Malawi	2009	MDR	Blood	Enteritidis	11	4772938	43	110998.56	947044	314753	5	100
8987_8#88	4	D17739	Malawi	2002	Unknown	Blood	Enteritidis	11	4841972	41	118096.88	944283	259741	5	100
8987_8#89	4	A7828	Malawi	2000	MDR	Blood	Enteritidis	11	4794139	37	129571.32	820049	405107	4	100
8987_8#9	4	A34142	Malawi	2005	MDR	Blood	Enteritidis	11	4779460	49	97540	532809	284393	6	100
8987_8#90	4	A53843	Malawi	2009	Sensitive	Blood	Enteritidis	11	4780516	34	140603.41	998005	306625	5	100
8987_8#91	4	A36950	Malawi	2005	Sensitive	Blood	Enteritidis	11	4835898	48	100747.88	813803	259020	6	100
8987_8#92	6	A45427	Malawi	2007	Sensitive	Blood	Enteritidis	11	4698021	28	167786.46	706759	375650	5	100
8987_8#93	4	A35261	Malawi	2005	MDR	Blood	Enteritidis	11	4778631	39	122529	837369	259059	6	100
8987_8#94	6	A41750	Malawi	2006	Sensitive	Blood	Enteritidis	11	4706427	24	196101.12	900491	405903	4	100
8987_8#95	6	1971	Malawi	1996	Sensitive	Blood	Enteritidis	11	4701977	21	223903.67	1132060	439643	3	100
8987_8#96	2	D5408	Malawi	2000	Sensitive	Blood	Enteritidis	11	4692281	49	95760.84	915743	283957	4	100
9032_4#1	4	31353	Congo	2010	MDR	Blood	Enteritidis	11	4786464	43	111313.12	519719	290048	6	100
9032_4#10	4	33013	Congo	2011	MDR	Blood	Enteritidis	11	4779820	46	103909.13	607219	281912	6	100
9032_4#11	4	33033	Congo	2011	MDR	Blood	Enteritidis	11	4782924	41	116656.68	813882	308930	5	100
9032_4#12	4	33078	Congo	2011	MDR	Blood	Enteritidis	11	4775467	62	77023.66	813828	208676	7	100
9032_4#13	4	33393	Congo	2011	MDR	Blood	Enteritidis	11	4788703	39	122787.26	900879	405331	4	100
9032_4#14	4	33453	Congo	2011	MDR	Blood	Enteritidis	11	4780802	46	103930.48	785551	258986	5	100
9032_4#15	4	33513	Congo	2011	MDR	Blood	Enteritidis	11	4788006	56	85500.11	899221	231195	6	100
9032_4#16	4	33523	Congo	2011	MDR	Blood	Enteritidis	11	4782720	45	106282.67	853168	260047	6	100

9032_4#17	4	33623	Congo	2011	MDR	Blood	Enteritidis	11	4777876	42	113758.95	937640	410768	5	100
9032_4#18	4	33653	Congo	2011	MDR	Blood	Enteritidis	11	4784513	40	119612.82	814906	410841	4	100
9032_4#19	4	33663	Congo	2011	MDR	Blood	Enteritidis	11	4774213	54	88411.35	511152	227725	8	100
9032_4#2	4	32363	Congo	2010	MDR	Blood	Enteritidis	11	4788632	34	140842.12	1056245	422987	4	100
9032_4#20	4	33673	Congo	2011	MDR	Blood	Enteritidis	11	4774203	67	71256.76	692670	152797	8	100
9032_4#21	4	33683	Congo	2011	MDR	Blood	Enteritidis	11	4794522	38	126171.63	1076049	477533	4	100
9032_4#22	4	33693	Congo	2011	MDR	Blood	Enteritidis	11	4785488	40	119637.2	813858	328474	5	100
9032_4#23	4	33703	Congo	2011	MDR	Blood	Enteritidis	11	4783392	44	108713.45	805239	306638	5	100
9032_4#24	4	33713	Congo	2011	MDR	Blood	Enteritidis	11	4779462	42	113796.71	814267	390161	4	100
9032_4#25	4	33873	Congo	2011	MDR	Blood	Enteritidis	11	5118772	4428	1156	944203	260520	6	100
9032_4#26	4	33893	Congo	2011	MDR	Blood	Enteritidis	11	4777803	46	103865.28	944672	282173	5	100
9032_4#27	4	33913	Congo	2011	MDR	Blood	Enteritidis	11	4786055	47	101830.96	1083857	405114	4	100
9032_4#28	4	33923	Congo	2011	MDR	Blood	Enteritidis	11	4785022	49	97653.51	955904	374756	4	100
9032_4#29	4	33943	Congo	2011	MDR	Blood	Enteritidis	11	4774553	63	75786.56	602660	212159	8	100
9032_4#3	4	32013	Congo	2010	MDR	Blood	Enteritidis	11	4787858	32	149620.56	1156904	567537	3	100
9032_4#30	4	33993	Congo	2011	MDR	Blood	Enteritidis	11	4783691	39	122658.74	903744	478692	4	100
9032_4#31	4	34083	Congo	2011	MDR	Blood	Enteritidis	11	4777814	52	91881.04	813562	261454	6	100
9032_4#32	4	34173	Congo	2011	MDR	Blood	Enteritidis	11	4779467	55	86899.4	823938	284224	5	100
9032_4#33	4	34193	Congo	2011	MDR	Blood	Enteritidis	11	4787054	53	90321.77	826696	255585	6	100
9032_4#34	4	34223	Congo	2011	MDR	Blood	Enteritidis	11	4783799	41	116678.02	997693	284362	5	100
9032_4#35	4	34233	Congo	2011	MDR	Blood	Enteritidis	11	4781862	48	99622.12	923349	284363	5	100
9032_4#36	4	34243	Congo	2011	MDR	Blood	Enteritidis	11	4785570	42	113942.14	620931	284396	6	100
9032_4#37	4	34253	Congo	2011	MDR	Blood	Enteritidis	11	4783234	41	116664.24	692742	314653	5	100
9032_4#38	4	34393	Congo	2011	MDR	Blood	Enteritidis	11	4787805	41	116775.73	814008	263525	5	100
9032_4#39	4	34403	Congo	2011	MDR	Blood	Enteritidis	11	4788818	45	106418.18	913326	284361	5	100
9032_4#4	4	32963	Congo	2011	MDR	Blood	Enteritidis	11	4825769	55	87741.25	815251	284200	5	100
9032_4#40	4	34413	Congo	2011	MDR	Blood	Enteritidis	11	4782571	41	116648.07	944204	284320	5	100
9032_4#41	4	34423	Congo	2011	MDR	Blood	Enteritidis	11	4779639	55	86902.53	826726	255645	6	100
9032_4#42	4	34443	Congo	2011	MDR	Blood	Enteritidis	11	4794641	43	111503.28	737197	404944	5	100
9032_4#44	4	34483	Congo	2011	MDR	Blood	Enteritidis	11	4783522	48	99656.71	899286	259775	6	100
9032_4#45	4	34493	Congo	2011	MDR	Blood	Enteritidis	11	4776536	62	77040.9	813474	221808	6	100
9032_4#46	4	34503	Congo	2011	MDR	Blood	Enteritidis	11	4780640	39	122580.51	813597	439242	4	100
9032_4#47	4	34563	Congo	2011	MDR	Blood	Enteritidis	11	4781742	46	103950.91	692795	284224	6	100
9032_4#48	4	34683	Congo	2011	MDR	Blood	Enteritidis	11	4787204	44	108800.09	1075239	405109	4	100
9032_4#49	4	34853	Congo	2011	MDR	Blood	Enteritidis	11	4786300	39	122725.64	1307307	405246	4	100
9032_4#5	4	32523	Congo	2010	MDR	Blood	Enteritidis	11	4786467	49	97683	944606	314641	5	100
9032_4#50	4	34863	Congo	2011	MDR	Blood	Enteritidis	11	4794182	37	129572.49	1063255	405333	4	100
9032_4#51	4	34873	Congo	2011	MDR	Blood	Enteritidis	11	4781897	39	122612.74	813483	284423	5	100
9032_4#52	4	34893	Congo	2011	MDR	Blood	Enteritidis	11	4786119	44	108775.43	806047	410761	4	100
9032_4#53	4	34933	Congo	2011	MDR	Blood	Enteritidis	11	4785452	44	108760.27	813831	328489	5	100
9032_4#55	4	35203	Congo	2011	MDR	Blood	Enteritidis	11	4776074	52	91847.58	813547	255590	6	100
9032_4#56	4	35213	Congo	2011	MDR	Blood	Enteritidis	11	4785949	38	125946.03	710795	404786	5	100
9032_4#57	4	35223	Congo	2011	MDR	Blood	Enteritidis	11	4776551	60	79609.18	813913	189398	7	100
9032_4#58	4	35263	Congo	2011	MDR	Blood	Enteritidis	11	4777177	51	93670.14	693020	253518	7	100
9032_4#59	4	35433	Congo	2011	MDR	Blood	Enteritidis	11	4782807	56	85407.27	824024	255643	6	100
9032_4#6	4	32633	Congo	2011	MDR	Blood	Enteritidis	11	4781244	45	106249.87	813869	314645	5	100
9032_4#60	4	35443	Congo	2011	MDR	Blood	Enteritidis	11	4783873	46	103997.24	813682	256349	6	100
9032_4#61	4	35473	Congo	2011	MDR	Blood	Enteritidis	11	4778233	61	78331.69	813880	183781	7	100
9032_4#62	4	35563	Congo	2011	MDR	Blood	Enteritidis	11	4787797	44	108813.57	1794309	306638	3	100
9032_4#63	4	36803	Congo	2011	MDR	Blood	Enteritidis	11	4786796	42	113971.33	813644	259030	6	100
9032_4#64	4	36723	Congo	2011	MDR	Blood	Enteritidis	11	4791177	50	95823.54	828836	284320	5	100
9032_4#65	4	36343	Congo	2011	MDR	Blood	Enteritidis	11	4761593	41	116136.41	810632	403023	4	100

9032_4#66	4	36313	Congo	2011	MDR	Blood	Enteritidis	11	4778343	61	78333.49	621811	227685	7	100
9032_4#67	4	36183	Congo	2011	MDR	Blood	Enteritidis	11	4778855	52	91901.06	805563	230110	7	100
9032_4#68	4	36173	Congo	2011	MDR	Blood	Enteritidis	11	4788077	43	111350.63	985888	306639	5	100
9032_4#69	4	36163	Congo	2011	MDR	Blood	Enteritidis	11	4781621	57	83888.09	546962	259005	6	100
9032_4#70	4	36103	Congo	2011	MDR	Blood	Enteritidis	11	4775653	90	53062.81	692522	150022	9	100
9032_4#71	4	35693	Congo	2011	MDR	Blood	Enteritidis	11	4777361	56	85310.02	692743	221631	7	100
9032_4#72	4	35773	Congo	2011	MDR	Blood	Enteritidis	11	4786921	44	108793.66	838566	314635	5	100
9032_4#73	4	35723	Congo	2011	MDR	Blood	Enteritidis	1632	4787580	43	111339.07	985712	405235	4	100
9032_4#74	4	35713	Congo	2011	MDR	Blood	Enteritidis	11	4792231	40	119805.77	836648	405188	5	100
9032_4#8	4	32863	Congo	2011	MDR	Blood	Enteritidis	11	4791208	37	129492.11	1079266	405133	4	100
9032_4#9	4	32853	Congo	2010	MDR	Blood	Enteritidis	11	4787212	42	113981.24	987539	281801	5	100
9032_4#92	4	1020983	Malawi	2011	MDR	Blood	Enteritidis	11	4804694	40	120117.35	819822	477518	4	100
9032_4#96	2	A59130	Malawi	2010	Unknown	Blood	Enteritidis	11	4699845	22	213629.32	1161320	439610	4	100
9716_8#10	5	119_2012K_187	USA	2012	Unknown	Stool	Enteritidis	11	4740791	23	206121.35	1551977	478981	3	100
9716_8#11	6	120_2021	USA	Unknown	Unknown	Unknown	Antarctica	11	4713775	18	261876.39	1551722	686807	3	100
9716_8#12	3	121_67	USA	Unknown	Unknown	Unknown	Blegdam	11	4751476	23	206585.91	1070428	480137	4	100
9716_8#13	3	122_68	USA	Unknown	Unknown	Unknown	Blegdam	11	4705109	16	294069.31	1151008	771144	3	100
9716_8#15	6	124_2711	USA	Unknown	Unknown	Unknown	Rosenberg	11	4704194	19	247589.16	1780173	694579	2	100
9716_8#16	5	125_81_2625	USA	1981	Unknown	Blood	Enteritidis	11	4708018	21	224191.33	1517478	489959	3	100
9716_8#17	5	126_88_303	USA	1988	Unknown	Stool	Enteritidis	11	4705580	22	213890	1508449	490375	3	100
9716_8#18	6	127_85_98	USA	1985	Unknown	Stool	I 9,12:g,m,p:-	11	4704177	18	261343.17	1549495	970211	2	100
9716_8#2	2	111_2012K_181	USA	2011	Unknown	Stool	Enteritidis	814	4726855	21	225088.33	1020585	489698	4	100
9716_8#20	2	129_88_415	USA	Unknown	Unknown	Unknown	Enteritidis	11	4719290	18	262182.78	2001305	892626	2	100
9716_8#21	5	130_88_408	USA	Unknown	Unknown	Unknown	Enteritidis	11	4700838	22	213674.45	1496424	969257	2	100
9716_8#23	1	132_88_406	USA	Unknown	Unknown	Unknown	Enteritidis	136	4726879	22	214858.14	1818589	682801	2	100
9716_8#24	5	133_48_0565	USA	1948	Unknown	Animal	Enteritidis	11	4643929	25	185757.16	1346289	479006	3	100
9716_8#25	5	135_50_3079	USA	1949	Unknown	Unknown	Enteritidis	11	4736691	18	263149.5	1346309	709907	3	100
9716_8#26	6	136_50_4630	USA	1950	Unknown	Unknown	Enteritidis	11	4712042	17	277178.94	1786231	695475	2	100
9716_8#27	5	137_50_5306	USA	1950	Unknown	Animal	Enteritidis	11	4702553	21	223931.1	1492764	516779	3	100
9716_8#28	5	138_50_5646	Japan	1950	Unknown	Animal	Enteritidis	11	4702658	23	204463.39	1514885	490384	3	100
9716_8#29	4	SAEN4	Congo	1950	Amp+Chlor	Blood	Enteritidis	11	4818934	31	155449.48	1071985	407077	4	100
9716_8#3	6	112_2012K_180	USA	2012	Unknown	Stool	Enteritidis	11	4710637	20	235531.85	1549409	527888	3	100
9716_8#30	4	SAEN8	Congo	1988	Amp+Chlor	Blood	Enteritidis	11	4780691	34	140608.56	1074264	407077	4	100
9716_8#31	4	SAEN13	Congo	1988	Amp+Chlor	Blood	Enteritidis	11	4793531	30	159784.37	1074286	405369	4	100
9716_8#32	4	SAEN19	Congo	1989	Amp+Chlor	Blood	Enteritidis	11	4848573	38	127594.03	1080672	406258	4	100
9716_8#33	4	SAEN26	Congo	1989	Amp+Chlor	Blood	Enteritidis	11	4805794	33	145630.12	1157393	559817	3	100
9716_8#34	4	SAEN27	Congo	1989	Amp+Chlor	Blood	Enteritidis	11	4816414	28	172014.79	1161154	401394	4	100
9716_8#35	5	SAEN62	Congo	1989	Sensitive	Blood	Enteritidis	11	4716059	19	248213.63	1509198	972080	2	100
9716_8#36	4	SAEN84	Congo	1989	Amp+Chlor	Blood	Enteritidis	11	4792199	35	136919.97	1484552	405378	3	100
9716_8#37	4	SAEN87	Congo	1989	Amp+Chlor	Blood	Enteritidis	11	4803359	30	160111.97	1304158	559874	3	100
9716_8#38	4	SAEN111	Congo	1990	Amp+Chlor	Blood	Enteritidis	11	4789960	37	129458.38	1074530	401491	4	100

9716_8#39	4	SAEN128	Congo	1990	Amp+Chlor	Blood	Enteritidis	11	4789883	28	171067.25	1711146	405714	3	100
9716_8#4	3	113_2012K_220	USA	2012	Unknown	Blood	Pending	11	4733190	34	139211.47	647874	312743	6	100
9716_8#40	4	SAEN146	Congo	1990	Amp+Chlor	Blood	Enteritidis	11	4788719	38	126018.92	1079417	405725	4	100
9716_8#41	4	SAEN152	Congo	1990	Amp+Chlor	Blood	Enteritidis	11	4790070	35	136859.14	1078686	401394	4	100
9716_8#42	4	SAEN180	Congo	1990	Amp+Chlor	Blood	Enteritidis	11	4787509	34	140809.09	1157337	559814	3	100
9716_8#43	4	SAEN199	Congo	1991	Amp+Chlor	Blood	Enteritidis	11	4788877	35	136825.06	1309787	477748	4	100
9716_8#44	4	SAEN203	Congo	1991	Chloramphenicol	Blood	Enteritidis	11	4755070	31	153389.35	1311558	405379	4	100
9716_8#45	4	SAEN220	Congo	1991	Chloramphenicol	Blood	Enteritidis	11	4799438	33	145437.52	1163241	405711	4	100
9716_8#46	4	SAEN256	Congo	1991	Chloramphenicol	Blood	Enteritidis	11	4770035	37	128919.86	1074524	407077	4	100
9716_8#47	4	SAEN280	Congo	1991	MDR	Blood	Enteritidis	11	4789983	33	145151	1466691	559865	3	100
9716_8#48	4	SAEN284	Congo	1991	MDR	Blood	Enteritidis	11	4787975	34	140822.79	1741455	708796	2	100
9716_8#49	5	SAEN378	Congo	1992	Sensitive	Blood	Enteritidis	11	4756794	33	144145.27	1508340	489881	3	100
9716_8#5	5	114_2012K_174	USA	2012	Unknown	Stool	Enteritidis	11	4697952	21	223712	1508825	492796	3	100
9716_8#50	4	SAEN390	Congo	1992	Chloramphenicol	Blood	Enteritidis	11	4783842	35	136681.2	1075987	477724	4	100
9716_8#51	5	SAEN392	Congo	1992	Sensitive	Blood	Enteritidis	11	4752879	20	237643.95	1739574	864533	2	100
9716_8#52	5	SAEN437	Congo	1992	Sensitive	Blood	Enteritidis	11	4796585	21	228408.81	1508638	492806	3	100
9716_8#53	5	SAEN1975	Uganda	1996	Sensitive	Blood	Enteritidis	11	4695541	14	335395.79	2001354	724769	2	100
9716_8#54	4	SAEN2209	Uganda	1996	Amp+Chlor	Stool	Enteritidis	11	4628989	28	165321.04	954635	407080	4	100
9716_8#55	5	SAEN2862	Uganda	1997	Sensitive	Blood	Enteritidis	11	4814269	85	56638.46	1349360	736939	3	100
9716_8#56	5	SAEN4582	Uganda	1997	MDR	Blood	Enteritidis	11	4665441	15	311029.4	1915850	970336	2	100
9716_8#57	4	SAEN6442	Uganda	1998	MDR	Blood	Enteritidis	11	4797777	47	102080.36	1074532	405729	4	100
9716_8#58	4	SAEN6474	Uganda	1998	MDR	Stool	Enteritidis	11	4813829	48	100288.1	1074525	642073	3	100
9716_8#6	6	115_2012K_175	USA	2012	Unknown	Stool	Enteritidis	11	4706241	15	313749.4	2042162	694436	2	100
9716_8#7	4	116_2012K_184	USA	2012	Unknown	Stool	Enteritidis	11	5041756	51	98857.96	1334498	401394	4	100
9716_8#8	5	117_2012K_185	USA	2012	Unknown	Stool	Enteritidis	11	4697212	24	195717.17	1229604	479362	3	100
9716_8#9	2	118_2012K_186	USA	2012	Unknown	Stool	Enteritidis	11	4876051	43	113396.53	1106646	412579	4	100
9803_3#1	6	P2	Unknown	2007	Unknown	Unknown	Enteritidis	11	4710182	17	277069.53	1043672	694988	3	100
9803_3#10	6	P12	Unknown	2007	Unknown	Unknown	Enteritidis	11	4753285	18	264071.39	1386912	694722	3	100
9803_3#11	6	P13	Unknown	2007	Unknown	Unknown	Enteritidis	11	4753761	17	279633	1865599	694744	2	100
9803_3#12	6	P14	Unknown	2007	Unknown	Unknown	Enteritidis	11	4705627	17	276801.59	1555468	970254	2	100
9803_3#13	6	P15	Unknown	2007	Unknown	Unknown	Enteritidis	11	4704634	13	361894.92	1549431	694858	3	100
9803_3#14	6	P16	Unknown	2007	Unknown	Unknown	Enteritidis	11	4705257	21	224059.86	1555486	489951	3	100
9803_3#15	5	P17	Unknown	2007	Unknown	Unknown	Enteritidis	11	4699608	17	276447.53	1508510	708327	3	100
9803_3#16	5	P19	Unknown	2007	Unknown	Unknown	Enteritidis	11	4774986	23	207608.09	1508513	490097	3	100
9803_3#17	6	RD2	UK	2005	Unknown	food	Enteritidis	11	4787941	19	251996.89	1549441	489950	3	100
9803_3#18	6	RD3	UK	2005	Unknown	food	Enteritidis	11	4749452	17	279379.53	1549464	694578	3	100
9803_3#19	6	RD6	UK	2005	Unknown	food	Enteritidis	11	4744492	19	249710.11	1320849	694686	3	100
9803_3#2	6	P4	Unknown	2007	Unknown	Unknown	Enteritidis	11	4711089	17	277122.88	1956440	970653	2	100
9803_3#20	6	RD26	UK	2005	Unknown	food	Enteritidis	11	4700012	16	293750.75	1543028	489945	3	100
9803_3#21	6	RD30	UK	2005	Unknown	food	Enteritidis	11	4712354	13	362488.77	1632697	695001	3	100
9803_3#22	6	RD36	UK	2005	Unknown	food	Enteritidis	11	4737021	19	249316.89	1630272	694557	3	100
9803_3#23	6	RD40	UK	2005	Unknown	food	Enteritidis	11	4736693	17	278629	1546878	970246	2	100

9803_3#24	6	RD42	UK	2005	Unknown	food	Enteritidis	11	4708164	17	276950.82	1549425	489948	3	100
9803_3#25	6	RD44	UK	2005	Unknown	food	Enteritidis	11	4708148	14	336296.29	1549413	694513	3	100
9803_3#26	6	RD46	UK	2005	Unknown	food	Enteritidis	11	4705700	16	294106.25	1386808	516449	3	100
9803_3#27	6	RD45	UK	2005	Unknown	food	Enteritidis	11	4705661	15	313710.73	1549415	695007	3	100
9803_3#28	6	RD49	UK	2005	Unknown	food	Enteritidis	11	4607755	15	307183.67	1451821	694528	3	100
9803_3#29	6	RD50	UK	2005	Unknown	food	Enteritidis	11	4718937	17	277584.53	1549437	516575	3	100
9803_3#3	6	P5	PTG	2007	Unknown	Unknown	Enteritidis	11	4744803	17	279106.06	1549433	489959	3	100
9803_3#30	6	RD51	UK	2005	Unknown	food	Enteritidis	11	4703446	19	247549.79	1546880	489956	3	100
9803_3#31	6	RD54	UK	2005	Unknown	food	Enteritidis	11	4706865	18	261492.5	1382459	489945	3	100
9803_3#32	6	RD55	UK	2005	Unknown	food	Enteritidis	11	4705085	14	336077.5	1549421	489943	3	100
9803_3#33	6	RD57	UK	2005	Unknown	food	Enteritidis	11	4657157	28	166327.04	1549446	972764	2	100
9803_3#34	6	RD72	UK	2005	Unknown	food	Enteritidis	11	4648503	18	258250.17	1555498	489951	3	100
9803_3#35	6	RD76	UK	2005	Unknown	food	Enteritidis	11	4749975	18	263887.5	1549487	970253	2	100
9803_3#36	6	RD79	UK	2005	Unknown	food	Enteritidis	11	4748573	14	339183.79	1555204	970234	2	100
9803_3#37	6	RD84	UK	2005	Unknown	food	Enteritidis	11	4707964	15	313864.27	1549421	699880	3	100
9803_3#39	5	SEA	USA	1994	Unknown	food	Enteritidis	11	4916549	38	129382.87	1110789	593725	3	100
9803_3#4	6	P6	Unknown	2007	Unknown	Unknown	Enteritidis	11	4735014	21	225476.86	1508333	491279	3	100
9803_3#40	5	SEC	USA	1994	Unknown	food	Enteritidis	11	4744658	18	263592.11	1549380	489959	3	100
9803_3#41	5	SED	USA	1994	Unknown	food	Enteritidis	11	4683224	20	234161.2	1508411	490085	3	100
9803_3#42	6	EX4_PT21b	PTG	1996	Unknown	Unknown	Enteritidis	11	4740535	24	197522.29	1508451	490083	3	100
9803_3#43	5	9508_195	USA	1994	Unknown	Unknown	Enteritidis	11	4702264	15	313484.27	1543033	490416	3	100
9803_3#44	5	9508_211	USA	1994	Unknown	Unknown	Enteritidis	11	4731023	25	189240.92	761440	490648	4	100
9803_3#45	5	S4726_04	UK	2006	Unknown	Unknown	Enteritidis	11	4739192	20	236959.6	1745016	732302	2	100
9803_3#46	6	S1750_06	PTG	2006	Unknown	Unknown	Enteritidis	11	4704624	17	276742.59	1345995	490224	3	100
9803_3#47	6	S6669_03L	PTG	2006	Unknown	Unknown	Enteritidis	11	4709427	13	362263.62	1632713	970159	2	100
9803_3#48	6	S6669_03N	PTG	2006	Unknown	Unknown	Enteritidis	11	4643455	16	290215.94	1544887	490361	3	100
9803_3#5	6	P7	Unknown	2007	Unknown	Unknown	Enteritidis	11	4647733	14	331980.93	1549431	694978	3	100
9803_3#50	6	99_CDC_H9556	USA	Unknown	Unknown	Stool	Enteritidis	11	4708520	17	276971.76	1549399	490057	3	100
9803_3#51	5	100_8_403	USA	2008	Unknown	Stool	Enteritidis	11	4807267	19	253014.05	1540775	721096	3	100
9803_3#52	6	101_AM27283_B	USA	2006	Unknown	Stool	Enteritidis	11	4809078	60	80151.3	1508677	283181	4	100
9803_3#53	5	102_AM36958_B	USA	2008	Unknown	Unknown	Enteritidis	1632	4830692	16	301918.25	1632734	694954	3	100
9803_3#54	5	103_08_639	USA	2008	Unknown	Stool	Enteritidis	11	4797004	17	282176.71	1193913	489798	3	100
9803_3#55	6	104_AM25928_B	USA	2006	Unknown	Blood	Enteritidis	11	4744914	21	225948.29	1986155	729081	2	100
9803_3#56	2	105_2011K_1654	USA	2011	Unknown	Stool	Enteritidis	11	4705178	11	427743.45	1549180	489950	3	100
9803_3#57	5	106_2011K_1681	USA	2011	Unknown	Stool	Enteritidis	11	4961268	21	236250.86	1106558	489046	4	100
9803_3#58	6	107_2012K_147	USA	2005	Unknown	Stool	Enteritidis	11	4717577	17	277504.53	1508854	695044	3	100
9803_3#59	6	108_2012K_179	USA	2006	Unknown	Stool	Enteritidis	11	4758829	16	297426.81	1549429	702244	3	100
9803_3#6	6	P8	Unknown	2007	Unknown	Unknown	Enteritidis	11	4743119	21	225862.81	1549392	708211	3	100
9803_3#7	5	P9	Spain	2007	Unknown	Unknown	Enteritidis	11	4712168	20	235608.4	1549432	489959	3	100
9803_3#8	5	P10	Spain	2007	Unknown	Unknown	Enteritidis	11	4706629	19	247717.32	1508535	970501	2	100
9803_3#9	5	P11	Spain	2007	Unknown	Unknown	Enteritidis	11	4705024	22	213864.73	1508501	492295	3	100
P125109	6	P125589	UK	1988		Food	Enteritidis	11							